J. gen. Microbiol. (1967), **48**, 161–169
Printed in Great Britain

Enumeration of Cellulolytic Cocci in Sheep Rumen by Using a Fluorescent Antibody Technique

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(*Accepted for publication* 28 *February* 1967)

SUMMARY

Strains of *Ruminococcus albus* were isolated from sheep fed lucerne chaff once daily. Antisera to these bacteria were prepared in rabbits and labelled with fluorescein isothiocyanate. A specific labelling reagent was produced from these conjugates by chromatography on Sephadex G-25 and two absorptions with mouse liver powder. The Breed smear technique was adapted for counting fluorescent cocci. Background fluorescence in rumen fluid samples was controlled by making suitable dilutions. A sample taken 10 hr after feeding had a mean value of 5·76 (S.E. 1·16) $\times 10^6$ cocci/ml. Samples taken from different parts of the rumen at the same time showed random but significant variation in the number of fluorescent cocci/ml. The natural fluorescence of some particles of digesta was bright enough to obscure the specific fluorescence and attempts were made to release fluorescent cocci by homogenization; this was partially successful, and the average ratio of untreated to homogenized counts was 1 : 1·17. Diurnal variations in the number of cellulolytic cocci were followed over a 4-day period. Minimum numbers were recorded 6 hr after feeding and maximum numbers 18 hr later. There was a measurable day-to-day variation.

INTRODUCTION

Several cultural methods have been described for the enumeration of cellulolytic cocci in the rumen (Hungate, 1950; Kistner, 1960). These methods usually depend on the use of ball-milled cellulose as the sole substrate in a habitat-simulating medium. After incubation cellulolytic colonies are detected by cleared zones in the opaque medium. Cellulolytic bacteria have also been recovered from high dilutions of rumen liquor by picking colonies from a non-selective medium and observing whether they hydrolyse cellulose in liquid culture (Bryant & Burkey, 1953).

Serological studies of ruminococci (Jarvis, 1967) demonstrated immunological relationships among strains of both *Ruminococcus albus* and *R. flavifaciens* and serologically related strains persisted in the rumen. It was concluded that the fluorescent antibody technique could be adapted to permit direct microscopic examination of rumen contents for cellulolytic cocci. This paper describes the development and use of a simple counting method for *R. albus*, based on the direct fluorescent antibody technique.

METHODS

Sheep. Two fistulated merino sheep, C6 and C18, were housed indoors in separate pens on a slatted floor and fed rations which would maintain them at roughly constant body weight. Each sheep received 800 g. chaffed lucerne hay at 9.00 a.m. daily and had free access to water. Both sheep had been established on their diets more than 6 months before bacterial isolations were made.

Bacterial strains. Ruminococcus albus strains LY3 and LY4 were isolated from sheep C6 using a modification of the anaerobic culture technique described by Hungate (1950). The characteristics of these strains were described by Jarvis & Annison (1967). *Escherichia coli* and *Staphylococcus aureus* were obtained from stock cultures in a collection maintained at the School of Rural Science.

Production of antiserum. Antisera to LY3 and LY4 were produced in rabbits using the methods described by Jarvis (1967).

Preparation of fluorescein-labelled antiserum

Labelled antiserum was prepared by the method of Marshall, Eveland & Smith (1958) as described by Hobson, Mann & Smith (1962) with the exception that the proportion of fluorescein isothiocyanate (Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Maryland, 21204, U.S.A.) to protein used was 0·05 mg./mg. protein.

Estimation of total serum protein. Serum protein was determined spectrophotometrically from the extinction of a 1/100 dilution of serum in a 1 cm. cuvette. Readings were taken at 260 mμ and at 280 mμ and applied to a nomograph based on data given by Warburg & Christian (1941) (prepared by California Corporation for Biochemical Research, Los Angeles 63).

Conjugation of antiserum with fluorescein isothiocyanate. A magnetic stirrer was used to mix 2·5 ml. whole serum, 4·2 ml. 0·15 M-NaCl and 1·7 ml. 0·5 M-carbonate + bicarbonate buffer (pH 9·0) at 2° in a cold room. Cold acetone (1 ml.) containing fluorescein isothiocyanate (FITC) was added dropwise to the stirred solution from a syringe fitted with a 26-gauge needle. Stirring was continued for 18 hr at 2°.

Separation of fluorescein-conjugated protein from unreacted fluorescein. When dialysis was used for this purpose (Hobson *et al.* 1962) the solution of serum proteins in buffered saline (conjugated antiserum) was dialysed against litre volumes of 0·01 M-phosphate buffered saline (pH 7·0) at 2°. The dialysing solution was stirred continually and changed twice daily. After 3 days an ultraviolet lamp was required to detect dialysed fluorescein but a slight amount was present in the saline when dialysis was stopped after 9 days. Chromatography on Sephadex G-25 (Gordon, Edwards & Tompkins, 1962; Peters, 1963) was found to be a rapid and efficient means of separating conjugated antiserum and unreacted FITC.

Estimation of labelling efficiency. After the unconjugated fluorescein had been separated on a Sephadex column the relative fluorescein:protein ratios of different conjugates were compared by reading the extinction at 280 mμ and 495 mμ (Tokumaru, 1962). It was confirmed that FITC was degraded during prolonged storage at 2° (McKinney, Spillane & Pearce, 1964). Use of degraded FITC resulted in the production of conjugate with a low fluorescein:protein ratio. Fluorescence with such conjugates was dull although the titre was high.

Absorption of conjugated antiserum. Dialysed solutions were cleared by centrifugation at 22,000 *g* for 35 min. at 2° and then absorbed. Sephadex-treated conjugates were not cleared before adsorption. Acetone-dried mouse liver powder (100 mg./ml. of conjugate) was added to conjugated antiserum and the mixture was shaken for 1 hr at room temperature, then centrifuged at 22,000 *g* for 20 min. at 0°. The supernatant absorbed conjugated antiserum was recovered, preserved with Thiomersal (British Drug Houses Ltd., England) and deep frozen until required.

Antibody titre of labelled antiserum. The effect of fluorescein labelling treatments on the antibody activity of the antiserum was followed by a tube agglutination test against homologous antigen (Jarvis, 1967).

Preparation of slides from pure cultures

A thin smear of bacteria was spread on an ultraviolet transmitting glass slide (Shandon Scientific Co., Cromwell Place, London) and allowed to dry on a warm surface. The smear was fixed by immersing the slide in acetone for 20 min. The acetone was allowed to evaporate from the slide and it was then immersed in 0·1 M-phosphate buffered saline (pH 7·0) for 1 min. Excess saline was drained from the slide and two drops of absorbed conjugated antiserum were spread over the moist smear with a glass rod. The slide was incubated 30 min. at 37° in a moist chamber. After incubation the slide was rinsed with a phosphate buffered saline and washed by immersion in three lots of buffered saline for 3–5 min. each. Excess saline was drained off and a drop of redistilled glycerol containing 10 % (v/v) buffered saline added to the smear. A coverslip was applied (Chance No. 1) and the slide blotted to remove excess buffered glycerol. These smears were examined using the methods and apparatus described earlier (Jarvis, 1967).

Counting ruminococci in samples of rumen liquor

A wide-bore glass tube was used to collect samples of rumen content from fistulated sheep. Each sample was transferred to a 25 × 150 mm. test-tube, stoppered and allowed to stand for 20 min. Counts were made on liquid drawn from a point 4 mm. below the surface and diluted 1:1 in 0·01 M-phosphate-buffered saline. After thorough mixing 0·01 ml. of the dilution was pipetted on to a slide and immediately spread over an area of 1 cm.^{2} with the end of a thin glass rod. Smears were dried on a warm horizontal surface (5–10 min.) and then fixed in methanol (10 min.). The methanol was allowed to evaporate, and smears were moistened in phosphate-buffered saline (1–2 min.), drained and covered with conjugated specific antiserum (1–2 drops). After incubation at 37° for 30 min. in a moist chamber the slides were removed, rinsed, washed and mounted as described above.

A × 100 objective and × 10 eyepiece were used for counting. The field diameter was measured with a slide micrometer and a microscope factor was computed (American Public Health Assoc., 1948) allowing for the appropriate dilution 1/1, 1/5 or 1/10. With a dilution of 1/1 the average count per field × 10^6 represented the count/ml. of rumen fluid. Fields were counted at approximately equal distances along two diameters of the smear at right angles to one another. Individual cocci were counted in the occasional small colonies encountered. Diplococci were considered separate cocci when any clear division between daughters could be distinguished.

RESULTS AND DISCUSSION

Development of a fluorescent antiserum specific for ruminococci

Cherry, Goldman & Carski (1960) discussed the steps necessary to demonstrate the serological specificity of observed immunofluorescence; and the following list is taken from their work. (1) Uninfected tissue or uninoculated culture media should not stain. (2) Conjugates of normal serum should not stain the antigen. (3) Staining should be inhibited by pretreatment of the smear with unlabelled specific antibody or by dilution of the conjugate in unlabelled specific antibody. (4) Staining should be inhibited by adsorption of the antiserum with homologous antigen prior to staining. These criteria were applied to show the specificity of antisera to strains of *Ruminococcus albus* for their homologous antigen.

Unstained antigen suspensions were found to have absolutely no natural fluorescence. Unstrained smears of rumen content contained particles which fluoresced red or yellow (Hobson & Mann, 1957) and also green-fluorescing particles, some of which were small enough to be confusing. These particles could be distinguished from bacteria by their lack of regular shape.

Table 1. *Staining of Escherichia coli and Ruminoccus albus* LY3 *by conjugated normal serum fractions*

Normal serum	*E. coli*	*R. albus* LY3
Liver-absorbed protein fraction	Positive but dull	Negative. No visible cocci by blue-light fluorescence
Liver-absorbed fractions immediately behind the protein peak	Negative. No visible rods	Very faint labelling

Conjugates of normal serum were prepared by the method of Hobson *et al.* (1962), dialysed for 7 days and absorbed with mouse liver powder. When smears of *Escherichia coli* and *Ruminococcus albus* LY3 were treated with this material *R. albus* LY3 fluoresced but *E. coli* did not. The labelled normal serum was passed through a Sephadex G-25 column and this revealed considerable fluorescence which did not move with the protein peak. The extinction of successive fractions was measured at 280 mμ and 495 mμ and the values obtained were corrected for absorption at 280 mμ due to FITC (Tokomaru, 1962). Most of the FITC travelled with the protein peak but the extinction at 280 mμ decreased much more rapidly than that at 495 mμ, indicating a tail of unconjugated FITC. Table 1 records the results obtained when the liver absorbed protein fraction from the Sephadex column was used to label *E. coli* and *R. albus* LY3. It was concluded that dialysis would not remove traces of FITC which were sufficient to cause the observed non-specific staining. When unconjugated FITC was removed labelled normal serum could not cause appreciable fluorescence.

The blocking effect of unlabelled specific antiserum was shown by comparing two treatments: (*a*) 2 vol. homologous unlabelled antiserum to 1 vol. homologous labelled antiserum; (*b*) 2 vol. normal serum to 1 vol. homologous labelled antiserum. There was a considerable decrease in fluorescence in (*a*) as compared with (*b*) because of the blocking effect of unlabelled homologous antibodies.

Smears of *Ruminococcus albus* LY3 were treated with conjugated LY3 antiserum

which had been absorbed with LY3 organisms. Examination by blue-light fluorescence showed a slight glow visible only where the cocci were closely packed. On the other hand, three successive absorptions of conjugated LY3 antiserum with a suspension of *Escherichia coli* did not decrease the fluorescence imparted to LY3 organisms appreciably. It was concluded that the fluorescence observed was due to a specific serological reaction.

Gordon *et al.* (1962) showed that conjugates specific for bacterial antigens could be obtained by Sephadex treatment of labelled γ-globulin solutions. The column separated unconjugated dye and low molecular weight fractions from labelled protein. A conjugate of whole serum (titre 1/20,000) prepared in this way stained non-specifically. Smears of *Escherichia coli* stained as brightly as the homologous antigen *Ruminococcus albus* LY3. *Staphylococcus aureus* did not fluoresce with this conjugate. The antibody titre of the protein fraction was 1/5000. Visible fluorescence in solution did not extend beyond a dilution of 1/640, suggesting that the non-specific material was present in considerably lower concentration than the specific labelled antibody. Subsequently it was shown that complete removal of unconjugated FITC by a second Sephadex treatment did not give a specific antiserum. Two successive absorptions with liver powder were required to produce a conjugate specific for *R. albus* LY3. After this treatment the agglutination titre of labelled antiserum was 1/1300 and fluorescence in the supernatant fluid of the agglutination tubes was absent above a dilution of 1/80. Table 2 records the results obtained when the twice absorbed conjugate was used to stain smears of *E. coli* and *R. albus* LY3.

Although Sephadex G-25 is not intended to fractionate proteins, three fractions

Table 2. *Staining of Escherichia coli and Ruminoccus albus LY3 by conjugated antiserum specific for Ruminococcus albus LY3*

Diluent	*E. coli*	*R. albus* LY3
Phosphate buffered saline (pH 7·0)	Fluorescence just visible. Very hard to distinguish rods	Bright fluorescence of cocci against a black background
Rumen liquor	No identifiable fluorescent rods	Bright fluorescence of cocci against a faintly fluorescent background

Table 3. *Staining of Escherichia coli and Ruminococcus albus LY3 in rumen fluid by conjugated protein fractions specific for Ruminococcus albus LY3*

Fraction*	*E. coli*	*R. albus* LY3
1	No fluorescent rods	Bright fluorescence of cocci on a faintly staining background
2	*E. coli* visible but much less fluorescent than *R. albus* LY3 cocci	Brilliant fluorescence of cocci on a black background
3	No fluorescent rods but a few fluorescent cocci which would be present in rumen fluid were observed	Bright fluorescence of cocci on a faintly staining background

* These protein fractions are described in the text.

were recognized in the protein band: *fraction* 1, a small volume having the brightest fluorescence and travelling ahead of the main band, *fraction* 2, the main protein band, and *fraction* 3, a small volume behind the main peak and seemingly more dilute. An attempt was made to determine whether these three fractions could be bulked for use as specific labelled antiserum. Each fraction was used to stain homologous and heterologous cells in rumen liquor. The results are recorded in Table 3. It was concluded that the non-specific reaction was associated with protein travelling in the middle of the band and that this could be decreased to a satisfactory degree by two absorptions with liver powder without impairing the specificity of the antiserum.

Counting ruminococci in rumen fluid

Precision of the counting method. A sheep was sampled 10 hr after being fed 800 g. lucerne chaff. Eight parallel dilutions of the sample were each smeared once and 30 fields were counted on each smear. The resulting estimates of numbers of ruminococci were: $6{\cdot}76 \times 10^6$, $4{\cdot}90 \times 10^6$, $4{\cdot}17 \times 10^6$, $5{\cdot}30 \times 10^6$, $7{\cdot}8 \times 10^6$, $7{\cdot}37 \times 10^6$, $5{\cdot}34 \times 10^6$, $4{\cdot}43 \times 10^6$, mean $5{\cdot}76 \times 10^6$/ml., standard deviation $1{\cdot}38 \times 10^6$ (corrected for the small number of observations); standard error of the mean (95 % confidence interval), $\pm 1{\cdot}16 \times 10^6$/ml.

Wilson (1935) examined the errors inherent in the Breed smear technique and concluded that it was unsuitable for low count samples since an average of at least one organism per field was required for reasonable accuracy. The main source of random error in the method was considered to be the irregular distribution of the bacteria in the smear. Subsequently Hanks & James (1940) recommended the use of circular smears, and the selection of fields at regular intervals along two diameters at right angles to each other in order to minimize these errors. In the method described here an additional source of error was the masking effect of fluorescent background material. Sources of variation in the background fluorescence included: extent of sedimentation before dilutions were prepared, thickness of the smear, evenness of drying, extent of digestion within the rumen before sampling. Samples taken after extensive digestion showed more intense background fluorescence and higher counts than those taken soon after feeding. Consequently the background fluorescence could be controlled by suitable dilution of the sample without introducing excessive statistical errors. The total number of fluorescent cocci/ml. rumen content varied between 2×10^6 and 12×10^6. A counting method with a precision of approximately $\pm 1{\cdot}0 \times 10^6$ was considered adequate to resolve the differences encountered.

Variation between sampling sites in the rumen. At intervals of several days samples were obtained from three sites in the rumen 11 hr after feeding. The regions sampled were: anterior ventral, anterior dorsal, posterior ventral. Duplicate dilutions were made from each sample and each was smeared once: 30 fields were counted on each smear. The average for each sample is recorded in Table 4. It was concluded that sometimes there were significant differences in the concentration of ruminococci at different points in the rumen. Samples intended to represent the average population should be drawn from various parts of the organ.

Effect of homogenizing rumen contents. Davies (1965) used an indirect fluorescent antibody technique to study the distribution of cellulolytic bacteria isolated from the large intestine of a horse. She concluded that 'free' and 'fixed' bacteria could be enumerated. But the present study indicated that the natural fluorescence of many

particles of ingesta was too bright to permit fluorescent bacteria on them to be distinguished. Homogenization was intended to release 'fixed' bacteria. The whole rumen content was macerated in a tissue grinder at 0° for 2 min. This caused partial destruction of food particles but viable protozoa still remained. Counts were made on homogenized whole rumen content and compared with those on untreated rumen liquor (prepared as described in Methods). Homogenized rumen content was diluted 1/10 to decrease the background fluorescence to an acceptable degree. These results are recorded in Table 5 where each figure represents the mean of duplicate dilutions smeared once. The increase in count due to homogenization is not as much as expected (Bryant, 1959; Warner, 1962*a*). This may have been due to the particular antiserum used or to the technique used, but it is interesting to note that Hungate (1947) found no increase in cellulolytic colony counts after similar treatment. Qualitative examination of rumen contents indicates that the fluorescent organisms do not occur as large colonies and are often associated with highly degraded material, consequently blending might not release many.

Table 4. *Concentration of ruminococci at selected sites in the rumen of sheep fed chaffed lucerne hay once daily and sampled 11 hr after feeding, determined by a direct fluorescent antibody technique*

Series*	Anterior ventral (10^{-6}/ml.)	Anterior dorsal (10^{-6}/ml.)	Posterior ventral (10^{-6}/ml.)
1	6·68	7·00	7·08
2	4·68	8·18	8·13
3	2·75	1·79	4·81
4	2·42	1·40	3·33
5	3·78	3·93	12·26
Average	4·06	4·46	7·10

* Successive sets of samples were collected at intervals of several days.

Table 5. *Effect of maceration on the number of fluorescent ruminococci observed by a direct fluorescent antibody technique in the rumen contents of sheep fed chaffed hay once daily*

	No. of cocci ($\times 10^{-6}$ ml.)		
Sheep sampled*	Untreated Control	Homogenized	Ratio, homogenized: control
C_6 12 hr after feeding	7·18	6·35	0·89
C_{18} 4 hr after feeding	5·43	6·78	1·25
C_{18} 4 hr after feeding	7·38	8·69	1·19
C_6 12 hr after feeding	12·05	16·37	1·36
Average	8·01	9·55	1·19

* Successive samples were collected at intervals of several days.

Diurnal variation in the concentration of fluorescent ruminococci. Results obtained with one sheep are presented here as an indication of the fluctuation in numbers to be expected (Fig. 1). Sheep C18 was fed 800 g. lucerne chaff each morning Food was withdrawn after 2 hr. Samples of ingesta were taken before feeding and also at 3.00 p.m., 9.00 p.m. and 3.00 a.m. The samples were preserved with 1/10,000 Thiomersal and held at 2°. Each count represents the mean of counts made on duplicate

dilutions smeared once. Thirty fields were usually counted on each smear, but 60 or 90 fields were counted on smears from 3.00 p.m. samples because of the small number of fluorescent cocci.

The numbers of fluorescent ruminococci fluctuated in accordance with a recognizable pattern. The full extent of these fluctuations is not known, but the lowest values were always observed at 3.00 p.m. and the highest at 3.00 a.m. There was also a noticeable day-to-day variation in the concentration of cocci at a given sampling time. A decrease in total bacterial numbers after feeding has been observed by Christian & Williams (1957), Bryant & Robinson (1961) and Warner (1962*b*). The subsequent increase in

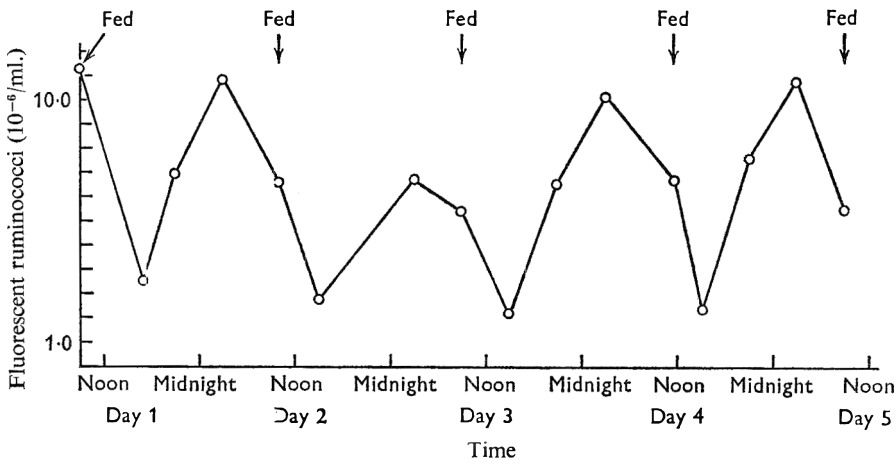

Fig. 1. Diurnal and daily changes in the concentration of fluorescent ruminococci obtained from a sheep fed chaffed lucerne hay once daily. The sheep was fed 800 g. lucerne chaff daily, between 8.00 a.m. and 12.00 noon, after it had been sampled.

numbers found here appeared to parallel the pattern of rumination (Pearce, 1965); this is in accordance with the observations of Pearce & Moir (1964) and Dehority & Johnson (1961) which indicated that trituration played an important part in the digestion of roughage.

It is probable that the use of labelled antiserum to one antigen would not reveal all the ruminococci although most strains of *Ruminococcus albus* would have been counted (Jarvis, 1967). Over a period of 39 days samples were taken on seven occasions among five sheep fed chaffed lucerne hay. A total of 14 strains were collected, only half of which were subsequently identified as *R. albus* (Jarvis & Annison, 1967). This suggests that a count restricted to *R. albus* would enumerate about half the ruminococci present.

REFERENCES

American Public Health Association. (1948). *Standard Methods for the Examination of Dairy Products*. New York: American Public Health Assoc.

Bryant, M. P. (1959). Bacterial species of the rumen. *Bact. Rev.* **23**, 125.

Bryant, M. P. & Burkey, L. A. (1953). Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* **36**, 205.

Bryant, M. P. & Robinson, I. M. (1961). An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. *J. Dairy Sci.* **44**, 1446.

CHERRY, W. B., GOLDMAN, M. & CARSKI, T. R. with the collaboration of MOODY, M. D. (1960). Fluorescent antibody techniques in the diagnosis of communicable diseases. *Publ. Hlth Serv. Publs. Wash. no.* 727.

CHRISTIAN, K. R. & WILLIAMS, V. J. (1957). Rumen Studies in Sheep. 4. The rumen metabolism of fresh and dried grass. *N.Z. Jl Sci. Technol.* **38**, sect. A, p. 1003.

DAVIES, E. M. (1965). Cellulolytic bacteria in some ruminants and herbivores as shown by fluorescent antibody. *J. gen. Microbiol.* **39**, 139.

DEHORITY, B. A., & JOHNSON, R. R. (1961). Effect of particle size upon the *in vitro* digestibility of forages by rumen bacteria. *J. Dairy Sci.* **44**, 2242.

GORDON, M. A., EDWARDS, M. R. & TOMPKINS, V. N. (1962). Refinement of fluorescent antibody by gel filtration. *Proc. Soc. exp. Biol. Med.* **109**, 96.

HANKS, J. H. & JAMES, D. F. (1940). The enumeration of bacteria by the microscopic method. *J. Bact.* **39**, 297.

HOBSON, P. N. & MANN, S. O. (1957). Some studies on the identification of rumen bacteria with fluorescent antibodies. *J. gen. Microbiol.* **16**, 463.

HOBSON, P. N., MANN, S. O. & SMITH, W. (1962). Serological tests of a relationship between rumen selenomonads *in vitro* and *in vivo*. *J. gen. Microbiol.* **29**, 265.

HUNGATE, R. E. (1947). Studies of cellulose fermentation. III. The culture and isolation of cellulose-decomposing bacteria from the rumen of cattle. *J. Bact.* **53**, 631.

HUNGATE, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bact. Rev.* **14**, 1.

JARVIS, B. D. W. (1967). Antigenic relations of cellulolytic cocci in the sheep rumen. *J. gen. Microbiol.* **47**, 309.

JARVIS, B. D. W. & ANNISON, E. F. (1967). Isolation, classification and nutritional characteristics of cellulolytic cocci in the sheep. *J. gen. Microbiol.* **47**, 295.

KISTNER, A. (1960). An improved method for viable counts of bacteria of the ovine rumen which ferment carbohydrates. *J. gen. Microbiol.* **23**, 565.

MARSHALL, J. D., EVELAND, W. C. & SMITH, C. W. (1958). Superiority of fluorescein isothiocyanate (Riggs) for fluorescent antibody technique with a modificaton of its application. *Proc. Soc. exp. Biol. Med.* **98**, 898.

MCKINNEY, R. M., SPILLANE, J. T. & PEARCE, G. W. (1964). Determination of purity of fluorescein isothiocyanates. *Analyt. Biochem.* **7**, 74.

PEARCE, G. R. (1965). Rumination in sheep. 2. The circadian pattern of rumination. *Aust. J. agric. Res.* **16**, 635.

PEARCE, G. R. & MOIR, R. J. (1964). Rumination in sheep. 1. The influence of rumination and grinding upon the passage and digestion of food. *Aust. J. agric. Res.* **15**, 635.

PETERS, H. (1963). Construction and use of a small Sephadex column for the separation of fluorescent antibodies. *Stain Technol.* **38**, 261.

TOKUMARU, T. (1962). A kinetic study on the labelling of serum globulin with fluorescein isothiocyanate by means of gel filtration technique. *J. Immunol.* **89**, 195.

WARBURG, O. & CHRISTIAN, W. (1941). Isolierung und Kristallisation des Gärungsferments Enolase. *Biochem. Z.* **310**, 384.

WARNER, A. C. I. (1962*a*). Enumeration of rumen micro-organisms. *J. gen. Microbiol.* **28**, 119.

WARNER, A. C. I. (1962*b*). Some factors influencing the rumen microbial population. *J. gen. Microbiol.* **28**, 129.

WILSON, G. S. (1935). The bacteriological grading of milk. *Spec. Rep. Ser. med. Res. Coun.* no. 206.

J. gen. Microbiol. (1967), **48**, 171–177
With 4 plates
Printed in Great Britain

Use of a Scanning Electron Microscope for the Examination of Actinomycetes

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(*Accepted for publication* 28 *February* 1967)

SUMMARY

Organisms belonging to several genera of the order Actinomycetales were examined using the 'Stereoscan' electron microscope of the Cambridge Instrument Company. Unlike the transmission electron microscope, this instrument provides surface views of fine structures without the need for preparation of replicas. Using a simple preparation procedure, surface views of intact sporing structures of several actinomycetes were observed over a range of magnifications. The potential value of using scanning electron microscopes for the examination of actinomycetes was assessed.

INTRODUCTION

Investigations of the structure of organisms belonging to various genera of the Actinomycetales with transmission electron microscopes have provided much useful information. Details of the internal structure of vegetative and reproductive structures of several genera have been obtained from the examination of ultra-thin sections. Details of the development of spores in sporangia-forming genera have been given by Rancourt & Lechevalier (1963) who studied *Microellobospora*, Lechevalier & Holbert (1965) who examined *Actinoplanes*, and Lechevalier, Lechevalier & Holbert (1966) who examined *Streptosporangium*, *Spirillospora* and *Actinoplanes*.

The fine structure of reproductive and vegetative organs of the genus *Streptomyces* has been investigated by many workers (Moore & Chapman, 1959; Stuart, 1959; Glauert & Hopwood, 1960; Chen, 1964; Painter & Bradley 1965). Rancourt & Lechevalier (1964) were able to outline the developmental sequence of spores of *Streptomyces viridochromogenes* and *S. violaceus* by the examination of sections. Studies of other genera include those of Overman & Pine (1963) on Actinomyces species and Kawato & Inoue (1965) on *Nocardia asteroides*.

Examination of actinomycetes with the transmission electron microscope has also provided taxonomic criteria. Observation of the silhouettes of whole spores of various species of the genus *Streptomyces* provides details of their surface ornamentation and this has been shown to be a useful taxonomic character (Küster, 1953; Preobrazhenskaya *et al.* 1960; Tresner, Davies & Backus, 1961).

Surface views of electron-dense structures, such as actinomycete spores, have until recently been obtainable only by the examination of carbon replicas of the structures. Replication techniques have had only limited application in studies of actinomycetes. Hopwood & Glauert (1961) examined carbon replicas of spores of *Streptomyces violaceoruber* and, using similar techniques, other species of this genus were examined by Dietz & Matthews (1962) and Preobrazhenskaya *et al.* (1965).

We have examined organisms belonging to several actinomycete genera, using the 'Stereoscan' electron microscope developed by the Cambridge Instrument Company. This is a scanning electron microscope which has a larger depth of focus even than the transmission type, providing a surface view of whole structures. The procedures used for the examination of actinomycetes are described, and from the results obtained the potential value of such an instrument for studies of this group of micro-organisms is assessed.

METHODS

Strains used. These were as follows: *Actinoplanes* sp., *Microbispora rosea* (RIA 477), *Microellobosporia flavea* (IMRU 3858), *Micromonospora* sp., *Nocardia rubra* (CBS), *Streptomyces finlayi* (ISP 5218), *S. viridosporus* (ISP 5243), *Streptomyces* sp., *Streptosporangium* sp., and *Thermoactinomyces vulgaris* (Küster P 121). (RIA, U.S.S.R. Research Institute for Antibiotics, Moscow; IMRU, Institute of Microbiology, Rutgers, New Brunswick, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; ISP, Code number of culture in International Streptomyces Project; Prof. E. Küster, Dept. of Industrial Microbiology, Dublin, Eire.)

Media. With the exception of *Nocardia rubra* and *Thermoactinomyces vulgaris*, which were grown on Oxoid Nutrient Agar, all strains were grown on oatmeal agar (Waksman, 1961).

Culture Method. After preliminary tests, the following procedure was found to be satisfactory for providing a growth of actinomycetes which could be examined, with the minimum of disturbance, in the scanning electron microscope. Circular glass coverslips, $\frac{1}{2}$ in. in diameter, were sterilized by autoclaving. Each of these was then inserted at an angle of about 45° into solidified medium in a Petri dish until about half the coverslip was in the medium. An inoculum of actinomycetes from slope cultures was then spread along the line where the upper surface of the coverslip met the medium, using a fine wire needle. The plates were then incubated, the length of incubation period needed for each strain to produce mature sporing structures having been previously determined. All were incubated at 25° except for *Thermoactinomyces vulgaris* which was grown at 45°. After incubation the actinomycetes were growing both on the medium and in a line across the upper surface of the coverslips. This line of growth remained attached to the coverslips when they were carefully withdrawn from the medium. A similar culture method for actinomycetes was described by Kawato & Shinobu (1959).

Preparation of cover-slip cultures for examination. With the actinomycetes uppermost the coverslips were attached by 'Durofix' adhesive, to solid metal specimen stubs which had a circular face of the same diameter as the coverslips. The upper surface of each coverslip was then coated, under vacuum, with a film, about 500 Å thick, of gold–palladium alloy. This process was completed in 15 min. and four preparations were coated at a time. During examination in the scanning electron microscope, the film allowed excess charge built up on the specimens to leak away, but was not thick enough to decrease the resolution. The coverslips were coated within 10 min. of their removal from the medium and thus the actinomycetes were alive until they were placed under vacuum and distortions from slow air drying were minimized. Once coated with gold–palladium, the specimens were ready for examination in the microscope.

Examination with the scanning electron microscope. The metal stubs with their attached coated coverslips were placed in the specimen chamber of the microscope. The field was scanned at low magnification until the line of growth was detected. Areas with clear, intact sporing structures were then selected for examination at higher magnification. Suitable subjects were photographed using Ilford H.P. 3 film.

In this instrument the specimen is scanned by a beam of electrons and these generate secondary electrons by the ionization of atoms of the specimen. Some of these have sufficient energy to escape into the vacuum and are collected, together with some reflected primary electrons, to give the screen image. The 'shadowed' appearance of the specimen, which emphasizes surface details, is due to the positioning of the collector for the secondary electrons at an angle to the original beam.

RESULTS

The results obtained are shown in Plates 1–4. It can be seen that the images produced by the scanning electron microscope were similar to those seen with a binocular light microscope but at greater magnification. The instrument can be used over a wide range of magnification but for actinomycetes the most useful range is from 1000 to 30,000 times. Thus in Pl. 1, figs. 1–3, views of the same streptomyces species are shown at three different magnifications. At lower magnifications the general morphology of the intact spore chains is well illustrated, while the smooth indented surface of the spores can be seen at higher magnifications.

Examination of Streptomyces species. Three species of Streptomyces were examined. It was known from previous examinations with a transmission electron microscope that they had variously ornamented spore surfaces. *Streptomyces viridosporus* had spores with spines, those of *S. finlayi* had hairs and those of another *Streptomyces* sp. were smooth. As previously stated, the ornamentation of spore surfaces is an important taxonomic character for species of the genus *Streptomyces*. It is considered to be one of the more stable attributes of streptomycetes and was included in the criteria used in the International Streptomyces Project for the redescription of type cultures (Shirling & Gottlieb, 1966). The ornamentation of the spores of *S. viridosporus* and *S. finlayi* was clearly revealed here (Pl. 1, fig. 4; Pl. 2, fig. 5). Whole surface views of ornamented Streptomyces spores were obtained by Preobrazhenskaya *et al.* (1965) from examination of carbon replicas. They obtained replicas of smooth spores and of spores with wart-like projections and spines, but no example of a carbon replica of a spore with hairs was shown. To obtain carbon replicas of such delicate and flexible structures would be extremely difficult.

Using the scanning electron microscope it was also possible to examine intact chains of spores, and observations of those of *S. viridosporus* revealed some interesting features. Some chains were composed of spores all of which had spiny surfaces (Pl. 2, fig. 5); in others both smooth and spiny spores occurred (Pl. 2, fig. 7), while in some the chains were composed entirely of spores with smooth surfaces (Pl. 2, fig. 6). Similar findings were made by Baldacci, Balduzzi & Amici (1957) and Hodgkiss & Mitchell (1965) from their examinations of silhouettes of spores with the transmission electron microscope. Baldacci *et al.* (1957) suggested that this variation in appearance was because some of the spores had lost their outer coat on which the spines were located. This suggestion agrees with the details of spore development in

S. viridochromogenes given by Rancourt & Lechevalier (1964). They showed that the spines were on a superficial layer which did not adhere strongly to the surface of mature spores. We obtained no additional evidence on the cause of this phenomenon and further study is needed. When ornamentation of spores is used as a taxonomic character it is important to take account of this variation and examine a wide range of spore chains. This can be easily done using the scanning electron microscope at relatively low magnifications (Pl. 1, fig. 2), when details of the overall spore-chain morphology, another important taxonomic character, can also be observed.

In Pl. 2, fig. 6 it can be seen that the smooth spores of *Streptomyces viridosporus* had lateral depressions; these are also evident in the smooth spores of *Streptomyces* sp. (Pl. 1, fig. 3). Recently, Tresner, Davies & Englert (1966) noted that the smooth spores of some streptomycetes had a 'phalange' shape in outline and in section; it seems likely that these workers observed spores with similar depressions to those shown here. Care must be taken when interpreting such structures in the electron microscope, as the depressions may be artifacts caused by the partial collapse of spore walls under vacuum.

Examination of sporangia-forming genera. Examples of three genera forming sporangia were examined: *Actinoplanes* sp., *Streptosporangium* sp. and *Microellobosporia flavea.* The internal structure of sporangia of Actinoplanes was studied by Lechevalier & Holbert (1965). From their examination of ultra-thin sections they suggested that spores in a mature sporangium gave it a bulging appearance; this was confirmed in this study (Pl. 2, fig. 8). Sections of sporangia of Streptosporangium examined by Lechevalier *et al.* (1966) indicated that the outer sporangial envelope was a loosely wrinkled structure and this was also evident from the observations of intact sporangia with the scanning electron microscope (Pl. 3, fig. 9). Sections of Microellobosporia were examined by Rancourt & Lechevalier (1963) who also noted that the sporangial wall had a wrinkled membranous form. This was true of the mature three-spored sporangium observed here (Pl. 3, fig. 11). In younger sporangia the wall was more regular (Pl. 3, fig. 10), possibly because it was still attached to tissues inside the sporangium.

Examination of other genera. The spores of the thermophilic actinomycete, *Thermoactinomyces vulgaris*, had a polygonal shape when viewed with the scanning electron microscope (Pl. 3, fig. 12). Other workers, examining silhouettes of spores of thermophilic actinomycetes with the electron microscope, have noted their angled outline. Agre (1962) observed 8- to 10-sided spore silhouettes in a thermophilic *Micromonospora vulgaris* and Kudrina & Maksimova (1963) noted similar spores in a thermophilic *M. thermolutea.* As both of these actinomycetes formed their spores on aerial mycelium above the medium, they would not be included in the genus *Micromonospora* by some workers, but placed in *Thermoactinomyces.* Thus it is possible that the shape of Thermoactinomyces spores may be a useful diagnostic character for this genus.

Some of the stages in the development of the paired spores of *Microbispora rosea* were evident in Pl. 4, fig. 13. Younger spores were rather elongated, without any external indication of a paired structure. Gradually they became broader and the central constriction became more marked until a typical paired spore was formed. This pattern of development is similar to the one indicated by the drawings of Nonomura & Ohara (1957).

In the micrographs of *Nocardia rubra* (Pl. 4, figs. 14, 15) can be seen its hyphae at

various stages of fragmentation. The Micromonospora species studied produced spores which were mainly sessile, arising directly from the hyphae without a sporophore. Two sessile spores at different stages of development are shown in Pl. 4, fig. 16.

DISCUSSION

The value of the scanning electron microscope for the examination of actinomycetes

Using this instrument, surface views of reproductive and vegetative structures at high magnification were obtained. The method of preparing specimens for observation was simple and rapid, up to 12 could be prepared in an hour. In addition, intact sporing structures could be examined. The only other way of obtaining surface views of these electron-dense structures at high magnification is by examination of carbon replicas with a transmission electron microscope. While the resolving power of such instruments is higher than that of the scanning type, about 5 Å as opposed to about 500 Å, the preparation of carbon replicas is a laborious procedure needing great care. Also it is impossible to obtain replicas of certain types of surface and the technique can seldom provide views of intact sporing structures. Therefore, for most purposes, a scanning electron microscope would be more useful.

Using this instrument at relatively low magnifications (1000–6000), general morphological details of intact sporing structures were easily observed. Normally such features, which are important in the taxonomy of these organisms, are observed with the light microscope. However, the small size of actinomycetes requires the light microscope to be used near the limit of its resolving power and the application of stains and immersion oil to preparations often results in the disruption of important structures. The scanning electron microscope provided views similar to those seen when intact specimens are examined with the lower-power objectives of the light microscope but at considerably higher magnification.

From this preliminary study, several possible applications of the scanning electron microscope to studies of actinomycetes can be suggested. As the preparation procedure is so simple and rapid, this type of instrument could be most useful for the routine examination and identification of specimens. This can be achieved more quickly and more accurately than when a light microscope is used, and at the same time features hitherto seen only with the transmission electron microscope (e.g. ornamentation of spore surfaces) are visible.

In studies of developmental processes, the scanning electron microscope would be a useful tool, especially if used to supplement information obtained from the examination of thin sections with the transmission microscope. Other applications which can be envisaged include investigations of the distribution and growth of actinomycetes in their natural habitats, such as the soil, and study of the occurrence of hyphal fusions in genetical studies.

The authors wish to express their gratitude to the Cambridge Instrument Company for permitting the use of their 'Stereoscan' microscope. The kind assistance of Miss P. J. Killingworth, Miss C. E. Moss and Mr K. W. Trevatt is gratefully acknowledged.

REFERENCES

AGRE, N. S. (1962). Electron microscopic investigations of spores of *Micromonospora vulgaris*. *Mikrobiologiya* **31**, 77.

BALDACCI, E., BALDUZZI, P. & AMICI, A. M. (1957). Observations of some Actinomyces isolated from soil samples from Israel, and their morphology under electronic microscopy. *Bull. Res. Coun. Israel* **5**, 263.

CHEN, P. L. (1964). The membrane system of *Streptomyces cinnamonensis*. *Am. J. Bot.* **51**, 125.

DIETZ, A. & MATHEWS, J. (1962). Taxonomy by carbon replication. I. An examination of *Streptomyces hygroscopicus*. *Appl. Microbiol.* **10**, 258.

GLAUERT, A. M. & HOPWOOD, D. A. (1960). The fine structure of *Streptomyces coelicolor*. I. The cytoplasmic membrane system. *J. biophys. biochem. Cytol.* **7**, 479.

HODGKISS, W. & MITCHELL, T. G. (1965). Variation in spore morphology in a streptomycete. *J. gen. Microbiol.* **41**, xix.

HOPWOOD, D. A. & GLAUERT, A. M. (1961). Electron microscope observations on the surface structures of *Streptomyces violaceoruber*. *J. gen. Microbiol.* **26**, 325.

KAWATA, T. & INOUE, T. (1965). Ultrastructure of *Nocardia asteroides* as revealed by electron microscopy. *Jap. J. Microbiol.* **9**, 101.

KAWATO, M. & SHINOBU, R. (1959). On *Streptomyces herbaricolor* nov.sp. Supplement: A simple technique for the microscopical observation. *Mem. Osaka Univ. lib. Arts Educ.* **8**, 114.

KUDRINA, E. S. & MAKSIMOVA, T. S. (1963). Some species of thermophil Actinomyces from soils of China and their antibiotic properties. *Mikrobiologiya* **32**, 623.

KÜSTER, E. (1953). Beitrag zur Genese und Morphologie der Streptomyceten-sporen. *VIth Int. Congr. Microbiol.* (Rome), p. 114.

LECHEVALIER, H. & HOLBERT, P. E. (1965). Electron microscopic observation of the sporangial structure of a strain of Actinoplanes. *J. Bact.* **89**, 217.

LECHEVALIER, H. A., LECHEVALIER, M. P. & HOLBERT, P. E. (1966). Electron microscopic observation of the sporangial structure of strains of Actinoplanaceae. *J. Bact.* **92**, 1228.

MOORE, R. T. & CHAPMAN, G. B. (1959). Observations of the fine structure and modes of growth of a streptomycete. *J. Bact.* **78**, 878.

NONOMURA, H. & OHARA, Y. (1957). Distribution of actinomycetes in the soil. II. *Microbispora*, a new genus of Streptomycetaceae. *J. Ferment. Technol. Osaka.* **35**, 307.

OVERMAN, J. R. & PINE, L. (1963). Electron microscopy of cytoplasmic structures in facultative and anaerobic Actinomyces. *J. Bact.* **86**, 656.

PAINTER, B. G. & BRADLEY, S. G. (1965). Observation on the structure of *Streptomyces venezuelae*. *Bact. Proc.* p. 5.

PREOBRAZHENSKAYA, T. P., MAKSIMOVA, T. S., LUKIVANOVICH, V. M. & EVKO, E. I. (1965). Application of the carbon replica method to the electron microscopic study of the surface of actinomycete spores. *Mikrobiologiya* **34**, 519.

PREOBRAZHENSKAYA, T. P., KUDRINA, E. S., MAKSIMOVA, T. S., SVESHNIKOVA, M. A. & BOYARSKAYA, R. V. (1960). Electron microscopy of spores of various actinomycete species. *Mikrobiologiya* **29**, 51.

RANCOURT, M. & LECHEVALIER, H. A. (1963). Electron microscopic observations of the sporangial structure of an actinomycete, *Microellobosporia flavea*. *J. gen. Microbiol.* **31**, 495.

RANCOURT, M. & LECHEVALIER, H. A. (1964). Electron microscopic study of the formation of spiny conidia of Streptomyces. *Can. J. Microbiol.* **10**, 311.

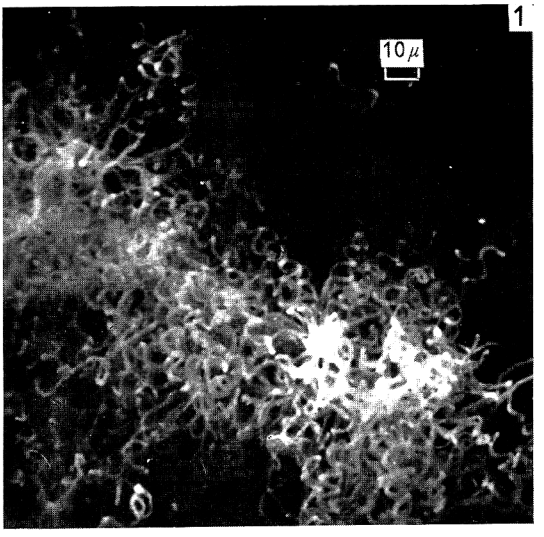
SHIRLING, E. B. & GOTTLIEB, D. (1966). Methods for characterization of Streptomyces species. *Int. J. Syst. Bact.* **16**, 313.

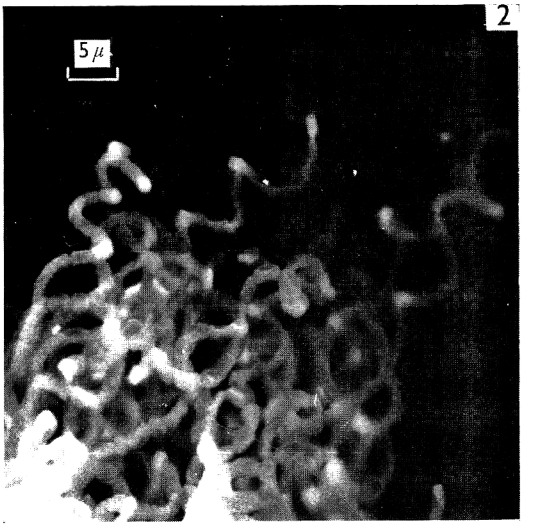
STUART, D. C. (1959). Fine structure of the nucleoid and internal membrane systems of Streptomyces. *J. Bact.* **78**, 272.

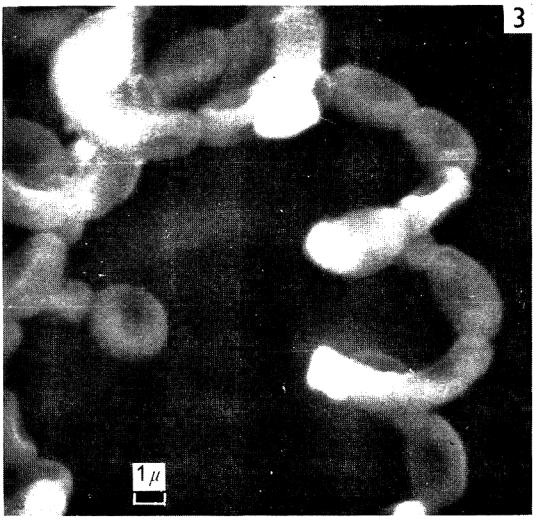
TRESNER, H. D., DAVIES, M. C. & BACKUS, E. J. (1961). Electron microscopy of Streptomyces spore morphology and its role in species differentiation. *J. Bact.* **81**, 70.

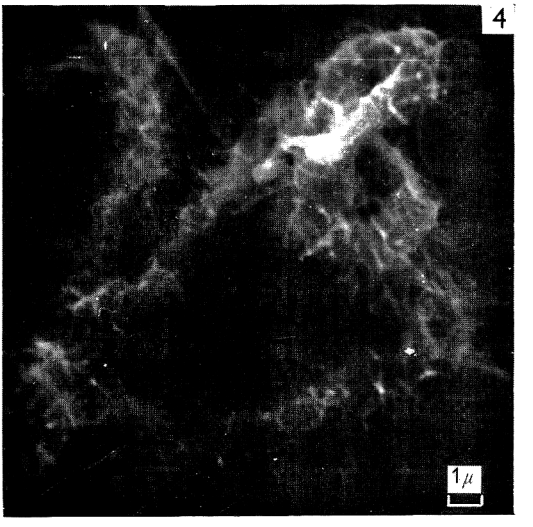
TRESNER, H. D., DAVIES, M. C. & ENGLERT, M. E. (1966). Morphological subtype of the smooth-spored streptomycetes. *J. Bact.* **91**, 1998.

WAKSMAN, S. A. (1961). *The Actinomycetes*, vol. 2. *Classification, Identification and Description of Genera and Species*. Baltimore: The Williams and Wilkins Co.









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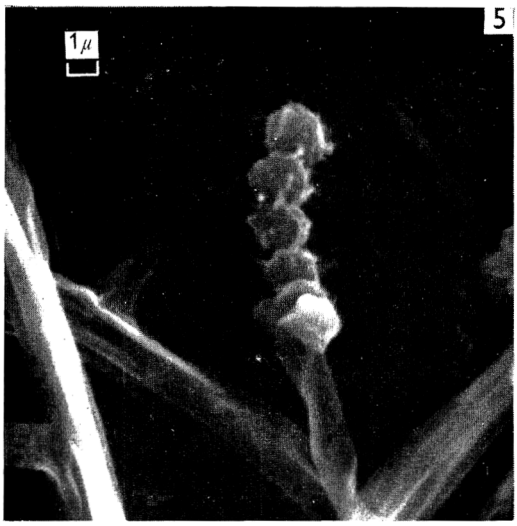
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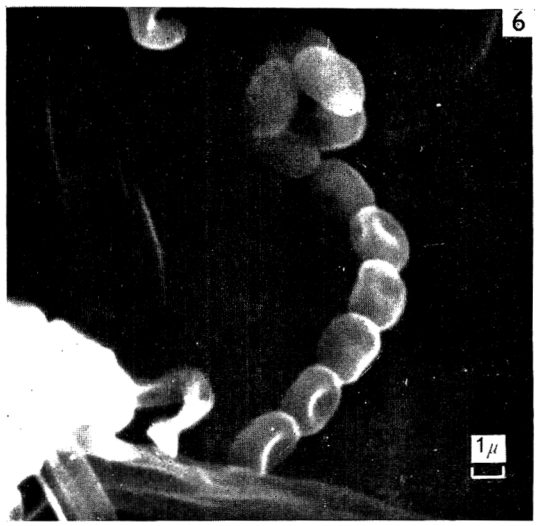
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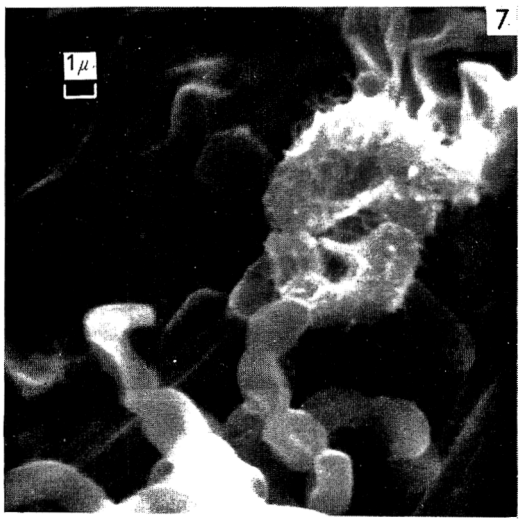
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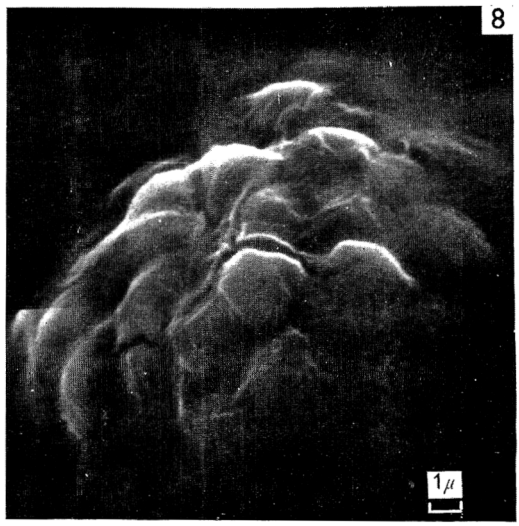
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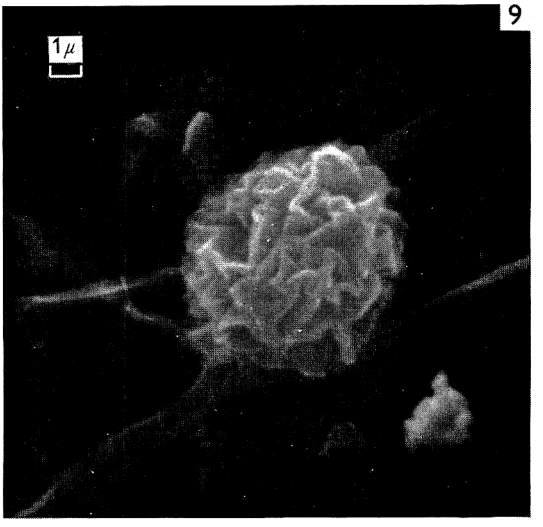
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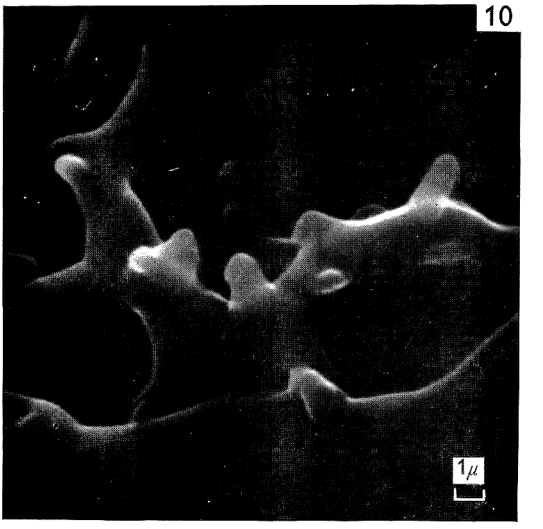
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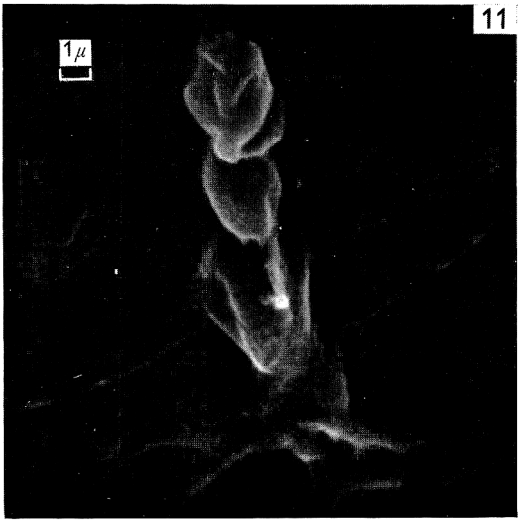
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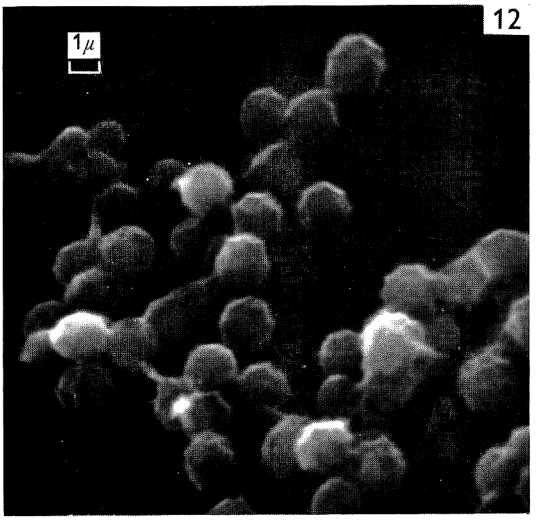
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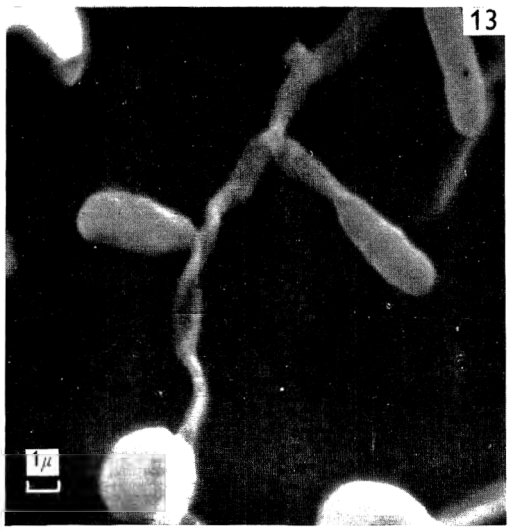
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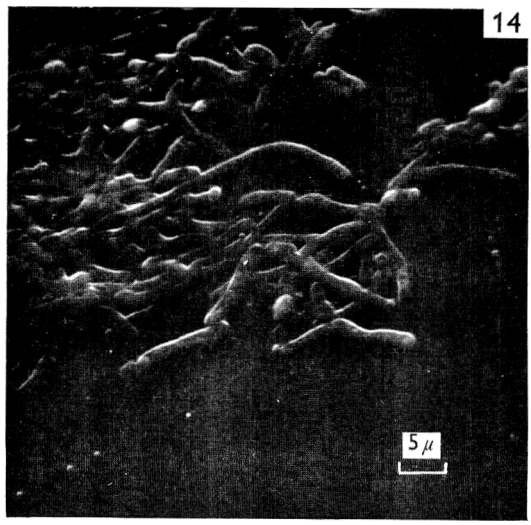
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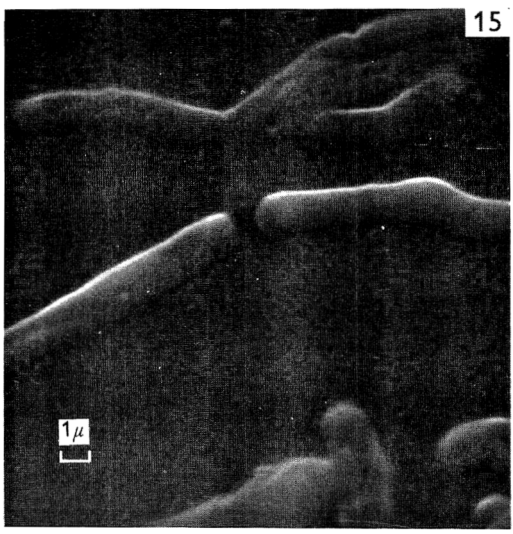
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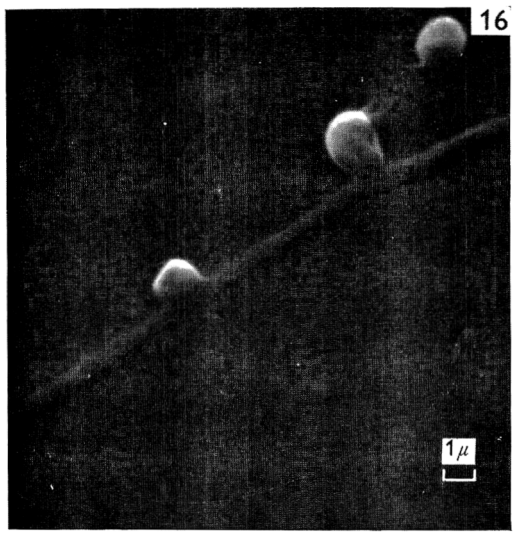
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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Streptomyces* sp., spiral spore chains. × 700.
Fig. 2. *Streptomyces* sp., spiral spore chains. × 2100.
Fig. 3. *Streptomyces* sp., spiral spore chain. × 7250.
Fig. 4. *Streptomyces finlayi*, chains of hairy spores. × 7000.

PLATE 2

Fig. 5. *Streptomyces viridosporus*, chain of spiny spores. × 6500.
Fig. 6. *S. viridosporus*, chain of smooth spores. × 6500.
Fig. 7. *S. viridosporus*, chain with smooth and spiny spores. × 6500.
Fig. 8. *Actinoplanes* sp., surface of sporangium. × 6750.

PLATE 3

Fig. 9. *Streptosporangium* sp., sporangium on sporangiophore. × 6250.
Fig. 10. *Microellobosporia flavea*, developing sporangia. × 6250.
Fig. 11. *M. flavea*, mature sporangium. × 7000.
Fig. 12. *Thermoactinomyces vulgaris* spores. × 7000.

PLATE 4

Fig. 13. *Microbispora rosea*, spores at various stages of development. × 6250.
Fig. 14. *Nocardia rubra*, fragmenting hyphae. × 1800.
Fig. 15. *N. rubra*, fragmenting hyphae. × 6250.
Fig. 16. *Micromonospora* sp., sessile spores. × 6250.

J. gen. Microbiol. (1967), **48**, 179–188
Printed in Great Britain

Analyses of Lipopolysaccharides Extracted from Penicillin-Resistant, Serum-Sensitive Salmonella Mutants

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(*Accepted for publication* 1 *March* 1967)

SUMMARY

Serum-sensitive mutants have been derived from serum-resistant smooth virulent *Salmonella typhimurium* and *S. enteritidis* strains by selection for resistance to cephalosporin or penicillin. Chemical analyses of the lipopolysaccharides of these mutants reveal that they belong to at least three different rough or semi-rough classes. Partial or total loss from the lipopolysaccharide of the sugars responsible for O antigenicity resulted in loss of virulence, as well as increased sensitivity to the bactericidal effect of antibody plus complement. However, such loss is not necessary for serum sensitivity because two serum-sensitive mutants possessed lipopolysaccharides indistinguishable from the smooth serum-resistant parents and were nearly as virulent.

INTRODUCTION

Rough mutants derived from smooth virulent strains of Salmonella species are less virulent than their parents (Lingelsheim, 1913; Topley & Ayrton, 1924) and are more susceptible to killing by antibody and complement (Thjøtta & Waaler, 1932; Rowley, 1956). Recently much has been learned about the composition of cell walls of Enterobacteriaceae, particularly those of Salmonella species (Lüderitz, Staub & Westphal, 1966), and it seems appropriate to reinvestigate the effect of various mutations to roughness on the virulence and serum-sensitivity of some Salmonellas.

New knowledge of the polysaccharide substructure of the lipopolysaccharides (LPS) of Salmonellas is largely attributable to a combination of chemical and immunochemical analyses (Lüderitz, Kauffmann, Stierlin & Westphal, 1960; Staub, 1960) and the investigation of mutants unable to synthesize or attach one or another of the monosaccharides which constitute the polysaccharide (Osborn *et al.* 1964; Lüderitz & Westphal, 1966). The results of these studies make it reasonably certain that the lipopolysaccharides of some Salmonella species of different O antigenic groups contain a common rough polysaccharide core. This consists of a polyheptose phosphate backbone (probably attached to lipid by a keto-deoxyoctonate) and an attached pentasaccharide side-chain of the composition:

← glucose ← galactose ← glucose ← *N* acetyl glucosamine
↑
galactose

In fully smooth strains of *Salmonella typhimurium* a repeating unit is attached to the distal end:

← (galactose ← rhamnose ← mannose)
↑
abequose

It has been shown (Staub, Tinelli, Lüderitz & Westphal, 1959) that the monosaccharides abequose, O-acetyl galactose and rhamnose are those principally responsible, respectively, for the O antigenic factors 4, 5, and 12 in the Kauffmann–White classification. The antigenic factor 1 is present in those *Salmonella typhimurium* strains lysogenized with P22 or other A phages and is dependent upon a glucose attached to the galactose in the repeating unit (Stocker, Staub, Tinelli & Kopacka, 1960). The repeating unit of *S. enteritidis* differs from that of *S. typhimurium* in containing tyvelose in place of abequose.

If one disregards the mutants unable to synthesize the activated donor forms of the proximal sugars of the rough core side-chains, two main classes of rough mutants have been described.

Strains of one class are unable to synthesize the oligosaccharide repeating unit of the distal part of the side-chains responsible for O specificity. The LPS of such mutants has the antigenic character R II (Beckmann, Subbaiah & Stocker, 1964) presumably determined by the exposed terminus of the proximal part ('R-stub') of the side-chain. In *Salmonella typhimurium*, inability to manufacture the O repeat unit may result either from mutation in the *rouB* gene cluster near *his* (Subbaiah & Stocker, 1964) or, less commonly, from a mutation mapping between *gal* and *try* resulting in loss of ability to synthesize phosphomannose isomerase and consequent loss of the mannose component of the repeating unit (Rosen *et al.* 1965).

Mutants of the other main rough class are unable to attach to their LPS the O-specific polysaccharide which is found in the supernatant (L-1) fraction after the LPS has been sedimented in the Westphal extraction procedure (Beckmann *et al.* 1964). It is presumed (or in some instances proven) that this class is unable to complete the synthesis of the 'R-stub' to which the O-specific polysaccharide is usually attached. Many mutants of *Salmonella typhimurium* belonging to this class map in the *rouA* cluster near *xyl* (Subbaiah & Stocker, 1964); some, but not all, make LPS of antigenic character R I (Beckmann *et al.* 1964).

In addition, two forms of 'semi-rough' mutants have been described (Naide *et al.* 1965). Their LPS contains much less of the oligo-saccharide responsible for O antigenicity than does that of smooth strains. The LPS of one type of semi-R mutant probably has only one O-specific unit attached to each of the rough stubs whereas that of the other has some long O-specific side-chains, but has also many R stubs which lack repeating units.

An opportunity to study the virulence of some classes of rough strains occurred when it was found that certain penicillin-resistant mutants derived from virulent, serum-resistant *Salmonella enteritidis* and *S. typhimurium* strains were sensitive to serum. Most of them were less virulent for mice than the parent strains (Roantree & Steward, 1965). Among these mutants, some showed typical character of rough strains, i.e. growth as a deposit in broth, auto-agglutinability in physiologic saline, loss of specific agglutinability in anti-O sera and rough colonial appearance on eosin-methylene-blue agar. Other mutants, although serum-sensitive, could not be distinguished from the smooth parents in these respects; one single-step mutant of *S. enteritidis* grew in broth like a rough strain but retained its O-agglutinability. The penicillin-resistant, serum-sensitive strains were isolated as series of mutants, each one more antibiotic-resistant than its predecessor, by serial passage on gradient plates containing either benzylpenicillin or α-aminobenzylpenicillin. Subsequent work with

the virulent parent strains of *S. typhimurium* and *S. enteritidis* has shown that if picks from single colonies of the parent strain were grown overnight in broth cultures and 0·1 ml. portions of these spread on gradient plates containing just one concentration of penicillin or cephalosporin, a variety of single-step mutants were isolated, some of which were serum-sensitive and less virulent than the parent. This method avoided the confusion of multiple mutations to antibiotic resistance.

This paper describes the chemical composition of the polysaccharide of the cell walls of these serum-sensitive mutants. Some previously reported observations on the virulence of the mutants and their sensitivity to serum are recapitulated here (Roantree & Steward, 1965).

MATERIALS

Bacterial strains

Salmonella typhimurium 173 (O = 1, 4, 5, 12) was originally obtained from Professor D. Rowley, University of Adelaide, Adelaide, Australia.

Salmonella enteritidis 203 (O = 9, 12) was obtained from the Division of Laboratories, California Department of Public Health.

Mutants derived on penicillin gradient plates are given the prefix P-; those on α-aminobenzylpenicillin (ampicillin) the prefix BR, and those on cephalothin the prefix K. The numeral immediately following the prefix denotes the serial mutational step to antibiotic resistance represented by the strain. A small letter following the strain number indicates that the strain is one of a series of mutants. For example, the designation BR 3–173a means that this is a third-step mutant derived by passage on α-aminobenzylpenicillin and is a member of the 'a' series. A capital letter following the strain number indicates a particular one-step mutant to antibiotic resistance.

Testing of resistance to penicillin

Nutrient agar plates containing various concentrations of benzylpenicillin or α-aminobenzylpenicillin were inoculated with 100–200 organisms of the strain to be tested. The greatest concentration of antibiotic allowing the consistent appearance of colonies was taken as the value for degree of resistance. Mutant strains within any one series were plated on the same batches of agar on the same day. Concentrations of antibiotic were varied by increments of 5 u./ml. for benzylpenicillin and 2 μg./ml. for α-aminobenzylpenicillin in testing strains of *Salmonella typhimurium* (Table 1). Increments of α-aminobenzylpenicillin used to test *S. enteritidis* strains were 0·2 μg./ml. (Table 2).

Determination of virulence of bacteria for the mouse

Ten mature CF-1 mice housed together were each inoculated intraperitoneally with the same volume of one serial 10-fold dilution in saline of an overnight broth culture. Sufficient 10-fold dilutions were used to permit the calculation of the LD 50 from survivals to 30 days (Reed & Muench, 1938). The LD 50, when greater than 100, is reported to the closest whole power of 10.

Determination of sensitivity to serum

This has been described in detail (Roantree & Steward, 1965). Briefly, 0·1 ml. amounts of 10^{-2} and 10^{-5} dilutions of overnight broth cultures were added to 0·4 ml. amounts of active human serum and incubated at 37°. The number of bacteria

surviving in 0·1 ml. volumes of the suspensions was determined by colony counts from pour plates made at 1 and 2 hr. The number of bacteria at zero time was determined by colony counts made from pour plates prepared from 0·1 ml. volumes of 10^{-6} dilutions of overnight culture. For the purpose of the present paper, strains showing a survival of greater than 10 % from an inoculum of 10^2 organisms per 0·1 ml. after 2 hr are considered resistant; those showing survival of less than 0·1 % from an inoculum of 10^5 are considered sensitive.

Table 1. *Characteristics of parent and mutant strains of Salmonella typhimurium and the sugars related to the O antigens found in their lipopolysaccharides*

Strain	Penicillin resistance*	Sensitivity to serum†	O Antigens (1, 4, 5, 12)	LD 50 for mice	Deposit in broth	Rhamnose	Abequose	Mannose	Galactose
173	1 μg. 5 u.	Res.	+ +	10^1	0	+ + +	+ + +	+ + +	+ + +
P 1–173	10 u.	Res.	+ +	10^2	0	+ + +	+ + +	+ + +	+ + +
P 2–173	15 u.	Sen.	+	10^4	0	+	+	+	± +
P 3–173	40 u.	Sen.	+ +	10^5	0	+	+	+	± +
P 5–173	50 u.	Sen.	+ +	10^5	0	+	+	+	± +
BR 1–173 a	2 μg.	Res.	+ +	10^1	0	+ + +	+ + +	+ + +	+ + +
BR 2–173 a	4 μg.	Sen.	0	n.d.	±	0	0	0	± +
BR 3–173 a	8 μg.	Sen.	0	n.d.	+ +	0	0	0	± +
BR 6–173 a	10 μg.	Sen.	0	10^7	+ +	0	0	0	± +
BR 1–173 c	2 μg.	Res.	+ +	n.d.	0	+ + +	+ + +	+ + +	+ + +
BR 2–173 c	8 μg.	Res.	+ +	n.d.	0	+ + +	+ + +	+ + +	± + +
BR 4–173 c	16 μg.	Res.	+ +	n.d.	0	+ + +	+ + +	+ + +	+ + +
BR 6–173 c	20 μg.	Res.	+ +	10^5	0	+ + +	+ + +	+ + +	+ + +
P 173 C	10 u.	Sen.	+ +	10^1	0	+ + +	+ + +	+ + +	+ + +
K 173 A		Sen.	0	10^4‡	+ +	0§	0§	0§	± +§

*u. = units benzylpenicillin/ml. nutrient agar. μg. = micrograms α-aminobenzylpenicillin/ml. nutrient agar.
†Serum sensitivity is defined as less than 0·1 % survival of an inoculum of 10^5 organisms in 0·1 ml. of active human serum in 2 hr; serum resistance as greater than 10 % survival of an inoculum of 10^2.
‡10^4 was highest challenge dose used.
§These sugars were present in the supernatant (L-1) fraction but not in the LPS.

Table 2. *Characteristics of parent and mutant strains of Salmonella enteritidis and the sugars related to the O antigens found in their lipopolysaccharides*

Strain	Penicillin resistance†	Sensitivity to serum†	O Antigens (9, 12)	LD 50 for mice	Deposit in broth	Rhamnose	Abequose	Mannose	Galactose
203	1·0 μg.	Res.	+ +	10^2	0	+ + +	+ + +	+ + +	+ + +
BR 1–203 b	2·0 μg.	Sen.	+	10^5	+ + +	+	+	+	± +
BR 203 C	1·8 μg.	Sen.	+ +	10^2	0	+ + +	+ + +	+ + +	+ + +

*μg. = micrograms α-aminobenzylpenicillin/ml. nutrient agar.
†Serum sensitivity is defined as less than 0·1 % survival of an inoculum of 10^5 organisms in 0·1 ml. of active human serum in 2 hr; serum resistance as greater than 10 % survival of an inoculum of 10^2.

Analysis of lipopolysaccharides

Mass cultures of the organisms were grown on brain–heart infusion agar (Difco) in Roux bottles at 37° for 18 hr. Bacteria were washed from the surface with saline, centrifuged in the cold, resuspended in fresh saline and again centrifuged. They were then killed by immersion in approximately 10 vol. acetone for 24 hr.

Lipopolysaccharide was extracted from acetone-dried organisms by the method of Westphal, Lüderitz & Bister (1952). Approximately 10 g. dried organisms were suspended in 600 ml. of 45 % phenol at 65–68° and agitated for 20 min. The water phase was separated from the phenol phase by centrifugation in the cold; the phenol phase was re-extracted with an additional 300 ml. water. The water-phase material from both extractions was washed with ether and dialysed overnight against running tap water. After concentration of the water phase by pervaporation and removal of debris by low-speed centrifugation, the LPS was separated from contaminating nucleic acid, etc., by spinning the concentrate for 2 hr at 100,000 *g* in a Beckman preparative ultracentrifuge. The pellet containing the LPS was resuspended in distilled water and the ultracentrifugation repeated twice. Sediment from the last ultracentrifugation was lyophilized. Greater than fivefold differences in yields (mg. LPS/g. dry wt bacteria) from different smooth strains occurred. The supernatant from the first ultracentrifugation (L_1 fraction of Beckman *et al.* 1964) was saved and lyophilized.

Degraded polysaccharide was prepared directly from dried cells by the method of Freeman (1942). The crude polysaccharide was extracted with dilute acetic acid at 100°. Purification consisted of 6 alcoholic and 3 acetic acid fractionations except that the LPS from P2–173 failed to precipitate in the designated concentrations of acetic acid so ten alcoholic fractionations were substituted in this instance.

To determine the sugars present in either the LPS, polysaccharide or L_1 fraction, 10–20 mg. dried material was hydrolysed for 4 hr in 1 ml. N-H_2SO_4 at 100° in a sealed ampoule. Samples were neutralized with saturated $Ba(OH)_2$, dried and then redissolved in a small volume (0·2–0·4 ml.) of water: 5 and 10 μl. samples were then spotted on plates for thin-layer chromatography (TLC). The silica gel on the plates had been emulsified with 0·1 M-boric acid. The solvent systems were butanol + acetic acid 4 + 1 (v/v), and butanol + pyridine + water 6 + 4 + 3 (v/v); the plates were developed with a diphenylamine, aniline mixture. The butanol + pyridine + water system was also used for paper chromatography. Sugars were identified by comparison with commercially available sugars with the exception of abequose and tyvelose. These sugars were identified by (1) comparison of experimental R_F values with published values (Kabat & Mayer, 1961); (2) a failure of the material eluted from TLC plates to produce an absorption band in the Dische reaction, and (3) the close similarity of the R_F value of the spot identified as tyvelose to that of a spot from hydrolysates containing colitose. Both spots were of characteristic purple colour and migrated considerably farther than rhamnose. Good separation of rhamnose and dideoxyhexose from the other sugars was obtained by thin-layer chromatography. Paper chromatography for 18 hr at room temperature was used to separate mannose, glucose, and galactose. Mannose was also determined by paper electrophoresis of the LPS in a Spinco cell at 320 v for 4 hr. The buffer used was 0·025 N-sodium tetraborate (pH 9·3) and the sugars were detected by spraying the paper with aniline hydrogen phthalate.

Glucose was determined quantitatively by the glucose oxidase test (Glucostat, Worthington Biochemical Co.) and galactose by the galactose oxidase test (Galactostat, Worthington Biochemical Co.). Rhamnose was determined quantitatively and heptose both qualitatively and quantitatively by the 10 min. modification of the Dische Cysteine–sulphuric acid reaction (Dische, 1953). The sugar standard used for the determination of heptose was α-D-manno-heptose.

RESULTS

Certain characteristics of the virulent *Salmonella typhimurium* parent strain 173, three series of mutants and two single-step mutants derived from it are included in Table 1. Sugar contents of their lipopolysaccharides were estimated from the results of thin-layer chromatography and paper electrophoresis by comparing the densities of spots obtained from equal amounts by weight of the LPS from each member of the group of mutants.

The first-step mutant in the P 173 series differed from the parent strain mainly in its greater resistance to penicillin, but the second-step strain, P 2–173, was sensitive to serum and relatively avirulent. It was considered a semi-rough mutant because its LPS contained much less rhamnose, mannose and abequose and somewhat less galactose than the parent. Further multi-step mutants in this line led to no change in the tested characteristics, other than greater resistance to penicillin.

Similarly, in the BR 173a series, obtained by passage of 173 on α-aminobenzylpenicillin, the first-step mutant differed little from the parent strain but the second-step mutant showed the qualities of a completely rough strain and its LPS lacked detectable rhamnose, mannose or abequose, and galactose appeared reduced. Further mutants in this series shared these rough characteristics and the sixth-step mutant was tested and found avirulent for mice.

The BR 173c series originated from a different colony on the same initial gradient plate from which the colony giving rise to the BR 173a series was picked. Although the sixth mutant in the 'c' line was more resistant to α-aminobenzylpenicillin than its counterpart in the 'a' line (20 to 10 μg./ml.), the 'c' line showed no loss of smooth characteristics or 0 specific sugars and remained resistant to serum. The sixth-step mutant in the 'c' line was much less virulent than the parent, but its LD 50 (10^5) was considerably less than that of the rough BR 6–173a (10^7).

The P 173C one-step mutant seemed identical to the parent in the characteristics tested except for being more resistant to penicillin and sensitive to serum.

The one-step mutant to cephalosporin resistance, K 173A, was sensitive to serum, culturally rough, and relatively avirulent. Although its LPS lacked the smooth-specific sugars, the strain differed from the rough mutants described above because O-specific sugars were found in the L 1 supernatant fraction.

Characteristics of the virulent serum-resistant *Salmonella enteritidis* 203 and two one-step mutants resistant to α-aminobenzylpenicillin are summarized in Table 2. The BR 1–203b strain looked like a typical rough strain when grown in broth, but weak agglutination was obtained with anti-09 and -012 sera. The LPS contained substantially reduced amounts of smooth-specific sugars; it was therefore considered to be a semi-rough mutant.

Salmonella enteritidis BR 203C is like the P 173C strain in the *S. typhimurium* group because its LPS seems to contain the same amounts of sugars as that of the parent strain even though it is sensitive to serum.

The lipopolysaccharides of the more interesting strains were analysed further by obtaining absorption spectra of the products of the Dische reaction. In Fig. 1, curves from analyses of the LPS derived from the smooth *Salmonella typhimurium* 173, the semi-rough mutant P 2–173, and the rough mutant BR 6–173a are compared. The sharp peak at 400 mμ represents rhamnose and the broader peak at 505 mμ is mainly

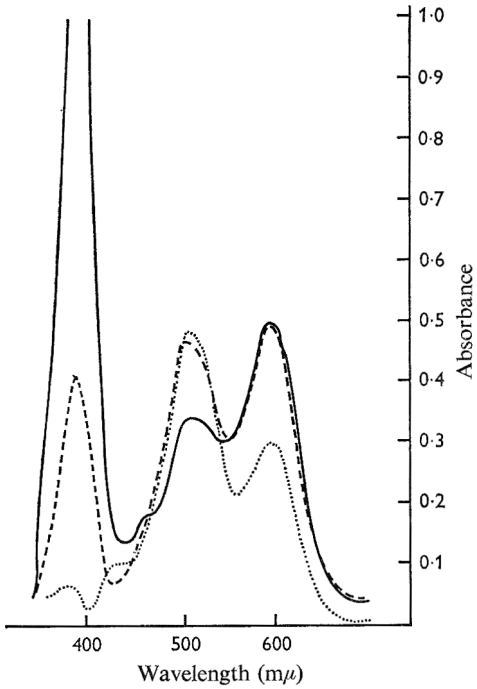

Fig. 1

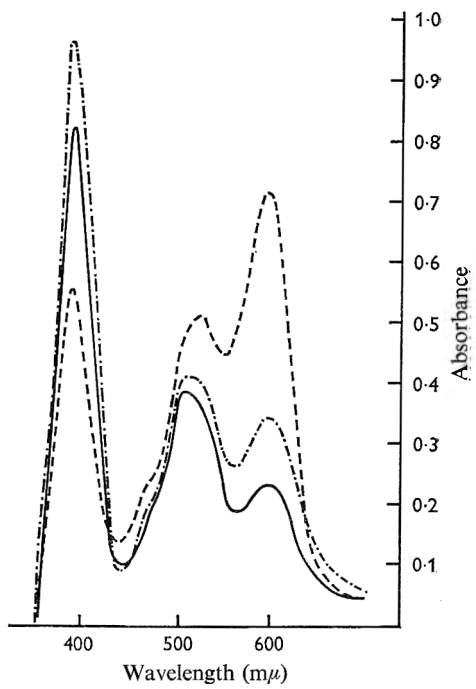

Fig. 2

Fig. 1. Absorption spectra of products derived from 750 μg. LPS 24 hr after the Dische reaction. Peak at 400 mμ indicates the amount of rhamnose; that at 505 mμ is principally caused by heptose. LPS from *Salmonella typhimurium*: ———, smooth parent 173, — — —, semi-rough mutant P2-173; ....., and rough mutant BR6-173*a*.

Fig. 2. Absorption spectra of products derived from 750 μg. LPS 24 hr after the Dische reaction. Peak at 400 mμ indicates amount of rhamnose; that at 505 mμ is principally caused by heptose. LPS from *Salmonella enteritidis*: ———, smooth parent 203; — · —, smooth serum-sensitive mutant BR203C; — — —, semi-rough mutant BR203b.

Table 3. *Rhamnose, glucose and heptose composition of strain P2–173*

	Molar ratios			
Lipopolysaccharide	Rhamnose / Heptose	Glucose / Heptose	Galactose / Heptose	Rhamnose / Glucose
Parent strain 173	2·0	2·0	3·5	1·0
Mutant strain P 2-173	0·23	0·72	0·82	0·3
Polysaccharide				
Parent strain 173	3·1	3·6	5	0·85
Mutant strain P 2-173	0·50	0·84	0·78	0·55

caused by heptose. It is evident that the rhamnose:heptose ratio is greatly reduced in the P2–173 LPS as compared with the parent and that no rhamnose peak is detectable in the LPS from the rough mutant BR6–173a. The curve obtained from the LPS of the smooth serum-sensitive mutant P173C is not included since it is virtually identical with that of the parent.

LPS and degraded polysaccharides isolated from the parent 173 and the semi-rough

mutant P2–173 were analysed for their glucose and galactose contents, and the results are shown in Table 3, together with the rhamnose:heptose ratios derived from the curves in Fig. 1. These values confirm the previous results in indicating that the LPS of P2–173 contains reduced amounts of the sugars determining O specificity.

Absorption spectra of the products from the Dische reactions upon lipopolysaccharides from *Salmonella enteritidis* 203, its serum-sensitive smooth mutant, BR203C, and the suspected semi-rough BR1–203b, are shown in Fig. 2. The greatly diminished rhamnose:heptose ratio of BR1–203b as compared with the parent is evident, confirming its classification as a semi-rough strain. Curves of the two smooth strains are very similar.

DISCUSSION

At least five classes of mutants have been derived from virulent serum-resistant *Salmonella typhimurium* and *S. enteritidis* strains by selection of colonies resistant to antibiotics which interfere with the synthesis of the bacterial cell wall.

(1) The late mutants in the BR173a series are rough and neither their LPS nor their L_1 fractions contain detectable amounts of O-specific material as evidenced by the absence of mannose, rhamnose and abequose. By chemical analysis, this class could fit into the hapten-negative (*rouB*) class (Beckmann *et al.* 1964), but antigenic analyses of LPS from BR6–173a, kindly done by Dr O. Lüderitz, did not identify it as of R II antigenic character or other rough antigenic subclasses (Lüderitz & Westphal, 1966). Tests of the sensitivity of this mutant to various R-specific phages by Dr Peter Gemski confirmed the classification of roughness, but did not aid in sub-classification. It is not a mutant lacking ability to synthesize phosphomannose isomerase because it can ferment mannose (see Rosen *et al.* 1965).

(2) The K173A one-step mutant to cephalosporin resistance represents a rough class different from the above because it apparently makes polysaccharide containing the ← galactose ← rhamnose ← mannose ← abequose repeating unit but does not attach it. In this respect it is similar to the hapten-positive (*rouA*) mutants of *Salmonella typhimurium* (Beckmann *et al.* 1964). However, antigenic analyses by Dr Lüderitz and phage analyses by Dr Gemski did not conclusively subclassify this type of mutant.

(3) The two mutants, *Salmonella typhimurium* P2–173 and *S. enteritidis* BR1–203b, clearly belong in the semi-rough category (Naide *et al.* 1965). The two are very different from each other; BR1–203b would probably be classified as rough by traditional criteria whereas P2–173 is indistinguishable from the smooth parent by such criteria.

(4) The existence of semi-rough, serum-sensitive strains, such as P2–173 which are very like smooth strains, made us suspect that every seemingly smooth strain sensitive to serum was actually some form of semi-rough. However, the mutants *Salmonella typhimurium* P173C and *S. enteritidis* BR203C represent a chemically smooth class markedly more sensitive to antibody and complement than the parent.

(5) The *Salmonella typhimurium* BR173c line represents a group of penicillin-resistant mutants which show no significant changes in sensitivity to serum, antigenic character or sugar composition of the lipopolysaccharide, although they are less virulent for mice than the parent strain.

Selection of mutants of enteric bacilli resistant to penicillin reveals a minority which are more sensitive to serum than the parent strain (Michael & Braun, 1958; Roantree

& Steward, 1965). The present work shows that among the serum-sensitive mutants are rough and semi-rough strains as well as 'chemically smooth' mutants. It is possible that the latter strains have a chemical change in their lipopolysaccharide too minor to detect by our means of analysis.

Our work with these mutants and a number of rough strains not obtained by selection for antibiotic resistance indicates that any loss of sugars from the lipopolysaccharide of enteric bacilli is accompanied by increased sensitivity to the bactericidal effect of antibody and complement. The rough strains are uniformly avirulent (see also Herzberg & Green, 1964) whereas the semi-rough strains, *Salmonella enteritidis* and *S. typhimurium* P2–173, are of intermediate virulence.

The smooth serum-sensitive mutants are nearly as virulent for mice and guinea pigs as the parent serum-resistant strains (Roantree & Steward, 1965; Steward, Collis & Roantree, 1966). The investigation of their virulence uncovered the fact that most guinea pigs and mice used in this laboratory did not possess measurable natural bactericidal antibody against *Salmonella typhimurium* or *S. enteritidis*. Immunization with the homologous species led to high levels of bactericidal antibody and excellent protection against either the serum-resistant parents or the serum-sensitive mutants (Steward *et al.* 1966). Present information, then, indicates that virulence of the serum sensitive smooth mutants is very like that of the parent strains. Perhaps a difference in virulence might be observed if animals with intermediate levels of antibody were tested.

It is well established that penicillin interferes with the synthesis of the mucopeptide portion of the cell wall (Park & Strominger, 1957; Rogers & Mandelstam, 1962; Izaki, Matsuhashi & Strominger, 1966). We have derived serum-sensitive mutants by selection with cephalosoporin and bacitracin, as well as with penicillin. Less extensive selections on neomycin, streptomycin and chloramphenicol have yielded no such mutants.

In our present state of ignorance of the relationship of the rigid mucopeptide layer to the lipopolysaccharide, it is difficult to see why some mutations to penicillin resistance should involve a change in the latter layer.

This investigation was supported by United States Public Health Service Research Grant no. 02755 and Training Grant no. 82, from the National Institute of Allergy and Infectious Diseases.

REFERENCES

BECKMANN, I., SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhimurium*. 2. Serological and chemical investigations. *Nature, London.* **301**, 1299.

DISCHE, Z. (1953). Qualitative and quantitative colorimetric determination of heptoses. *J. biol. Chem.* **204**, 983.

FREEMAN, G. G. (1942). The preparation and properties of a specific polysaccharide from *Bact. typhosa* Ty 2. *Biochem. J.* **36**, 340.

HERZBERG, M. & GREEN, J. H. (1964). Characteristics of cell walls of smooth strains of *Salmonella typhimurium* and derived rough variants. *J. gen. Microbiology.* **35**, 421.

IZAKI, K., MATSUHASHI, M. & STROMINGER, J. L. (1966). Glycopeptide transpeptidase and D-alanine carboxy-peptidase: a penicillin-sensitive enzymatic reactions. *Proc. natn. Acad. Sci., U.S.A.* **55**, 566.

KABAT, E. A. & MAYER, M. M. (1961). *Experimental Immunochemistry*, 2nd ed. Springfield, Illinois: C. C. Thomas.

LINGELSHEIM, W. v. (1913). Zur Frage der Variation der Typhusbacillen und verwandter Gruppen. *Zentbl. Bakt. ParasitKde*, Abt. I Orig. **68**, 577.

LÜDERITZ, O., KAUFFMANN, F., STIERLIN, H. & WESTPHAL, O. (1960). Zur Immunochemie der O-Antigene von Enterobacteriaceae. II. Vergleich der Zuckerbausteine von Salmonella S–, R–, und T-Formen. *Zentbl. Bakt. ParasitKde, Abt. T. Orig.* **179**, 180.

LÜDERITZ, O., STAUB, A. M. & WESTPHAL, O. (1966). Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae. *Bact. Rev.* **30**, 192.

LÜDERITZ, O. & WESTPHAL, O. (1966). The significance of enterobacterial mutants for the chemical investigation of their cell-wall polysaccharides. *Angew. Chem. int. Edit.* **5**, 198.

MICHAEL, J. G. & BRAUN, W. (1958). Relationships between bacterial resistance to serum and penicillin. *Proc. Soc. exp. Biol. Med.* **97**, 104.

NAIDE, Y., NIKAIDO, H., MÄKELÄ, P. H., WILKINSON, R. G. & STOCKER, B. A. D. (1965). Semi-rough strains of Salmonella. *Proc. natn. Acad. Sci., U.S.A.* **53**, 147.

OSBORN, M. J., ROSEN, S. M., ROTHFIELD, L., ZELEZNICK, L. D. & HORECKER, B. L. (1964). Lipopolysaccharide of the Gram-negative cell wall. *Science, N.Y.* **145**, 783.

PARK, J. T. & STROMINGER, J. L. (1957). Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. *Science, N.Y.* **125**, 99.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**, 493.

ROANTREE, R. J. & STEWARD, J. P. (1965). Mutations to penicillin resistance in Enterobacteriaceae that affect sensitivity to serum and virulence for the mouse. *J. Bact.* **89**, 630.

ROGERS, H. J. & MANDELSTAM, J. (1962). Inhibition of cell-wall-mucopeptide formation in *Escherichia coli* by benzylpenicillin and 6 [D(−)-α-aminophenylacetamido] penicillanic acid (ampicillin). *Biochem. J.* **84**, 299.

ROSEN, S. M., ZELEZNICK, L. D., FRAENKEL, D., WIENER, I. M., OSBORN, M. J. & HORECKER, B. L. (1965). Characterization of the cell wall lipopolysaccharide of a mutant of *Salmonella typhimurium* lacking phosphomannose isomerase. *Biochem. Z.* **342**, 375.

ROWLEY, D. (1956). Rapidly induced changes in the level of nonspecific immunity in laboratory animals. *Br. J. exp. Path.* **37**, 223.

STAUB, A. M. (1960). Bases chimiques de la spécifité immunologique des antigènes des Salmonella. *Annls Inst. Pasteur, Paris* **98**, 814.

STAUB, A. M., TINELLI, R., LÜDERITZ, O. & WESTPHAL, O. (1959). Role de quelques sucres et en particular des 3–6 dideoxyhexoses dans la spécifité des antigènes O du tableau de Kauffmann–White. *Annls Inst. Pasteur, Paris* **96**, 303.

STEWARD, J. P., COLLIS, L. R. & ROANTREE, R. J. (1966). Effects of immunization of guinea pigs lacking bactericidal antibody against *Salmonella enteritidis*. *J. Immunol.* **97**, 224.

STOCKER, B. A. D., STAUB, A. M., TINELLI, R. & KOPACKA, B. (1960). VI. Étude de l'antigène l présent sur deux Salmonella des groups B et E. *Annls Inst. Pasteur, Paris* **98**, 505.

SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhimurium*. 1. Genetics. *Nature, Lond.* **201**, 1298.

THJØTTA, R. & WAALER, E. (1932). Dissociation and sensitiveness to normal serum in dysentery bacilli of type III. *J. Bact.* **24**, 301.

TOPLEY, W. C. C. & AYRTON, J. (1924). The segregation of biological factors in *B. enteritidis* (*Aertrycke*). *J. Hyg., Camb.* **22**, 305.

WESTPHAL, O., LÜDERITZ, O, & BISTER, F. (1942). Über die Extraktion von Bacterien mit Phenol/Wasser. *Z. Naturforsch.* 7*b*, 148.

J. gen. Microbiol. (1967), **48**, 189–203
Printed in Great Britain

Analogue Computer Studies of the Growth Characteristics of *Escherichia coli* Following Dihydrostreptomycin Treatment

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(*Accepted for publication* 2 *March* 1967)

SUMMARY

Cultures of *Escherichia coli* B which had received pretreatment with dihydrostreptomycin were considered to consist of two subpopulations corresponding to the two fractions of culture samples that did and did not survive through plating and incubation to produce visible colonies. No initial assumptions were made about the viability of the subpopulations at the time that the samples were taken. A general-purpose analogue computer was then used both in mathematical analysis and mathematical synthesis to investigate the growth characteristics of the survivors and non-survivors as functions both of time and of pretreatment.

INTRODUCTION

Previous investigations into the effects of dihydrostreptomycin (DHS) upon cultures of *Escherichia coli* B have established a technique (Kogut, Lightbown & Isaacson, 1965*a*) which allows intracellular effects to be studied in a situation uncomplicated by continuous uptake of antibiotic. This involves removal of antibiotic from the culture medium, by filtration and washing, after various periods of aerobic growth in the presence of DHS. By using this technique, growth rates at the time of removal of DHS have been shown to be decreased by an amount dependent upon the concentration of antibiotic and the duration of prior treatment. These decreased growth rates are maintained for periods from 2 to 4 hr and constitute an initial phase of inhibited but logarithmic growth, which then gives way to a second phase, during which the growth rates recover to that observed for untreated control cultures. In a further paper (Kogut, Lightbown & Isaacson, 1965*b*) viable counts determined during the various stages of inhibition and recovery were reported and showed that the viable count per unit population was also initially decreased by an amount dependent upon the preceding DHS treatment. Despite the numerical scatter encountered with viable count data, it was evident that the viable count per unit population remained relatively constant during the period of constant decreased growth rate, but subsequently increased during the period of growth rate recovery.

The association of constant viable count per unit population with the initial phase of constant growth rate implies that those bacteria which do and those which do not give rise to visible colonies may be dividing at similar rates. Direct microscopic observation of *E. coli* B after removal from DHS treated cultures adds support to this conjecture. The present paper reports closer investigation of the possibility that the growth rates of all the bacteria are initially equally affected by the form of DHS treatment described, and it examines the subsequent growth rate behaviour of that

subpopulation which gives rise to visible colonies and that subpopulation which does not. A preliminary account of this work has been communicated (Hammond, Kogut & Lightbown, 1966).

METHODS

Biological methods

The organism used, *Escherichia coli* strain B, its maintenance, preparation of inocula, growth media used for experiments (mineral medium of McQuillen & Roberts, 1954, plus 0·2 % (w/v) trisodium citrate and 0·2 % (w/v) glucose) and growth conditions (32° and aerobiosis obtained by adequate shaking) were as described by Kogut *et al.* 1965*a*).

The growth of the bacterial population was measured as increase in extinction (E) at 500 mμ (E_{500}) in a Hilger 'Uvispeck' spectrophotometer with optical cells of 5 mm. light path, and recorded as logarithms to the base two (Finney, Hazlewood & Smith, 1955) which allowed direct determination of the specific growth rate in doublings/hr (μ), (Herbert, Elsworth & Telling, 1956). It had been shown previously (Kogut *et al.* 1965*a*, table 1) that the relationships between E_{500} values, total counts and dry weight determinations for samples taken at different times during growth in the presence or absence of dihydrostreptomycin are reasonably constant. Dihydrostreptomycin sulphate (Distillers Biochemicals Co. Ltd., Speke, Liverpool, 1; potency 800 i.u./mg.) was added to cultures in the early exponential stage of aerobic growth (E_{500} = 0·1–0·3). Removal of dihydrostreptomycin from treated cultures was achieved by rapid filtration on membrane filters (Millipore; diameter 47 mm. A.P.D. 0·45 μ) followed by three washings (with half the original volume each time) and resuspension in antibiotic-free medium pre-warmed to the growth temperature. Viable (colony) counts were made by diluting appropriate samples in sterile growth medium without glucose and plating on complete growth medium solidified with 1 % (w/v) agar. Pour-plates were made in triplicate from each of two different dilutions and incubated 33–35° for 48–72 hr. The colony counts for each sample were determined from the dilution giving totals of between 150 and 400 colonies and expressed as visible colonies/ml. original sample. Those organisms in the original sample which do not give rise to visible colonies are usually described as 'non-viable', but since the possibility arises that they initially retain a finite growth rate, the less anomalous term 'non-survivor' has been adopted. This term, when applied to the appropriate fraction of a culture, is intended to imply that a sample will show upon incubation under the defined conditions the corresponding loss of viable units, but it does not directly imply that the fraction is non-viable at the time when the sample is taken. The conjugate term 'survivor' is also used, and replaces the designation 'viable' in reference to those bacteria which do produce visible colonies upon incubation.

For convenience of use with a computer the measured values of extinction and survivor counts were embodied as data in the following forms. The values of E_{500} for each experiment were numerically normalized to provide a value of unity at the time of removal of dihydrostreptomycin, and this value then defined on a linear scale as unit 'population'. The resulting values, in population units, were then used as the first of two variables representing the experimental data. Also for each experiment the survivor counts were divided by the corresponding values of E_{500} and this provided an index proportional to the number of survivors per unit population. The value of this

index (number of colonies obtained from 1 ml. culture/E_{500} value) in the case of control cultures was evaluated from seventeen experiments covering all stages of exponential growth (i.e. E_{500} from 0·15 to 0·95) and was found to be $1{\cdot}9 \times 10^9$ per E_{500} with a standard deviation of 7·6 %. The value of each index obtained from survivor counts for treated cultures, when divided by the above value for control cultures, was termed the 'survivor fraction'. Since it is impracticable to determine directly the standard deviations for each index obtained from treated cultures it had to be assumed that the standard deviation, expressed as a percentage, was the same in treated cultures and in controls. Error theory then gives the standard deviation for 'survivor fraction' as 11 %. Survivor fraction was taken as the second of the two variables embodying the experimental data.

Computer methods

The analogue computer installation that was used comprised a PACE TR 48 computer with both display oscilloscope and X–Y recorder output facilities. These were used at 0·1 and 50 sec. compute times, respectively. Two different approaches, employing mathematical analysis and mathematical synthesis, were formulated on the computer; the methods are described in later sections where the various functions of time are described by the following symbols:

P = Total population (in population units)
P_s = Survivor population (in population units)
P_n = Non-survivor population (in population units)
S = Survivor fraction (dimensionless)
G = Growth rate of total population (doublings/hr)
G_s = Growth rate of survivors (doublings/hr)
G_n = Growth rate of non-survivors (doublings/hr)
K_s = Rate constant at which survivor growth rate changes (per min.)
K_n = Rate constant at which non-survivor growth rate changes, per min.

Method of mathematical analysis. Since the growth of a culture at any time may be described by

$$dP/dt = 0{\cdot}693GP,$$

the evaluation of the growth rates of the survivors and non-survivors from the available data requires the numerical evaluation of

$$G_s = \frac{1{\cdot}44}{P_s}\frac{dP_s}{dt} \quad \text{where} \quad P_s = PS$$

and

$$G_n = \frac{1{\cdot}44}{P - P_s}\frac{d(P - P_s)}{dt}.$$

It is thus necessary to present to the computer for the analysis of each experiment the relevant data on total population and survivor fraction as measured at various times during the experiment. In the former case it was found sufficient to produce a voltage analogue of the data by setting a variable diode function generator (VDFG) to produce a straight-line approximation through the data curve at 40 min. intervals. However the scatter of the data points of survivor fraction, which originates from the technique of viable counting, made it necessary first to draw a smooth curve through the survivor fraction data points and then approximate this latter curve by using a second VDFG. This method of analysis thus involves some pre-interpretation of the

data. The required analysis also involves differentiation of functions derived from the VDFG outputs, and the straight-line approximations introduced by these devices, give rise to stepped derivative. The plotted solutions for the growth rates are correspondingly stepped and only the horizontal sections of the steps represent mathematically accurate solutions.

Method of mathematical synthesis. Since the preceding analytical approach involved a certain degree of subjective pre-treatment of the data the alternative approach using mathematical synthesis was also explored and a system devised which allowed a suitable range of hypotheses about the growth rates of two sub-populations, P_1 and P_2, to be implemented in the computer. For each set of hypotheses, P_1 and P_2 were provisionally considered as models of the subpopulations P_s and P_n, respectively, and the validity of the assumption checked by comparing P_1+P_2 with total population data and $P_1/(P_1+P_2)$ with the data on survivor fraction. The general mathematical form adopted for the model populations was:

$$dP_1/dt = 0{\cdot}693 G_s P_1 \quad (t = 0; \quad P_1 = P_s(0)),$$

where

$$G_s = G_s(0)+[G_s(\infty)-G_s(0)]\,(1\text{-exp. } K_s(T-t)),$$

and

$$dP_2/dt = 0{\cdot}693\, G_n P_2 \quad (t = 0; \quad P_2 = P_n(0)),$$

where

$$G_n = G_n(0)+[G_n(\infty)-G_n(0)]\,(1\text{-exp. } K_n(T-t)) \quad (T < t; \quad K_s = \quad K_n = 0).$$

Examination of these equations as implemented in the patching diagram of Fig. 1 shows that apart from the initial sizes of the two populations, $P_s(0)$ and $P_n(0)$, all other parameters refer to the growth rates of the populations and each is represented on the computer by an independent potentiometer setting. Particular growth rate hypotheses may thus be readily implemented on the computer by appropriate settings of these potentiometers. For example, when constant growth rates are required the T potentiometer may be set to correspond to a time interval greater than that of the duration of the experiment and the growth rate of P_1 will remain constant at any value set by the $G_s(0)$ potentiometer. Correspondingly, P_2 will grow at any constant rate set by the $G_n(0)$ potentiometer. When the growth rates are required to vary from time zero, the T potentiometer may be set to zero and the growth rate of P_1 will then change exponentially from the value set for $G_s(0)$ to any value set for $G_s(\infty)$ where the rate constant of the change is determined independently by the setting of the K_s potentiometer. Similarly, the growth rate of P_2 will change from $G_n(0)$ to $G_n(\infty)$ at a rate constant of K_n.

The method used for checking the results of growth rate hypotheses against data was simply to pre-plot on paper the total population and survivor fraction data for any given experiment, and to superimpose over these, by using the computer X–Y plotter, a trace of the corresponding curves computed for the particular set of growth rate hypotheses. In the case of pre-plotting the data on survivor fraction the limits set by its standard deviation were taken as the significant values.

RESULTS

Experimental data

As reported in preceding papers (Kogut *et al.* 1965*a*, *b*; 1966), addition of low concentrations of dihydrostreptomycin or streptomycin to aerobically growing cultures of *Escherichia coli* B during the early exponential phase led to intracellular accumulation of the antibiotic (also Hancock, 1962) and a gradual decline in growth rate as

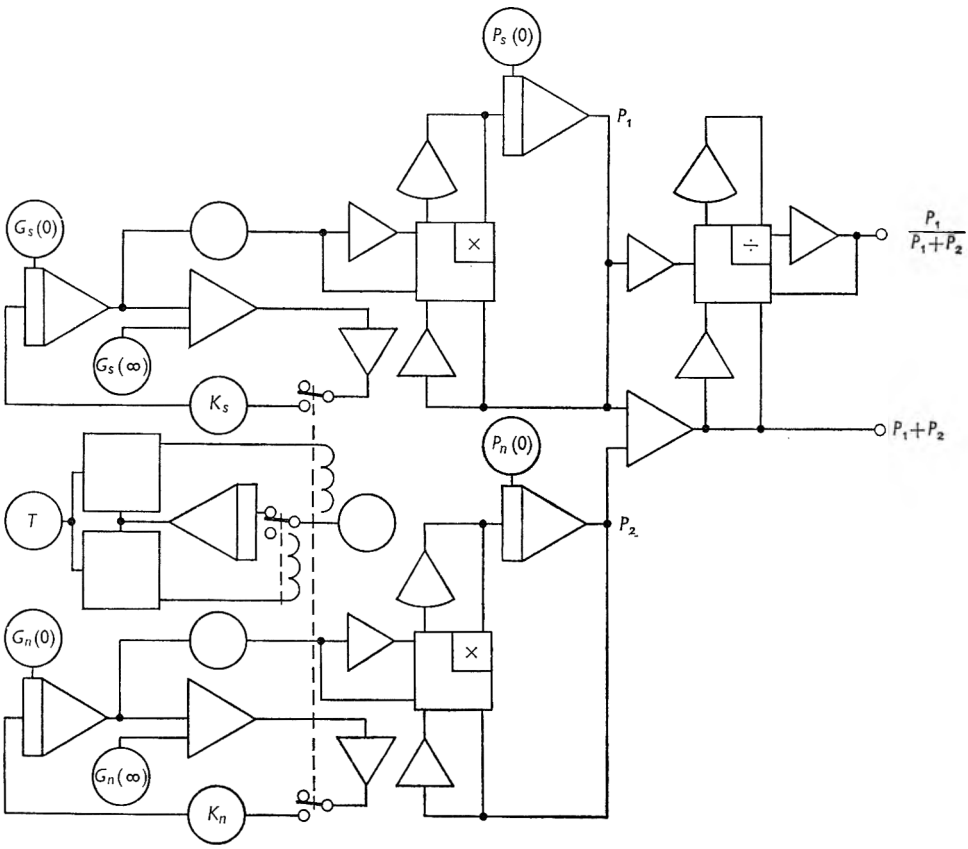

Fig. 1. Computer patching diagram. The above interconnexions of computer components generates curves for the theoretical populations P_1 and P_2 according to the equations given in the text. Outputs corresponding to (P_1+P_2) and $P_1/(P_1+P_2)$ are also provided for comparison with experimental data. The constants of the equations appear as settings of the computer potentiometers as annotated.

illustrated in Fig. 2*a* (see Kogut *et al.* 1965*a*). Removal of antibiotic from the culture medium at various times before growth had entirely ceased, prevented further intracellular accumulation and resulted in cultures which continued to grow at apparently exponential though decreased rates for several hours, followed by gradual recovery to normal growth rates; this is illustrated in Fig. 2*b*. We have already shown (Kogut *et al.* 1965*b*) that concomitant with the decrease in growth rate, the ratio of colony-forming units/ml. to E_{500} values, i.e. the index of survivor count per E_{500} value and hence the 'survivor fraction', as defined above, is decreased. After removal of extra-

cellular antibiotic, the index of survivor count per E_{500} was shown to remain more or less constant during the period when growth rate appeared constant, followed by gradual increase to the control value.

To obtain more complete data for mathematical analysis, we have now made measurements of survivor counts and E_{500} values at frequent intervals to cover the whole of the initial logarithmic growth phase and the subsequent recovery phase of

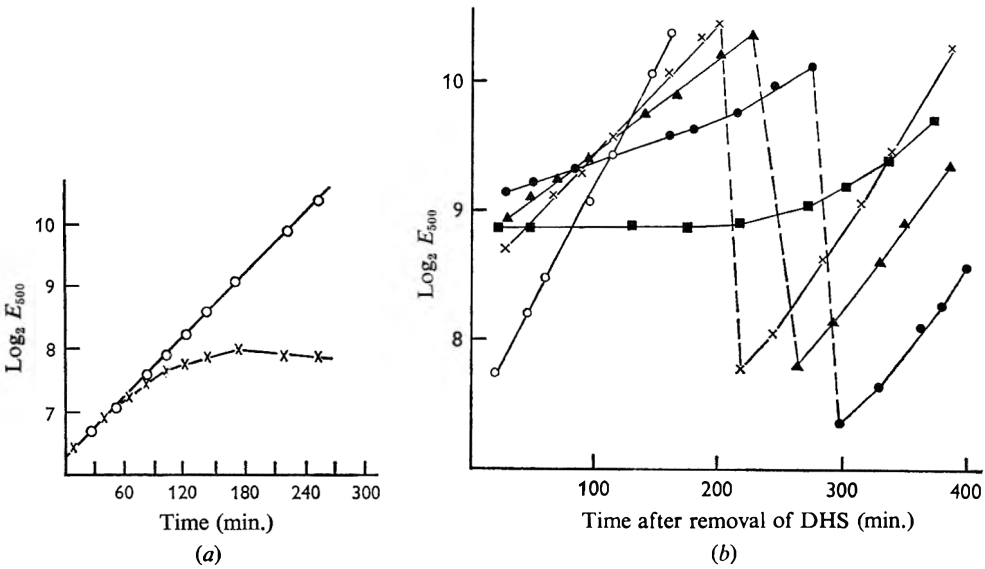

Fig. 2 (*a*) Growth curve of *Escherichia coli* B with and without the addition of dihydrostreptomycin sulphate, 20 μg./ml. at time zero. Growth conditions and measurements as described under Methods. ○ — ○, control culture; × — ×, culture with dihydrostreptomycin sulphate. (*b*) Aerobic growth of *E. coli* B in antibiotic-free medium after various periods of aerobic growth in the presence of dihydrostreptomycin sulphate, 20 μg./ml. Conditions as described in Methods. The dashed lines indicate culture dilution with fresh medium to allow for continued growth. ○ — ○ represents a control culture and the remainder, treated cultures subjected to various periods of pre-treatment and filtered after: × — ×, 65 min.; ▲ — ▲, 85 min.; ● — ●, 105 min; ■ — ■, 125 min.

Table 1. *Initial growth rates, survivor counts and survivor fractions of Eschericha coli* B *cultures following treatment with dihydrostreptomycin (DHS) of various concentrations for various times*

Expt. no....	1	2	3	4	5	6	Control
			Before filtration				
Concentration of DHS treatment (μg./ml.)	30	30	20	30	20	30	0
Duration of DHS treatment (min.)	70	65	105	75	110	65	0
			After filtration				
Initial constant growth rate (μ)	0·17	0·32	0·35	0·37	0·45	0·56	1·0
Decrease from control (%)	83	68	65	63	55	44	0
Initial survivor count/E_{500} ($\times 10^9$)	0·37	0·76	0·96	1·1	1·3	1·4	1·9
Survivor fraction	0·20	0·40	0·57	0·60	0·70	0·74	1·0

increasing growth rates and survivor fractions, on cultures treated with dihydrostreptomycin for various times and then freed from extracellular antibiotic. Figure 3 shows the time course for a representative experiment, and the relevant details for the six sets of experiments are tabulated in Table 1.

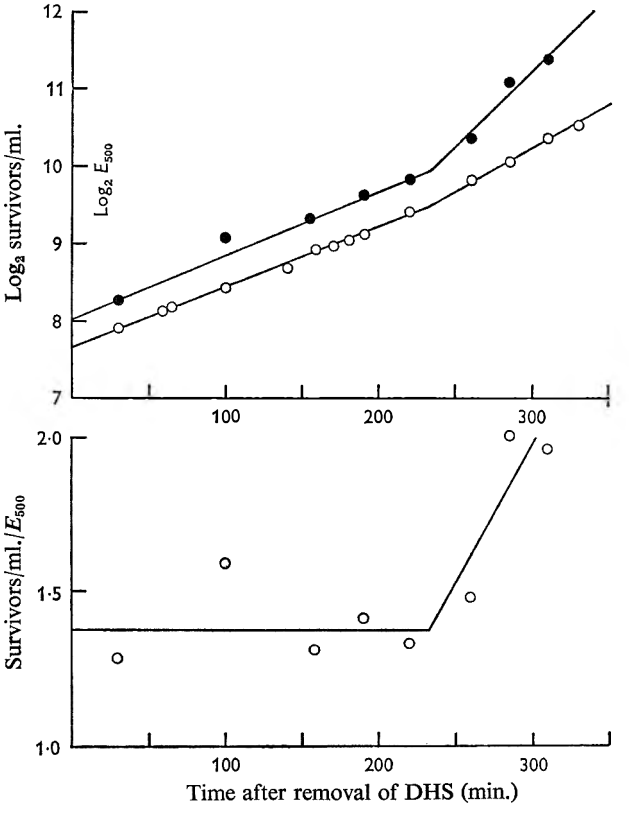

Fig. 3. The upper graph shows the increase in total population, measured as E_{500}, and the corresponding number of survivors/ml. in a culture of *Escherichia coli* B after treatment with dihydrostreptomycin sulphate, 20 μg./ml. for 110 min. followed by removal of extracellular antibiotic (Expt. 5 of Table 1). ○ — ○, $\text{Log}_2 E_{500}$; ● — ●, $\log_2$ survivors/ml. The lower graph shows the ratio between these variables, namely the number of survivors/ml./E_{500}.

Results of mathematical analysis

The method of mathematical analysis previously described was applied to the six sets of experimental data and the corresponding stepped solutions for growth rates as functions of time obtained. A typical result is that of Fig. 4, where the derived values of growth rates of survivors and non-survivors are shown as points taken from the horizontal sections of the stepped solution curve. The numerical values of such points depend upon the particular smooth curve chosen to represent survivor fraction data in the computer and the results are taken to be qualitative rather than quantitative. The majority of solution curves obtained were of the same basic structure as Fig. 4 and it was noted that in each experiment the growth rate of the non-survivor population eventually assumed a negative and not merely a zero growth rate thus indicating a non-survivor population which decreased in size at later stages of the experiments.

Results of mathematical synthesis: 1; growth-rate parameters as functions of time

The method of mathematical synthesis was initially used to investigate certain simple hypotheses involving constant growth rates. The first of these hypotheses was the case where the non-survivors are assumed to be completely non-viable, that is, their growth rate is zero, whilst the survivors have a constant growth rate throughout the experiment. This assumption covers the possibility that the survivors continued to grow at control growth rates. The hypothesis does not specify the initial sizes of the survivor and non-survivor populations, but these are substantially established by the initial

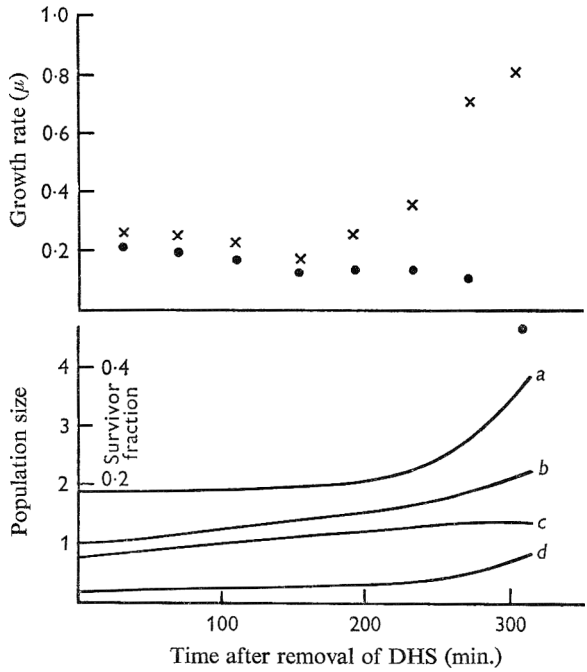

Fig. 4. Curve *a* is the computer representation of the survivor fraction data from Expt. 1 of Table 1, and is obtained from a suitably preset VDFG unit: curve *b* is the representation of the corresponding total population data similarly obtained. These data curves, analysed according to the equations given in 'Methods of mathematical analysis', provide the following solutions: curve *c*, size of non-survivor population; curve *d*, size of survivor population; ● — ●, growth rate of non-survivor population; × — ×, growth rate of survivor population. The latter growth-rate solutions are shown only as the mathematically accurate points of the computed curve in which steps occur due to mathematical artifact.

values of total population and survivor fraction data. The only variable is thus survivor growth rate and this was varied on the computer until the hypothesis produced a match to total population data. The situation is illustrated in Fig. 5*a*, which relates to Expt. 1 where a DHS concentration of 30 μg./ml. had been administered 70 min. before time zero. A value of survivor growth rate of 0·540 doublings/hr was found necessary to produce a match to total population data, and the corresponding computed survivor fraction curve was as shown. Any attempt to bring the latter into closer agreement with its data involved an unacceptable divergence of the total population curve.

The second of the hypotheses involving constant growth rates removed the restriction that the value of the constant for the non-survivor population should be zero, and thus presumed a total population that was the sum of two independent logarithmic subpopulations. Neither of the growth rates is specified in the hypothesis, but these were adjusted on the computer to determine the combination which gave a total

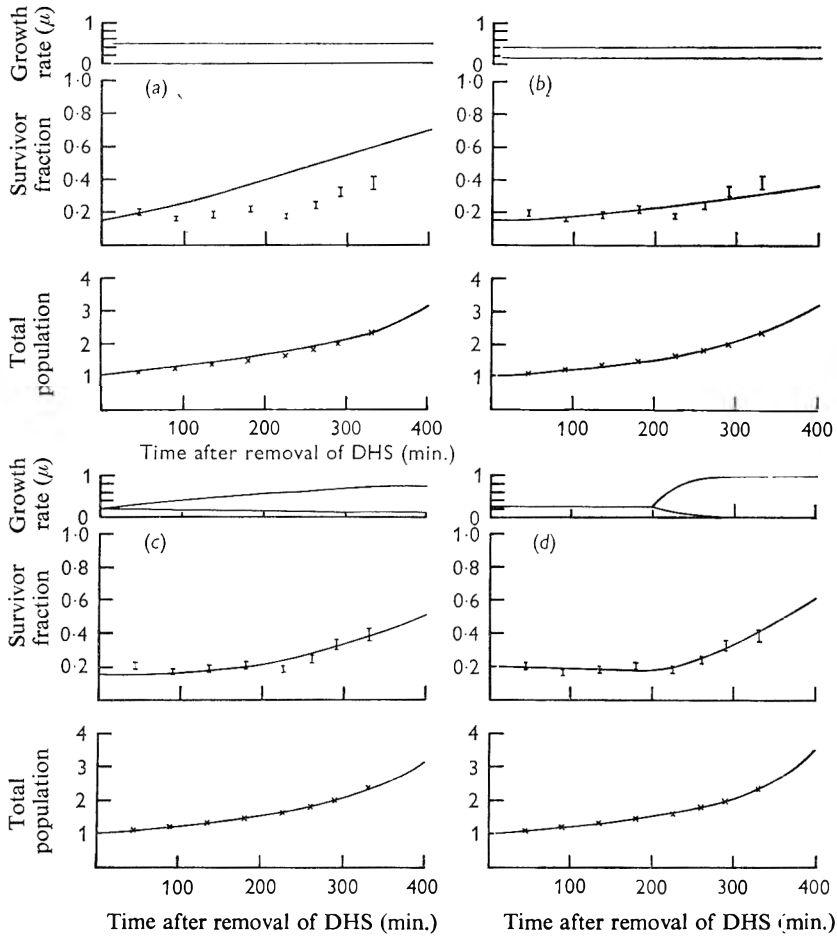

Fig. 5. Four comparisons between the curves synthesized from various growth-rate hypotheses and the experimental data of Expt. 1, Table 1. In all cases the time axes refer to time after removal of extracellular antibiotic, and in each case the growth-rate hypotheses are embodied in the upper curves of each section. The centre graphs of the four sections show the experimental data points for survivor fraction (I) and the solid curves indicate the computed result of growth-rate hypotheses. The corresponding computed curves for total population are shown in the lower graphs of each section together with the total population experimental data (×).

population curve that fitted the data. Figure 5*b* shows the resultant curves for the same experiment as previously considered, where the determined values of growth rates were 0·400 and 0·160 doublings/hr for survivors and non-survivors respectively.

The remaining hypotheses that were tested all involved varying growth rates and these were implemented on the computer as exponential changes from some initial

value towards a second and final value. The final value for the survivor growth rate was made equal to the approximate control value of 1 doubling/hr, thus allowing for the observed eventual recovery of a treated population; that for the non-survivors was made zero, thus allowing for their eventual non-viability. Since the rates of the growth rate changes could be varied by potentiometer control on the computer and the adjustment included the possibility of setting the rates to zero, this system allowed the possibility that the non-survivor growth rate was zero.

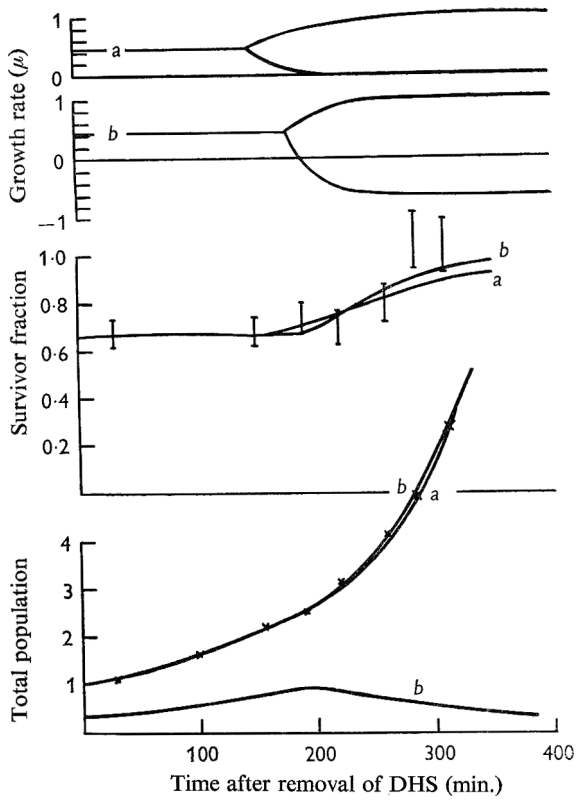

Fig. 6. Comparison of growth-rate hypotheses with the data of Expt. 5, Table 1. The upper graphs, *a* and *b*, represent variant hypotheses regarding the ultimate non-survivor growth rate and the centre graph compares the survivor fraction data (I) with the computed implications of these hypotheses. The lower graph makes simultaneous comparison between the total population data (×) and the computed curves. The bottom curve records the non-survivor population corresponding to the second hypothesis (*b*).

Figure 5*c* shows the situation that resulted from a hypothesis which allowed the growth rates of survivors and non-survivors to start their exponential changes as soon as DHS was removed from the culture medium. The experiment illustrated is the same as that previously used and the best match to its data was achieved by adjusting the value of initial growth rate and the subsequent rate constants of the changing survivor and non-survivor growth rates. The value of the initial growth rate was found to be 0·20 doublings/hr and those of the rate constants were 0·18(4)/min. and 0·11(2)/min., respectively. These gave rise to the recorded growth rate and survivor fraction curves.

The final type of hypothesis considered differed only from the above in that an un-

specified period was allowed before the onset of growth rate change, during which the survivor and non-survivor growth rates were constant and equal. The period of this delay, together with the initial value of growth rate and the rate constants of ensuing growth rate changes were treated as parametric variables in finding the set of values which not only fitted the total population data but also gave the best fit to survivor fraction data. The values so determined in the case of Expt. 1 were 200 min. for the delay time, an initial growth rate of 0·20 doublings/hr and 2·2(2)/min. and 2·0(9)/min.

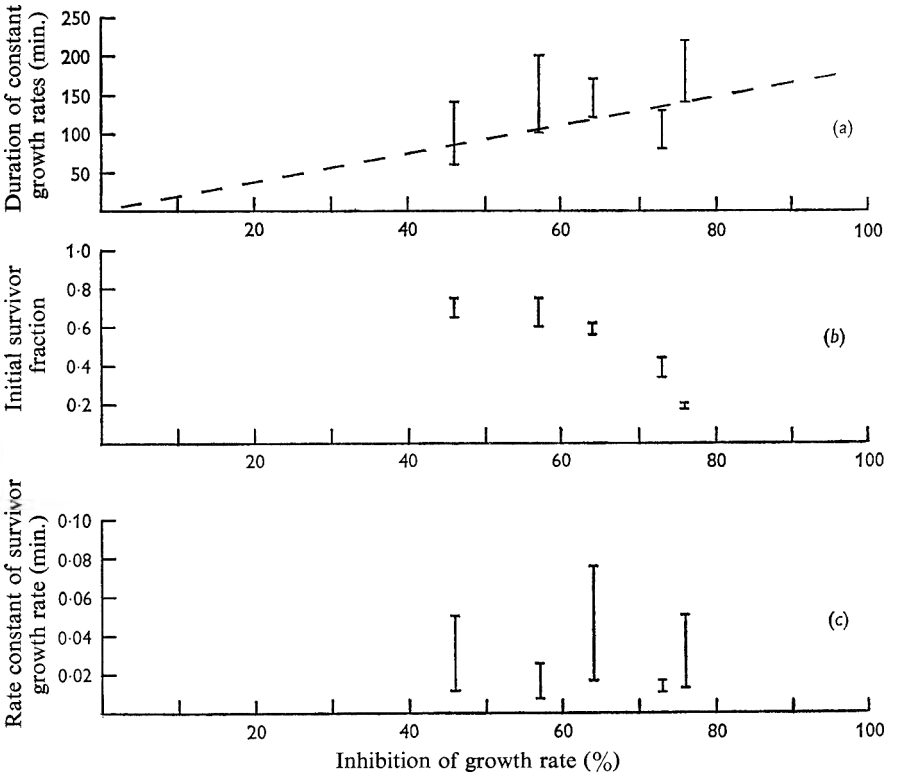

Fig. 7. The maximum and minimum values of the indicated parameters were determined as described in 'Results of mathematical synthesis, 2' and the values so determined are represented by bars at the ends of the vertical lines. These values are presented as functions of the growth-rate inhibition that results from various concentrations and times of dihydrostreptomycin pretreatment. A maximum value of one parameter is associated with maxima of the other two parameters. Results from only five of the six experiments are shown since those from experiments three and four were almost identical.

for the rate constants of the ensuing survivor and non-survivor growth rates changes, respectively. The forms of the resultant growth rate curves, total population and survivor fraction curves are shown in Fig. 5*d*.

Similar results to these were obtained for all six sets of data with, however, a tendency for less satisfactory final fits to be obtained for those experiments involving higher initial survivor fractions. Such an experiment is no. 5 of Table 1; the situation is shown in Fig. 6 which illustrates the improvement in fit that resulted when the eventual growth rate of the non-survivors was allowed to become negative and not merely zero.

Results of mathematical synthesis: 2; growth rate parameters as function of treatment

Each set of data was re-examined by using the computer simulation of the hypothesis involving a 'delay' period of equal and constant survivor and non-survivor growth rates before the onset of exponential changes. In this study however, the maximum and minimum values for the delay period, which by suitable adjustment of other parameters would still allow acceptable fits to both total population and survivor fraction data, were examined. It was found that to obtain a maximum delay period which still allowed such acceptable fits to experimental data, it was necessary to use on the computer a maximum value of initial survivor fraction and a maximum value of the rate constant of survivor growth rate recovery. The converse situation applied when a minimum initial period of constant growth rate was enforced. Other parameters were inconsistently related to the maximum and minimum delay situations. It was thus possible to determine for each experiment maximum and minimum values for initial survivor fraction, duration of period of constant growth rates, and the rate constant of survivor growth rate. These values are plotted as functions of the initial value of constant growth rate in Fig. 7*a–c*: only five sets of values are given since those from Expts. 3 and 4 of Table 1 were found to be almost identical.

DISCUSSION AND CONCLUSIONS

Growth rate parameters as functions of time

The original suggestion (Kogut *et al.* 1965*b*) that the early effects of dihydrostreptomycin upon growth rates of *Escherichia coli* B cultures were similar in all individuals was based upon consideration of growth characteristics and viable counts of cultures freed from extracellular antibiotics, and was supported by direct microscopic observation. This proposition, which contrasts with the more common assumption that loss of viability consequent upon antibiotic treatment implies immediate and complete cessation of growth (Hurwitz, Landau & Doppel, 1962) was made despite the ensuing separation of a bacterial population into two fractions, according to the retention or loss of the capability to form a colony when incubated for 72 hr.

The apparent anomaly that bacteria whose growth rates were initially equally affected, nevertheless ended as members of one or other of these two opposed categories ('viable' and 'non-viable') was recognized at the time and resolution of the anomaly sought in attributing genuine changes of growth rate to the two groups. It was also observed that the loss of ability to go on dividing did not occur throughout the 72 hr period of incubation, but seemed to become established within the first 3–4 generation times after removal of extracellular dihydrostreptomycin.

The nature of the changes in growth rates, postulated for the two subpopulations, may in principle be determined analytically, by using the simple equations previously described; but errors inherent in one of the independent variables of the equations, namely the survivor fraction data, eventually limits use of the method to qualitative interpretation. It should not be overlooked, however, that the solutions obtained do approximate to the unique solution for each experiment, and such results as those in Fig. 4 do strongly support the original contentions about the form of growth rate variations for survivors and non-survivors.

Further indirect support for the proposed growth characteristics is obtained by

means of mathematical synthesis, which shows that some simpler conjectures involving constant growth rates are untenable, on the basis that their quantitative results cannot be made to match experimental data (Figs. 5*a*, *b*). Good agreement with data, however, can be obtained (Fig. 5*d*) with the mathematical formulation of the hypothesis that the growth rates of all individuals in the culture have an initial depressed value which remains constant for a period, followed by a second phase when the growth rate of the survivors increases exponentially to the control value, and that of the non-survivors decreases exponentially to zero. No intrinsic significance is attributed to the exponential form of growth-rate change as this form was merely chosen for convenience; but the overall sigmoid form of growth rate changes is proposed as being a close representation of the true situation.

Both the methods used, mathematical analysis and mathematical synthesis, give indication that the growth rates of non-survivors may eventually assume negative values, implying that the number of non-survivors in such cultures actually decreases towards the end of the experimental period. This tendency is most apparent in those cultures where the initial common growth rate and the size of the initial survivor fraction were least severely depressed. The simplest explanation of such decrease in the number of non-survivors would lie in the eventual lysis of some, if not all, of the non-surviving bacteria. Lysis of even substantial fractions (20–40 %) of the total population, occurring during the period when growth rates of total population and of the survivor fraction were increasing would be impossible to detect by extinction measurements. (We only occasionally observed decrease in E_{500} values of cultures that had stopped growing and were left in the presence of extracellular dihydrostreptomycin.) In four determinations of E_{500} values and total counts on cultures which were left in the presence of extracellular antibiotic after growth had completely ceased, the ratio between these two increased, i.e. the bacteria appeared to shrink or become denser. Alternative explanations for the possible decrease in number of non-survivors can be derived on the basis of differences between growth characteristics of *Escherichia coli* in liquid culture and on solid media. It could, for instance, be assumed that a loss or excretion of antibiotics from some of the bacteria during growth in liquid culture, where extracellular dilution would be rapid might lead to recovery of the ability to go on dividing, whilst such recovery would be prevented by the slower diffusion of such excreted antibiotic in solid media. In such a situation successive samples from a liquid culture would score decreasing numbers of non-survivors upon incubation on solid media. Moreover, such a phenomenon would be most apparent in cultures which show less severe depression of initial growth rate and survivor fraction, and presumably have smaller total intracellular concentrations of antibiotic (Kogut, Lightbown & Isaacson, 1966).

Growth parameters as functions of treatment

A consistent relationship between pre-treatment with dihydrostreptomycin, resultant survivor fraction and the initial growth rate of the corresponding total population was previously shown (Kogut *et al.* 1965*b*). The duration of common constant growth rates in cultures with various degrees of initial growth-rate inhibition cannot be determined with accuracy, but the results obtained here (Fig. 7*a*) are consistent with a linear increase from zero for an untreated culture at a mean rate of 15–20 min./10 % increase in growth-rate inhibition. Such a linear relationship would

imply that the duration of common constant growth rate is not dependent on bacterial multiplication or on the amount of growth, but is consequent upon the same or related factors(s) which govern(s) the initial inhibition of growth rate.

The rate constants of survivor growth-rate recovery in some cases display very wide limits. The results in Fig. 7*c*, which gives these values as functions of initial growth rate inhibition, allow no specific relationship between these variables to be postulated.

The relationship between initial growth-rate inhibition and size of the survivor fraction, initially postulated, is not supported by the results shown in Fig. 7*b*. Any curve drawn through these points must also conform to the conditions of a control culture and have a unity value of survivor fraction at zero inhibition; the relationship between these two variables then becomes evidently non-linear. Extrapolation of any simple curve through the points also implies that pre-treatment with higher levels of dihydrostreptomycin for longer periods will produce bacterial populations consisting entirely of non-survivors, which can nevertheless retain an apparent initial growth rate (rate of increase in E_{500} value) of approximately 20 % of the control value. It must be remembered, of course, that this refers to the initial period of common constant growth rate which persists for about 3 hr, so that the corresponding increase in bacterial mass for that period would be about 50–60 %. Occasional direct determinations of protein (Lowry, Rosebrough, Farr & Randall, 1951): E_{500} ratios of cultures whose growth rate was severely inhibited (by 80–90 %) showed no significant decrease from control values. This makes it unlikely that such 'residual' growth rates merely reflect synthesis of carbohydrates or reserve materials.

It remains to consider mechanisms which might account for the growth patterns of populations of *Escherichia coli* B treated with streptomycin or dihydrostreptomycin in the manner described. If such treated populations were already differentiated in their growth behaviour at the time of removal of extracellular antibiotic, then one portion (the survivor fraction) should have growth rates which continuously increase and the other (the non-survivor fraction) should exhibit continuously decreasing growth rates. This situation is contradicted by the results of analogue computer simulations presented here. What then is the mechanism which results in approximately equal and constant growth rates which are maintained for periods extending through 3 to 4 generations? The present study confirms the previous suggestions that 'loss of viability' does not occur as a sudden and irreversible cessation of growth and multiplication, i.e. a unique 'lethal' event, at the time when the antibiotic enters the organism, but seems to be consequent upon a series of events taking place in the presence of intracellular antiobiotic. It has been suggested, on the basis of earlier studies, (Kogut *et al.* 1965*b*; 1966) that some of the intracellular antiobiotic accumulated by treated populations is not immediately inhibitory, but constitutes a 'pool' from which transfer and combination with the sites of inhibition can take place during subsequent growth in the absence of further intracellular accumulation. The distribution of such pools within a treated population need not be uniform. Elucidation of the factor(s) which may govern the transfer of intracellular antibiotic between 'pool' and the sites of inhibition (e.g. the ribosomes) would involve a better understanding of the nature and distribution of such non-inhibitory 'pool' streptomycin within the bacteria, as well as the nature of the effective growth-inhibitory combination between streptomycin and ribosomes and the control of ribosome synthesis and assembly.

Our thanks are due to Miss Maureen Harris for able and conscientious technical assistance.

REFERENCES

FINNEY, D. J., HAZLEWOOD, T. & SMITH, M. J. (1955). Logarithms to base two. *J. gen. Microbiol.* **12**, 222.

HAMMOND, B., KOGUT, M. & LIGHTBOWN, J. W. (1966). Analogue computer studies of the growth characteristics of survivors and non-survivors in dihydrostreptomycin-treated cultures of *Escherichia coli*: the concept of lethality. *J. gen. Microbiol.* **44**, vii.

HANCOCK, R. (1962). Uptake of ^{14}C-streptomycin by some micro-organisms and its relation to their streptomycin sensitivity. *J. gen. Microbiol.* **28**, 493.

HERBERT, D., ELSWORTH, R. & TELLING, R. C. (1956). The continuous culture of bacteria; a theoretical and experimental study. *J. gen. Microbiol.* **14**, 601.

HURWITZ, C., LANDAU, J. V. & DOPPEL, H. W. (1962). Effect of exposure of *Escherichia coli* to streptomycin on ability to undergo cell division. *J. Bact.* **84**, 1116.

KOGUT, M., LIGHTBOWN, J. W. & ISAACSON, P. (1965*a*). Streptomycin action and anaerobiosis. *J. gen. Microbiol.* **39**, 155.

KOGUT, M., LIGHTBOWN, J. W. & ISAACSON, P. (1965*b*). Effects of dihydrostreptomycin treatment on the growth of *Escherichia coli* after removal of extracellular antibiotic. *J. gen. Microbiol.* **39**, 165.

KOGUT, M., LIGHTBOWN, J. W. & ISAACSON, P. (1966). The intracellular accumulation of ^{14}C-streptomycin by *Escherichia coli* strain B in relation to its growth-inhibitory effect. *J. gen. Microbiol.* **42**, 333.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., & RANDALL, R. J. (1951). Protein measurement with Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MCQUILLEN, K. & ROBERTS, R. B. (1954). The utilization of acetate for synthesis in *Escherichia coli*. *J. biol. Chem.* **207**, 81.

J. gen. Microbiol. (1967), **48**, 205–213
Printed in Great Britain

Metabolic Disorders in Thiamineless Dwarf Strains of *Staphylococcus aureus*

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(*Accepted for publication* 28 *February* 1967)

SUMMARY

Dwarf colony variants of *Staphylococcus aureus* have been described as a causative agent of bovine mastitis in Israel. These strains were reported as auxotrophic for thiamine, being unable to synthesize the vitamin from its pyrimidine and thiazole moieties. In the present paper it is shown that strains with two different deficiencies are involved. One type is unable to concentrate the thiazole moiety and the other is unable to phosphorylate the pyrimidine portion of the vitamin. The results indicate the existence of independent permeases in *S. aureus* for these thiamine moieties.

INTRODUCTION

Dwarf-colony variants of *Staphylococcus aureus* have been reported by several authors. They were generally obtained *in vitro* from normal strains kept under adverse conditions, such as exposure to chemicals (Hoffstadt & Youmans, 1932; Youmans & Delves, 1942; Hale, 1947; Browning & Adamson, 1950), to antibiotics (Barbour, 1950; Browning & Adamson, 1950; Wise & Spink, 1954), or to irradiation (Hale, 1949; Gause, 1961). Similar variants have been isolated from infectious lesions, especially after treatment with antibiotic drugs. In the latter case the cultures developed as normal colonies when incubated under increased CO_2 pressure (Goudie & Goudie, 1955; Thomas & Cowlard, 1955; Sherris, 1952; Hale, 1951).

We have previously reported dwarf variants of *Staphylococcus aureus* to cause bovine mastitis in Israel. The growth of these strains was unaffected by CO_2, but when thiamine hydrochloride was added to the nutrient medium after sterilization by autoclave normal staphylococcal colonies developed (Lernau & Sompolinsky, 1962; Sompolinsky & Levy, 1961). As known, the thiamine required for growth of *S. aureus* may normally be replaced by the pyrimidine + thiazole moieties of the vitamin (Knight, 1937). In the present communication, further studies of the metabolic disorders of these variants are reported.

METHODS

Bacterial strains

The dwarf strains were all coagulase positive and produced α and β hemolysins. No differences in phage pattern and antibiotic spectra were observed between dwarf strains isolated from cows of a single herd, but strains from different herds varied.

On most nutrient media the dwarf strains formed minute transparent colonies. On

these colonies dark orange sectors developed after a few days at 37°. These were composed of mutant clones which could easily be isolated and their growth was typical of *Staphylococcus aureus*. In some strains the dwarf colonies lying around the periphery of mutant colonies were significantly larger than when they occurred alone. This satellite growth suggested cross-feeding. This effect was not observed with other strains. Strains D 551 and D 552 were of the former type and D 1560 of the latter. These three strains were from different herds. Other dwarf strains from these herds as well as from other herds were metabolically identical with one of these two types. Only experiments with these strains will therefore be described.

Strain D 1560. Phage pattern 75. For the present study we used a streptomycin-resistant mutant developed in this laboratory.

Strain D 551. Phage pattern 42 E. Some of the experiments were made with D 552 resistant to the typing phages in general use.

Strain N 1560. Normally growing mutant of D 1560. The identity was checked by streptomycin resistance, phage sensitivity, and production of α and β hemolysins.

For the sake of comparison several strains isolated from human pathological conditions were included in some of the experiments as described below.

Media and chemicals used; cultivation

Saline buffer: 0·15 M-Na_2HPO_4, 61·1 ml.; 0·15 M-KH_2PO_4, 38·9 ml.; NaCl, 0·8 %, 100 ml.

Bacto brain–heart infusion agar (BHA).

Nutrient broth prepared from: Bacto peptone, 5 g.; beef extract (Baltimore Biol. Lab., Inc.), 3 g.; distilled water to 1 l.; pH 7·2.

Vitamin-free casamino acids medium (CVF): Bacto vitamin-free casamino acids, 25 g.; NaCl, 5 g.; nicotinic acid, 1 mg.; distilled water to 1 l.; pH 7·2.

Defined medium, modified from Gladstone (1937): KH_2PO_4, 0·91 g.; $Na_2H\,PO_4$, 3·83 g.; $Mg\,SO_4.7\,H_2O$, 0·13 g.; $Fe\,SO_4.(NH_4)_2\,SO_4.6\,H_2O$, 0·01 g.; glucose, 5·0 g.; nicotinic acid, 1·0 mg.; distilled water to 1 l.; pH 7·4. The following amino acids were added: glycine, L-valine, L-leucine, L-proline, L-aspartic acid, L-glutamic acid to a final concentration of 1/1500 M; L-phenylalanine, L-arginine, L-histidine to 1/4000 M; L-cystine to 1/10000 M. Thiamine or its thiazole and pyrimidine moieties were added as indicated. The KH_2PO_4, Na_2HPO_4 and $MgSO_4.7H_2O$ were autoclaved, the other ingredients were sterilized by filtration and added aseptically.

Thiamine hydrochloride (Sigma), thiamine pyrophosphate (Sigma), sodium pyruvate, A grade (Calbiochem), 2-methyl 4-amino 5-aminomethylpyrimidine from E. Merck, Darmstadt, were used and 4-methyl-5-(β hydroxyethyl)-thiazole synthesized by Professor Dr B. Prijs, Institute for Inorganic Chemistry, Basel University, Switzerland. The two thiamine moieties will be referred to as the pyrimidine and the thiazole moieties.

Phosphorylated derivatives of the thiazole and pyrimidine moieties. Due to lack of authentic samples the following preparations were used. Staphylococcal strain N 1560 was grown under aeration in CVF medium without thiamine, and the starved cocci harvested, washed and transferred to Warburg respiration vessels (see later). To one vessel was added 6×10^{-3} M of the pyrimidine moiety, to another an equimolar amount of the thiazole moiety. The vessels were incubated at 37° for 20 hr. After separation of the cocci, the supernatant solutions were designated 'crude pyrimidine pyrophos-

phate' and 'crude thiazole phosphate', respectively. It was assumed that these solutions probably contained a certain amount of phosphorylated end-products synthesized by the cocci. The solutions were virtually free of thiamine, since they did not stimulate oxygen consumption with pyruvate by starved bacteria of the strain N 156C.

Growth. To determine growth requirements, the cultures were grown with aeration in a water bath at 37° for 20 hr. in CVF medium enriched with 3×10^{-9} M-thiamine. The cocci were harvested, washed five times in cold 0·05 M phosphate-buffer (pH 7·0), resuspended in Gladstone's defined medium to a concentration giving 20 Klett units (filter 540 mμ) and incubated for 24 hr. Growth was indicated by an increase in turbidity of at least 100 Klett units.

Oxygen uptake was measured by standard Warburg techniques, Q_{O_2} values are expressed as μl. O_2/hr/mg. bacterial dry wt. The cocci were grown in broth, harvested, washed five times in saline buffer (pH 7·0) and resuspended to a concentration equivalent to 600–900 Klett units. Bacterial dry weight was determined on samples dried for 18 hr at 105°. Each Warburg vessel contained 0·5 ml. of phosphate buffer as above and 1 ml. of bacterial suspension. The centre well contained 0·2 ml. 20 % KOH. After 20 min. incubation, 0·5 ml. of a sodium pyruvate solution was added from the side arm. With a 0·1 M solution (50 μmoles) respiratory activity was maximal and linear during the first 30–50 min., with 1·0 M the linear period extended for several hours (Fig. 1). Oxygen uptake reached values of 15–22 μmoles O_2/50 μmoles pyruvate, which approached the theoretical value in oxidative decarboxylation. The endogenous respiration was 8–12 % of that found with an exogenous substrate.

RESULTS

Growth requirements

Growth of staphylococcal strain N 1560 depended on supplementation of Gladstone's defined medium with either thiamine or with both moieties of the vitamin. When the medium was enriched with thiamine both types of dwarf variants grew as well. Strain D 551 gave no growth on defined medium supplemented with the thiazole or the pyrimidine singly or together even at high concentrations. On the other hand, strain D 1560 grew as well as strain N 1560 when both the thiazole and pyrimidine were added together, provided that the thiazole concentration was about 10,000 times higher than that required by strain N 1560 (Tables 1, 2). Further experiments showed that the pyrimidine requirement for growth was quantitatively similar in strains N 1560 and D 1560.

Respiration with pyruvate as substrate

Further information on the enzymic defects which cause the thiamine requirement of the dwarf variants was obtained by measuring the respiration of oxygen uptake with pyruvate as substrate, in Warburg manometers under various conditions.

Strain N 1560. When grown in nutrient broth, the cocci of the normal mutant showed a marked O_2 uptake with pyruvate as substrate. Addition of thiamine had no influence on respiration. The same was true for six recently isolated human strains of *Staphylococcus aureus.* This indicates that the cocci after growth in nutrient broth had an intracellular store of thiamine or thiamine equivalent (e.g. cocarboxylase).

On the other hand, the cocci of strain N 1560 were depleted of thiamine, and they

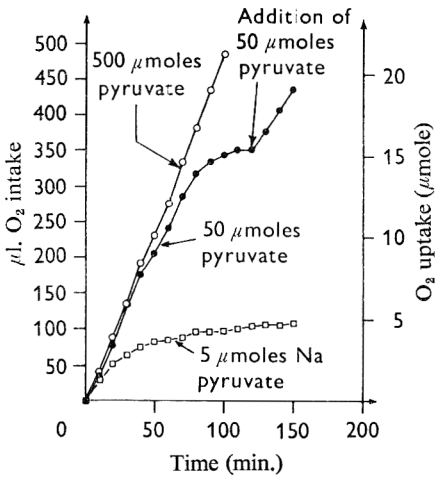

Fig. 1

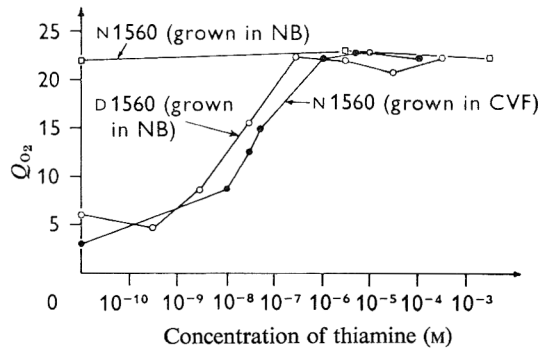

Fig. 2

Fig. 1. Dependence of oxygen uptake by *Staphylococcus aureus* D 1560 on the amount of sodium pyruvate added. Each vessel contained 2·5 μg. thiamine hydrochloride. □—□, 5 μmole pyruvate; ●—●, 50 μmole pyruvate, a further 50 μmole added at 120 min.; ○—○, 500 μmole pyruvate.

Fig. 2. Relationship between oxygen uptake (Q_{O_2}) with pyruvate as substrate by *Staphylococcus aureus* and the thiamine concentration. □—□, strain N 1560 grown in nutrient broth (NB); ●—●, strain N 1560 grown in vitamin free casamino acid medium; ○—○, strain D 1560 grown in nutrient broth (NB).

Table 1. *Growth of dwarf variants and a normal (mutant) strain of Staphylococcus aureus in Gladstone's (1937) defined medium with addition of thiamine or its moieties*

Supplements	Concentration of thiamine or of its moieties (M)	Strain: D 1560	Strain: D 551	Strain: N 1560
		Growth	Growth	Growth
Thiazole + pyrimidine*	4×10^{-3}	+	−	+
	1×10^{-3}	+	−	+
	1×10^{-4}	+	−	+
	1×10^{-5}	−	−	+
	1×10^{-8}	−	−	+
Thiamine HCl	1×10^{-7}	+	+	+
Nil		−	−	−

* The thiazole and pyrimidine moieties of thiamine.

Table 2. *Growth of a dwarf variant (D 1560) of Staphylococcus aureus in Gladstone's defined medium supplemented with the thiamine moieties*

Thiazole (M)*	Pyrimidine (M)*	Thiamine (M)	Growth
1×10^{-3}	1×10^{-5}	.	+
1×10^{-5}	1×10^{-3}	.	−
1×10^{-8}	.	.	−
.	.	1×10^{-7}	+
No supplement	.	.	−

* The thiazole and pyrimidine moieties of thiamine.

respired with pyruvate as substrate only when thiamine was added to the Warburg vessel, when the bacteria had been grown in CVF medium without supplementation or when supplemented with only a minute amount of thiamine (3×10^{-10} M). The relation between Q_{O_2} and thiamine concentration was linear in the range of approximately 10^{-8}–10^{-7} M (Fig. 2). Respiration was likewise stimulated by addition to the Warburg vessel of both moieties of thiamine together at similar concentrations. When growth in CVF medium was maximal, the cocci were starved for both these compounds.

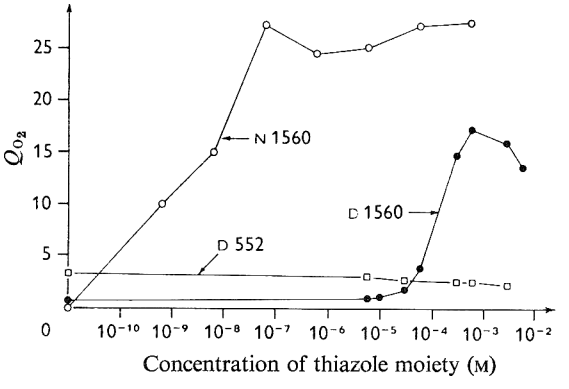

Fig. 3

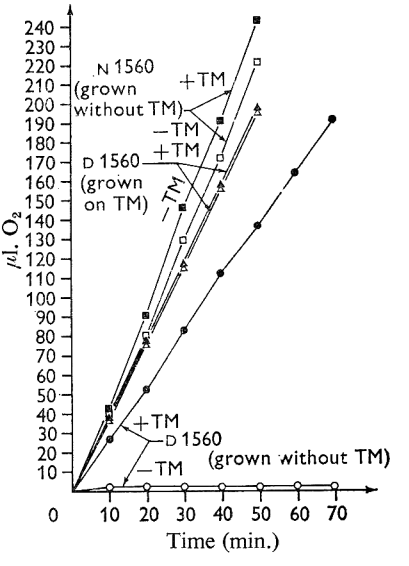

Fig. 4

Fig. 3. Relation between oxygen uptake with pyruvate as substrate by different *Staphylococcus aureus* strains and concentration of the thiazole moiety of thiamine. Concentration of the pyrimidine moiety was 6×10^{-4} M throughout. Strain N1560 was deficient in thiamine after growth in vitamin free casamino acid medium. □—□, strain D552; ●—●, strain D1560; ○—○, strain N1560.

Fig. 4. Oxygen uptake with pyruvate as substrate by *Staphylococcus aureus*, strain N1560 and strain D1560 with and without addition of extraneous thiamine (TM) (3×10^{-6} M). ■—■, Strain N1560 grown without thiamine, with thiamine added. □—□, strain N1560 grown without thiamine, without thiamine. ▲—▲, strain D1560 grown with thiamine, with thiamine added; △—△, strain D1560 grown with thiamine, without thiamine. ●—●, strain D1560 grown without thiamine, with thiamine added; ○—○, strain D1560 grown without thiamine, without thiamine.

By variation of the concentration of the pyrimidine with a constant concentration of the thiazole and vice versa, it was found that the requirement for both moieties was approximately equivalent to the required concentration of thiamine (Fig. 3). Respiration was maximal and linear from the beginning of the experiment, indicating a high ability of the non-proliferating cocci to concentrate the pyrimidine and the thiazole and to synthesize cocarboxylase.

Strain D1560. This variant, when grown in nutrient broth, was unable to respire with pyruvate as substrate unless thiamine was added. The concentration of thiamine needed was equivalent to that required by strain N1560 after growth in CVF medium. Supplementation of nutrient broth with thiamine had the effect that the harvested

cocci respired with pyruvate as substrate, without addition of more thiamine (Fig. 4).

Stimulation of oxygen uptake was obtained to the same extent when the thiazole moiety was substituted for thiamine, but the concentration required was approximately 500 times higher, on a molar basis. The pyrimidine had no influence on respiration with strain D 1560, not even at concentrations required by strain N 1560 (Fig. 5). The 'crude thiazole phosphate' preparation did not stimulate respiration when added at concentrations significantly lower than those at which the thiazole moiety was effective, at higher concentrations its effect on oxygen consumption was as with the unphosphorylated thiazole compound.

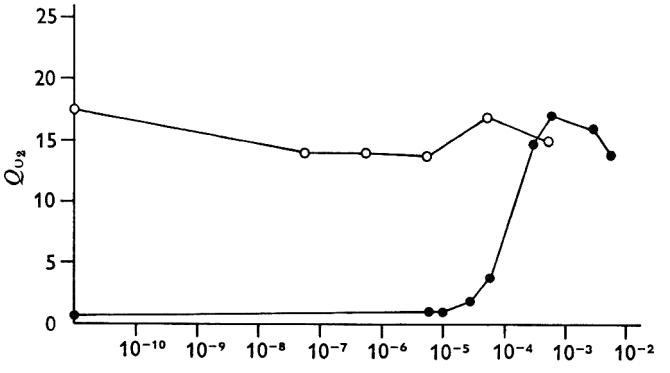

Fig. 5. Oxygen uptake with pyruvate as substrate by *Staphylococcus aureus* D 1560, grown in nutrient broth. ○—○, Concentration of the thiazole moiety of thiamine 6×10^{-4} M; concentration of the pyrimidine moiety is shown on the abscissa. ●—●, Concentration of pyrimidine moiety 6×10^{-4} M; concentration of thiazole moiety shown on the abscissa.

Table 3. *Stimulation of respiration of strain D 551 by 'crude thiazole phosphate' and 'crude pyrimidine pyrophosphate'*

Supplement (M) added to Warburg vessel	Relative Q_{O_2}*
3×10^{-6} thiamine	100
None	5·4
4×10^{-5} crude thiazole phosphate + 6×10^{-4} pyrimidine moiety	7·3
4×10^{-6} crude thiazole phosphate	0·3
4×10^{-6} crude thiazole phosphate + 6×10^{-4} pyrimidine moiety	4·1
2×10^{-5} crude pyrimidine pyrophosphate	45·7
2×10^{-5} crude pyrimidine pyrophosphate + 6×10^{-4} thiazole moiety	38·2
2×10^{-6} crude pyrimidine pyrophosphate	24·0
2×10^{-6} crude pyrimidine pyrophosphate + 6×10^{-4} pyrimidine moiety	6·5
2×10^{-8} crude pyrimidine pyrophosphate	4·9
3×10^{-6} thiamine + 6×10^{-3} pyrimidine moiety	95·2

* Relative Q_{O_2} values are calculated as % of the Q_{O_2} observed with 3×10^{-6} M-thiamine.

Strains D 551 and D 552. These variants behaved exactly like strain D 1560 with respect to requirement for thiamine after growth in nutrient broth. On the other hand, neither the pyrimidine moiety nor the thiazole moiety, nor both together, stimulated oxygen uptake at any concentration up to 4×10^{-3} M. However, a significant stimulation was obtained with the 'crude pyrimidine pyrophosphate' preparation at a concen-

tration corresponding to 2×10^{-6} M pyrimidine. Oxygen uptake with the 'crude pyrimidine phosphate' was never more than 25–50 % of that obtained with thiamine and was strongly antagonized by the unphosphorylated pyrimidine. The thiazole moiety or the 'crude thiazole phosphate' did not stimulate O_2 uptake by themselves and had no influence on O_2 uptake when added to the 'crude pyrimidine phosphate' (Table 3).

DISCUSSION

Cocarboxylase synthesis by *Staphylococcus aureus* (Tsubota, 1964) follows the same pathway as that in baker's yeast (Camiener & Brown, 1960). This may be outlined as follows:

Pyrimidine moiety → pyrimidine pyrophosphate
Thiazole moiety → thiazole phosphate
→ thiamine phosphate → thiamine → thiamine pyrophosphate

Since stimulation of respiration by starved cocci of strain N 1560 with exogenous thiazole and pyrimidine moieties of thiamine was maximal from the moment the substrate (pyruvate) was added, the synthesis of cocarboxylase is apparently rapid, and was probably performed during the pre-incubation period (20 min, see Methods).

During growth in unsupplemented nutrient broth the normal strain, N 1560, builds up an intracellular reserve of thiamine or thiamine pyrophosphate. This reserve must be synthesized from the thiazole and pyrimidine moieties, since thiamine present in the nutrient medium is destroyed during sterilization. The strain D 1560 was deficient for thiamine after growth in nutrient broth, growth of this strain depended probably on traces of thiamine or on a slow synthesis of the vitamin from its breakdown products. The cocci needed extraneous thiazole at a molar concentration about 500 times that of thiamine for maximal respiratory activity. It is, however, of interest that this strain concentrated the pyrimidine moiety intracellularly during growth in nutrient broth, and needed no extraneous supply of this compound for respiration of pyruvate (Fig. 5).

The failure to concentrate thiazole could be explained by (*a*) a defect in the enzyme which phosphorylates the thiazole moiety, or (*b*) lack of permease for this compound. Proof of the first assumption would automatically deny the existence of a permease for the thiazole moiety, since otherwise a defective phosphorylating enzyme would lead to a maximal packing of the cocci with the thiazole moiety. But the fact that respiration of strain D 551 with 'crude pyrimidine phosphate' was independent of extraneous thiazole suggests that in this strain (and probably also in other *Staphylococcus aureus* strains) a permease system for the thiazole moiety does exist. The second explanation is therefore the more likely, and is also in accordance with the fact that 'crude thiazole phosphate' did not stimulate respiration of strain D 1560 at concentrations significantly lower than that needed when the nonphosphorylated thiazole was used.

Strain D 551 was probably deficient in enzyme which converts the pyrimidine moiety to the pyrimidine pyrophosphate. This is indicated by (*a*) failure to decarboxylate pyruvate when given thiazole + pyrimidine moieties even at high concentration, and (*b*) stimulation of oxygen uptake by the product designated 'crude pyrimidine pyrophosphate', which logically should contain a certain amount of the phosphorylated pyrimidine.

The antagonistic effect of non-phosphorylated pyrimidine on 'crude pyrimidine pyrophosphate' might most probably indicate competition for a permease, since the non-phosphorylated compound should have no influence on the thiamine phosphate synthesizing enzyme. That this antagonism was not merely due to a contamination of our pyrimidine compound with some inhibitory chemical was demonstrated by its failure to inhibit the stimulation by thiamine of pyruvate respiration by the same strain (D 551). The suggestion that a permease for the pyrimidine moiety does exist in *Staphylococcus aureus* is also consistent with the intracellular gathering of this compound by the cells of strain D 1560 during growth in nutrient broth (Fig. 5). Although these results offer no conclusive proof, they seem to indicate that permeases for the pyrimidine moiety and the thiazole moiety are present in *Staphylococcus aureus*. These two permease systems are most probably independent of each other since cocci of strain D 1560, which are unable to concentrate the thiazole moiety, nevertheless have enough intracellular pyrimidine moiety for co-enzyme synthesis. The existence of a permease for thiamine in *Lactobacillus fermenti* has recently been shown in a convincing way (Neujahr, 1966).

The authors are indebted to Professor B. Prijs (Basel University) for the generous gift of 4-methyl-5-(β-hydroxyethyl)-thiazole and to Dr S. Yurugi, Research and Development Division, Takeda Chemical Industries, Ltd., Osaka, Japan, and to Dr J. S. Tzoumertis, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, U.S.A., who kindly provided other samples of thiazoles and pyrimidines. Warm appreciation is expressed to Dr H. Lernau, V.M.D., of the Laboratory for Milk Hygiene, Veterinary Institute, Bet Dagon, Israel, for giving us the opportunity to examine D variants isolated by him, as well as for his constant interest in the work, and to Professor A. Pinsky for his help and critical advice in preparing the manuscript.

A part of this investigation was supported by Research Grant 66/81 to Z.E.G. from Bar-Ilan University.

REFERENCES

BARBOUR, R. G. H. (1950). Small colony variants ('G' forms) produced by *Staphylococcus pyogenes* during the development of resistance to streptomycin. *Aust. J. exp. Biol. med. Sci.* **28**, 411.

BROWNING, D. H. & ADAMSON, H. S. (1950). Stable dwarf colony forms produced by *Staphylococcus pyogenes*. *J. Path. Bact.* **62**, 499.

CAMIENER, G. W. & BROWN, G. M. (1960). The biosynthesis of thiamine. II. Fractionation of enzyme system and identification of thiazole monophosphate and thiamine monophosphate as intermediates. *J. biol. Chem.* **235**, 2411.

GAUSE, G. F. (1961). Biochemical changes accompanying impaired respiration in *Staphylococcus aureus*. *Nature, Lond.* **190**, 978.

GLADSTONE, G. P. (1937). The nutrition of *Staphylococcus aureus*: nitrogen requirements. *Br. J. exp. Path.* **18**, 322.

GOUDIE, J. G. & GOUDIE, R. B. (1955). Recurrent infections by a stable dwarf-colony variant of *Staphylococcus aureus*. *J. clin. Path.* **8**, 284.

HALE, J. H. (1947). Studies on staphylococcus mutation: characteristics of the G (gonidial) variant and factors concerned in its production. *Br. J. exp. Path.* **28**, 202.

HALE, J. H. (1949). Studies on staphylococcus mutation: an investigation of the growth requirements of a 'G' (gonidial) variant. *Br. J. exp. Path.* **30**, 297.

HALE, J. H. (1951). Studies on staphylococcus mutation: a naturally occurring 'G' (gonidial) variant and its carbon dioxide requirements. *Br. J. exp. Path.* **32**, 307.

HOFFSTADT, R. E. & YOUMANS, G. P. (1932). *Staphylococcus aureus* dissociation and its relation to infection and immunity. *J. inf. Dis.* **51**, 216.

KNIGHT, B. C. J. G. (1937). The nutrition of *Staphylococcus aureus*. The activities of nicotinamide, aneurin (vitamin B 1), and related compounds. *Biochem. J.* **31**, 966.

LERNAU, H. & SOMPOLINSKY, D. (1962). Bovine mastitis due to dwarf colony variants of *Staphylococcus aureus*. *Cornell Vet.* **52**, 445.

NEUJAHR, H. Y. (1966). Transport of B-vitamins in micro-organisms. III. Chromatographic studies on the radioactivity extracted from non-proliferating cells of *Lactobacillus fermenti* after exposure to labelled thiamine. *Acta chem. scand.* **20**, 786.

SHERRIS, J. C. (1952). Two small colony variants of *Staphylococcus aureus* isolated in pure culture from closed infected lesions and their carbon dioxide requirements. *J. clin. Path.* **5**, 354.

SOMPOLINSKY, D. & LEVY, M. (1961). Further studies on the metabolism of D-colony forms of *Staphylococcus aureus*. *Bull. Res. Coun. Israel*, sect. E, **9**, 3.

THOMAS, M. E. M. & COWLARD, J. H. (1955). Studies on a CO_2-dependent Staphylococcus. *J. clin. Path.* **8**, 288.

TSUBOTA, Y. (1964). Studies on the enzymatic synthesis of thiamine by *S. aureus*. *Bitamin* (*Kyoto*) **29**, 510 (Japan). Quoted from *Chem. Abstr.*, 1965, **62**, 1990.

WISE, R. J. & SPINK, W. W. (1954). The influence of antibiotics on the origin of small colonies (G variants) of *Micrococcus pyogenes* var. *aureus*. *J. clin. Invest.* **33**, 1611.

YOUMANS, G. P. & DELVES, E. (1942). The effect of inorganic salts on the production of small colony variants by *Staphylococcus aureus*. *J. Bact.* **44**, 127.

J. gen. Microbiol. (1967), 48, 215–224
Printed in Great Britain

Development of Competence in Cultures of *Bacillus subtilis* Inoculated with Different Numbers of Bacteria

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(*Accepted for publication* 8 *March* 1967)

SUMMARY

The influence of casein hydrolysate, tryptophan, glucose, magnesium sulphate and different buffer solutions on the rate of multiplication of *Bacillus subtilis* strain 168 was determined. Inoculum size was shown to influence the generation time and the increase in the mass of bacteria was found to be inversely proportional to the size of the inoculum.

Bacillus subtilis 168 forms chains especially in minimal media. In complete media chains are formed by only a small proportion of the bacteria and sometimes not at all.

A simple transformation method was used to follow the development of competence in cultures of *Bacillus subtilis* 168 in media inoculated with different numbers of bacteria.

The estimation of the number of transformants was subject to greater error than estimation of the viable count.

This error in the estimation of the number of transformants is due mainly to the thread-like character of DNA, which is difficult to dilute accurately. The smaller the inoculum of bacteria, the shorter the time for the rise phase of competence. Optimal competence is reached when about 10^6 bacteria/ml. are inoculated into the medium.

The rise phase of competence takes a shorter time than the decline phase.

An equation was developed from which it is easy to calculate approximately the onset of the peak of competence. Precise determination of this peak can be achieved by controlling the density of the bacterial suspension.

INTRODUCTION

Genetic transformation has been demonstrated in: *Streptococcus pneumoniae* (Hotchkiss, 1957); *Haemophilus influenzae* (Goodgal & Herriott, 1961); *Neisseria meningitidis* (Alexander & Redman, 1953); *N. catarrhalis* (Catlin & Cunningham, 1964); Agrobacterium (Klein & Klein, 1953); Xanthomonas (Corey & Starr, 1957); Rhizobium (Balassa, 1954; Balassa, 1960); Streptococcus (Bracco, Krauss, Roe & MacLeod, 1957); and in *Bacillus subtilis* (Spizizen, 1958). DNA isolated from donor bacteria is taken up by the recipient organisms during the physiological state known as competence.

From the literature concerning competence it is apparent that several investigators have found different optimal conditions for transformation (Jensen & Haas, 1963; Ephrussi-Taylor & Freed, 1964; Tomasz, 1965). It seems that the factors influencing transformation are not completely known.

Competent bacteria can be transformed by DNA isolated from donor bacteria. DNA from bacteriophage (Földes & Trautner, 1964; Harm & Rupert, 1963) and

virus (Abel & Trautner, 1964; Bayreuther & Romig, 1964) can be incorporated into competent bacteria and can replicate in them. Competence is therefore an important field of investigation.

In media inoculated with a small number of *Bacillus subtilis* 168, (Burkholder & Giles 1947) we found that the organisms divided more times than when the medium was inoculated with a large number of bacteria. Of course, the increase in the mass of bacteria was also greater in media inoculated with a smaller number of bacteria. Their evidence also suggested that there were differences in generation time for bacteria in cultures inoculated with different numbers of organisms. These changes in generation time were of interest to us because it seemed that they may play an important part in the development of competence.

Therefore experiments were carried out to investigate in detail the relationship between generation time and the development of competence.

METHODS

Bacterial strains. For transformation the recipient strain *Bacillus subtilis* 168 *try*$^-$, and the donor strain *B. subtilis* MARBURG were both kindly provided by Dr B. S. Strauss. (See Burkholder & Giles, 1947; Spizizen, 1958; Nester, 1964).

Media. (*a*). Bacterial strains were maintained on potato agar (Spizizen, 1958; Nester, 1964).

(*b*) Minimal glucose yeast agar medium (MGY agar): 0·2 % $(NH_4)_2SO_4$, 1·4 % (w/v) K_2HPO_4, 0·6 % KH_2PO_4, 0·1 % Na-citrate.$2H_2O$, 0·02 % $MgSO_4.7H_2O$, 0·5 % glucose, 5 μg./ml. L-tryptophan, 0·1 % casein hydrolysate (Difco), 0·2 % yeast extract (Difco), and 1 75 % (w/v) agar.

(*c*) The development of competence was investigated in MGY liquid medium.

(*d*) T-medium was used for transformation: 1·4 % (w/v) K_2HPO_4, 0·6 % KH_2PO_4, 0·5 % glucose, 0·1 % casein hydrolysate, 2·5 μg./ml. L-tryptophan and 5 μmoles/ml. $MgSO_4.7H_2O$.

(*e*) MG agar: MGY agar without L-tryptophan and yeast extract, containing 0·02 % casein hydrolysate was used for the selection of transformants.

Chain formation. Smears of the bacteria were made, fixed and stained with gentian violet for 2 min. and examined by the light microscope. The results were analysed statistically by expressing the number of bacteria per chain in log to the base 2 units since chain lengths were related by a factor of two.

Preparation of transforming DNA. 10^7 organisms of *Bacillus subtilis* Marburg grown in log. phase were inoculated into 250 ml. MGY medium without tryptophan in four 1 l. Erlenmeyer flasks and incubated at 30° for 15 hr. with mechanical shaking. When the cultures contained about 10^9 bacilli/ml., the suspensions were centrifuged and DNA was isolated by the phenol extraction method of Saito & Miura (1963).

Determination of competence. Bacteria from potato agar were inoculated onto MGY agar slopes and incubated for 16 hr at 37°. The growth was scraped off, suspended in MGY nutrient medium and adjusted to a suitable optical density: 10 ml. of this suspension were measured into 100 ml. Erlenmeyer flasks fitted with side arms for optical density measurements. The bacterial suspensions were incubated in a water bath at 37° on a reciprocal shaker at 100 rev./min.

During growth, samples were taken and assayed for the number of competent

bacteria by measuring the efficiency of transformation to prototrophy under standard conditions.

Transformation procedure. The samples were adjusted with T-medium to 0·1 optical density (equivalent to $5{\cdot}9 \times 10^7$ colony forming units/ml.). To 0·25 ml. of this suspension 0·75 ml. of DNA solution in T-medium was added to give a final concentration of DNA about 1 μg./ml.

This mixture was shaken for 15 min. in a water bath at 37° and 0·2 ml. was then plated on MG agar about 1 or 2 cm. from the edge of the Petri dish. The dish was kept on an inclined plane and gently shaken to distribute the suspension over the agar surface, avoiding the use of a glass spreader. This procedure took 10–15 sec. The surface of the plates inoculated in such a way was dried off at 37° for 20–25 min. The final dilution of the bacterial suspension was at least 1/1500 at which dilution the final concentrations of tryptophan and yeast extract from the inoculum were not sufficient to permit the growth of the recipient *Bacillus subtilis* 168 *try*$^-$ strain. Only prototrophic *try*$^+$ transformants could grow; the number of such transformants was counted on the following day.

RESULTS

The effect of casein hydrolysate, tryptophan and pH on the growth of Bacillus subtilis 168.

Since it has been suggested that casein hydrolysate plays an essential role in the development of competence in *Bacillus subtilis* (Anagnostopoulos & Spizizen, 1961), the effects of various concentrations of casein hydrolysate on the growth of *B. subtilis* were investigated. Optical density measurements on cultures after 8 hr growth in casein hydrolysate show that the lag phase is shorter and the log rise steeper in high concentrations of casein hydrolysate compared to low concentrations (Fig. 1).

5 μg./ml. L-tryptophan was sufficient to give maximum growth of *Bacillus subtilis* 168 *try*$^-$ and the density of cultures did not change until the tryptophan concentration exceeded 80 μg./ml. Glucose and $MgSO_4 . 7H_2O$ had very little or no effect on the growth of *B. subtilis*. Nutrient media containing 0·02% casein hydrolysate, 0·5% glucose and 50 μg./ml. L-tryptophan and buffered with phosphate at different pH values from 5·59 to 8·04 were used for the cultivation of *Bacillus subtilis* 168. Optimal multiplication of the bacteria was found in nutrient medium at pH 6·8–7·0.

The effect of inoculum size on the growth of Bacillus subtilis

To test the effect of inoculum size on the growth of *Bacillus subtilis* different numbers of bacteria were seeded into media containing 0·5% glucose, 0·04% casein hydrolysate, 5 μg/.ml. L-tryptophan and 80% (v/v) M/20 tris-HCl buffer at pH 7·1. Incubation was continued for 10 hr and the growth curves obtained are shown in Fig. 2.

The biggest increase in bacterial mass was found when the lowest numbers of bacteria ($2{\cdot}95 \times 10^6$) were inoculated into the medium (Fig. 3). The data also show that the bacteria had to divide at least four times in the case of the smallest inoculum, and only once in the case of the largest one. These results are in agreement with the estimates of division made from the lengths of the bacterial chains found during cultivation.

The changes in the generation times of these bacteria were determined from the growth curves obtained in this same experiment and are shown in Fig. 4. This figure

shows clearly that there are big differences in generation time for bacteria in cultures grown from different sized inocula.

Chain formation by Bacillus subtilis

We paid special attention to chain formation because *B. subtilis* 168 forms long chains under certain conditions. To obtain accurate colony counts in transformation experiments therefore it is important to use suspensions of single bacteria. In liquid MGY nutrient meduim chains increased up to the beginning of the log. phase, but thereafter single bacteria were produced (Fig. 5). Colony counts give an accurate estimate of the numbers of viable bacteria in these suspensions.

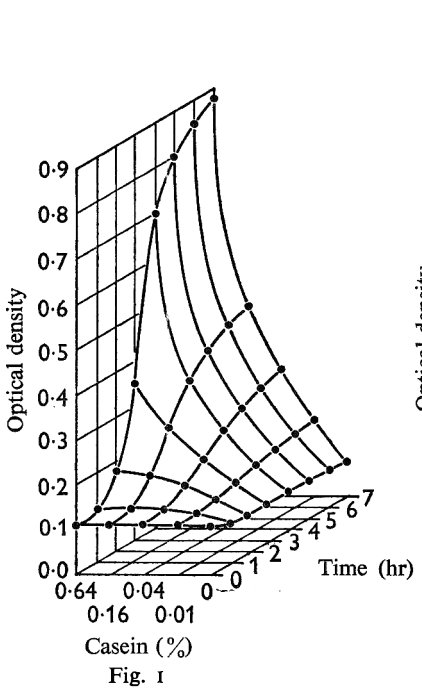

Fig. 1

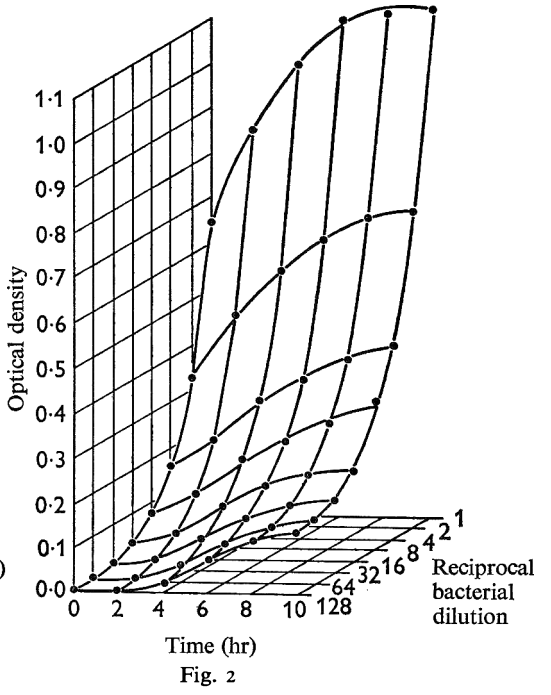

Fig. 2

Fig. 1. Growth curve of *Bacillus subtilis* in 80 % (v/v) tris-HCl buffer at pH 7·1, 0·5 % (w/v) glucose, 5 μg./ml. L-tryptophan and various concentrations of casein hydrolysate.

Fig. 2. The effect of inoculum size on the growth of *Bacillus subtilis* in 80 % (v/v) tris-HCl buffer at pH 7·1 containing 0·5 % (w/v) glucose, 0·04 % casein hydrolysate and 5 μg./ml. tryptophan. The medium was inoculated from serial twofold dilutions of a suspension of $3{\cdot}8 \times 10^8$ bacteria/ml.

Transformation procedure

DNAse was not used after the 15 min. incubation period with DNA. If the transformation system does not contain tryptophan, transformants do not appear. This was demonstrated by the following experiment.

To samples of T-medium, containing different quantities of tryptophan and 2 μg. DNA/ml., equal volumes of bacterial suspensions (0·2 optical density units) in T-medium without tryptophan were added. These suspensions were shaken for 15 min. at 37° and plated on MG agar for the recovery of transformants. The results are illustrated in Fig. 6. It appears that transformation does not occur in media containing very

small amounts of tryptophan. Plating slowly stops further transformations, because the tryptophan became diluted. In control experiments in which the bacterial suspension did not contain any DNA, *try*$^+$ colonies were not found at tryptophan concentra-

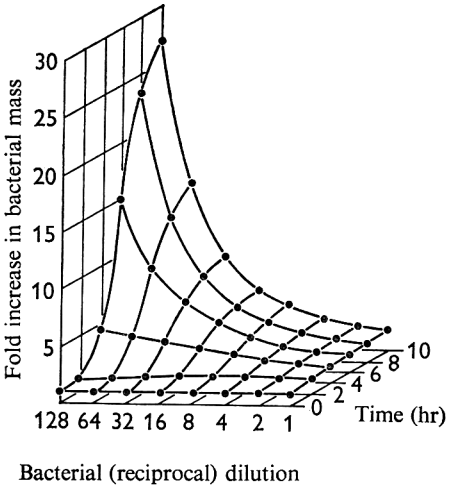

Fig. 3

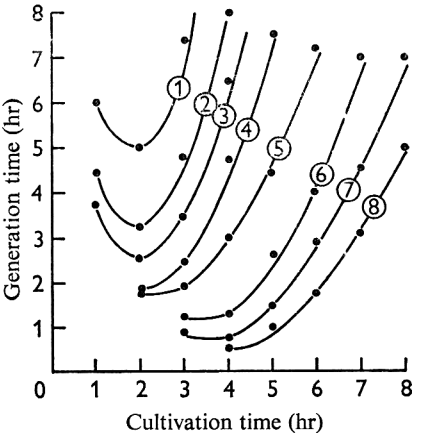

Fig. 4

Fig. 3. Increase in the bacterial mass of *Bacillus subtilis* cultures started from different inocula calculated from the data in Fig. 2.

Fig. 4. Changes in the generation time of *Bacillus subtilis* in cultures started from different inocula calculated from the growth curves in Fig. 2.

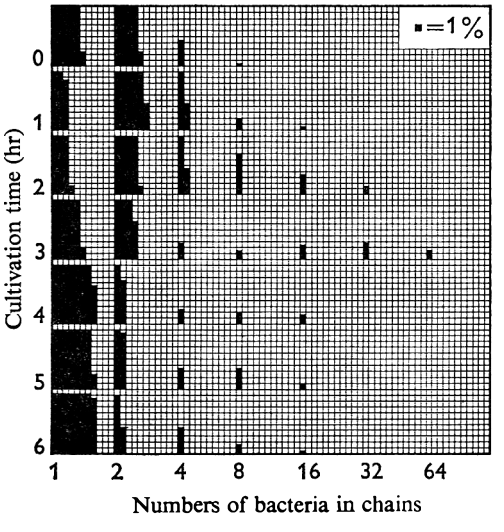

Fig. 5

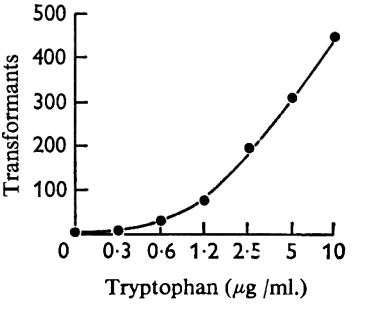

Fig. 6

Fig. 5. Percentage distribution of chain length in 6 hr cultures of *Bacillus subtilis*. The bacteria were grown in MGY nutrient medium.

Fig. 6. The effect of various concentrations of tryptophan on the yield of *try*$^+$ transformants from *Bacillus subtilis* 168 *try*$^-$.

tions up to 10 μg./ml. For this reason 2·5 μg. tryptophan/ml. was used in T-medium for transformation.

The thread-like nature and viscosity of the DNA make it very difficult to dilute accurately, and even after diluting with great care the numbers of transformants vary greatly in parallel experiments. To determine this error 0·75 ml. of DNA diluted in T-medium was measured into each of 20 tubes and 0·25 ml. of a competent cell suspension (0·1 optical density units) added to each tube. The final concentration of DNA was 1 μg./ml. After incubation the mixture was plated out on MG agar for recovery of transformants. The average number of transformants per plate was 146 with a standard deviation of ±85.

Viable counts were determined on 27 samples from the same suspension. The average number of colonies per plate was 100 with a standard deviation of ±23. These results show that in estimating the number of transformants there are greater errors than in estimating the number of viable bacteria. If there are chains of bacteria the accuracy of the viable count will be even less.

Determination of the time of development of competence

A bacterial suspension was inoculated into MGY medium at 0·1 optical density units and incubated at 37°. At regular intervals samples were moved and the competence of the culture assayed by measuring the number of transformants obtained by the standard transformation procedure.

The development of competence is shown in Fig. 7 together with the changes in the generation times of the bacteria in these cultures. The shaded area of the figure represents the results obtained from 75 samples. It is clear that there was some regularity in the development of competence and that it was related to the changes in generation time. Three phases can be differentiated in the development of competence, the 'silent' phase (Fig. 7A, B), the 'rise' phase (Fig. 7B, C) and the 'decline' phase (Fig. 7C, D). The most interesting points are B, the beginning, and C, the peak of competence.

The steepness of the rise phase of competence was characterized by the time which was necessary to obtain a twofold increase in competence. This value can be calculated from the following equation:

$$C_i = t/N,$$

where

C_i = the twofold increase of competence in min.
t = the time of the rise phase of competence in min.
N = the number of transformants at the peak of competence in log. to the base 2 units.

In all the experiments the rise periods of competence were not quite straight lines on a semilogarithmic plot, but slightly curved. This clearly shows that more than one factor influences competence.

The development of competence in cultures inoculated with different numbers of bacteria

Different numbers of bacteria were inoculated into MGY medium. The bacterial suspensions were incubated in a water bath at 37° on a reciprocal shaker. At regular intervals samples were removed and adjusted with Y-medium to 0·1 optical density units. To 0·25 ml. of this suspension 0·75 ml. DNA solution was added; the final

concentration of DNA was about 1 μg./ml. This mixture was shaken for 15 min. at 37° and 0·2 ml. was then plated on MG agar for transformation. Figure 8 shows the changes in optical density and the shaded area of the figure shows the rise phase of competence. From this experiment the generation times were determined and results in Fig. 9 show that the smaller the inoculum of bacteria, the shorter the time for the

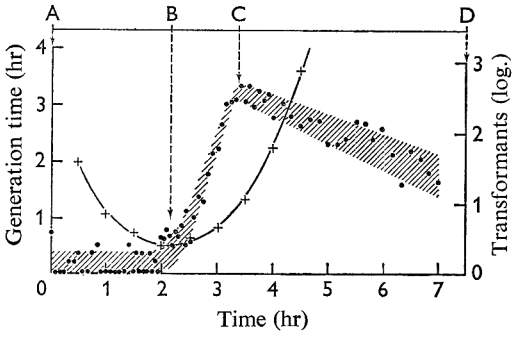

Fig. 7

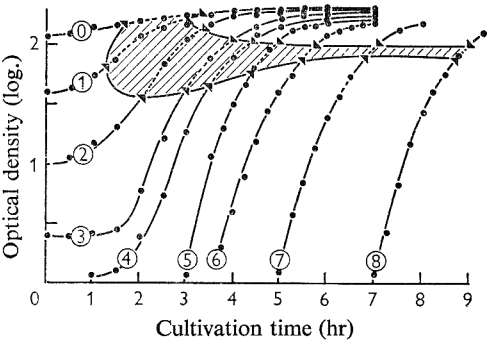

Fig. 8

Fig. 7. Changes in generation time and the development of competence in cultures of *Bacillus subtilis* 168 *try*⁻. The shaded area represents the development of competence.

Fig. 8. Changes in optical density and the development of competence in cultures of *Bacillus subtilis* 168 *try*⁻ inoculated with different numbers of bacteria. Bacteria/ml. at the start of the culture: curve 0, $6{\cdot}93 \times 10^8$; curve 1, $2{\cdot}35 \times 10^8$; curve 2, $5{\cdot}90 \times 10^7$; curve 3, $1{\cdot}49 \times 10^7$; curve 4, $3{\cdot}72 \times 10^6$; curve 5, $9{\cdot}35 \times 10^5$; curve 6, $1{\cdot}18 \times 10^5$; curve 7, $7{\cdot}45 \times 10^3$; curve 8, $1{\cdot}00 \times 10^2$. The shaded area represents the rise phase of competence.

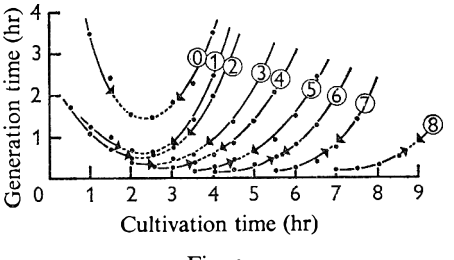

Fig. 9

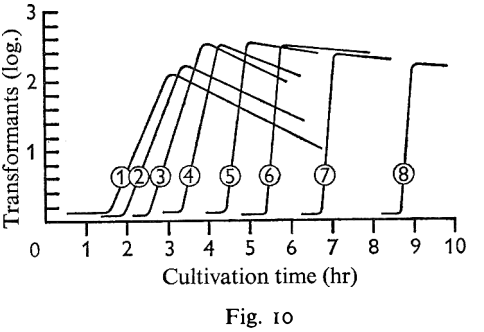

Fig. 10

Fig. 9. The changes in generation time and rise phase of competence in cultures of *Bacillus subtilis* 168 *try*⁻ inoculated with different numbers of bacteria. The dotted line represents the rise phase of competence. For details of inocula see Fig. 8.

Fig. 10. Development of competence in cultures of *Bacillus subtilis* 168 *try*⁻ inoculated with different numbers of bacteria. For details of inocula see Fig. 8.

rise phase of competence. The generation time was about 45–60 min. except for the culture with the largest inoculum. Nester also found that optimal competence was obtained for cultures with a generation time of about 45 min. (Spizizen, 1958).

Curves showing the development of competence in cultures from this experiment are shown in Fig. 10. The rise phase of competence was steep and appeared late in

cultures inoculated with a small quantity of bacteria. The time which was necessary for a twofold increase in competence in cultures inoculated with different numbers of bacteria is shown in Fig. 11; it was very short for cultures started with a small inoculum of bacteria. The numbers of transformants scored at the peaks of competence are also given in Fig. 11. Optimal competence appeared when about 10^6 bacteria/ml. were inoculated at the beginning of the experiment. The number of transformants obtained during the rise phase of competence is shown in Fig. 12, as % of the total.

The rise phase of competence is shown in Fig. 13 for cultures started with different bacterial inocula. The upper line connecting the peaks of competence is straight; except for cultures inoculated with a very large number of bacteria, it was calculated by the least square method.

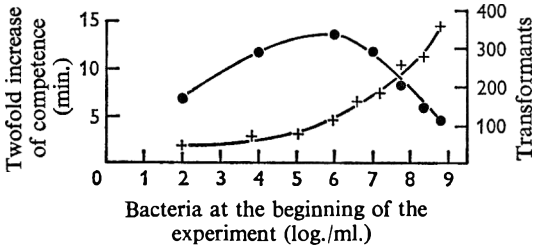

Fig. 11

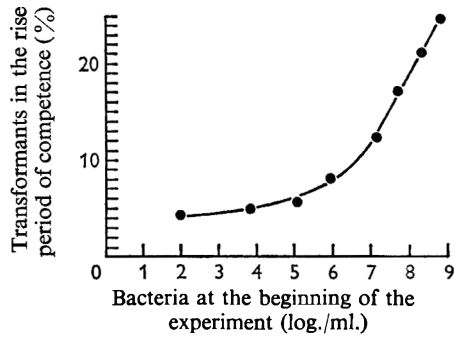

Fig. 12

Fig. 11. The time required for a twofold increase in competence and the number of transformants at the peak of competence for cultures of *Bacillus subtilis* 168 *try*$^-$ inoculated with different numbers of bacteria. +—+, increase in competence; ●—●, number of transformants.

Fig. 12. The number of transformants in the rise phase of competence expressed as a percentage of the total number of transformants for cultures of *Bacillus subtilis* 168 *try*$^-$ inoculated with different numbers of bacteria.

On the basis of simple considerations an equation was developed from which it is easy to calculate approximately the time necessary to obtain optimal competence in a culture:

$$C_p = (a-b).tg\alpha,$$

where

C_p = the time of incubation in hr to reach the peak in competence.
a = the distance in log. units where the straight line intersects the abscissa (in Fig. 13 approximately 11·26),
b = the number of bacteria/ml. in log. units inoculated into the medium,
$tg\alpha$ = the tangent of the straight line (in Fig. 13 approximately 0·95).

The values of a and $tg\alpha$ show some variation in different media, so it is important to standardize experimental conditions in these experiments. But even under standard conditions there is some variation in the time required to reach optimal competence when calculated according to the equation. The higher the inoculum of bacteria the smaller the error. When a small number of bacteria were inoculated, the error was about ±5 to 10 min. In these circumstances the peak of competence can only be calculated approximately because the rise phase of competence is short—about 15–20 min.

In practice it was easy to determine optimal competence by controlling the optical density of the bacterial suspension. In Fig. 14 the optical density values are shown at the start and at the peaks of competence for cultures inoculated with different numbers of bacteria. The lower curve in Fig. 14 connects the points at the beginning of competence and the upper curve connects the points at the peaks of competence. Using the upper curve the optimal values of competence can be determined with a deviation of ± 2 to 3 min.

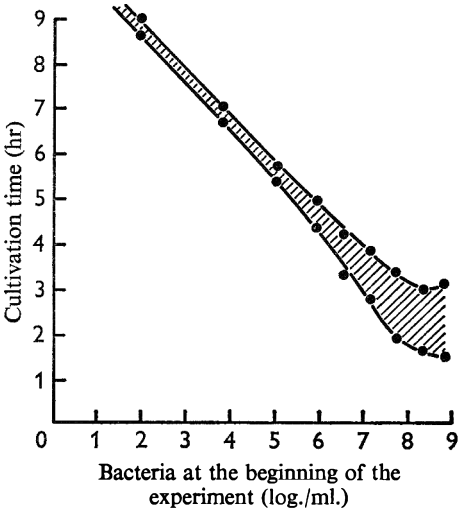

Fig. 13

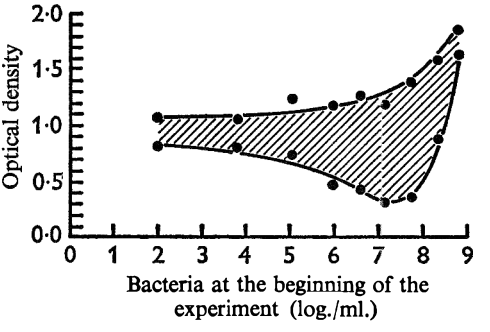

Fig. 14

Fig. 13. Time required to reach the rise phase of competence in cultures of *Bacillus subtilis* 168 *try*$^-$ inoculated with different numbers of bacteria.

Fig. 14. Optical density values at the start and at the peaks of competence for cultures of *Bacillus subtilis* inoculated with different numbers of bacteria.

I should like to express my gratitude to Director Dr B. Györffy for his valuable instruction and to I. Kállay for her expert technical assistance.

REFERENCES

ABEL, P. & TRAUTNER, T. A. (1964). Formation of an animal virus within a bacterium. *Z. VererbLehre* **95**, 66.

ALEXANDER, H. E. & REDMAN, W. (1953). Transformation of type specificity of meningococci. Change in heritable type induced by type specific extracts containing desoxyribonucleic acid. *J. exp. Med.* **97**, 797.

ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *J. Bact.* **81**, 741.

BALASSA, R. (1954). Transformationsmechanismen der Rhizobien. *Acta microbiol. hung.* **2**, 51.

BALASSA, R. (1960). Transformation of a strain of *Rhizobium lupini*. *Nature, Lond.* **188**, 246.

BAYREUTHER, K. E. & ROMIG, W. R. (1964). Polyoma virus: production in *Bacillus subtilis*. *Science, N.Y.* **146**, 778.

BRACCO, R. M., KRAUSS, M. R., ROE, A. S. & MACLEOD, C. M. (1957). Transformation reactions between pneumococcus and three strains of streptococci. *J. exp. Med.* **106**, 247.

BURKHOLDER, P. R. & GILES, N. H. (1947). Induced biochemical mutations in *Bacillus subtilis*. *Am. J. Bot.* **34**, 345.

CATLIN, B. W. & CUNNINGHAM, L. S. (1964). Genetic transformation of *Neisseria catarrhalis* by deoxyribonucleate preparations having different average base composition. *J. gen. Microbiol.* **37**, 341.

COREY, R. R. & STARR, M. P. (1957). Genetic transformation of Neisseria by culture slime containing deoxyribonucleate. *Science, N.Y.* **131**, 608.

EPHRUSSI-TAYLOR, H. & FREED, B. A. (1964). Incorporation of thymidine and amino acids into deoxyribonucleic acid and acid-insoluble cell structures in pneumococcal cultures synchronized for competence to transform. *J. Bact.* **87**, 1211.

FÖLDES, J. & TRAUTNER, T. A. (1964). Infectious DNA from a newly isolated *Bacillus subtilis* phage. *Z. VererbLehre.* **95**, 57.

GOODGAL, S. H. & HERRIOTT, R. M. (1961). Studies on transformation of *Haemophilus influenzae*. I. Competence. *J. gen. Physiol.* **44**, 1201.

HARM, W. & RUPERT, C. S. (1963). Infection of transformable cells of *Haemophilus influenzae* by bacteriophage and bacteriophage DNA. *Z. VererLehre* **94**, 336.

HOTCHKISS, R. D. (1957). Criteria for quantitative genetic transformation of bacteria. In *The Chemical Basis of Heredity*, p. 321. Baltimore: John Hopkins Press.

JENSEN, R. A. & HAAS, F. L. (1963). Electrokinetics and cell physiology. II. Relationship of surface change to onset of bacterial competence for genetic transformation. *J. Bact.* **86**, 79.

KLEIN, D. T. & KLEIN, R. M. (1953). Transmittance of tumorinducing ability to avirulent crown-gall and related bacteria. *J. Bact.* **66**, 220.

NESTER, E. W. (1964). Penicillin resistance of competent cells in deoxyribonucleic acid transformation of *Bacillus subtilis*. *J. Bact.* **87**, 867.

SAITO, M. & MIURA, K. I. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. biophys. Acta* **72**, 619.

SPIZIZEN, J. (1958). Transformation of a biochemically deficient strain of *Bacillus subtilis* by deoxyribonucleate. *Proc. natn. Acad. Sci. U.S.A.* **44**, 1072.

TOMASZ, A. (1965). Control of the competent state in Pneumococcus by a hormone like cell product: an example for a new type of regulatory mechanism in bacteria. *Science, N.Y.* **208**, 155.

J. gen. Microbiol. (1967), **48**, 225–233
With 2 plates
Printed in Great Britain

Studies of a Receptor for Felix 0–1 Phage in *Salmonella minnesota*

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(*Accepted for publication* 8 *March* 1967)

SUMMARY

Isolated lipopolysaccharide from a rough mutant of *Salmonella minnesota* was shown to act as a receptor for Felix 0–1 bacteriophage. The isolated receptor structure could be dissociated by sodium deoxycholate with subsequent loss of receptor activity. When the deoxycholate was removed by dialysis the receptor activity was restored. Ultrasonic treatment destroyed the receptor activity and electron micrographs indicated that the ultrasonic treatment had altered the structure of the lipopolysaccharide. The experiments suggest that the inactivation of phages can be used as a test of the biological activity of a lipopolysaccharide. The interaction between the 0–1 phage and the receptor structure was also studied with the aid of electron microscopy.

INTRODUCTION

The somatic polysaccharide antigens in the cell wall of enterobacteria were shown to act as receptors for bacteriophages by Levine & Frisch (1933*a*, *b*, 1934) and Burnet (1934). Weidel (1951) and Hotchin, Dawson & Elford (1952) observed the direct attachment of phages to isolated cell walls by examination in the electron microscope. Since then, the binding of phages to isolated cell walls as well as to purified receptor structures has been observed for a variety of Gram-positive and Gram-negative bacteria.

Felix 0–1 phage (Felix & Callow, 1943) lyses 96–99·5% of Salmonella strains studied (Cherry, Davis, Edwards & Hogan, 1954; Thal & Kallings, 1955; Seidel, 1956). Some details of the particular relationship between the 0–1 phage and salmonellas have been investigated (Kallings, 1967; Kallings & Lindberg, 1967). The 0–1 phage adsorbs to and lyses the rough mutant *Salmonella minnesota* R60, while it does not adsorb to or lyse another rough mutant *S. minnesota* R345. According to chemical analysis (Lüderitz *et al.* 1965) the difference between these two rough organisms is that the polysaccharide of strain R60 contains 2-keto-3-deoxyoctonate (KDO), heptose, galactose, glucose and glucosamine, whereas the polysaccharide of strain R345 contains only KDO, heptose, galactose and glucose.

As shown by Oroszlan & Mora (1963) and Ribi *et al.* (1966) surface-active agents such as sodium laurylsulphate and sodium deoxycholate have the ability to dissociate endotoxins. The polysaccharide and lipopolysaccharide complexes are dissociated into subunits with concomitant loss of biological activity. When the agent is removed the subunits reaggregate to macromolecular complexes and the biological activity reappears.

The present paper forms part of the studies on phage 0–1 and Salmonella initiated by Dr L. O. Kallings, and describes the isolation from *Salmonella minnesota* of a lipopolysaccharide receptor structure for phage 0–1. The receptor activity of the isolated material was measured by its inactivation of the 0–1 phage. The depolymerization of the receptor structure by sodium deoxycholate was found to be correlated with a decrease in the 0–1 phage-inactivating capacity of the preparation. Upon removal of the surface-active agent the 0–1 phage-inactivating ability was partly restored. The effect of ultrasonic treatment of the receptor structure was examined and the interaction between the 0–1 phage and the receptor structure studied by electron microscopy.

METHODS

Bacteria. Salmonella minnesota strains R60 and R345 were obtained from Dr L. O. Lüderitz (Max-Planck-Institut für Immunbiologie, Freiburg, Germany).

Phage. Felix 0–1 phage was propagated on *Salmonella paratyphi* strain B76 by the agar layer method (Adams, 1959). The phage was purified and concentrated by alternate cycles in a Christ Omega preparative ultracentrifuge at 5000 *g* to sediment debris, and at 30,000 *g* to sediment phage. The phage pellet was suspended in 0·01 M-phosphate buffer (pH 7·2). The purified stock had a titre of $2{\cdot}6 \times 10^{10}$ plaque-forming units (p.f.u./ml.). The phage suspension was stored at +4°.

Media. The defined glucose+salts medium M 9 Adams (1959) was used for continuous cultivation of salmonella strains. This medium without glucose was used in the 0–1 phage inactivation experiments and as a diluent. Nutrient broth (Difco) was used for the growth of *Salmonella paratyphi* B76.

Preparation of lipopolysaccharide. Acetone-killed and dried bacteria were extracted by a modification of the phenol+water method of Westphal, Lüderitz & Bister (1952). The collected aqueous layers were washed five times with chilled ether to remove residual phenol. Nitrogen was passed through the solution to remove ether. The opalescent solution was freeze-dried. A 2 % (w/v) suspension of the material in distilled water was centrifuged at 105,000 *g* for 4 hr in a Christ Omega preparative ultracentrifuge. The sediment was washed twice with water and recentrifuged. The pellet was freeze-dried. This lipopolysaccharide preparation (LPS) was used in the experiments.

Inactivation of phage 0–1 by the lipopolysaccharide. The LPS was diluted in medium M 9 in twofold dilution steps. The 0–1 phage was diluted in medium M 9 to 2×10^3 p.f.u./ml., which was the concentration used for inactivation experiments. One ml. of each LPS dilution + 1·0 ml. 0–1 phage suspension were mixed and incubated in a water bath at 37° for 60 min. Assay of unadsorbed 0–1 phage was performed by adding 1·0 ml. of the LPS+phage mixture to 8·5 ml. melted 0·6 (w/v) agar and 0·5 ml. of a 4 hr broth culture of the indicator strain (*Salmonella paratyphi* B76). Two ml. of this mixture was poured on agar plates. After overnight incubation at 37°, the plaque numbers were recorded. The experiments were done in duplicate. The inactivation of the 0–1 phage was determined by comparing with controls where the LPS was replaced by the defined M 9 medium. The activity of the lipopolysaccharide was expressed as the concentration of LPS (dry wt/volume) causing 50 % inhibition of the 0–1 phage under the experimental conditions (PhI 50; Beumer & Dirkx, 1960).

Treatment of lipopolysaccharide with sodium deoxycholate (SDC). The LPS was

dissolved as completely as possible in 0·1 M-tris + HCl buffer (pH 8·0). SDC dissolved in 0·1 M-tris buffer was added to the LPS solution to a final concentration of 0·5 %. The mixture was kept at 4° overnight. The LPS + SDC suspension was dialysed three times against 0·1 M-tris-buffer (pH 8·0) followed by dialysis five times against medium M 9 (pH 7·4 at 4°).

Ultrasonic treatment of lipopolysaccharide. The LPS was dissolved as completely as possible in distilled water to give a 0·8 % (w/v) suspension. The suspension was kept in an ice-water bath and was treated in a water-cooled M.S.E. (Measuring & Scientific Equipment Ltd, London) 500 W. ultrasonic disintegrator at 20 kc./s. for 5 min. During this procedure the temperature did not rise above 46°. A sample was removed and tested immediately for 0–1 phage inactivating ability and examined in the electron microscope.

Electron microscopy. The negative-staining technique (Brenner & Horne, 1959) with 2 % (w/v) sodium tungstic silicate (STS; pH 7·2) as the staining medium was used. For increasing the spreading quality of the suspension 0·1 % (w/v) bovine serum albumin was added to the STS solution. A thin layer of carbon was evaporated on to the surface of 400-mesh Formvar-coated specimen grids, from which the Formvar was subsequently dissolved away with amyl acetate. The phage and lipopolysaccharide materials were mixed with STS and applied as a drop on to the specimen grid. The solution remained on the grids for 60–120 sec. before the excess liquid was withdrawn, leaving a thin film. The specimens were examined with a JEM–5 Y electron microscope at a magnification of × 40,000.

RESULTS

Lipopolysaccharide preparations from *Salmonella minnesota* strains R 60 and R 345 were tested for their inactivating capacity towards phage 0–1. The experiments were made in a salt solution (M 9 medium without glucose) which gave a maximum adsorption of the 0–1 phage. Fig. 1 shows that the concentration of LPS from strain R 60 necessary to inactivate 50 % of the phage (PhI 50) varied from 0·4 to 0·9 μg. LPS/ml. for different preparations. Repeated experiments with LPS from strain R 345 showed no inactivation of the 0–1 phage, even in suspensions containing 1·0 mg. LPS/ml. These experiments suggest that LPS from strain R 60 acted as a receptor for the 0–1 phage while LPS from strain R 345 did not.

The interaction between the phage and receptor was observed with the electron microscope. Plate 1, figs. 1, 2 show the 0–1 phage with a polyhedral head, 60 mμ in diameter, a tail 90–100 mμ length with a base plate 25 mμ wide with 6 tail fibres, generally extended. Plate 1, fig. 3 shows the 0–1 phage adsorbed on to LPS of strain R 60. The LPS is seen as long filaments several microns in length and 60–90 Å wide. The 0–1 phage head is empty, clearly showing its polyhedral shape, the tail sheaths have contracted to a length of about 50 mμ. The contracted sheaths display longitudinal striations in contrast to the cross striations seen in extended tail sheaths. No tail fibres are visible. Plate 2, fig. 4 shows the 0–1 phage and LPS from strain R 345. The LPS from R 345 shows the same modal pattern as the LPS of R 60, with long filaments of a rather uniform width. None of the phages has been adsorbed on to the LPS; they are seen with uncontracted tail sheaths.

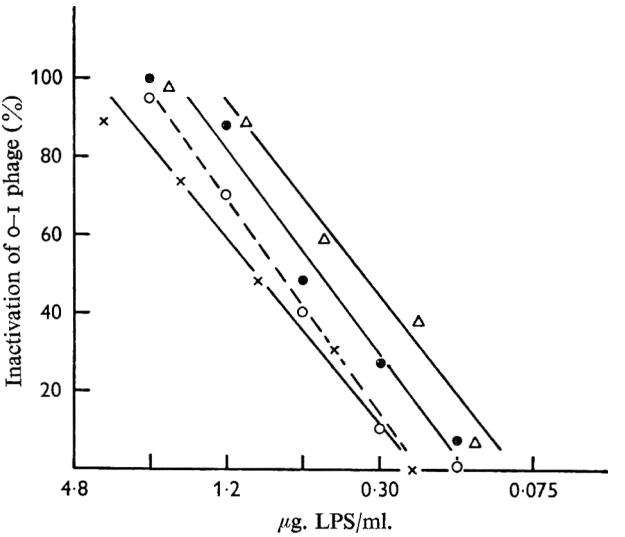


Fig. 1. The o–1 phage inactivating capacity of different samples of phenol+water-extracted lipopolysaccharides from *Salmonella minnesota* strain R60. The activity of the lipopolysaccharide (LPS) is expressed as the concentration of LPS (dry wt./vol.) necessary to inactivate 50 % of the phage (PhI 50). ×—×, PhI 50 = 0·91 μg. LPS/ml.; ○—○, PhI 50 = 0·73 μg. LPS/ml. ●—●, PhI 50 = 0·51 μg. LPS/ml.; △—△, PhI 50 = 0·36 μg. LPS/ml.

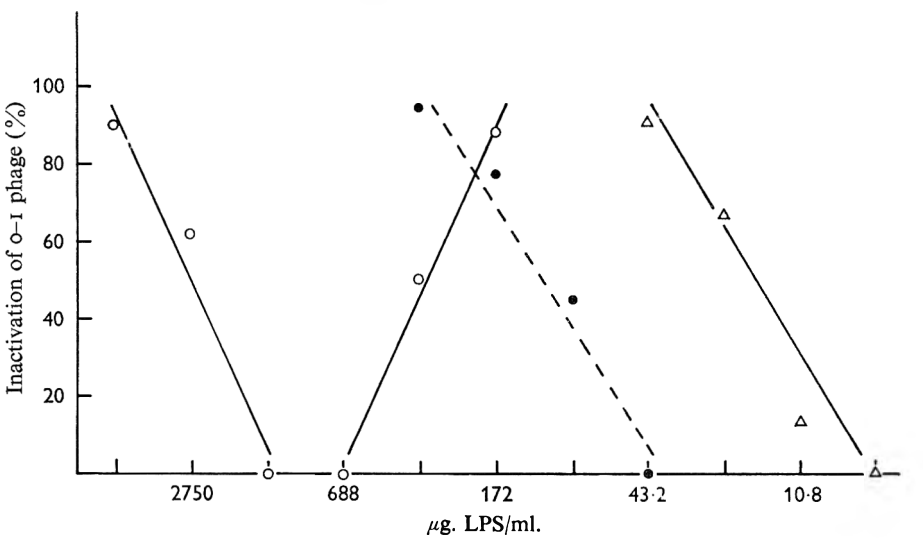


Fig. 2. Action of sodium deoxycholate on lipopolysaccharide (LPS) from *Salmonella minnesota* strain R60. The o–1 phage inactivating capacity of the LPS is expressed as PhI 50. ○—○, LPS of strain R60 treated with deoxycholate, PhI 50 = 2750 μg. LPS/ml. When the deoxycholate concentration was 0·06 % (corresponding to LPS 1375 μg./ml.) no inactivation of the o–1 phage was observed. After dilution of deoxycholate to less than 0·03 % the phage inactivating ability was restored. ●—●, LPS of strain R60 where the deoxycholate was removed by dialysis; PhI 50 = 110 μg. LPS/ml.; △—△, LPS of strain R60 after further treatment by phenol+water extraction, PhI 50 = 17 μg. LPS ml.

Treatment of lipopolysaccharide from strain R60 with sodium deoxycholate (SDC)

Lyophilized LPS from strain R60 was dissolved in 0·1 M-tris-buffer (pH 8·0) and SDC added to a final concentration of 0·5 % (w/v). After addition of SDC the opalescence of the LPS suspension disappeared almost completely. The PhI 50 of the LPS from strain R60 preparation was 0·5 μg./ml. Addition of SDC resulted in a dramatic decrease in the 0–1 phage inactivating ability to a PhI 50 value corresponding to 2750 μg. LPS/ml. (Fig. 2). When the concentration of SDC was diluted with M 9 medium to less than 0·03% the receptor activity of the LPS was restored. No decrease in the infectivity of the 0–1 phage was observed in control experiments with 0·5 % (w/v) SDC, nor did this concentration interfere with the adsorption of the phage to sensitive bacteria.

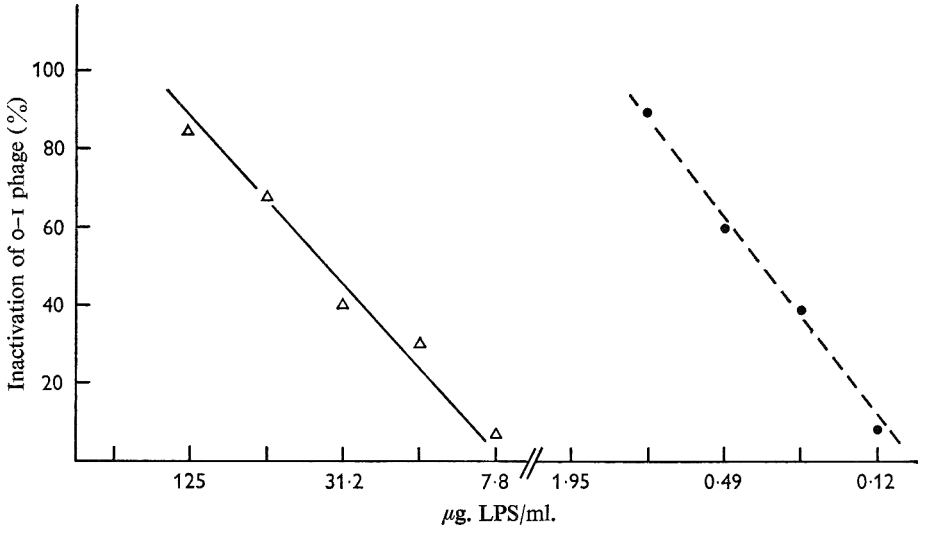


Fig. 3. Effect of ultrasonic treatment of lipopolysaccharide from *Salmonella minnesota* strain R 60. The 0–1 phage inactivating capacity of the LPS is expressed as PhI 50. ●—●, LPS of strain R 60 before ultrasonic treatment; PhI 50 = 0·35 μg. LPS/ml.; △—△, LPS of strain R 60 after 5 min. ultrasonic treatment, PhI 50 = 35 μg. LPS/ml.

The deoxycholate was removed by dialysis against 0·1 M-tris buffer and against the defined medium M 9. The dialysis against medium M 9 restored the optimal conditions for 0–1 phage adsorption. The inactivating capacity of the LPS suspension increased after dialysis to a PhI 50 corresponding to 110 μg. LPS/ml. After treatment of the dialysed LPS suspension with phenol, the inactivating ability was further increased to a PhI 50 value corresponding to 17 μg. LPS/ml.

The processes were directly observed by electron microscopy. Intact LPS of R 60 appeared as long filaments with a uniform width (Pl. 1, fig. 3). When this LPS of R 60 was treated with 0·5 % SDC the filaments dissociated into elements which could not be seen with the electron microscope, i.e. the deoxycholate which had dried on the grid covered them. However, when the deoxycholate was removed by dialysis the elements re-associated into units which could be observed as a population of short rods, spherical and irregular particles (Pl. 2, fig. 5). The size varied from 100 to 400 Å.

The dissociated R 60 lipopolysaccharide was further aggregated when the suspension

was treated by phenol + water extraction. Plate 2, fig. 6 shows a representative field; some filaments are more than 1000 Å long and there are rods from 300 to 500 Å long. The width of the filaments varied from 60 to 100 Å. There were also many spherical particles measuring around 250 Å in diameter. The smallest observed units of lipopolysaccharide R60 which adsorbed phages measured from 200 to 300 Å.

Ultrasonic treatment of lipopolysaccharide of strain R60

Ultrasonic treatment of LPS of strain R60 decreased its O–1 phage inactivating capacity to about 1 % of that of the original preparation (Fig. 3). The PhI 50 value increased from 0·4 μg. LPS/ml. to 35 μg. LPS/ml.

Electron micrographs of preparations before and after ultrasonic treatment showed great variations. The filaments had disintegrated into rods and spherical particles. Plate 2, fig. 7 is an electron micrograph showing a representative field with some rods from 600 to 900 Å long. The majority of particles were rods from 150 to 240 Å long and from 60 to 80 Å wide. Spherical particles of diameter about 100 Å could be seen.

DISCUSSION

Investigations carried out in many laboratories (Beckmann *et al.* 1964; Nikaido *et al.* 1964 Osborn *et al.* 1964; Lüderitz *et al.* 1965) led to the hypothesis first proposed by Davies (1960), Kauffmann *et al.* (1960) and Kauffmann *et al.* (1961) that the O antigen in Salmonella contains a central core of lipopolysaccharide common to all the salmonellas examined so far. Antigenic determinants attached to this core give the different Salmonella species their serological specificity. The O–1 phage, which adsorbs to both S forms and to a certain class of R-mutants, probably attaches to a cell wall structure which is common in Salmonella. As shown here in the inactivation experiments and the electron micrographs the isolated lipopolysaccharide from *Salmonella minnesota* strain R60 acts as a receptor structure for the O–1 phage.

The non-reducing *N*-acetyl-D-glucosamine unit which probably terminates the polysaccharide core in *Salmonella minnesota* R60 may determine the specificity of the O–1 phage receptor. The only difference between *S. minnesota* R60 and strain R345 so far detected is that R345 lacks the glucosamine unit in the polysaccharide (Lüderitz *et al.* 1965). As suggested by the inactivation experiments and seen with the electron microscope the lipopolysaccharide of strain R345 does not cause contraction of the tail sheath of the O–1 phage. The terminal *N*-acetyl-D-glucosamine unit may in fact mediate the adsorption of the O–1 phage to the lipopolysaccharide. Taylor (1966) found that the adsorption of Vi-phage II to the Vi-polysaccharide of *S. typhi* was followed by an enzymic de-acetylation of the polysaccharide. The enzyme, situated on the tip of the Vi-phage tail, split *O*-acetyl as well as *N*-acetyl groups by hydrolysis.

Ribi *et al.* (1966) have found that sodium deoxycholate can dissociate lipopolysaccharide endotoxins from Gram-negative bacteria into subunits with molecular weights of about 20,000, with a subsequent loss of ability to produce fever in rabbits. The biological activity reappeared when the deoxycholate was removed. Their findings suggested another way of testing how the integrity of the lipopolysaccharide structure of *Salmonella minnesota* R60 is related to O–1 phage receptor activity. By treatment of the receptor structure with deoxycholate the phage inactivating capacity decreased to less than 0·1 % of that of the original lipopolysaccharide suspension (Fig. 2). The

failure of the subunits to adsorb the 0–1 phage can be explained in at least two different ways: (i) the adsorption of the phage to the subunits is blocked by the presence of deoxycholate; (ii) the subunits are too small to contain a single complete site of attachment to the phage. When the concentration of deoxycholate was diluted to less than 0·03 % the phage inactivating capacity was restored. This concentration may be too low either to block adsorption of phage or to keep the subunits dissociated. When the deoxycholate was removed by dialysis the subunits recombined to form complete receptor structures. The phage inactivating capacity could be further increased by phenol treatment. The experiments suggest that the inactivation of phages can be used as a simple test of the biological activity of a lipopolysaccharide along with other methods, e.g. toxicity and fever response in rabbits.

The smallest units which were found to cause ejection of the 0–1 phage nucleic acid and contraction of its tail sheath measured from 200 to 300 Å of length. Ribi *et al.* (1966) have found particles of the same size to have molecular weights ranging from 500,000 to 1,000,000. As a comparison, the soluble lipopolysaccharide receptor structure for coliphage T4 in *Shigella sonnei* varied from 50 to 100 Å in size (Jesaitis & Goebel, 1953). The *Escherichia coli* B receptor structure of phage T5, which contained a core of lipopolysaccharide covered with lipoprotein, has a spherical shape around 315 Å (Weidel & Kellenberger, 1955).

Ultrasonic treatment caused a decrease in the 0–1 inactivating capacity of *Salmonella minnesota* R60 lipopolysaccharide. The observed gross morphological changes of the lipopolysaccharide of strain R60 after ultrasonic treatment might explain the difference in 0–1 phage inactivating capacity of the two suspensions. Whereas untreated lipopolysaccharide offered a receptor structure for the phage, ultrasonic treatment disintegrated the lipopolysaccharide into smaller units with no inactivating capacity. The residual inactivating capacity after disintegration might be explained by the presence of the remaining rods still retaining intact receptor sites.

Investigations on the 0–1 phage receptor have been made by the author on some of the *Salmonella typhimurium* mutant strains used by Subbaiah & Stocker (1964). Isolated lipopolysaccharides from strains containing the terminal acetyl-glucosamine unit inactivated the 0–1 phage, whereas lipopolysaccharides from strains lacking the amino sugar did not. Thus, strains containing the central core in *S. minnesota* and *S. typhimurium* offer a receptor for the 0–1 phage provided that the terminal acetyl-glucosamine unit is present. If the central core in Salmonella represents a receptor for the 0–1 phage, the chemical configuration of the lipopolysaccharides of most enterobacteria must differ from that of Salmonella. Alternatively the cell wall of Enterobacteriaceae may contain the receptor which may be blocked by other surface components. Experiments are in progress to investigate the 0–1 phage inactivating capacity of isolated lipopolysaccharides from other Salmonella and enterobacteria strains.

This investigation was supported by a grant from Swedish Medical Research Council Project no. B 67 40X–786–02. The author thanks Miss Mary Anderzon for skilful technical assistance and Mr U. Schönning for handling the electron microscope.

REFERENCES

ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers, Inc.

BECKMANN, I., SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhimurium*. 2. Serological and chemical investigations. *Nature, Lond.* **201**, 1299.

BEUMER, J. & DIRKX, J. (1960). Isolement de substances réceptrices des bactériophages chez les Shigellae. *Annls Inst. Pasteur, Paris* **98**, 910.

BRENNER, S. & HORNE, R. W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochim. biophys. Acta* **34**, 103.

BURNET, F. M. (1934). The phage-inactivating agent of bacterial extracts. *J. Path. Bact.* **38**, 285.

CHERRY, W. B., DAVIS, B. R., EDWARDS, P. R. & HOGAN, R. B. (1954). A simple procedure for the identification of the genus *Salmonella* by means of a specific bacteriophage. *J. Lab. clin. Med.* **44**, 51.

DAVIES, D. A. L. (1960). Polysaccharides of Gram-negative bacteria. *Adv. Carbohyd. Chem.* **15**, 271.

FELIX, A. & CALLOW, B. R. (1943). Typing of paratyphoid B bacilli by means of Vi bacteriophage. *Br. med. J.* **2**, 127.

HOTCHIN, J. E., DAWSON, I. M. & ELFORD, W. J. (1952). The use of empty bacterial membranes in the study of the adsorption of Staphylococcus K phage upon its host. *Br. J. exp. Path.* **33**, 177.

JESAITIS, M. A. & GOEBEL, W. F. (1953). Mechanism of phage action. *Nature, Lond.* **172**, 622.

KALLINGS, L. O. (1967). Sensitivity of various salmonella strains to Felix O-1 phage. *Acta path. microbiol. scand.* (in the Press).

KALLINGS, L. O. & LINDBERG, A. A. (1967). Resistance to Felix O-1 phage in Salmonella bacteria. *Acta path. microbiol. scand.* (in the Press).

KAUFFMANN, F., LÜDERITZ, O., STIERLIN, H. & WESTPHAL, O. (1960). Zur Immunchemie der O-Antigene von Enterobacteriaceae. I. Analyse der Zuckerbausteine von Salmonella O-Antigenen. *Zentbl. Bakt. ParasitKde.* Abt. I. Orig. **178**, 442.

KAUFFMANN, F., KRÜGER, L., LÜDERITZ, O. & WESTPHAL, O. (1961). Zur Immunchemie der O-Antigene von Enterobacteriaceae. VI. Vergleich der Zuckerbausteine von Polysacchariden aus Salmonella-S-und R-Formen. *Zentbl. Bakt. ParasitKde*, Abt. I, Orig. **182**, 57.

LEVINE, P. & FRISCH, A. W. (1933*a*). Specific inhibition of bacteriophage by bacterial extracts. *Proc. Soc. exp. Biol. Med.* **30**, 993.

LEVINE, P. & FRISCH, A. W. (1933*b*). Further observations on specific inhibition of bacteriophage action. *Proc. Soc. exp. Biol. Med.* **31**, 46.

LEVINE, P. & FRISCH, A. W. (1934). On specific inhibition of bacteriophage action by bacterial extracts. *J. exp. Med.* **59**, 213.

LÜDERITZ, O., RISSE, H. J., SCHULTE-HOLTHAUSEN, H., STROMINGER, J. L., SUTHERLAND, I. W. & WESTPHAL, O. (1965). Biochemical studies of the smooth-rough mutation in *Salmonella minnesota*. *J. Bact.* **89**, 343.

NIKAIDO, H., NIKAIDO, K., SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhi-murium*. 3. Enzymatic synthesis of nucleotide sugar compounds. *Nature, Lond.* **201**, 1301.

OROSZLAN, S. I. & MORA, P. T. (1963). Dissociation and reconstitution of an endotoxin. *Biochem. biophys. Res. Commun.* **12**, 345.

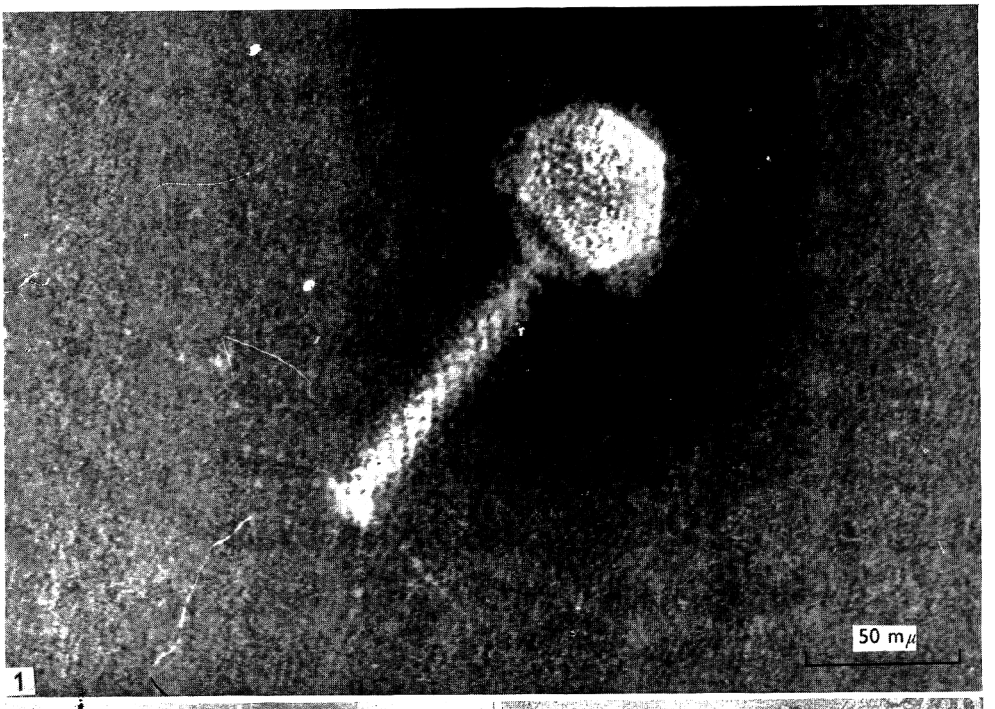
OSBORN, M. J., ROSEN, S. M., ROTHFIELD, L., ZELEZNICK, L. D. & HORECKER, B. L. (1964), Lipopolysaccharide of the Gram-negative cell wall. *Science, N.Y.* **145**, 783.

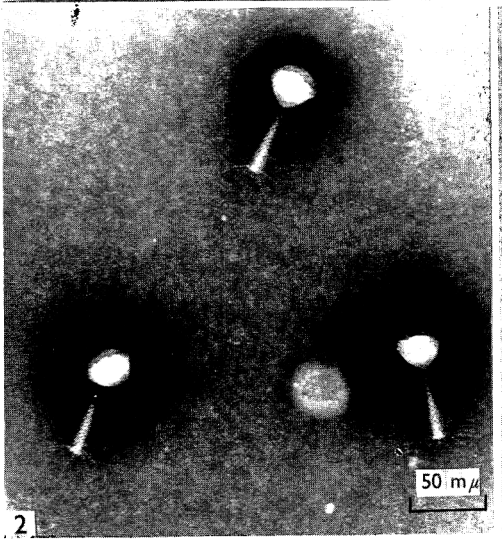
RIBI, E., ANACKER, R. L., BROWN, R., HASKINS, W. T., MALMGREN, B., MILNER, K. C. & RUDBACH, J. A. (1966) Reaction of endotoxin and surfactants. I. Physical and biological properties of endotoxin treated with sodium deoxycholate. *J. Bact.* **92**, 1493.

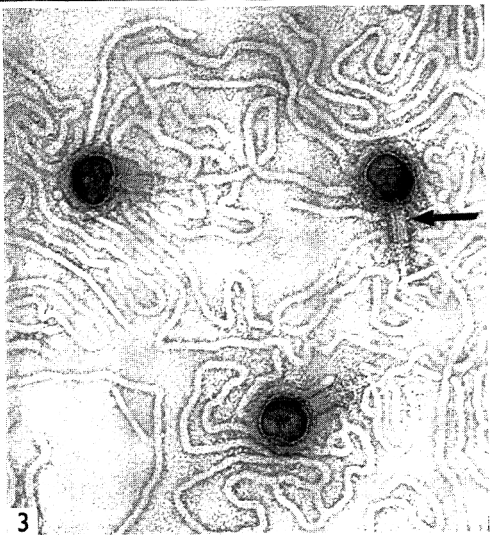
SEIDEL, G. (1956). Über die Spezifität des O-Phagentestes für die Salmonella-Diagnostik. 2. *Colloquium über Fragen der Lysotypie*, p. 69. Zentrallaboratorium für Lysotypie, Bezirks-Hygiene-Institut Wernigerode (Harz).

SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhi-murium*. 1. Genetics *Nature, Lond.* **201**, 1298.

TAYLOR, K. (1966). Physical and chemical changes of Vi-polysaccharide due to Vi-phage II action. *Acta biochim. pol.* **13**, 97.







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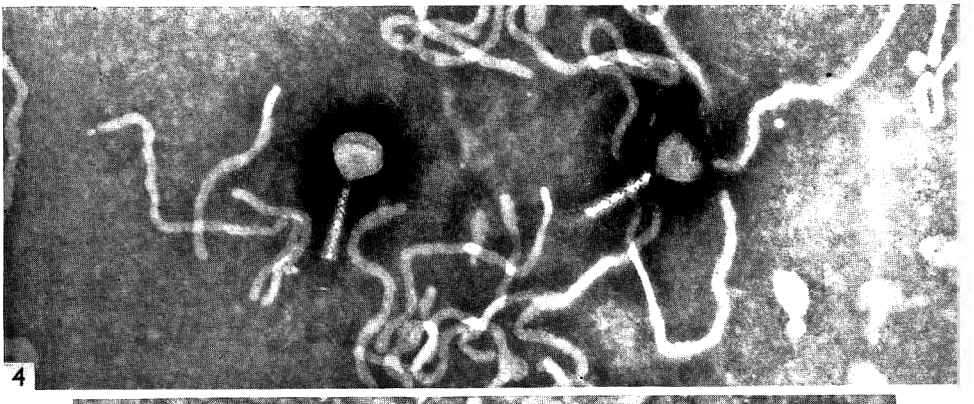

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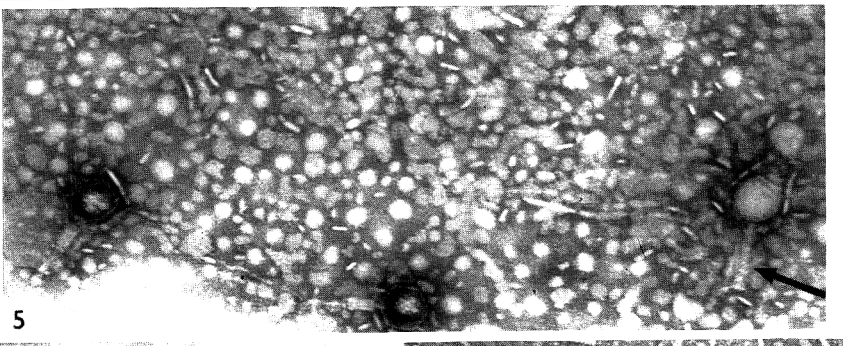

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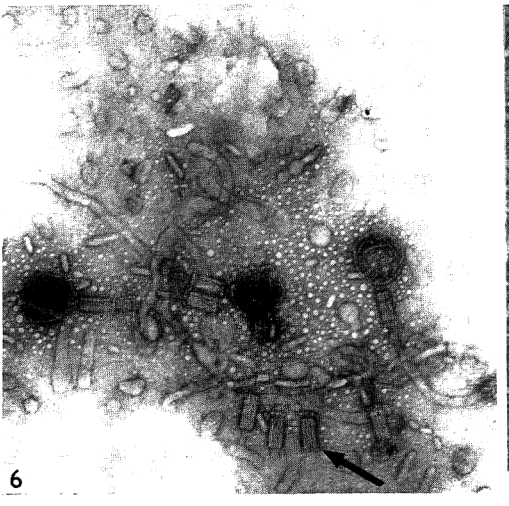

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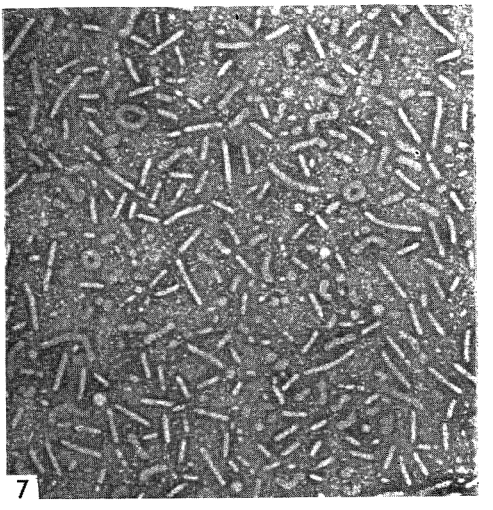

7

THAL, E. & KALLINGS, L. O. (1955). Zur Bestimmung des Genus *Salmonella* mit Hilfe eines Bakteriophagen. *Nord. VetMed.* **7**, 1063.

WEIDEL, W. (1951). Über die Zellmembran von *Escherichia coli* B. I. Präparierung der Membranen gegenüber den Bakteriophagen der T-Serie. *Z. Naturf*, 6*b*, 251.

WEIDEL, W. & KELLENBERGER, E. (1955). The *E. coli* B receptor for the phage T5. II. Electron microscopic studies. *Biochim. biophys. Acta* **17**, 1.

WESTPHAL, O., LÜDERITZ, O. & BISTER, F. (1952). Über die Extraktion von Bakterien mit Phenol Wasser. *Z. Naturf.* 7*b*, 148.

EXPLANATION OF PLATES

The magnification in figs. 5–10 is × 100,000 and in fig. 4 × 400,000. The 0–1 phage and the lipopolysaccharide were negatively stained with sodium tungstic silicate (STS).

PLATE 1

Figs. 1, 2. Felix 0–1 phage.

Fig. 3. Lipopolysaccharide isolated from *Salmonella minnesota* strain R 60 with adsorbed 0–1 phage particles. The tail sheaths (indicated by an arrow) are contracted.

PLATE 2

Fig. 4. Lipopolysaccharide isolated from *Salmonella minnesota* strain R 345 and 0–1 phage. No phage adsorbed on to the lipopolysaccharide.

Fig. 5. Lipopolysaccharide of strain R 60 after treatment with sodium deoxycholate and subsequent dialysis. Two of the 0–1 particles are seen with contracted tail sheaths, one has an uncontracted tail sheath (indicated by an arrow).

Fig. 6. The sodium deoxycholate treated lipopolysaccharide of strain R 60 after dialysis and phenol + water extraction. The 0–1 particles are adsorbed on to the LPS. Some detached contracted tail sheaths (indicated by an arrow) are also seen.

Fig. 7. Lipopolysaccharide from *Salmonella minnesota* strain R 60 after 5 min. ultrasonic treatment.

J. gen. Microbiol. (1967), **48**, 235–248
Printed in Great Britain

Glutamic Acid Dehydrogenases in Quiescent and Germinating Conidia of *Neurospora crassa*

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(*Accepted for publication* 7 *March* 1967)

SUMMARY

As conidia of *Neurospora crassa* aged, the activity of NADP-specific glutamic acid dehydrogenase (NADP-GDH) decreased to negligible values. Subsequent to this decrease, a significant increase occurred in the activity of the NAD-specific glutamic acid dehydrogenase (NAD-GDH). Incubation of aged conidia in basal medium resulted in over a 100-fold increase in NADP-GDH activity within 3 hr. Although no net protein synthesis occurred during these early stages of germination, a turnover in protein was observed. The data presented are consistent with *de novo* synthesis of NADP-GDH. Development of NADP-GDH activity was dependent upon an appropriate carbon source and pH value. An exogenous nitrogen source was not required. The data do not directly support reciprocal regulation of the synthesis of NAD-GDH and NADP-GDH in Neurospora as postulated by Sanwal & Lata (1962*a*, *b*).

INTRODUCTION

Changes in enzyme activity, presumably reflecting enzyme synthesis, in Neurospora, have been the subject of numerous investigations (Horowitz, Fling, MacLeod & Watanabe, 1961; Horowitz, 1965; Turian, 1961; 1963; Turian, Seydoux & Volkmann, 1962). Changes in the activity of NADP-GDH and NAD-GDH in Neurospora are of particular interest since the published evidence indicates that these enzymes are regulated reciprocally in their synthesis. To account for this reciprocality, Sanwal & Lata (1962*b*) proposed that the two enzymes are under the control of a single repressor substance which is active in repressing NAD-GDH, but is active against NADP-GDH only when combined with urea which serves as a co-repressor. The 'urea effect' is postulated as being specific for stages in the development of Neurospora following germination, since Sanwal & Lata (1962*a*) were not able to detect NAD-GDH in conidia until germination began, at which time NADP-GDH also was increasing. This obervation suggests that the regulation of the synthesis of the two GDH enzymes in Neurospora is qualitatively different during the germination process.

The data presented in this paper concerns the changes in the activity of NADP-GDH and NAD-GDH in Neurospora which occur in quiescent and germinating conidia. The changes in activities in germinating conidia were measured over a period up to 7 hr and showed that an increase in NADP-GDH enzyme was not precisely accompanied by a decrease in the activity of NAD-GDH which was found to be present in

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both quiescent and germinating conidia. These changes probably reflect *de novo* synthesis, rather than activation of NADP-GDH, which is independent of exogenous nitrogen but dependent on an energy source.

METHODS

Organism. The wild-type strain of *Neurospora crassa* used in these experiments was STA 4 (an asexual derivative of St Lawrence 74 A) obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire, U.S.A.

Chemicals. 2-Oxoglutarate (free acid or 0·1 M solution in phosphate buffer, pH 7·5), *cis*-oxalacetate (grade I), dihydronicotinamide adenine dinucleotide phosphate ($NADPH_2$, type II), dihydronicotinamide adenine dinucleotide ($NADH_2$, grade III) were obtained from Sigma Chemical Company, St Louis, Missouri. Amino acids and cycloheximide were obtained from the California Corporation for Biochemical Research (Los Angeles, California, U.S.A.). Uniformly labelled L-valine-^{14}C (208·5 mC/m-mole) was supplied by the New England Nuclear Corporation (Boston, Massachusetts, U.S.A.). Enzyme-hydrolysed casein (N-Z-Case) was obtained from Sheffield Chemical (a division of National Dairy Products Corporation, Norwich, New York). Yeast extract and Casamino acids were obtained from Difco Laboratories (Detroit, Michigan) and Tween 80 was purchased from Atlas Powder Company (Wilmington, Delaware).

Culture media. The basal medium used in these investigations was medium N+2 % sucrose (Vogel, 1956) supplemented with trace elements (Beadle & Tatum, 1945). Deletions, additions and other modifications made to the basal medium are described in connexion with specific experiments. For certain experiments, buffers or buffers with additions were utilized (Gomori, 1955). Wainwright's medium (Wainwright, 1959) supplemented with trace elements was used for the production of conidia. For the determination of conidial viability, the basal medium was modified by the substitution of sorbose 0·1 % and glycerol 0·5 % for sucrose and the addition of peptone 0·5 %, yeast extract 0·5 % and agar 1·5 % (w/v).

Determination of conidial viability and germination. Conidial viability was determined in one of two ways. In the experiment with ageing conidia viability was ascertained by a direct count of the fraction of conidia having germ tubes after a dilute culture had been incubated for a suitable period. Evidence for germination in experiments involving the incubation of dense conidial suspensions was obtained by direct inspection of conidia for the presence of germ tubes, at the end of the experiment. However, under the conditions used throughout these studies (conidial concentrations of $1·0$ to $6·2 \times 10^7$ viable conidia/ml.) no germ tubes were found to occur within the experimental period. Thus, in these investigations conidial viability was not equivalent to visible germination during the experiment. Under these circumstances viability was determined by plating a suitable dilution of the dense conidial suspension on supplemented sorbose + glycerol agar and counting the resulting colonies.

Preparation of large batches of conidia. The methods for obtaining large batches of conidia described by Barratt (1963) were used, with the following modifications. The inverted Fernbach flasks were kept at 32° for 36–48 hr rather than 24 hr. Subsequently the flasks were incubated at 22° and high relative humidity (range 70–85 %, mean 80 %). Aged conidia were harvested in 0·85 % NaCl solution containing 0·1 %

Tween 80. Conidia were loosened from the mycelial mat with the aid of a sterile stirring bar and a magnetic stirrer, and were separated from mycelial fragments as described by Barratt (1963).

Conditions for conidial incubation. Following concentration by centrifugation at 10,500 *g* for 25 min. at 0°, the total conidial wet weight was ascertained, and samples were removed for the determination of dry weight and initial enzyme activities. The remaining conidia were resuspended in a minimum volume of saline (final volume of the suspended conidia from 8 Fernbach flasks varied from 30 to 40 ml.). Samples of suspended conidia were inoculated into 500 ml. Erlenmeyer flasks containing 250 ml. of the medium being tested. In two experiments with uniformly labelled L-valine-^{14}C, 0·5 μC/ml. or 0·25 μC-ml. of the labelled valine were added to either 15 ml. or 30 ml. of medium, and in one instance to 120 ml. of medium. The inoculated flasks were aerated on a rotary shaker at approximately 150 rev./min. at 32° for 0·5–7 hr. Following shaking, the conidia were centrifuged and a sample was removed to determine dry weight. The remainder were resuspended in 9 volumes of cold (0°) 0·1 M-Na phosphate buffer (pH 7·0) in preparation for extraction. The extraction buffer contained 2-mercaptoethanol (1·0 mM) to stabilize the NAD-GDH activity. In those experiments involving L-valine-^{14}C, the conidia were thrice washed in 0·85 % (w/v) NaCl solution containing unlabelled valine and then suspended in cold (0°) buffer.

Preparation of extracts. Conidia were disrupted at maximum output for 10 min. with a model S-125 Sonifier (Branson Instruments Inc., Stanford, Connecticut, U.S.A.), generating sonic oscillations of 20 kc./sec. The instrument was equipped with a microtip which has a power output 3·5 times that of the standard horn. 10–15 ml. volumes were treated ultrasonically in a Rosette cooling cell (Model 25) suspended in an ice bath ($-4°$ to $-8°$). The temperature of the extracts at the termination of ultrasonic treatment varied between 6° and 12°. The resulting extract was clarified by centrifugation at 17,000 *g* for 25 min. at 0° (Servall Superspeed RC-2) and the supernatant fluid was assayed immediately.

Enzyme assays. All assays were done by following the oxidation of the appropriate coenzyme ($NADH_2$ or $NADPH_2$) in a quartz spectrophotometer cell in a Cary model 15 spectrophotometer at 340 mμ at 25°.

NADP-GDH (L-glutamate: NADP oxidoreductase E.C. 1.4.1.4) was assayed as described previously (Barratt, 1963) with the exception that the concentration of 2-oxoglutarate was decreased by 40 %. Since it has recently been shown that NADP-GDH is in the inactive form when extracted at pH 7·0 (West, Tuveson & Barratt, 1966), the supernatant fluids were pre-incubated in the presence of tris-HCl buffer (pH 7·8) and 2-oxoglutarate for 20 min., a condition shown to achieve full activation. The reaction was initiated by adding NH_4Cl and $NADPH_2$. When the free acid was used it was neutralized to pH 7·0.

The assay mixture for NAD-GDH (L-glutamate: NAD oxidoreductase E.C. 1.4.1.2) consisted of the following in a 4 ml. cell: 2·1 ml. of 0·1 M-Na phosphate buffer (pH 8·0) containing 1·0 mM-2-mercaptoethanol; 0·2 ml. of 0·1 M-2-oxoglutarate solution; 0·15 ml. of 3·0 M-NH_4Cl in the phosphate buffer; sufficient volume of a 1·5 mM solution of $NADH_2$ to contribute an extinction of 0·5 to the final reaction mixture (volume varied between 0·16 and 0·20 ml.). The reaction was initiated by adding 0·1 ml. of enzyme solution.

The assay for malate dehydrogenase (MDH; L-malate: NAD oxidoreductase

E.C. 1.1.1.37) was a modification of the procedure described by Munkres (1965). The assay mixture consisted of the following in a 4 ml. cell: 2·25 ml. of 0·111 M-K phosphate buffer (pH 7·4); 0·2 ml. of 0·012 M-*cis*-oxalacetate solution; sufficient volume of a 1·5 mM solution of $NADH_2$ to contribute an extinction of 0·5 to the final reaction mixture (volume varied between 0·16 and 0·20 ml.). The reaction was initiated by adding 0·1 ml. of enzyme solution.

Since crude preparations were assayed in all but one experiment, correction for non-specific oxidation of the coenzymes was made by subtracting rates obtained in the absence of substrate.

One enzyme unit is defined as that amount of enzyme which will catalyse the transformation of 1 μmole substrate/min. A unit of enzyme activity is equivalent to a change in extinction at 340 mμ of 2·27/min. For convenience, the activities are presented in terms of milli-units (m.u.).

Protein determinations. Protein determinations were made on the soluble proteins by the Folin phenol method of Lowry, Rosebrough, Farr & Randall (1951). Specific activities are expressed as m.u./mg. protein.

Counting of radioactive samples. Supernatant material from ultrasonically treated conidia incubated in the presence of L-valine-^{14}C was spotted on 2·3 cm. filter-paper disks and treated by a modification of the procedure described by Mans & Novelli (1961). Disintegrations were detected in a liquid scintillation counter (Nuclear Chicago model 725, Nuclear-Chicago Corporation, Des Plaines, Illinois).

Electrophoresis. The methods used for electrophoresis on cellulose acetate strips were as described previously (Barratt & Strickland, 1963).

RESULTS

Effects of conidial ageing.

Preliminary experiments had suggested that NAD-GDH and NADP-GDH activity in conidia varied with conidial age (West *et al.*, 1966; Barratt *et al.*, 1966; Stine, 1966). A systematic investigation was undertaken to determine NAD-GDH and NADP-GDH in conidia from cultures of different ages. Malate dehydrogenase (MDH) was also assayed in the extracts obtained from the conidia to determine whether an enzyme metabolically separated from the glutamate dehydrogenase system varied with conidial age. The results of this experiment are presented in Table 1. Four days after inoculation was the earliest time when sufficient conidiation had occurred to permit harvesting. NADP-GDH activity decreased dramatically in conidia between 4 and 8 days of age. The inoculum for the 4-, 12-, 14-, 18- and 21-day cultures was prepared from a single agar slope. The inoculum for the remaining cultures was prepared from a duplicate slope 3 days previously. The slightly elevated NADP-GDH activities in the 14- and 18-day cultures may indicate some slight difference in the duplicate agar slopes used for inoculum which affected the decline of NADP-GDH during ageing. However, in more than 20 experiments NADP-GDH activity in 21-day (or older) conidia was never observed to be significantly greater than that reported for the 21-day culture in Table 1 (less than 2·5 % of that in young conidia). The NADP-GDH activity was determined in 4-day conidia in four independent experiments (see footnote, Table 1). It is apparent from Table 1 that the extracts of Neurospora conidia did exhibit significant NAD-GDH activity, irrespective of culture age

and contrary to the observations of Sanwal & Lata (1962*a*). The NAD-GDH activity remained relatively constant in conidia up to 14 days, after which there was a marked increase.

Table 1. *The relationship of culture age to specific activity in crude extracts of conidia of Neurospora crassa* STA *4*

Culture age (days)	Protein (mg./g. dry wt.)	Specific activity (m.u./mg.) NADP-GDH	NAD-GDH	MDH
4*	158	278	46·2	7500
8	173	15·1	37·3	5290
12	173	17·7	41·7	6420
13	200	2·7	45·3	5950
14	164	11·2	37·6	5760
15	219	2·7	60·4	6090
16	179	2·7	142	5400
17	174	1·8	128	6200
18	147	10·6	140	6940
20	166	4·4	118	6430
21†	114	6·2	242	7360
23†	54	6·2	500	7400

* Specific activities for NADP-GDH in crude extracts of conidia from 4-day cultures determined in 3 additional experiments were: 228, 372, 382.

† Extractable protein from 21- to 23-day conidial cultures averaged 153 mg./g. dry weight (Tables 2–6).

The MDH activity in the conidial extracts was unrelated to conidial age.

Parallel to these enzyme activity studies in ageing conidia, conidial viability was determined. Conidia from the 4-day culture showed essentially 100 % germination within 6 hr, in agreement with the findings of Ryan (1948). The % germination remained high (85 %) for cultures up to 18 days old. However, as the cultures aged, there was a marked tendency for germination to be non-uniform and delayed. Thus the conidia from the 23-day culture showed visible germination only after incubation for 8 hr. In such conidia germination increased up to 12 hr, by which time mycelial masses began to interfere with counting. For conidia from the 23-day culture, it was estimated that germination was between 50 and 70 %. Thus, ageing resulted in some loss of conidial viability.

Observations on incubating aged conidia

Neurospora mycelium shows appreciable degrees of NADP-GDH activity (Barratt, 1963). This activity developed during the process of germination or shortly thereafter. Therefore, aged conidia were incubated in basal medium for up to 3 hr and the NADP-GDH activity assayed. After 3 hr of incubation no visible evidence of germination was observed at the conidial concentrations used. Neither the initial inoculum nor conidia incubated for 0·5 hr in basal medium exhibited significant NADP-GDH activity (Fig. 1; Table 2). After shaking for 3 hr, however, NADP-GDH activity increased over 100-fold (Table 2). It was also apparent that absence of the carbon source (sucrose) severely restricted the development of NADP-GDH activity. The activity which did develop was probably supported by carbon reserves in the conidia. The omission of a nitrogen source from the basal medium did not decrease the

development of NADP-GDH activity significantly. The simultaneous omission of both carbon and nitrogen sources gave an NADP-GDH activity equivalent to that found when a carbon source was omitted. In this experiment the extractable soluble protein decreased after the first 0·5 hr of shaking and remained low for all treatments up to 3 hr. This initial decrease in extractable protein was observed in all experiments. Although the absolute quantity of extractable protein varied from experiment to experiment, the decrease in treated conidia shaken for as long as 4 hr, as opposed to untreated conidia, remained qualitatively equivalent. The NAD-GDH activity did not vary significantly throughout the experiments (Tables 2–6).

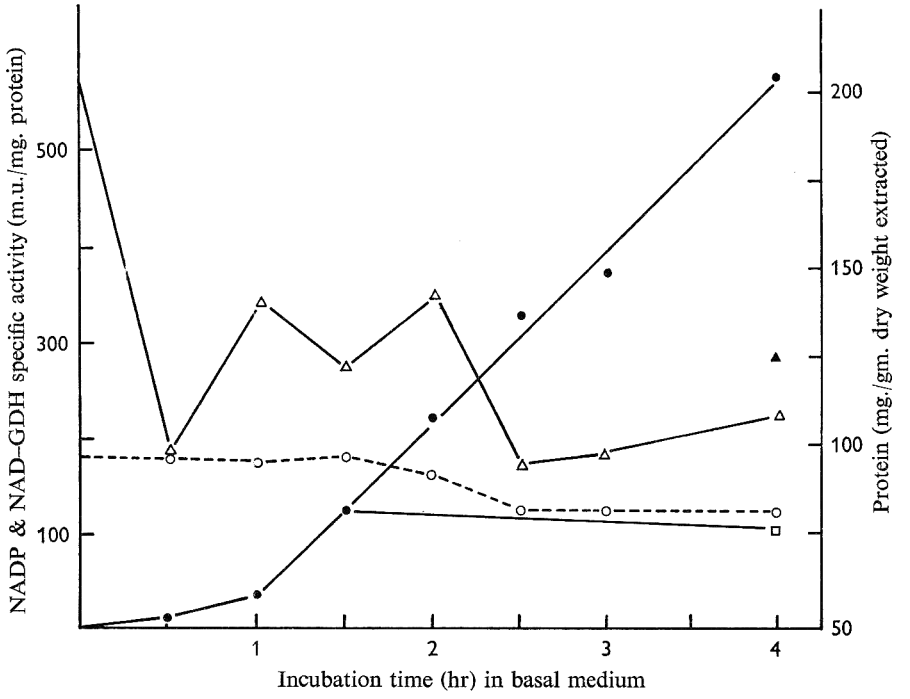

Fig. 1. ●, NADP-GDH; □, NADP-GDH, cycloheximide (10 μg./ml.) added at 1·5 hr; ○, NAD-GDH; △, protein; ▲, protein, cycloheximide-treated sample.

The kinetics of NADP- and NAD-GDH development were followed over a 4 hr period in basal medium. As expected, in the first 0·5 hr there was little development of NADP-GDH activity, but there was a marked decrease in extractable protein (Fig. 1). From 1·0 hr on, the development of NADP-GDH activity appeared to be linear with time. These same kinetics were observed in two similar experiments. Although the amounts of extractable protein varied widely in this experiment, they were consistently lower for the treated conidia up to 4 hr than for the conidia of the inoculum. The fact that the specific activity of NADP-GDH increased in a regular manner suggests that the variations in extractable protein were random, except for the initial decline, a fact also confirmed in subsequent experiments (Fig. 1; Tables 2–6). During the 4 hr period of incubation there appeared to be a slight decline in NAD-GDH activity, beginning between 1·5 and 2·0 hr.

Subsequent experiments were directed towards determining what other factors might influence the development of NADP-GDH in incubating aged conidia. Substitution of phosphate buffer (pH 5·7) + sucrose for basal medium resulted in an NADP-GDH activity approximately 60 % of that attained in the basal medium in 3 hr (Table 3). The addition of trace elements to the pH 5·7 buffer + sucrose increased NADP-GDH activity, but still not to the degree attained in the basal medium. Similar experiments with phosphate buffer (pH 7·0) + sucrose resulted in negligible NADP-GDH activity, while in citrate + phosphate buffer (pH 5·0) + sucrose NADP-GDH activity was

Table 2. *NADP-GDH specific activity in crude extracts of conidia of Neurospora crassa* STA *4 shaken in various media*

Viable conidia 10^7/ml. from a 23-day culture were inoculated into each flask containing 250 ml. of medium

Medium	Time of shaking (hr)	Protein (mg./g. dry wt)	Specific activity (m.u./mg.)
Inoculum	0	160	1·7
Basal medium*	0·5	58	4·4
Basal medium	3·0	67	300
Basal medium minus sucrose	3·0	62	42
Basal medium minus NH_4NO_3	3·0	95	280
Basal medium minus sucrose minus NH_4NO_3	3·0	87	32

* Vogel's minimal N containing 2 % sucrose (58·5 mM).

Table 3. *NADP-GDH and NAD-GDH specific activities in crude extracts of conidia of Neurospora crassa* STA *4 incubated in various media*

Viable conidia $3{\cdot}4 \times 10^7$/ml. from a 21-day culture were inoculated into each flask containing 250 ml. of medium.

Medium	Time of shaking (hr)	Protein (mg./g. dry wt)	Specific activity (m.u./mg.) NADP-GDH	Specific activity (m.u./mg.) NAD-GDH
Inoculum	0	135	Undetectable	195
Basal medium*	2·0	55	210	200
Basal medium	3·0	69	460	210
Basal medium	7·0	108	1050	93
0·1 M-phosphate buffer (pH 5·7)	3·0	78	32	170
0·1 M-phosphate buffer (pH 5·7) + sucrose (58·5 mM)	3·0	58	280	190
0·1 M-phosphate buffer (pH 5·7) + trace elements	3·0	49	34	250
0·1 M-phosphate buffer (pH 5·7) + sucrose (58·5 mM) + trace elements	3·0	47	340	220
Basal medium + cycloheximide (10 μg./ml.) added at 2 hr	3·0	54	240	220
Basal medium + cycloheximide (10 μg./ml.) added at 2 hr	7·0	57	270	190

* Vogel's minimal N containing 2 % sucrose (58·5 mM).

about 40 % of that attained in the basal medium in 3 hr. Again NAD-GDH activity showed no significant changes within 3 hr, irrespective of treatment. However, when the conidia had been shaken for 7 hr there was a decided increase in extractable protein, accounting for the exhibited decline in NAD-GDH specific activity.

The quantitative requirements for sucrose in the induction of NADP-GDH activity were investigated. 5·85 and 58·5 mM-sucrose supported equally the development of NADP-GDH activity (Table 4). The minimal concentration of sucrose necessary for maximal development was found (see Table 5). This experiment, in which phosphate buffer (pH 5·7) + sucrose was used in place of basal medium, showed that the limiting sucrose concentration was between 0·58 and 1·45 mM.

Table 4. *NADP-GDH and NAD-GDH specific activities in crude extracts of conidia of Neurospora crassa* STA *4 shaken in variously modified basal media*

Viable conidia $4{\cdot}6 \times 10^7$/ml. from a 21-day culture were inoculated into each flask containing 250 ml. of medium.

Carbon source	Time of shaking (hr)	Protein (mg./g. dry wt.)	Specific activity (m.u./mg.) NADP-GDH	Specific activity (m.u./mg.) NAD-GDH
Inoculum	0	147	4·4	119
Sucrose (58·5 mM)	3·0	80	800	96
Sucrose (5·85 mM)	3·0	89	920	110
Sucrose (58·5 mM)	4·0	77	860	92
Na acetate (58·5 mM)	3·0	81	58	150
Na acetate (5·85 mM)	3·0	115	174	109
Sucrose (58·5 mM) + Casamino acids (1·4 %)	3·0	69	380	97
Sucrose (58·5 mM) + Casamino acids (2·8 %)	3·0	64	420	94
Sucrose (58·5 mM) + arginine (3·2 mM) + lysine (6·8 mM) + threonine (5·0 mM)	3·0	87	390	120

The role of other carbon sources and certain amino acids in the induction of NADP-GDH activity was examined. When Na acetate was substituted for sucrose no appreciable development of NADP-GDH occurred. In this experiment the highest concentration of Na acetate (58·5 mM) exhibited NADP-GDH activity approximately 33 % of that found with the low concentration of Na acetate (5·85 mM), neither activity being comparable to that attained in buffer + sucrose or in the basal medium (Table 4). This experiment was repeated with phosphate buffer to which Na acetate was added at three concentrations. The results confirmed the original observations and indicated that the inhibition by Na acetate was not peculiar to the addition of Na acetate to basal medium in place of sucrose (Table 6). MDH was also assayed in this experiment to verify the result (Table 1) that its activity did not vary systematically under conditions which allowed the development of NADP-GDH activity. The results of this experiment were unusual in that after 3 hr of shaking conidia in the basal medium and in buffer + sucrose, the NADP-GDH activities found were identical within the limits of experimental error. However, if one takes the NADP-GDH activity at 4 hr and constructs a graph using 0·8 hr as the point where NADP-GDH activity should begin to develop (Fig. 1), the 3 hr point determined experimentally

was about 50 % under what might have been predicted. It seems probable that the NADP-GDH specific activity determined for conidia shaken for 3 hr in basal medium was low as the result of a technical error. If this were true, then the experiment would

Table 5. *NADP-GDH and NAD-GDH specific activities in crude extracts of conidia of Neurospora crassa* STA *4 shaken in various media*

Viable conidia 10^7/ml. from a 21-day culture were inoculated into each flask containing 250 ml. of medium. All cultures were shaken for 3 hr with the exception of the untreated conidia which were not shaken.

Medium	Protein (mg./g. dry wt)	Specific activity (m.u./mg.) NADP-GDH	NAD-GDH
Inoculum	143	Undetectable	196
0·1 M-phosphate buffer (pH 5·7)	88	20	170
0·1 M-phosphate buffer (pH 5·7)+ sucrose (5·85 mM)	68	220	230
0·1 M-phosphate buffer (pH 5·7)+ sucrose (2·9 mM)	74	240	130
0·1 M-phosphate buffer (pH 5·7)+ sucrose (1·45 mM)	100	254	183
0·1 M-phosphate buffer (pH 5·7)+ sucrose (0·58 mM)	63	130	180
0·1 M-phosphate buffer (pH 5·7)+ sucrose (5·85 mM)+biotin (0·08 μg./ml.)	65	240	130
0·1 M-phosphate buffer (pH 5·7)+ glucose (5·8 mM)	68	170	150

Table 6. *NADP-GDH, NAD-GDH and MDH specific activities in crude extracts of conidia of Neurospora crassa* STA *4 shaken in various media*

Viable conidia $2{\cdot}7 \times 10^7$/ml. from a 21-day culture were inoculated into each flask containing 250 ml. of medium.

Medium	Time of shaking (hr)	Protein (mg./g. dry wt)	Specific activity (m.u./mg.) NADP-GDH	NAD-GDH	MDH
Inoculum	0	181	0·9	188	5480
Basal medium* (5·85 mM sucrose)	3·0	118	176	188	6150
Basal medium	4·0	Not determined	430	240	5270
0·1 M-phosphate buffer (pH 5·7)	1·5	100	1·9	55·8	7660
0·1 M-phosphate buffer (pH 5·7)	3·0	42	25	190	6900
0·1 M-phosphate buffer (pH 5·7)+ sucrose (58·5 mM)	3·0	68	160	170	7000
0·1 M-phosphate buffer (pH 5·7)+ Na acetate (58·5 mM)	3·0	59	0·8	240	6800
0·1 M-phosphate buffer (pH 5·7)+ Na acetate (5·85 mM)	3·0	53	28	150	5900
0·1 M-phosphate buffer (pH 5·7)+ Na acetate (1·0 mM)	3·0	53	36	180	6100

* Vogel's minimal N containing 2 % sucrose (58·5 mM) except where indicated.

conform to all other experiments in which buffer+sucrose was always less effective in supporting the development of NADP-GDH than was the basal medium (Table 3 and unpublished experiments). In another experiment glucose was used in place of

sucrose, and buffer+sucrose was further supplemented with biotin (0·08 μg/ml.) in another case. If sucrose, pH value and biotin were the critical factors in the basal medium allowing for the development of maximal NADP-GDH activity, the addition of biotin would be expected to double the NADP-GDH activity. However, biotin did not enhance the effect of sucrose alone and equimolar glucose did not support the development of NADP-GDH activity as well as did sucrose. Concurrent with the experiment in which Na acetate was substituted for sucrose in basal medium, certain amino acids were tested for their effects on NADP-GDH activity. As can be seen from Table 4, Casamino acids or a mixture of amino acids in the basal medium depressed the development of NADP-GDH about 50 %. The NAD-GDH activity again remained about the same in most of the extracts, showing what can only be interpreted as random fluctuations.

Two experiments were done in an attempt to explain the differences in degrees of NADP-GDH activity attained in basal medium in different experiments after 3 hr shaking (Tables 2–4). In the first of these, the speed of the shaker was increased to 180 rev./min. from the usual 150 for one set of flasks, and decreased to 114 rev./min. for a second set. Shaking for 1·5 or 3·0 hr at these two speeds showed that the NADP-GDH activities attained were not significantly different. The amounts of viable conidia varied from experiment to experiment, as indicated in the individual tables; this might explain the observed differences in final NADP-GDH activity. The conidial concentration was varied systematically in the basal medium. The results indicated that NADP-GDH activity was restricted in its development when the conidial concentration exceeded $6{\cdot}2 \times 10^7$ viable conidia/ml. Since concentrations above this were not used in any experiments detailed in this paper, the variations in maximal NADP-GDH activity cannot be attributed to variations in viable conidial concentration.

In summary, sucrose appeared to be the most effective carbon source, and some other factor, as yet undetermined, in the basal medium other than sucrose in concert with the proper pH value is essential for maximum NADP-GDH development. Shaking speed and the concentration of viable conidia were not important factors in the experiments reported here.

Evidence for de novo NADP-GDH synthesis in incubating conidia

The effect of inhibition of protein synthesis on the observed changes in NADP-GDH activity was investigated. The addition at 1·5 hr (Fig. 1) or 2 hr (Table 3) of cycloheximide (10 μg/ml.), which effectively inhibits protein synthesis in fungi (Haidle & Storck, 1966; Pall, 1966), resulted in the total cessation of development of NADP-GDH activity. Sodium azide (5 mM), which inhibits oxidative phosphorylation and thus secondarily protein synthesis, markedly restricted the further development of NADP-GDH activity when added at 2 hr. Such data, while suggestive, do not constitute unequivocal proof that NADP-GDH was being synthesized *de novo* during the incubation period. It was essential to show that protein synthesis occurred during the standard incubation period. L-valine-^{14}C incorporation was selected to ascertain protein turnover during incubation. Before doing an experiment with L-valine-^{14}C, it was necessary to investigate the effects of L-valine on the development of NADP-GDH activity. In two experiments it was found that L-valine used at concentrations equivalent to that of the labelled valine in one experiment, and twice the concentration of the label in a second experiment, restricted maximal NADP-GDH

development by about 50 %. The inhibition was not sufficient to preclude the proposed experiment. Since the amino acid inhibition of NADP-GDH development seemed rather non-specific (Table 4), no attempt was made to find an amino acid other than valine to use in the proposed labelling experiment.

Conidia from a 21-day culture were incubated in basal medium containing L-valine-^{14}C and either kept cold in an ice bath with constant stirring or shaken for 4 hr with or without cycloheximide (10 μg./ml.) at 32° (Table 7). The conidia in basal medium exhibited a significant uptake of L-valine-^{14}C, substantial (85·8 %) incorporation of the label into protein (measured as trichloroacetic acid-precipitable material), and a large increase in NADP-GDH specific activity. The culture containing cycloheximide showed considerable uptake of ^{14}C, but negligible incorporation into protein, and a very low specific activity of NADP-GDH. The control flask maintained at 0° showed negligible uptake of ^{14}C, less than 1 % incorporation into protein, and again a negligible NADP-GDH specific activity.

Table 7. *Evidence for protein synthesis in incubating conidia from a 21-day culture of Neurospora crassa* STA 4

Viable conidia 5·1–6·2 × 10^7/ml. from a 21-day culture were inoculated into each flask. Experimental conditions: 4 hr incubation in basal medium + 0·15 mg./ml. L-valine-^{14}C (U.L.). Each flask contained 0·25 mc. L-valine-^{14}C/ml.

Incubation temp.	Medium	Total c.p.m.*	TCA insoluble c.p.m.†	Incorporation (%)	NADP-GDH specific activity (m.u./mg.)
0	Basal‡	296·0	2·6	0·90	1·2
32	Basal + cycloheximide (10 μg./ml.)	5077·9	52·2	1·03	Negligible
32	Basal	9524·1	8170·8	85·80	190

* Per 100 μl. supernatant fluid of sonically treated conidia.

† TCA insoluble fraction from 100 μl. of sonically treated conidia (by a modification of the method of Mans & Novelli, 1961).

‡ Vogel's minimal N containing 2 % sucrose (58·5 mM).

NADP-GDH extracted from the conidia incubated with L-valine-^{14}C at 32° for 4 hr was purified tenfold and subjected to electrophoresis on cellulose acetate strips under conditions known to permit migration of NADP-GDH. The cellulose acetate strip was sectioned, each section divided in half, one half counted on a scintillation counter, and the other half assayed for enzyme activity. Enzyme activity was found to be associated with a diffuse radioactive peak. This result does not preclude the possibility that association of radioactivity and NADP-GDH activity might represent contamination of the unlabelled NADP-GDH by another radioactive protein. However, the isotope and protein synthesis inhibitor experiments strongly suggest that NADP-GDH was synthesized *de novo* during the incubation period, and thus was induced, in the sense defined by Jacob & Monod (1961).

DISCUSSION

Neurospora crassa conidia retain a viability of greater than 50 % when stored for 23 days under conditions of high humidity and constant temperature. High humidity has been shown to maintain conidial viability in other investigations (Klingmüller 1963). Ageing conidia rapidly lost NADP-GDH activity, decreasing to a minimal value within 15 days. At that time NAD-GDH activity began to increase. Malate dehydrogenase (MDH) activity remained constant during the course of conidial ageing. Extractable protein exhibited only random fluctuations during conidial ageing; the two values obtained in the ageing experiment for 21- and 23-day conidia are considered to be spurious in the light of other experiments (see footnote to Table 1). Despite the constancy of total extractable protein during the ageing process, the changes in enzyme activity indicate that significant selective protein turnover was taking place; and that the degradation of NADP-GDH was not reciprocal with the appearance of NAD-GDH, either in magnitude or in the time course of the event.

In incubating conidia the enzyme complement is undergoing rapid change, presumably in preparation for germination. This was reflected by the marked decrease in extractable protein coincident with the rapid increase in NADP-GDH within 3 hr. The synthesis of NADP-GDH was dependent upon an exogenous carbon source (Tables 2, 3, 5, and 6). The carbon requirement was notable in that the concentration required for maximal induction was only 0·05 %. This might be interpreted as meaning that the sucrose was primarily acting to produce a specific metabolite such as $NADPH_2$ (through the dehydrogenation of glucose-6-phosphate) or 2-oxoglutarate, which in turn could serve as a specific inducer of NADP-GDH. Acetate, which would also be expected to serve as an energy source, was much less effective in the induction process. Acetate is known to induce the glyoxalate cycle in Neurospora (Turian, 1963), and this would be likely to result in diminished intracellular concentrations of 2-oxoglutarate as well as a decrease in glucose catabolism.

Exogenous ammonium ions neither repressed nor de-repressed NADP-GDH (Table 2), while exogenously supplied amino acids restricted NADP-GDH development (Table 4). The fate of the amino acids derived from the protein degraded before the induction of NADP-GDH was not investigated but the following possibilities may be considered: (*a*) deamination, (*b*) expansion of the free amino acid pool, (*c*) rapid conversion of amino acids into insoluble structural proteins. The only possibility which is difficult to reconcile with the data about exogenously supplied nitrogen sources is (*b*), the expansion of the amino acid pool. One analysis of a soluble protein fraction did not show significant quantities of free amino acids. Further investigations on the role of exogenously supplied amino acids as well as the fate and function of endogenously produced amino acids are in progress.

Although no net protein synthesis occurs within the first 4 hr of incubation, L-valine-^{14}C incorporation experiments established that protein turnover was taking place during this time; it is precisely during this time that NADP-GDH activity increased. The question remained whether the increase represented *de novo* synthesis of enzyme or activation of pre-existing enzyme. Since cycloheximide prevented the incorporation of L-valine-^{14}C into protein and simultaneously prevented the appearance of NADP-GDH activity (Tables 3, 7; Fig. 1), these data are consistent with *de novo* synthesis of NADP-GDH. However, proof of synthesis would require the

demonstration of radioactive label associated with NADP-GDH. Electrophoresis of a partially purified preparation obtained from conidia exposed to L-valine-^{14}C showed radioactivity associated with the region of NADP-GDH activity, but this can still not be considered unequivocal evidence since the label was distributed in overlapping bands along the cellulose acetate strip. The synthesis of NADP-GDH in the absence of net protein synthesis is not a unique situation in Neurospora. It has also been shown that the tyrosinase in Neurospora is synthesized *de novo* under conditions in which net protein synthesis is prevented by starvation or by certain inhibitors of growth (Horowitz *et al.* 1961).

In contrast to the report of Sanwal & Lata (1962*a*), the present experiments have shown that NAD-GDH was present in conidia under all conditions investigated. As conidia aged, in fact, the specific activity of NAD-GDH increased while the NADP-GDH activity decreased to negligible values. Since they were unable to detect NAD-GDH in conidia, Sanwal & Lata (1962b) postulated that conidia are qualitatively different with regard to the regulation of NAD-GDH and NADP-GDH. The postulated uniqueness of conidia was required since NADP-GDH and NAD-GDH were thought to be synthesized together during the process of germination, but the occurrence of these same enzymes appeared to be regulated reciprocally in mycelium. The data presented here concerning the early stages of germination clearly show that parallel synthesis of the two enzymes did not occur. It is still possible, however, that there is a difference between conidia and mycelium, in that the two enzymes do not appear to be reciprocally regulated in germinating conidia. Since it has recently been shown the NADP-GDH is an allosteric protein (West *et al.* 1966; West, Tuveson, Barratt & Fincham, 1967), further investigation of the inter-relationships in the regulation of NADP-GDH and NAD-GDH may be profitably investigated at the level of activity as well as synthesis.

This investigation was supported in part by a U.S. Public Health Fellowship (1-F3-GM-28,398-01) from the National Institute of General Medical Sciences and NSF Grant no. GB 1914, and National Institute of Health Training Grant no. STI-GM 961.

REFERENCES

BARRATT, R. W. (1963). Effect of environmental conditions on the NADP-specific glutamic acid dehydrogenase in *Neurospora crassa*. *J. gen. Microbiol.* **33**, 33.

BARRATT, R. W. & STRICKLAND, W. N. (1963). Purification and characterization of a TPN-specific glutamic acid dehydrogenase from *Neurospora crassa*. *Archs Biochem. Biophys.* **102**, 66.

BARRATT, R. W., TUVESON, R. W & WEST, D. J. (1966). NADP-glutamic acid dehydrogenase (NADP-GKN) and NAD-glutamic acid dehydrogenase (NAD-GDN) activities in wild-type resting and germinating conidia of Neurospora. *Genetics* **54**, 320. (Abstr.)

BEADLE, G. W. & TATUM, E. L. (1945). Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. *Am. J. Bot.* **32**, 678.

GOMORI, G. (1955). Preparation of buffers for use in enzyme studies. *Meth. Enzymol.* **1**.

HAIDLE, C. W. & STORCK, R. (1966). Inhibition by cycloheximide of protein and RNA synthesis in *Mucor rouxii*. *Biochem. biophys. Res. Comm.* **22**, 175.

HOROWITZ, N. H. (1965). Evidence for common control of tyrosinase and L-amino acid oxidase in Neurospora. *Biochem. biophys. Res. Comm.* **18**, 686.

HOROWITZ, N. H., FLING, M., MACLEOD, H. L. & WATANABE, Y. (1961). Structural and regulative genes controlling tyrosinase synthesis in Neurospora. *Cold Spr. Harb. Symp. Quant. Biol.* **26**, 233.

JACOB, F. & MONOD, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. molec. Biol.* **3**, 318.

KLINGMÜLLER, W. (1963). A damaging effect of drying on *Neurospora crassa* conidia and its reversibility. *Z. Naturforsch.* **18**, 55.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MANS, R. J. & NOVELLI, G. D. (1961). Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. *Archs Biochem. Biophys.* **94**, 48.

MUNKRES, K. D. (1965). An assay procedure for Neurospora malate dehydrogenase. *Neurospora Newsletter* **8**, 19.

PALL, M. L. (1966). The use of cycloheximide as an inhibitor of protein synthesis in Neurospora. *Neurospora Newsletter* **9**, 16.

RYAN, F. J. (1948). The germination of conidia from biochemical mutants of Neurospora. *Am. J. Bot.* **35**, 497.

RYAN, F. J., BEADLE, G. W. & TATUM, E. L. (1943). The tube method of measuring the growth rate of Neurospora. *Am. J. Bot.* **30**, 784.

SANWAL, B. D. & LATA, M. (1962*a*). Effect of glutamic acid on the formation of two glutamic acid dehydrogenases of Neurospora. *Biochem. biophys. Res. Comm.* **6**, 404.

SANWAL, B. D. & LATA, M. (1962*b*). Concurrent regulation of glutamic acid dehydrogenases of Neurospora. *Archs Biochem. Biophys.* **97**, 582.

STINE, G. J. (1965). *NADP- and NAD-dependent glutamic dehydrogenases, succinic dehydrogenase and NADase activities during the asexual cycle of Neurospora crassa.* Ph.D. thesis, University of Delaware, Newark, Delaware.

TURIAN, G. (1961). L'acétate et son double effet d'induction isocitratasique et de différenciation conidienne chez les Neurospora. *C. r. hebd. Séanc. Acad. Sci., Paris* **252**, 1374.

TURIAN, G. (1963). Sur le mécanisme de l'induction isocitratasique chez Allomyces et Neurospora. *Path. Microbiol.* **26**, 553.

TURIAN, G., SEYDOUX, J. & VOLKMANN, D. (1962). Activité isocitratasique et type de sporulation chez *Neurospora tetrasperma* et chez *N. sitophila*, souche normale et mutant microconidien. *Path. Microbiol.* **25**, 737.

VOGEL, H. J. (1956). A convenient growth medium for Neurospora. *Microbiol. Genet. Bull.* **13**, 42.

WAINWRIGHT, S. D. (1959). On the development of increased tryptophan synthetase activity by cell free extracts of *Neurospora crassa*. *Can. J. Biochem. Physiol.* **37**, 1417.

WEST, D. J., TUVESON, R. W. & BARRATT, R. W. (1966). Allosteric interactions in NADP-glutamic acid dehydrogenase (NADP-GDH) from wild type Neurospora. *Genetics* **54**, 368. (Abstr.)

WEST, D. J., TUVESON, R. W., BARRATT, R. W. & FINCHAM, J. R. S (1967). Allosteric effects in nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase from Neurospora. *J. bial. Chem.* **242**, (9), (in press).

J. gen. Microbiol. (1967), **48**, 249–259
Printed in Great Britain

Cell-Bound Penicillinase of *Bacillus licheniformis*; Properties and Purification

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(*Accepted for publication* 9 *March* 1967)

SUMMARY

The cell-bound penicillinase of *Bacillus licheniformis* strain 749/C was examined since this material appears to be an intermediate in the formation of the exopenicillinase. The enzyme remained attached to fragments of the cell membrane during lysis of the cell. It was not released from these fragments by a variety of physical or chemical treatments (urea, hydroxylamine, deoxycholate, ultrasound, etc.). Free penicillinase, very similar if not identical, to the exoenzyme, was produced by treatment with trypsin, *Streptomyces griseus* proteinase or papain, but not by chymotrypsin, lipases or nucleases. The cell-bound enzyme appeared to be covalently linked to the cell membrane through a peptide chain. Trypsin probably cleaved this chain at a linkage involving the carboxyl group of lysine or arginine. The enzymic characteristics of the bound penicillinase are essentially equivalent to those of the exoenzyme. Partial purification of the membrane-bound enzyme is described.

INTRODUCTION

In logarithmic phase cultures of *Bacillus licheniformis* (originally considered to be strains of *B. subtilis*; Pollock, 1963, 1965), either inducible or constitutive strains, about half of the penicillinase is present in the medium and the rest is bound to the organisms. This cell-bound enzyme is firmly held (Kushner & Pollock, 1961) and is external to the cell membrane, since it is partially accessible to antibodies to purified exopenicillinase and, in hypertonic solution, can be liberated by trypsin, which does not appear to penetrate the membrane (Kushner & Pollock, 1961). There is kinetic evidence that the cell-bound enzyme of *B. licheniformis* is an intermediate in the natural formation of the exoenzyme (reviewed by Lampen, 1965). Collins (1964) showed that during penicillinase induction the newly formed enzyme is cell bound. Also, release of penicillinase continued after synthesis of new enzyme had been arrested by removal either of inducer (Pollock, 1961*a*, *b*), of a source of nitrogen (Lampen, 1967), or of an essential amino acid (Segal, 1965).

Kushner & Pollock (1961) prepared 'disrupted spheroplasts' from *Bacillus licheniformis* by treatment with lysozyme in the presence of 0·05 M–Mg^{2+}. These preparations contained essentially all of the original bound penicillinase. The enzyme could be released by trypsin or by sodium deoxycholate (as activity not sedimenting in 10 min. at 20,000 *g*). The slow endogenous release was prevented by 0·05 M–Mg^{2+}. It has been found, however, that removal of the cell wall by lysozyme and the release of intracellular protein and nucleic acid are incomplete under the conditions previously

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used (Lampen, 1967). Thus the observed effects of Mg^{2+} and of deoxycholate probably reflected protection and fragmentation of the spheroplasts, respectively, rather than direct action on the liberation of free enzyme.

The properties of the cell-bound penicillinase, and especially the nature of its attachment, are important for an understanding of the formation and release of what eventually becomes exoenzyme. The present work was designed to isolate the bound penicillinase for comparison with the exoenzyme and to determine its location in the cell and means of attachment. In preliminary studies it was found that the cell-bound or membrane-bound enzyme could be readily differentiated from the exoenzyme by its behaviour during electrophoresis in starch gel. The previous criterion for free enzyme, i.e. failure to sediment in 10 min. at 20,000 *g*, did not distinguish between exoenzyme and enzyme bound to small pieces of membrane. For this reason, electrophoretic mobility is here used as the major criterion for identifying the several types of penicillinase. Earlier studies with *Bacillus licheniformis* utilized primarily strain 6346 (Kushner & Pollock, 1961), but strain 749/C was chosen here since it is less sensitive to 'anaerobic lysis' (see Pollock, 1961*a*) and produces greater amounts of penicillinase.

METHODS

Organisms. Bacillus licheniformis strain 749/C, a penicillinase-magnoconstitutive mutant of the penicillinase-inducible wild-type strain 749, was isolated by Pollock (1963). Stock cultures were preserved as spores in aqueous suspension at +2°. The casein hydrolysate+salts medium (CH/S) generally used for growth of the organism was described by Pollock (1965).

Growth conditions. The most convenient way to prepare stable logarithmic-phase organisms was as follows: Petri dishes (9 cm. diam.) containing Andrade agar (Kogut, Pollock & Tridgell, 1956) were inoculated with 10^6 spores and incubated overnight at 35°. These plates provided a routine check on purity of the culture and on penicillinase production, giving red colonies after flooding a plate with penicillin solution. The bacteria from two plates were washed off the agar with a little CH/S medium, added to 800 ml. CH/S medium (warmed to 35°) in a 5 l. flask and shaken at 30 cycles/min. until the population reached the equivalent of 0·7 mg. dry wt bacteria/ml. (5–6 hr). In early experiments, 100 ml. CH/S medium were inoculated with 3×10^8 viable spores and incubated (without shaking) overnight at 35°. The entire culture was then added to 800 ml. CH/S medium for the final period of growth. In a few experiments when glucose was added, it was necessary to add N-NaOH occasionally to keep the culture at pH 7·0–7·6. Certain bound-penicillinase preparations were derived from the single large batch described by Pollock (1965). The exopenicillinase used for comparisons with the free and bound forms of the enzyme was obtained from a culture filtrate by adsorption on and elution from cellulose phosphate and fractional precipitation with ammonium sulphate (Pollock, 1965). It had a specific activity of about 300 units/μg. protein. (The enzyme released into the medium during growth is termed exopenicillinase; that released from preparations of the bound enzyme under various test conditions is called 'free', although it may be identical to the naturally released enzyme.) All weights of organisms are expressed as the dry-weight equivalent.

Methods. Starch-gel electrophoresis was done according to the methods of Smithies (1955) with a bridge solution of 0·33 M-sodium borate buffer (pH 8·45) and run for 4 hr

at a voltage gradient of approximately 14 V./cm. Temperature was maintained at 10–15° by placing the gel on a water-cooled block. After electrophoresis, the gel was sliced. In one half, zones of enzymic activity were located by soaking the gel for 20–30 min. in a saturated aqueous solution of *N*-phenyl-1-napthylamine-azo-*o*-carboxybenzene (Pollock, 1965); the dye was then drained off and the gel flooded with an aqueous solution of approximately 1·0 M-benzylpenicillin. For detection of smaller amounts of the various enzyme types, the other half of the slice was sprayed directly with a solution of 0·15 M-benzylpenicillin in 0·05 M-phosphate buffer (pH 7·0) containing 0·016 N-iodine and 0·06 M-KI. By including in each run penicillinase samples of known activity, these procedures could be made semiquantitative.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Penicillinase assay. Penicillinase was usually assayed by the iodometric method of Perret (1954) and activity expressed as units (μmoles benzylpenicillin hydrolysed/hr at 30°). Benzylpenicillin was used at 2 mg./ml., other penicillins at equimolar concentrations. Dilutions of enzyme samples were made in 0·1 % gelatin containing 0·84 mM-8-hydroxyquinoline. In experiments with cephalosporin C, and for studies of the effect of antisera to purified penicillinase, activity was assayed manometrically at pH 7·0 and 30° by the method of Henry & Housewright (1947), 1 ml. of 1·0 % gelatin solution being added to the manometer cups before assay. In determining affinity constants for benzylpenicillin and methicillin the micro-iodometric method of Novick (1962) was used because of its greater sensitivity. (For a comparison of these methods see Pollock, 1965.)

Chemicals. N-Phenyl-1-naphthylamine-azo-*o*-carboxybenzene was bought from the British Drug Houses Ltd., Poole, England. Methicillin and cephalosporin C were gifts from Beecham Research Laboratories, Ltd. and Glaxo Laboratories, respectively.

Trypsin, chymotrypsin, bovine pancreatic ribonuclease and lysozyme were obtained from Armour Pharmaceutical Company, Ltd., Eastbourne, England; *Streptomyces griseus* proteinase (Pronase) from Calbiochem., Los Angeles, California, U.S.A.; deoxyribonuclease and trypsin inhibitor (soy bean) from Worthington Biochemical Corp., Freehold, N. J., U.S.A. Purified papain was a gift from Dr J. Mandelstam.

RESULTS

Existence of membrane-bound enzyme

A fraction consisting mainly of membrane fragments was obtained by lysing log-phase *Bacillus licheniformis* 749/C organisms (20–30 mg./ml.) with lysozyme, 50 μg./ml. in 0·01 M-sodium phosphate buffer (pH 7·0) at 30°. Once visible lysis had begun, ribonuclease and deoxyribonuclease were added at 10 and 5 μg./ml., respectively. $MgSO_4$ (0·01 M) was added after lysis whenever it was desirable to maintain large fragments (Lampen, 1967); it was not added when smaller fragments were needed for purification trials or for enzymic studies.

In a typical experiment without added Mg^{2+}, a culture (800 ml.) harvested at a concentration of 0·73 mg. organisms/ml. contained 4920 units penicillinase/ml. (7800 units/mg. organisms). Of this quantity, 68 % sedimented with the bacteria (5000 *g*, 10 min.); 32 % was in the culture fluid. The bacteria ($2·5 \times 10^6$ units penicillinase) were resuspended in 25 ml. phosphate buffer + lysozyme and later deoxyribonuclease and

ribonuclease were added. After 20 min. at 30° the mixture was centrifuged at 20,000 *g* for 20 min. at 0°. The sediment contained 60 % of the original activity, the supernatant fluid 31 %. A second treatment of the residue with lysozyme and nucleases released only another 3 % of the original activity.

Most of the penicillinase in the extract was in the bound form (Fig. 1B). Centrifugation at 80,000 *g* for 120 min. (Spinco, Model L) sedimented 40 % of the activity; after 105,000 *g* for 4 hr about 75 % of the enzyme was found in the pellet. The bulk of the enzyme in these fractions did not move upon starch-block electrophoresis at pH 8·45 (Fig, 1C, D) or, in other experiments, at pH 6·5 (0·02 M-phosphate buffer).

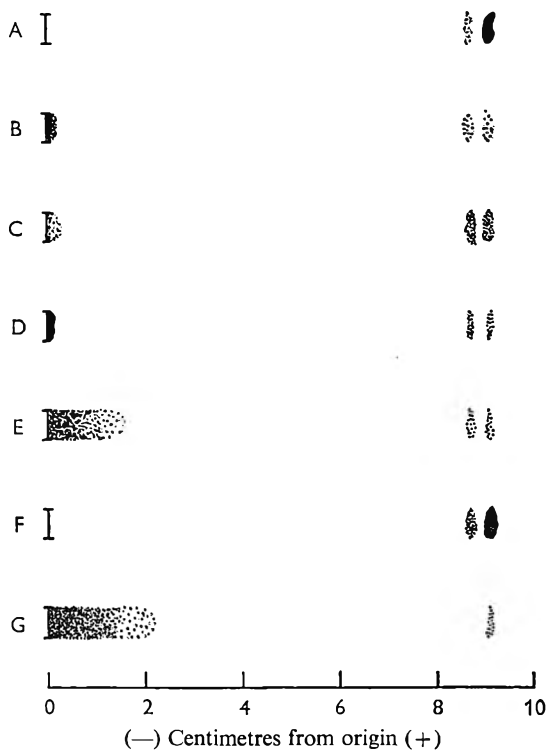

Fig. 1. Electrophoretic characteristics of free and bound penicillinase preparations from strain *Bacillus licheniformis* 749/C. Solutions containing 1500–2000 units activity/ml. were applied. The patterns were developed by soaking the gel in a saturated aqueous solution of *N*-phenyl-1-naphthylamine-azo-*o*-carboxybenzene and subsequently adding a concentrated solution of benzylpenicillin. A, 749/C exopenicillinase; B, 20,000 *g* supernatant fluid from lysed bacteria; C, 105,000 *g* supernatant fluid; D, 105,000 *g* sediment; E, as D after treatment with Na deoxycholate (Table 1); F, preparation (like E) treated with trypsin (Table 2); G, purified bound enzyme (Fig. 2).

In the final supernatant fluid about three quarters of the enzyme had the electrophoretic mobility of exopenicillinase. It is probable that this free enzyme (about 6 % of the original cell-bound activity) was released during the relatively long time required to sediment the slimy residues obtained when Mg^{2+} was not added following lysis.

When 0·01 M-$MgSO_4$ was added after lysis of the bacteria, the resulting membrane fraction remained aggregated and could be washed in 0·01 M-$MgSO_4$ or treated again

with lysozyme + nucleases without further rapid release of penicillinase. These residues contained about 30 % of the total cell protein and had a specific activity of 20–30 units penicillinase/μg. protein. Evidence is offered later that the exo- and bound forms of penicillinase have the same activity per mole of enzyme protein. The specific activity of pure *Bacillus licheniformis* 749/C penicillinase is about 325 units/μg. protein (Pollock, 1965). The bound penicillinase must therefore constitute 6–8 % of the protein of these membrane preparations. Typical logarithmic-phase organisms (70 % of the dry matter is protein) contain 4000 units of enzyme/mg. dry wt organism. Thus, the penicillinase protein is about 1·5 % of the total cellular protein.

Table 1. *Lack of release of free penicillinase of Bacillus licheniformis 749/C by physical treatment*

Bound penicillinase [2000–6000 units/ml.] as a washed sediment from centrifugation at 100,000 *g* for 4 hr was subjected to various procedures, usually in 0·01 M-potassium phosphate buffer (pH 7·0). Residual enzymic activity was measured, and samples were examined by starch gel electrophoresis for the possible presence of free enzyme.

Treatment		Formation of free enzyme*	Loss of activity
Na deoxycholate (1 %)	30° 30 min.	−†	.
Urea (7·2 M)	0° 40 min.	−†	< 20 %
Urea (7·2 M)	0° 100 min.	−	50 %‡
EDTA (pH 7·0; 0·01 M)	30° 20 min.	−†	.
Ultrasound, 25 kc	0° 12 min.	−	.
Frozen and thawed 4 times (in pH 4·1 acetate buffer; 0·05 M)	30° 20 min.	−	< 20 %
Butanol, 4 % (v/v) in water	30° 70 min.	−	.
Chloroform (saturated aqueous solution)	30° 60 min.	−†	58 %‡
Hydroxylamine (0·08 M)	0° 18 hr	−	.

* The symbol (−) indicates that no detectable amount of enzyme was formed which had a mobility one-third or more of that characteristic of exopenicillinase as present in culture filtrates. Treatment with deoxycholate, urea, EDTA or ultrasound produced a substantial amount of enzyme that moved a short distance from the origin in a diffuse band but was clearly retarded to a major extent in comparison with free enzyme (Fig. 1).

† There was no change in the mobility of exoenzyme following identical treatment.

‡ Inactivation of exoenzyme also took place.

Nature of binding to membrane

Lack of release by physical or chemical treatments. A variety of procedures designed to rupture hydrogen bonds or to fragment the particles which have attached penicillinase (Table 1) did not release enzyme activity in a form which exhibited the electrophoretic mobility of exopenicillinase. Treatment with sodium deoxycholate (Fig. 1 E), urea, ethylenediamine tetra-acetic acid (EDTA) or ultrasound did produce smaller particles, but no free enzyme was detectable. Even after an exposure to 7·2 M-urea, which was sufficient to destroy 50 % of the activity, the remaining activity had the low mobility characteristic of bound enzyme. Examination of the samples from the experiment of Table 1 by centrifugation in a 5–20 % sucrose gradient in 0·01 M-phosphate buffer (pH 7·0) gave results consistent with the above conclusions. It was clear that fragmentation of the particles had occurred during the treatment with urea, deoxycholate or ultrasound, but no significant amount of enzyme with the sedimentation properties of exopenicillinase was formed.

Action of proteolytic enzymes. In contrast to the ineffectiveness of physico-chemical

treatments, incubation of the bound penicillinase preparation with trypsin rapidly released enzyme which was electrophoretically identical with exopenicillinase (Table 2; Fig. 1 F). The reaction was prevented by adding trypsin inhibitor. With bound penicillinase equivalent to 30 μg. exoenzyme, 1 μg. trypsin released 80 % of the activity in 5 min. Pronase and papain were also highly effective. The chymotrypsin preparation

Table 2. *Release of free penicillinase by trypsin*

The penicillinase preparations were added at zero time to the other constituents (as indicated) in 0·01 M-phosphate buffer (pH 8·0). After incubation at 30° the mixtures were frozen until subjected to starch gel electrophoresis or to assay. The bound enzyme was a fraction which had sedimented in 1 hr at 100,000 *g* from an extract obtained by treatment with 0·5 % sodium deoxycholate and 6 M-urea (Fig. 2).

Penicillinase (μg. protein)	Incubation (min.)	Additions (per ml.)	Final activity (units/ml.)	% Free enzyme*
Bound (30)	70	.	1600	2–5
		1 μg. trypsin (0 min.)		
	70	+ 20 μg. trypsin inhibitor (0 min.)	.	2–5
	70	+ 20 μg. trypsin inhibitor (5 min.)	.	80
	70	+ 20 μg. trypsin inhibitor (70 min.)	1450	> 95
Free (15)	60	.	4370	.
	60	50 μg. trypsin	4100	.

* Detected by starch gel electrophoresis.

Table 3. *Release of penicillinase by proteinases*

The penicillinase samples were added at zero time to the indicated enzymes in a final volume of 2·0 ml. The bound enzyme preparation was that of Table 2. For tests with trypsin or chymotrypsin the buffer was 0·01 M-phosphate (pH 8·0); for Pronase 0·01 M-tris (pH 7·5) containing 0·01 M-$CaCl_2$; for papain 0·05 M-phosphate (pH 5·9) with 0·1 mg. cysteine HCl/ml. and 0·01 M-EDTA. After incubation at 30° the mixtures were frozen until used for assay and for the estimation of free enzyme by starch gel electrophoresis.

Penicillinase (μg. protein)	Proteinase	Trypsin inhibitor	Incubation (min.)	Final activity units/ml.	% Free enzyme
Bound (100)	.	.	60	4400	5
	Trypsin 2 μg.	20 μg. at 20 min.	60	.	80–90
	Chymotrypsin 5 μg.	.	60	.	10
	Chymotrypsin 50 μg.	50 μg. at 0 min.	60	.	5–10
	Chymotrypsin 50 μg.	50 μg. at 20 min.	60	.	50
	Chymotrypsin 50 μg.	50 μg. at 60 min.	60	4300	80
Bound (100)	.	.	5	5200	5–10
	Pronase 5 μg.	.	5	4000	90
Free (13)	.	.	90	4200	.
	Pronase 10 μg.	.	90	3820	.
	Pronase 10 μg.	.	360	2630*	.
Bound (100)	.	.	5	4330	10
	Papain 4 μg.	.	0	.	90†
	Papain 4 μg.	.	5	4550	95
Free (13)	.	.	60	3860	.
	Papain 4 μg.	.	60	3320*	.

* Electrophoretic mobility of remaining active materials was unchanged.

† Cleavage by the proteinases will take place in the starch gel during electrophoresis. Kinetic studies were therefore done only with trypsin or chymotrypsin whose action could be halted by trypsin inhibitor.

in high concentrations released free penicillinase slowly, but this release was prevented by adding an equal weight of trypsin inhibitor (chymotrypsin preparations usually contain small amounts of trypsin). Trypsin inhibitor combines almost instantly and irreversibly with trypsin on a 1:1 weight basis, but forms a dissociable complex with chymotrypsin (Kunitz, 1947) and would not inhibit completely the action of chymotrypsin under the conditions of Table 3. Thus one may conclude that chymotrypsin is inactive in releasing penicillinase from its bound form. Lysozyme, ribonuclease, deoxyribonuclease and wheat germ lipase were also without effect.

It is unlikely that trypsin acts by causing a gross disintegration of the membrane particles. Examination by electron microscopy did not reveal any major changes in the appearance of the particles during the period when at least 95 % of the bound penicillinase had been released.

Free penicillinase and exopenicillinase are both resistant to the action of proteolytic enzymes (Table 3). For examples, 13 μg. free penicillinase were incubated with 10 μg. of Pronase (10^{-3} M-$CaCl_2$ present); there was a decrease in activity of only 9 % during 90 min. and 37 % in 6 hr. This is in sharp contrast to the rapid cleavage of the denatured enzyme. Thus the penicillinase of *Bacillus licheniformis* 749/C though lacking disulphide bridges (Pollock & Richmond, 1962) must have extensive and stable tertiary structure.

Table 4. *Activity and accessibility of cell-bound penicillinase*

A culture of *Bacillus licheniformis* 749/C was harvested at a population equiv. 0·6 mg. dry wt/ml. and washed with 0·01 M-phosphate buffer (pH 7·0). The organisms were resuspended at twice the original concentration either in buffer alone or with various additions. After the indicated period at 30°, the penicillinase activity of the mixtures was determined at once, by the iodometric method with a 5 min. incubation period.

Additions	Incubation (min.)	Penicillinase activity (units/ml.)
None	0	5450
8-Hydroxyquinoline (0·0005 M)	0	5540
8-Hydroxyquinoline (0·0005 M)+ lysozyme (50 μg./ml.) ribonuclease (10 μg./ml.) deoxyribonuclease (5 μg./ml.) trypsin (20 μg./ml.)	20*	5600

* Lysis was complete within 10 min. After 20 min., a portion of the mixture received 100 μg. trypsin inhibitor/ml. to halt trypsin action. More than 90 % of the penicillinase was then present in the free form as determined by electrophoresis in starch gel.

Properties of bound penicillinase of Bacillus licheniformis 749/C

Accessibility. The cell-bound enzyme is completely available to substrate, as can be seen from the experiment of Table 4. Essentially identical values were obtained by using intact organisms, organisms treated with 8-hydroxyquinoline to halt penicillinase synthesis, or the suspension after lysis and release of free enzyme. One may also conclude that free and bound penicillinase probably have the same catalytic activity per mole of enzyme protein; further evidence for this is provided in Tables 2 and 3. There was no change in total penicillinase activity during a period when 95 % of the enzyme was released from the bound form.

Kinetic constants. The relative maximum velocities and affinity constants of the

exo- and bound enzymes for various substrates did not differ significantly (Table 5). Benzylpenicillin, cephalosporin C and methicillin were selected for the comparison, since the exopenicillinase of *Bacillus licheniformis* strain 749/C cleaves these substrates at widely different rates (Pollock, 1965). The affinity of penicillinase for methicillin is high and was most conveniently measured as K_i against benzylpenicillin, by using the procedure of Hunter & Downs (1945).

Table 5. *Kinetic characteristics of free and bound forms of penicillinase of Bacillus licheniformis 749/C*

V_{max} was determined by the manometric procedure (Henry & Housewright, 1947). The micro-iodometric method of Novick (1962) was used for measurement of affinity constants. K_i for methicillin was determined by its inhibition of the cleavage of benzylpenicillin (Hunter & Downs, 1945). The bound enzyme was prepared by ammonium sulphate precipitation and differential ultracentrifugation of the material released by lysis with lysozyme in the absence of Mg^{2+}. Its specific activity was 19 units/μg. protein. The washed organisms were harvested from a log-phase culture just before testing. In parallel flasks which did not receive substrate less than 15 % of the cell-bound enzyme was released during the 30 min. required for equilibration and determination of enzymatic rate. This quantity could not seriously influence the observed results.

	Substrate		
	Benzylpenicillin	Cephalosporin C	Methicillin
Penicillinase	V_{max} (relative)		
Exo	[100]	1·1	0·45
Bound	[100]	1·25	0·5
Washed organisms	[100]	0·9 1·3 *	0·56
	K_m		K_i
Exo	6×10^{-5} M		$1{\cdot}4 \times 10^{-6}$ M
Bound	$5{\cdot}0 \times 10^{-5}$ M $6{\cdot}4 \times 10^{-5}$ M *		$1{\cdot}1 \times 10^{-6}$ M

* Results of separate experiments.

Effects of antiserum to exopenicillinase. Pollock (1964) reported that antisera prepared in rabbits by the injection of purified exopenicillinase of *Bacillus licheniformis* strain 749 contain certain antibodies which stimulate and others which depress the activity ($V_{max.}$) of the enzyme, according to the substrate used. The antiserum to exopenicillinase used in the present studies produced (at the equivalent point) about a 50 % inhibition of the cleavage of benzylpenicillin and a 12–14-fold increase in the rate of hydrolysis of methicillin. Most preparations of bound enzyme responded to antiserum in the same way as did the exoenzyme. However, one preparation, an $(NH_4)_2SO_4$ precipitate from a sodium deoxycholate-urea extract, was inhibited to a lesser extent (only a 20 % decrease in activity) on benzylpenicillin. The stimulation of methicillin cleavage by this preparation was identical to that obtained with exopenicillinase. No explanation can be offered for the unusual result with the single preparation, but it is clear that attachment to the membrane fragment did not generally modify the conformational changes produced in the penicillinase molecule by antiserum.

Other properties. The sensitivity to iodine of the bound and exo-forms of penicillinase was compared by using the procedure of Garber & Citri (1962) as modified by Pollock

(1965). After 5 min. treatment at 0° with 3·8 mM-iodine there was 24 and 21 % inactivation, respectively, and after 30 min., 65 and 54 %. These variations are considered to be within experimental error.

Partial purification of bound enzyme. The procedure was based upon the finding (Table 1 and text) that a number of agents would partially disintegrate the membrane particles without releasing the bound penicillinase. $MgSO_4$ (0·01 M) was added after lysis of the organisms, since the membrane fraction could then be readily washed free of cytoplasmic proteins. Figure 2 presents the flow sheet of a typical preparation. The membranes were disintegrated with deoxycholate, which removed some lipid and produced a 'fluffy' particle (as observed with the electron microscope). Streptomycin sulphate precipitated a small amount of nucleic acid, and the particles were then further broken with urea (these fragments were difficult to visualize with the electron microscope and only 30–50 % of the activity sedimented in 4 hr at 100,000 *g*). Finally, the bound penicillinase was precipitated by saturating the solution with ammonium sulphate. At the penicillinase concentration used (about 50,000 units/ml.) very little of the free enzyme precipitated in 24 hr at 0°. The final preparation was dissolved in 0·01 M-phosphate buffer (pH 7·0) and dialysed against the same buffer.

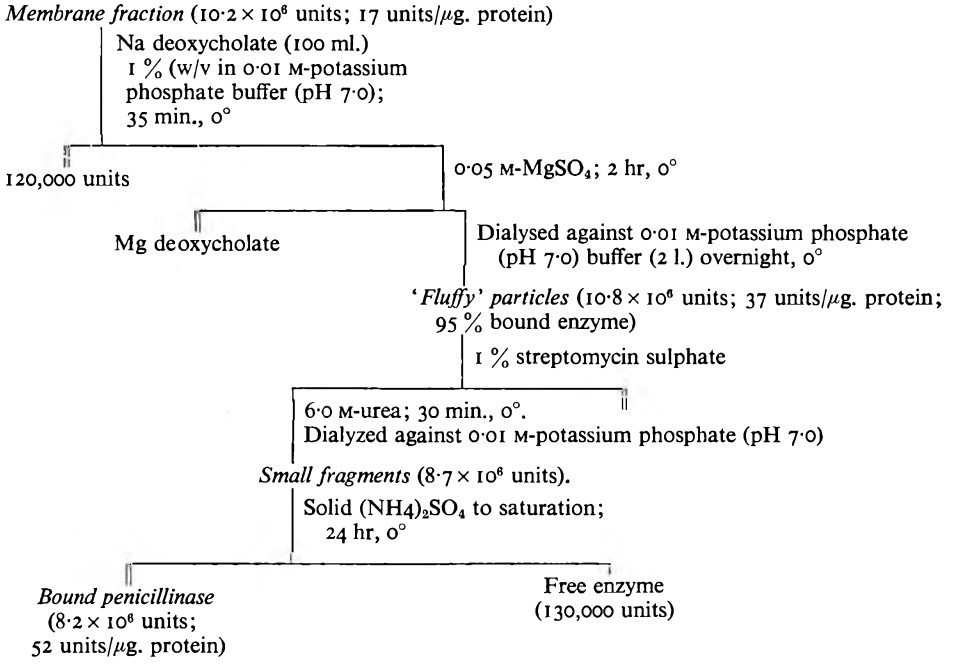

Fig. 2. Purification of bound penicillinase of *Bacillus licheniformis* 749/C

The specific activity was 52 units/μg. protein. This corresponds to a purity (in terms of protein) of about 15 %. The material was still heterogeneous in particle size, since about 10 % of the activity (and protein) sedimented in 1 hr at 100,000 *g* and 45 % in 4 hr. Also, on starch gel electrophoresis (Fig. 1 G) the bulk of the activity moved to some extent in the electric field and must represent a broad distribution of particle sizes.

The preparation still contained about 5 % nucleic acid, as determined by its extinction at 280 and 260 mμ. This material was removed only very slowly by ribonuclease.

DISCUSSION

From the results presented here and the earlier work of Kushner & Pollock (1961) it is clear that essentially all of the cell-bound penicillinase of *Bacillus licheniformis* 749/C remains attached to the cell membrane fraction during lysis of the cell. The enzyme is not removed from the membrane by a variety of physical or chemical treatments but can be rapidly released by trypsin, Pronase or papain, though chymotrypsin is ineffective. Tryspin appears to act exclusively on peptide bonds of the carboxyl groups of lysine or arginine, linkages which can also be cleaved by Pronase or papain, but not by chymotrypsin (Hill, 1965). One may conclude, therefore, that the binding of penicillinase to the cell membrane depends upon peptidic linkage through the carboxyl of either lysine or arginine. In an attempt to differentiate between the two possible linkages, a bound penicillinase preparation was treated with succinic anhydride under the conditions of Li & Bertsch (1960) in order to succinylate the ϵ-NH_2 group of the lysine residues. Trypsin cannot cleave the peptide bond adjacent to an ϵ-*N*-succinyl lysine; hence if the linkage is through the carboxyl of lysine, trypsin should be unable to release penicillinase from the modified protein. Unfortunately, succinylation caused complete inactivation of the penicillinase and no further information could be gained. The answer may be provided by studies now under way (R. Ambler, private communication), on the terminal amino acids of the exopenicillinase of *B. licheniformis* strain 749/C and the enzyme released by trypsin. Since the cell-bound enzyme appears to be an intermediate in the formation of the exopenicillinase (see Introduction), one may state that newly formed penicillinase is covalently linked to the outer side of the cell membrane and must subsequently be released from the cell by an enzymic process. The nature of this liberation process is the subject of the following paper (Lampen, 1967).

The enzymic properties of the bound penicillinase of *Bacillus licheniformis* strain 749/C (in the form of partially purified membrane fragments) appeared to be the same as those of the free enzyme, with the exception of the single preparation which was less sensitive to inhibition by antiserum to exopenicillinase. This is in contrast to the recent report by Ishimoto (1963) that the cell-bound penicillinase of a strain of *B. subtilis* differed significantly from the exoenzyme. The bound enzyme of *B. subtilis* was more sensitive to iodine than was the exo-form, and it was not inhibited by antiserum to the purified exoenzyme. The results with antiserum probably reflect a true difference between the two organisms, since even the penicillinase activity of intact organisms of *B. licheniformis* 749/C can be inhibited by antiserum to the exoenzyme (Kushner & Pollock, 1961). One should note, however, that Ishimoto's preparation of bound enzyme was a crude extract prepared by ultrasonic treatment, whereas ours consisted of purified membrane fragments. The sensitivity of bound penicillinase to various agents might differ in the two systems. Birnboim (1966) purified a nuclease from *B. subtilis* which appeared to be bound to the cell wall membrane fraction. It was released from the organisms during conversion to protoplasts with lysozyme, but in a form which could be solubilized by treatment with trypsin. The similarity between these properties and the characteristics of bound penicillinase (as outlined by Lampen, 1965) was noted.

I wish to thank Dr M. R. Pollock, F.R.S., for the hospitality of his laboratory during 1963–64 and for many valuable discussions. Dr Pollock provided the purified exo-penicillinase from *Bacillus licheniformis* strain 749/C and the antiserum to the purified exoenzyme from strain 749. Examination of the bound penicillinase preparations with the electron microscope was done with the generous co-operation of Dr R. Valentine. Miss Joan Fleming and Mr P. Thompson provided capable technical assistance. This work was supported in part by grants from the United States Public Health Service (AI–04572) and from the National Science Foundation (GB–1125).

REFERENCES

BIRNBOIM, H. C. (1966). Cellular site in *Bacillus subtilis* of a nuclease which preferentially degrades single-stranded nucleic acids. *J. Bact.* **91**, 1004.

COLLINS, J. F. (1964). The distribution and formation of penicillinase in a bacterial population of *Bacillus licheniformis*. *J. gen. Microbiol.* **34**, 363.

GARBER, N. & CITRI, N. (1962). The interaction of penicillinase with penicillins. I. Effect of substrates and of a competitive inhibitor of native and urea-treated enzyme. *Biochim. biophys. Acta* **62**, 385.

HENRY, R. J. & HOUSEWRIGHT, R. D. (1947). Studies on penicillinase. II. Manometric method of assaying penicillinase and penicillin, kinetics of the penicillin-penicillinase reaction, and the effects of inhibitors on penicillinase. *J. biol. Chem.* **167**, 559.

HILL, R. (1965). Hydrolysis of proteins. *Adv. Protein Chem.* **20**, 37.

HUNTER, A. & DOWNS, C. E. (1945). The inhibition of arginase by amino acids. *J. biol. Chem.* **157**, 427.

ISHIMOTO, M. (1963). A study of purification and properties of *Bacillus subtilis* exo-penicillinase. *Acta physiol. Acad. sci. hung.* **24**, 35.

KOGUT, M., POLLOCK, M. R. & TRIDGELL, E. J. (1956). Purification of penicillin-induced penicillinase of *Bacillus cereus* NRRL 569: a comparison of its properties with those of a similarly purified penicillinase produced spontaneously by a constitutive mutant strain. *Biochem. J.* **62**, 391.

KUNITZ, M. (1947). Crystalline soybean trypsin inhibitor. II. General properties. *J. gen. Physiol.* **30**, 291.

KUSHNER, D. J. & POLLOCK, M. R. (1961). The location of cell-bound penicillinase in *Bacillus subtilis*. *J. gen. Microbiol.* **26**, 255.

LAMPEN, J. O. (1965). Secretion of enzymes by micro-organisms. *Symp. Soc. gen. Microbiol.* **15**, 115.

LAMPEN, J. O. (1967). Release of penicillinase by *Bacillus licheniformis*. *J. gen. Microbiol.* **48**, 261.

LI, C. H. & BERTSCH, L. (1960). Adrenocorticotropins (ACTH). XX. Succinyl-adrenocorticotropin and its hydrolysis by trypsin. *J. biol. Chem.* **235**, 2638.

LOWRY, O. H., ROSEBROUGH, M. J., FARR, A. L. & RANDALL, R. S. (1951). Protein measurement with Folin phenol reagent. *J. biol. Chem.* **193**, 265.

NOVICK, R. P. (1962). Micro-iodometric assay for penicillinase. *Biochem. J.* **83**, 236.

PERRET, C. J. (1954). Iodometric assay of penicillinase. *Nature, Lond.* **174**, 1012.

POLLOCK, M. R. (1961*a*). The measurement of the liberation of penicillinase from *Bacillus subtilis*. *J. gen. Microbiol.* **26**, 239.

POLLOCK, M. R. (1961*b*). The mechanism of liberation of penicillinase from *Bacillus subtilis*. *J. gen. Microbiol.* **26**, 267.

POLLOCK, M. R. (1963). The differential effect of actinomycin D on the biosynthesis of enzymes in *Bacillus subtilis* and *Bacillus cereus*. *Biochim. biophys. Acta* **76**, 80.

POLLOCK, M. R. (1964). Stimulating and inhibiting antibodies for bacterial penicillinase. *Immunology* **7**, 707.

POLLOCK, M. R. (1965). Purification and properties of penicillinases from two strains of *Bacillus licheniformis*: a chemical, physicochemical and physiological comparison. *Biochem. J.* **94**, 666.

POLLOCK, M. R. & RICHMOND, M. H. (1962). Low cyst(e)ine content of bacterial extracellular proteins: its possible physiological significance. *Nature, Lond.* **194**, 446.

SEGAL, S. (1965). The formation of penicillinase by cysteine-starved auxotrophs of *Bacillus licheniformis*. *Biochim. biophys. Acta* **108**, 297.

SMITHIES, O. (1955). Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. *Biochem. J.* **61**, 629.

J. gen. Microbiol. (1967), 48, 261–268
Printed in Great Britain

Release of Penicillinase by *Bacillus licheniformis*

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(*Accepted for publication 9 March* 1967)

SUMMARY

Organisms (logarithmic growth phase) of *Bacillus licheniformis* strain 749/C when washed and resuspended in buffer ceased to produce penicillinase but continued to release their cell-bound enzyme as typical exopenicillinase. In the first 1–2 hr, 15–30 % of the bound enzyme was liberated; the process then became very slow. Liberation was dependent upon temperature and pH value; it was not inhibited by chloramphenicol. Release was apparently not a result of membrane damage, since there was no leakage of intracellular α-glucosidase. Lysis of the bacilli decreased sharply the rate of release of exoenzyme.

Membrane preparations active in releasing penicillinase were obtained by lysing the bacilli with lysozyme in the absence of added Mg^{2+} and subsequently adding 0·05 M-$MgSO_4$ to prevent dispersal of the membrane segments. The liberation of penicillinase by this fraction had the same general enzymic properties as had the process with intact bacilli. Disruption of the membranes also eliminated most of the penicillinase-releasing activity. The importance of membrane structure in the liberation process is discussed.

INTRODUCTION

In the preceding paper (Lampen, 1967) evidence is presented that the cell-bound penicillinase of *Bacillus licheniformis* strain 749/C is covalently linked to the exterior of the cell membrane by a peptide chain which can be cleaved by trypsin. This material appears to be a precursor of the natural exopenicillinase (Pollock, 1961*a*, *b*; Collins, 1964; Lampen, 1965). Although one may argue that the crucial stages in the production of an external or extracellular enzyme have been completed by the time a bound enzyme can be detected on the outer side of the membrane, the phenomenon of liberation may provide information from which one can infer characteristics of the formation of the enzyme.

Pollock (1961*a*) was able to separate to a large extent the liberation of penicillinase from its net production. He added a limiting amount of a metabolizable inducer (benzylpenicillin) to a growing culture of *Bacillus licheniformis* strain 6346. Release of enzyme into the medium continued after the induced formation of new enzyme had declined sharply. Liberation appeared to require an enzymic reaction (Pollock, 1961*b*), since it was temperature-dependent and did not occur at pH values less than 6·0, and the enzyme was not eluted from the bacilli by high concentrations of salts. Chloramphenicol at high concentrations produced a partial inhibition of exoenzyme release. In an attempt to obtain a simplified system, Kushner & Pollock (1961) investigated

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the liberation of penicillinase by 'disrupted spheroplasts' of *B. licheniformis* 6346. To produce these forms, bacilli were treated with lysozyme in the presence of 0·05 M-$MgSO_4$ + 0·3 M-sucrose and were subsequently centrifuged and resuspended in 0·05 M-Mg^{2+} without osmotic support. The residues still contained as much as 98 % of the original cell-bound penicillinase, but less than 5 % of the (normally 100 % cell-bound) α-glucosidase. Release of penicillinase from these particles was inhibited by 0·05 M-Mg^{2+} and by *p*-chloromercuribenzoate. Trypsin or sodium deoxycholate rapidly liberated the enzyme in a form not sedimentable in 10 min. at 20,000 *g*. Once it was known that the bound penicillinase of *B. licheniformis* strain 749/C is covalently linked to the cell membrane (Lampen, 1967), an attempt was made to extend the observations with *B. licheniformis* 6346 to the more active and stable strain 749/C. Initial studies showed that spheroplast formation was slow under the conditions previously used and that lysis was incomplete. An investigation of the liberation of penicillinase from strain 749/C was therefore undertaken. A summary of these results has been previously presented (Lampen, 1965).

METHODS

The preceding paper (Lampen, 1967) describes the procedures for maintaining spore stocks of *Bacillus licheniformis* 749/C, for preparing logarithmic-phase bacilli and the techniques for starch gel electrophoresis and detection and estimation of bound and free penicillinase. Formation of α-glucosidase was induced by adding maltose at 2 mg./ml. to cultures whose populations had reached the equivalent of 0·1 mg. dry wt bacilli/ml. α-Glucosidase activity was measured by the rate of hydrolysis of *p*-nitrophenyl-α-D-glucoside at 30° and pH 7·0 (Pollock, 1961*a*) and expressed as μmoles *p*-nitrophenol formed/hr. For the determination of total α-glucosidase activity, bacilli were shaken at 35° in stoppered tubes for 20 min., with 2 or 3 drops of butanol which was afterwards removed by a stream of air.

RESULTS

Release of penicillinase by washed organisms

The system for liberation of the cell-bound enzyme from strain *Bacillus licheniformis* 749/C resembles that of *B. licheniformis* strain 6346 in its dependence on temperature and pH value (Table 1), and thus also appears to be enzymic. At pH 5·0 the amount of free enzyme produced was slight, although a considerable amount of particle-bound enzyme was released, possibly as a result of cell lysis. An added energy source was not required for formation of free enzyme. In contrast to the results with strain 6346 chloramphenicol even at 100 μg./ml. did not inhibit release of enzyme by strain 749/C. The reason for this difference is not clear. However, in the present studies formation of new enzyme was arrested by removal of the nitrogen source, whereas with strain 6346 (Pollock, 1961*a*) the bacilli were in a complete growth medium but with their supply of inducer exhausted. A small net synthesis of penicillinase (and perhaps of the system for its release) was still taking place. Inhibition of these synthetic processes by chloramphenicol may have produced the observed partial decrease in the net amount liberated. There was no net synthesis of penicillinase by bacilli of strain 749/C placed in a nitrogen-free medium; under comparable conditions yeast protoplasts formed invertase for several hours at a rapid rate which was not increased by adding a mixture

of amino acids (Lampen, 1965). This difference may reflect the relatively large amino acid pool in yeast (Halvorson, Fry & Schwemmin, 1955).

The release of penicillinase was relatively rapid at first; 15–30 % of the total bound enzyme was generally liberated in the first 0·5–2 hr (typical results are given in Tables 1 and 2); later the rate decreased to a few %/hr. Release was not the result of membrane damage or lysis during incubation in the absence of growth or protein synthesis. Most of the active material released had the mobility of the exoenzyme and was not bound to membrane fragments. No leakage of the cell-bound α-glucosidase was detected during a period when about 20 % of the bound penicillinase was liberated (Table 2). Pollock (1961*a*) showed that leakage of this enzyme is an excellent indication of damage to the cell membrane.

Table 1. *Release of penicillinase by washed bacilli of Bacillus licheniformis strain 749/C*

Log-phase organisms were washed in 0·01 M-tris buffer (pH 7·5) and resuspended in the same buffer. Each incubation mixture contained (per ml.) 1·0 mg. (dry wt equiv.) bacilli, 50 μg. chloramphenicol and other supplements as indicated. After incubation for 2 hr, samples were frozen directly for measurement of total activity or centrifuged for 10 min. at 20,000 *g* and the supernatant fluids frozen for subsequent assay and starch gel electrophoresis.

			Penicillinase in supernatant fluid		
				Free enzyme*	
Additions	Temp. (°C.)	Total penicillinase units/ml.	Total units/ml.	% of total	units/ml.
—	—	4530 (0 time)	—	—	—
—	0	—	480	70	340
—	30	4770	1660	80	1330
Chloramphenicol omitted	30	4600	1650	80	1320
Na acetate buffer, (0·05 M, pH 5·0)	30	4460	780	25	200
Glucose, 0·5 %†	30	4700	1580	50	790

* Has the electrophoretic mobility of natural exopenicillinase.
† During incubation the pH fell to 6·5.

Nature of disrupted spheroplasts

When *Bacillus licheniformis* 749/C was treated with lysozyme in the presence of 0·05 M-Mg^{2+} + 0·3 M-sucrose and subsequently suspended in a medium lacking osmotic support, the residues contained more than 90 % of the original cell-bound penicillinase (Table 3). This was consistent with the observations of Kushner & Pollock (1961) who used *B. licheniformis* strain 6346. These residues consisted predominantly of rod-shaped Gram-variable forms which lysed only when the 0·05 M-Mg^{2+} was also removed. They contained 70–80 % of the protein and nucleotide material of the original bacilli. When Mg^{2+} was not present during treatment with lysozyme, most of the protein and polynucleotide was released upon removal of the osmotic support, along with large quantities of penicillinase, most of which was bound to membrane fragments. It is clear that 0·05 M-Mg^{2+} protected the cell wall against the action of lysozyme to a degree sufficient to prevent the production of true spheroplasts. The criterion of Kushner & Pollock (1961) for liberated enzyme (not sedimented in 10 min. at 20,000 *g*) would not

distinguish the true exo-form from enzyme bound to small fragments of membrane. It is suggested, therefore, that the protective effect of Mg^{2+} and release of penicillinase by sodium deoxycholate which Kushner & Pollock observed reflected the protection and the fragmentation, respectively, of the spheroplasts, rather than direct action on the cell-bound enzyme.

Table 2. *Release of penicillinase without leakage of β-glucosidase from washed Bacillus licheniformis 749/C*

The experimental conditions were those given in Table 1, except that 4·0 mg. (dry wt equiv.) of bacilli grown in the presence of maltose to induce formation of α-glucosidase were present per ml. Incubation at 30°.

	Penicillinase (units/ml.)		α-Glucosidase (units/ml.)	
Time (hr)	Total	Released*	Total	Released
0	26,000	200	18·2	< 0·2
0·5	24,300	1400	—	—
2	24,000	3000	18·1	< 0·2
3	25,100	3900	—	—
4	23,000	4400	18·7	< 0·2

* More than 90% of the released activity had the electrophoretic mobility of exopenicillinase.

Table 3. *Protection by 0·05 M-$MgSO_4$, against the disintegration of Bacillus licheniformis 749/C organisms during treatment with lysozyme*

Log-phase bacilli (24·4 mg. dry wt equiv.) containing 80,000 units of penicillinase, 14 mg. protein, and 160 units of total nucleotide (based on E_{260} value of a lysed preparation) were added to 60 ml. of 0·01 M-phosphate buffer (pH 7·5) + 0·3 M-sucrose. Lysozyme (100 μg./ml.) was added at zero time and the mixtures shaken gently for 2 or 5 min. at 30°. The suspensions were quickly cooled and centrifuged at 15,000 *g* for 15 min. The supernatant fluids represent the material released during formation of spheroplasts. The pellet was suspended in 0·01 M-phosphate buffer (± Mg^{2+}) at 0°, homogenized for 2 min. in a Potter–Elvehjem type of tube, and centrifuged 10 min. at 20,000 *g*. This supernatant fluid contained the material released by lysis of the spheroplasts. The residues were finally extracted by suspending them in 0·01 M-phosphate buffer (without Mg^{+2}) at 30° for 10 min. Ribonuclease (10 μg./ml.) and deoxyribonuclease (5 μg./ml.) were added to decrease the viscosity. The final sediment was removed by centrifugation at 3000 *g* for 10 min.

		% of total fraction released during indicated procedure		
		No Mg^{2+}		0·05 M-Mg^{2+}
Procedure	Cell fraction	2 min.	5 min.	5 min.
Formation of spheroplasts	Penicillinase	5	42 (B)*	4 (F)*
	Nucleotide	13	18	3
	Protein	13	18	5
Lysis of spheroplasts	Penicillinase	38	25 (B)	4 (F)
	Nucleotide	39	38	14
	Protein	67	55	25
Extraction of spheroplast residues	Penicillinase	65†	40†	68 (4B:1F)‡
	Nucleotide	25	20	74

* B = bound enzyme, F = free (based on electrophoretic mobility).
† No intact organisms or spheroplasts seen in these residues.
‡ Rod-like forms predominated in residue. Lysed during extraction.

Membrane structure and release of penicillinase

Bacillus licheniformis 749/C was suspended in the usual test mixture for determining release of bound penicillinase (as Table 1; no Mg^{2+}) and was lysed with lysozyme. Under these conditions (no added Mg^{2+} or osmotic support) the membrane disintegrated and much of the membrane-bound penicillinase did not sediment in 10 min. at 20,000 *g*. The liberation of free enzyme was sharply decreased following lysis,

Table 4. *Importance of membrane structure for release of penicillinase*

Preparations from *Bacillus licheniformis* 749/C. The penicillinase preparations were added at zero time to 0·01 M-tris buffer (pH 7·5) containing 50 μg. chloramphenicol/ml. and were incubated at 30°. At the stated intervals samples were frozen directly or were centrifuged at 20,000 *g* for 10 min. and the supernatant fluid frozen until subsequent assay and starch gel electrophoresis. Membrane fractions were prepared as described in the text.

Bound penicillinase preparation	Treatment	Incubation time (hr)	Penicillinase units/ml. Total	Penicillinase units/ml. 20,000 *g* supernatant fluid	Free enzyme % of total	Free enzyme % of enzyme in 20,000 *g* supernatant fluid
Log-phase bacilli	—	0	4000	140	3	90
	—	0·5	—	780	15	80
	—	2	3600	870	20	90
	Lysed with					
	Lysozyme, 50 μg./ml.	0·2*	3540	1840	1	1–2
	Ribonuclease, 10 μg./ml.	0·5	—	1480	1	1–2
	Deoxyribonuclease, 5 μg./ml.	2	3320	1440	5	5–10
Membrane fraction	—	0	9600	—	1	—
(15 min. lysis)	—	2	—	—	10	—
	—	18	—	—	50	—
(30 min. lysis)	—	0	4500	—	—	—
	—	2	—	190	4	90
	—	18	—	410	8	90
Membrane fraction (15 min. lysis)	Ultrasonic treatment 25 kc; 0°; 12 min.	0	9600	9000	5	—
		2	—	—	5	—
		18	—	—	5–10	—
	0·5 % Na deoxycholate extract†	0	6000	—	1	—
		18	—	—	2	—
	0·01 M-EDTA	0	9600	—	1	—
		18	—	—	5–10	—
Streptomycin $(NH_4)_2SO_4$ precipitate	—	0	2000	—	1	—
	—	24	—	—	5	—

* Lysis essentially complete in 10 min.

† This fraction did not prevent release of free enzyme by the membrane fraction (15 min. lysis) when present at a ratio of 2:1 (based on total units of penicillinase).

despite the fact that the bound enzyme was now free from the bacilli and susceptible to the action of any intracellular proteinases which could release penicillinase from its membrane-bound form (Lampen, 1967). Membrane fractions with moderate activity in releasing penicillinase were obtained, however, when disintegration of the membrane was prevented. Soon after lysis was complete, 0·01–0·05 M-Mg^{2+} was added and

the fraction washed with Mg^{2+} in tris buffer to free it from the added enzymes and cytoplasmic materials. As shown in Table 4, lysis occurred within 10 min. Addition of Mg^{2+} at 15 min. yielded an active preparation. When lysis was allowed to continue for 30 min. the resulting membrane material had a very low penicillinase-releasing activity. In the procedure chosen for subsequent work, $MgSO_4$ was added at 15 min., the mixture held for 10 min. at 0° and then centrifuged for 10 min. at 5000 *g*. The sediment was washed by suspension and centrifugation in 0·01 M-tris buffer (pH 7·5) containing 0·05 M-Mg^{2+}, and then in buffer without Mg^{2+}. The final material was essentially free from intact bacilli.

Table 5. *Characteristics of penicillinase release by membrane fraction*

Preparations from *Bacillus licheniformis* 749/C. Membrane fraction was prepared as described in the text. Each incubation mixture contained (per ml.) the membrane fraction from 3·6 mg. (dry wt equiv.) of bacilli, 50 μg. chloramphenicol and other supplements as indicated, in 0·01 M-tris buffer (pH 7·5). After 0, 2 or 18 hr at 30°, samples were frozen directly or centrifuged for 10 min. at 20,000 *g*. The supernatant fluids were frozen until estimation of penicillinase and starch gel electrophoresis was carried out.

			Released penicillinase*		
			Total enzyme	Free enzyme	
Addition	Incubation time (hr)	Temp.	units/ml.	%	units/ml.
—	0	—	200	—	—
—	2	0°	370	—	—
—	18	0°	760	30	230
—	2	30°	1220	75	915
—	18	30°	2520	75	1890
$MgSO_4$, 0·05 M	2	30°	1160	80	930
	18	30°	3620	80	2900
Na acetate buffer, 0·05 M, pH 5·0	2	30°	460	60	280
—	18	30°	920	50	460

* Total penicillinase, 9600 units/ml.

Liberation of penicillinase from the membrane fraction had the general characteristics of the system shown with intact organisms (Table 5). Thus it was dependent on temperature and pH value and insensitive to chloramphenicol. Added Mg^{2+} was not essential but, in some experiments, prolonged the period during which liberation occurred. At 0° or at pH 5·0 a substantial amount of the enzyme released from the membrane fragments was still in a bound form.

Disruption of the membrane fraction generally led to a severe loss of penicillinase-releasing activity (Table 4). After ultrasonic treatment essentially all of the fragments bearing bound enzyme were small enough to remain in the supernatant fluid after centrifugation for 10 min. at 20,000 *g*. Very little free enzyme was released during subsequent incubation. Sodium deoxycholate disintegrated the membrane preparations (see Lampen, 1967) and prevented release of free enzyme. (The deoxycholate-treated material did not inhibit release by the untreated preparation.) EDTA, which presumably removed Mg^{2+} and other cations from the membranes, was also inhibitory. Repeated rapid freezing and thawing did not disperse the membrane preparations to any noticeable degree and had no effect on the rate of release of free penicillinase.

The liberation of penicillinase, although diminished severely, was never abolished

by disruption of the membranes. Even a purified bound penicillinase preparation, the streptomycin-$(NH_4)_2SO_4$ precipitate used for Table 4, still released about 5 % of its enzyme in 24 hr at 30°. After storage for 2 weeks at 5° about half of the enzyme had been converted to the free form. All bound penicillinase fractions were, therefore, stored at −20°; they were stable under these conditions for up to one year.

Extracts from uninduced organisms

With the penicillinase-inducible strain 6346 of *Bacillus licheniformis* (Pollock, 1961*b*), crude extracts of uninduced organisms caused some release of cell-bound penicillinase, but this action could not be separated from cell damage. The availability of purified bound-penicillinase preparations from *B. licheniformis* 749/C made it possible to measure the formation of free penicillinase directly and without complications from autolytic enzymes released by cell damage. A culture of the penicillinase-inducible wild-type strain 749 of *B. licheniformis* was grown without inducer under the conditions used for the constitutive strain 749/C of *B. licheniformis* (Lampen, 1967). The culture was harvested at a population equiv. to 0·6 mg. dry wt organisms (4 units penicillinase)/ml.; at this stage of growth the constitutive strain would be rapidly forming and secreting penicillinase. The organisms were harvested and lysed as described earlier for preparation of the membrane fraction. The culture fluid, the membrane fraction and the cell-extract supernatant fluid were tested (as in Table 4) for ability to form free penicillinase from purified bound enzyme (the streptomycin + $(NH_4)_2SO_4$ precipitate was used at 2000 units/ml.—an amount of bound enzyme present in 0·6–0·8 ml. of an induced culture). The membrane fraction was inactive. The extract had a slight effect when present in an amount equivalent to the organisms from 5 ml. culture. The undiluted culture fluid released 5–10 % of the enzyme in 3 hr and 25 % in 8 hr. This fraction contained detectable quantities of a proteinase (active on casein). Thus all fractions were relatively ineffective in releasing free penicillinase, as compared with the quantities of penicillinase synthesized and released as exoenzyme in an induced culture or by the constitutive strain (approximately 50 % of the bound enzyme liberated/hr). The membrane fraction from the uninduced bacilli might still have contained a system for the liberation of exoenzyme but, if present, it was inactive on added bound penicillinase.

DISCUSSION

The results presented here, based upon the electrophoretic mobility of the various enzyme forms, emphasize the importance of a test for free (exo-type) enzyme which will differentiate it from enzyme attached to small membrane fragments. The previous criterion (lack of sedimentability in 10 min. at 20,000 *g*) did not distinguish between these forms, hence much of what was earlier considered to be release of exopenicillinase actually reflected disintegration of the cell membrane while its bound enzyme remained attached. The mechanism for releasing the membrane-bound enzyme also appears to reside in the membrane. The fraction obtained by lysis and subsequent addition of Mg^{2+} contained the bulk of the penicillinase-releasing activity. The characteristics of release from the membrane material were very similar to those shown by the intact organisms.

The loss of liberating activity upon distintegration of the membrane preparation may have a variety of explanations. There may be a direct inactivation of the essential

enzyme(s); this would be analogous to the destruction of the external alkyl-β-glucosidase of yeast upon rupture of the cell (Kaplan, 1965). It is unlikely that the decrease in activity reflects solely a dilution of the reactants, since both the penicillinase and the residual liberating activity continue to be bound to the particles. More probable is a requirement for a proper steric relationship between bound enzyme and liberating system, to obtain a reasonable reaction rate.

The only evidence about the nature of the enzyme(s) releasing penicillinase is the fact that added proteinase or proteinase-containing extracts or culture fluids (Pollock, 1961*b*; Lampen, 1967) release free enzyme which is indistinguishable from the natural exoenzyme (Pollock, 1965). The concentration of proteinase in the culture filtrates was too low to account for the normal rate of release of penicillinase; the process in the intact organism was not sensitive to several inhibitors of proteinase action (Pollock, 1961*b*). An investigation of the release phenomenon in protoplasts (where possible shielding of the releasing system from inhibitors is minimized) may indicate whether or not a membrane-bound proteinase, properly positioned in relation to the bound penicillinase, is the actual agent.

The findings presented in this and the preceding paper (Lampen, 1967) provide in part the basis for a working hypothesis of the mechanism of penicillinase formation and release (Lampen, 1965), which considers penicillinase formation to be a facet of membrane synthesis. It is suggested that the enzyme is formed as part of a larger unit which is incorporated into new membrane, with the enzyme oriented toward the outside of the permeability barrier and that the bound enzyme is initially in a protected site (membrane invagination?) and becomes susceptible to release once it has become fully external.

The author thanks Dr M. R. Pollock for his hospitality at the National Institute for Medical Research, Mill Hill, London, and to Mr P. Thompson for capable technical assistance. This work was supported in part by grants from the United States Public Health Service (AI-04572) and from the National Science Foundation (GB-1125).

REFERENCES

COLLINS, J. F. (1964). The distribution and formation of penicillinase in a bacterial population of *Bacillus licheniformis*. *J. gen. Microbiol.* **34**, 363.

HALVORSON, H., FRY, W. & SCHWEMMIN, D. (1955). A study of the properties of the free amino acid pool and enzyme synthesis in yeast. *J. gen. Physiol.* **38**, 549.

KAPLAN, J. G. (1965). An inducible system for the hydrolysis and transport of β-glucosides in yeast. I. Characteristics of the β-glucosidase activity of intact and of lysed cells. *J. gen. Physiol.* **48**, 873.

KUSHNER, D. J. & POLLOCK, M. R. (1961). The location of cell-bound penicillinase in *Bacillus subtilis*. *J. gen. Microbiol.* **26**, 255.

LAMPEN, J. O. (1965). Secretion of enzymes by micro-organisms. *Symp. Soc. gen. Microbiol.* **15**, 115.

LAMPEN, J. O. (1967). Cell-bound penicillinase of *Bacillus licheniformis* properties and purification. *J. gen. Microbiol.* **48**, 249.

POLLOCK, M. R. (1961*a*). The measurements of the liberation of penicillinase from *Bacillus subtilis* *J. gen. Microbiol.* **26**, 239.

POLLOCK, M. R. (1961*b*). The mechanism of liberation of penicillinase from *Bacillus subtilis*. *J. gen. Microbiol.* **26**, 267.

POLLOCK, M. R. (1965). Purification and properties of penicillinase from two strains of *Bacillus licheniformis*: a chemical, physico-chemical and physiological comparison. *Biochem. J.* **94**, 666.

J. gen. Microbiol. (1967), 48, 269–275
With 1 *plate*
Printed in Great Britain

The Role of Extracellular Melanoproteins of *Venturia inaequalis* in Host Susceptibility

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(*Accepted for publication* 14 *March* 1967)

SUMMARY

A series of fungal melanoproteins (mol. wt 10,000–70,000) was isolated from culture filtrates of *Venturia inaequalis*, and partially characterized by gel filtration and acid hydrolysis. Petiole injection of aqueous solutions of the melanoproteins with non-phytotoxic marker compounds, into apple shoots, produced specific effects on the transport of solutes within the leaves. The effects were not reproduced by substitution of melanoprotein by egg-white lysozyme, rabbit haemoglobin, bovine serum albumen or deoxyribonuclease (EC 3.1.4.5). Inhibition of leaf expansion by injected melanoproteins was observed.

The application of a melanoprotein with spore inoculum of *Venturia inaequalis* on to leaves of a susceptible apple variety caused a great increase in lesion development. This effect was not reproduced by the use of other proteins. The decreased lesion-stimulating activity of melanoprotein produced by *V. inaequalis* after storage of cultures at 0° was correlated with decreased effects on solute transport in the host. Radioactive material arising from spore inoculum labelled with [^{14}C]DL-alanine was detected in the vascular system of test plants. The distribution of this material in the infected leaf was similar to that given by petiole injection of an indicator compound with melanoprotein in healthy plants.

INTRODUCTION

Work on some obligate fungal parasites of plants has indirectly indicated that during lesion development an initial symbiotic phase occurs in which little or no damage is caused to the host. At an early stage in the formation of visible lesions, before the host tissue becomes necrotic, host metabolites accumulate at the sites of infection (Harvey, 1930; Yarwood & Jacobson, 1955; Shaw & Samborski, 1956; Shaw, 1961). It was suggested that products of the infection process contribute to this situation (Shaw & Samborski, 1956). *Venturia inaequalis*, being a very specialized yet facultative parasite easily grown in artificial media, was chosen as eminently suitable for studies on the host/pathogen interaction. This paper shows ways in which the pathogen redirects the host metabolism in favour of the developing lesion. Part of this work has already been reported in brief (Hignett & Kirkham, 1965).

METHODS

Venturia inaequalis (Cke.) Wint. (clone E1) was grown as described in a previous paper (Kirkham, 1957) single spore isolates on nutrient agar being subcultured on paper cylinders moistened with 10% (w/v) Oxoid malt extract solution (Oxoid Ltd.,

London). Deep cultures were grown in 500 ml. conical flasks containing 300 ml. aqueous Oxoid malt extract (10 %, w/v), with continuous magnetic stirring at 16–18°.

Radioactive spores were isolated from paper cylinder cultures grown on a basal medium augmented with [^{14}C]DL-alanine at the rate of 0·05 mC/ml. (specific activity 21·7 mC/m-mole). Loosely bound radioactivity was removed from the spores by thrice repeated washing with distilled water at 18° in the centrifuge. Labelled spores suspended in 0·5 ml. water were applied to the upper surface of leaves of test plants by means of a small paint brush. After incubation for the required period, the spores remaining on the leaf surface were stripped off using a solution of collodion in ether + ethanol (1 + 1, v/v) which was painted over the whole surface and allowed to dry. By careful peeling of the collodion film, and repetition of the process, over 90 % of the applied spores were removed, as estimated under the microscope.

Greenhouse methods. Test plants were propagated, inoculated and injected through the petioles as previously described (Kirkham & Hunter, 1965). Inoculation chambers were subdivided with polythene sheet for the application of different treatments.

Autoradiography. Plant material was dried rapidly under high vacuum at room temperature immediately after sampling. Samples were kept flat by application of weighted filter paper. Kodak Kodirex X-ray film was used for autoradiography, being protected from direct contact with the samples by Melinex sheet (gauge 25 type S, I.C.I. Ltd.). Exposure times varied from 7 days to 6 months at room temperature, according to the radioactivity of the sample.

Fungal melanoprotein products. Material was isolated from culture fluids and separated by ethanol fractionation. The fractions insoluble in 50 and 75 % (v/v) ethanol in water were designated N50 and N75, respectively. Fraction N75 was separated on Sephadex G100 into four subfractions, labelled 1 to 4 in order of decreasing molecular weight (range 70,000–10,000; Kirkham & Hignett, 1966).

Preliminary analysis of the fungal products was done after acid hydrolysis (6N-HCl, 107°, 18 hr). The black precipitate formed was removed from the mixture, and washed free from acid; it gave a positive reaction to all of the following tests for melanin (Fox & Kuchnow, 1965).

Colour reactions: (1) bleached by bromine–water, aqueous solutions of $KClO_3$ + HCl, $KMnO_4$ + oxalic acid, H_2O_2, CrO_3; (2) dark green colour developed with aqueous $K_3Fe(CN)_6$; (3) vapour given off by roasted pigment reddened a pine splinter moistened with concentrated HCl.

Solubility: the black precipitate was soluble in 6N-KOH, concentrated H_2SO_4, and insoluble in water, 6N-HCl and lipid solvents.

The acid-soluble products of the hydrolysis were dried to remove HCl. Paper chromatography (*n*-butanol + acetic acid + water (4 + 1 + 1, by vol.) descending) followed by electrophoresis (3 % formic acid + 6 % acetic acid) in the second dimension and development with ninhydrin showed the presence of at least 16 amino acids. On the basis of this evidence the products were designated as melanoproteins.

Staining procedure. Leaf discs taken at the suspected lesion sites (before sporulation had occurred) and from control regions were digested with pectinase (Light and Co. Ltd.) to isolate the cuticle (Preece, 1962). The presence of subcuticular mycelium was detected by using the Schiff-periodate stain (Preece, 1959).

Transpiration. Differential transpiration rates over infection sites were detected

by dusting the leaf with anhydrous copper sulphate. Increased transpiration through lesions caused blue patches to appear within 15 min. of application of the anhydrous copper sulphate.

RESULTS

One-year old potted apple plants (MM. 109 rootstocks), each with a single vigorously growing shoot, were injected with mixed solutions of the melanoproteins and non-phytotoxic indicator compounds (1 % acid fuchsin or [^{14}C]D-glucose). Two types of distribution pattern within the host leaves were observed. In type I high concentrations of indicator occurred in the vascular system, leaving the interveinal tissue entirely unpermeated. In contrast, control injections without melanoprotein resulted in even permeation of the entire leaf, followed after 24 hr by a decrease in the concentration of indicator in the vascular system. The type II distribution pattern showed fairly even permeation throughout the leaf but with the total uptake being strongly inhibited (see Plate; types I and II, and control).

The effects observed were not reproduced by replacing melanoprotein with bovine serum albumen, egg-white lysozyme, rabbit haemoglobin or deoxyribonuclease (EC 3.1.4.5). Type I effects were caused by material of fraction N75, also subfractions 3 and 4, and by a mixture of fractions N75 and N50. Type II effects were given by fraction N50, and by subfractions 1 and 2 of fraction N75. A variation of type I effect (type Ia, see Pl.) consisted in the accumulation of indicator in the secondary and tertiary veins only. This appearance was produced by the use of melanoprotein at a quarter of the concentration required to give the full type I effect. Type Ia effect was also observed in mature leaves after injection of melanoprotein at a dose rate high enough to give the major vein pattern in young leaves. Slow deterioration of the melanoprotein occurred (with increasing phytoxicity) during storage in aqueous solution at 0° under toluene.

Use of melanoprotein at concentrations lower than that required to give type Ia effect led to the escape of indicator from the vascular tissues, causing accumulations around the tertiary veins. Injection of melanoprotein at concentrations higher than that required to give the type I effect caused cupping of the leaves, followed by interveinal desiccation and necrosis. Melanoprotein injected at sublethal dose rates partially suppressed leaf growth but not shoot extension. Unevenly permeated leaves grew asymmetrically in the plane of the leaf round the permeated area.

The effects noted above were reproduced in sharply localized areas of the leaf by introducing the melanoprotein and indicator through small wounds. Discrete drops 3 mm. in diameter were applied to the upper surface of leaves between the primary veins, being retained *in situ* by means of previously applied rings of petroleum jelly. Uptake of liquid by the leaf was effected by piercing the area covered by the drop, with a group of four hypodermic needles mounted on a cork. The effects produced by this technique were restricted to less than one quadrant of the leaf. Pattern types I and II were observed and in addition the type II pattern showed a directional effect in that migration of indicator within the leaf was directed predominantly towards the leaf margin. Indicator applied alone migrated in all directions, and in particular, back to the midrib. The directional effect was investigated in a 10-times replicated experiment on young and on mature leaves. The solutions were applied in each quadrant as closely as possible to the margins of the leaves. In mature leaves only 42 %

of the controls (treated with indicator only) did not show migration of indicator right back to the midrib, as opposed to 100 % of the test leaves. Migration to the midrib occurred in all young control leaves, but in only 50 % of the young test leaves.

Experiments were done to determine whether metabolite transport in the infected host was altered in ways similar to those described above. For multiple sampling experiments, the second fully expanded leaf (at the time of inoculation) below the shoot tip was designated 0; leaves above this being designated +1, +2; and −1, −2, etc. below. A succession of shoots of Cox's Orange Pippin was injected with [^{14}C]D-glucose (5 μC total per shoot) at leaves −2, −3 and −4, 6 days after inoculation with *Venturia inaequalis*, and daily thereafter until lesions appeared. Leaf+1 was sampled

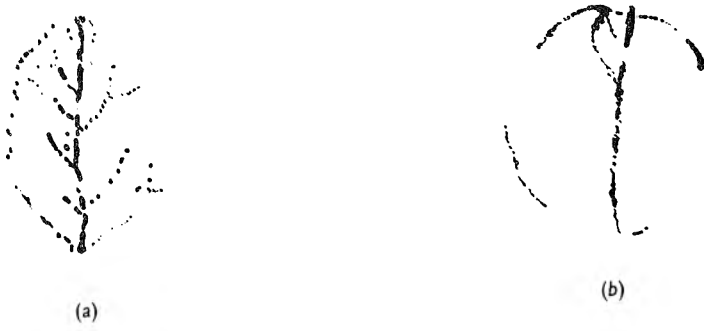


Fig. 1. Diagram of autoradiographs of labelled material injected into host leaf tissue by radioactive spores of *Venturia inaequalis*. (a) Apple variety MM. 109; upper surface of infected leaf 3 days after inoculation. (b) Apple variety Edward VII; lower surface of infected leaf 12 days after inoculation. Spores were removed in both cases before autoradiography.

24 hr after injection in each case. The plate (centre and bottom rows) shows a typical selection of autoradiographs from the samples. Type I activity was present at the time of the first sampling (2) and was fully developed 7 days after inoculation. After 12 days, before lesions were visible, [^{14}C]D-glucose had preferentially accumulated at the sites of infection (3) (confirmed by staining the cuticle as described under Methods). The final autoradiograph (4) indicates the direct and rapid transport of injected tracer to sporulating lesions, with very little labelling of the rest of the leaf.

The introduction of material into the host by infecting spores was demonstrated by using radioactive spores on shoots of apple varieties MM. 109 and Edward VII. Washed spores taken from cultures grown on 10 % (w/v) malt extract solution augmented with [^{14}C]DL-alanine were used as inoculum. Infected leaves were removed and processed as described earlier. Figure 1 is a diagram of autoradiographs (not reproduced for technical reasons) of the upper surface of an MM. 109 leaf sampled 3 days after inoculation, and of the lower surface of an Edward VII leaf removed 12 days after inoculation, by which time lesions were visible. Both show a type I effect, indicating the introduction of fungal products into the vascular system of the host.

The effect of melanoprotein on lesion development was tested by injecting it into inoculated shoots and by applying it with inoculum. In the former case injections were made 24 hr after inoculation, with a maximum of 1·5 ml. (containing up to 15 mg. melanoprotein/ml.) per shoot. In the latter case melanoprotein was added to the inoculum to give final concentrations up to 13 mg./ml. maximum.

The results show (Fig. 2) that melanoprotein subfractions 2 and 4 caused when injected into test plants a slight increase in the proportion of leaf area covered by lesions. These results tended to be erratic; stronger lesion stimulation was occasionally observed. In contrast, the same materials when applied with the spore inoculum to the leaf surface showed very consistent and marked lesion-stimulation activity. Culture variability expressed as differences in the patterning activity of the subfraction 4 produced has been noted earlier. Production of less active subfraction 4 was observed, particularly after cultures had been stored under oil for some months. Figure 3 shows the effect on lesion development of adding such material to inoculum used on test plants. Subfraction 4 produced by a freshly re-isolated culture showed markedly higher lesion-stimulation activity as compared to that produced by the same culture after 3 months storage under oil at 0°. The latter product gave results very little different from those of the control experiment. Bovine serum albumen applied with inoculum in a similar way did not influence lesion development. Melanoprotein added (final concentration up to 13 mg./ml.) to isolated conidia in culture had no effect on germination or growth.

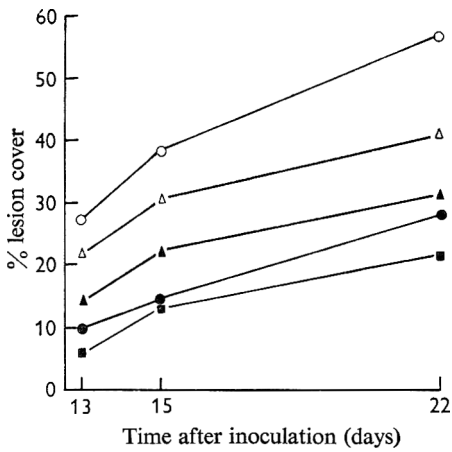


Fig. 2

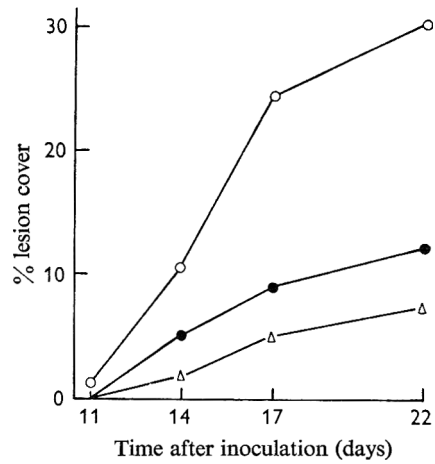


Fig. 3

Fig. 2. Effects of melanoproteins of *Venturia inaequalis* on lesion development on leaves of apple plants (variety MM. 109) after inoculation with *V. inaequalis*. ○, Subfraction 4 added to inoculum; ●, subfraction 4 injected 24 hr after inoculation; △, subfraction 2 added to inoculum; ▲, subfraction 2 injected 24 hr after inoculation; ■, control inoculation.

Fig. 3. Comparison of lesion-stimulation activity of melanoprotein produced by old and by freshly re-isolated cultures of *Venturia inaequalis*. Plants of apple variety MM. 109 were inoculated with spore suspensions containing, respectively, the different melanoprotein subfraction 4 preparations. ●, Subfraction 4 produced by cultures after storage; ○, subfraction 4 produced by re-isolated cultures; △, control inoculation. The re-isolated culture was used as a source of spore inoculum in all cases.

DISCUSSION

The accumulation of metabolites at the site of lesions in parasitized plants has been reported by various authors (Harvey, 1930; Yarwood & Jacobson, 1955; Shaw & Samborski, 1956; Shaw, 1961). No evidence was presented to show whether the effect was due either to the host, or to the pathogen. The present work shows that *Venturia inaequalis* is able to influence host metabolite transport by means of at least two types

of active extracellular material. The two types of effect on host metabolite transport shown by injected melanoprotein fractions appear to be due to specific properties possessed by them, since other enzymic and non-enzymic proteins tested gave negative results. The Type I activity detected *in vivo* by using radioactive conidia indicated that this effect was not an artifact of the deep culture method. This experiment also showed that material injected into the host by germinating conidia was not totally catabolized, and persisted in the vascular system and leaf margin up to and including the lesion stage. In contrast, injection of [^{14}C]D-glucose at various times after inoculation showed that development of type I activity against host metabolites only occurred during the intermediate stage of lesion development, between infection and sporulation. The vein pattern developed to a maximum intensity before visible lesions appeared, then declined rapidly, giving place to the accumulation of nutrients at infection sites.

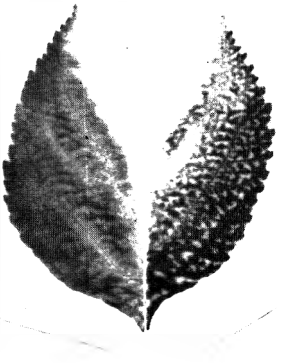
The appearance of a vein pattern in rust-infected barley, fed with radioactive compounds before lesion development is apparent in autoradiographs published by Shaw & Samborski (1956, p. 400, plate VI). As in the present work, a vein pattern developed and declined before visible lesions appeared, giving place to local accumulations of radioactivity. These similarities between the two systems suggest that similar mechanisms may be operating.

The results obtained by directly introducing melanoprotein with indicator compound into the leaf tissue through stab wounds showed that the effects described were not artifacts of the petiole injection method. In addition, the restrictive (type II) activity of melanoprotein fraction N 50 had directional properties in that, in contrast to the control experiments, migration of indicator compound towards the midrib was inhibited. The activity of fraction N 50 at a given dose-rate was more pronounced in mature leaves; this is to be compared with the greater effect shown by subfraction 4 material (type I activity) on younger leaves. In view of these observations it is suggested that in leaves approaching the lesion stage, i.e. mature leaves, movement of nutrients towards the midrib and out of the leaf is restricted by the action of fraction N 50. This explanation would be consistent with the stunted growth observed during earlier work with leaves permeated with fraction N 50 since the normal circulation of metabolites in the host would be restricted (Hignett & Kirkham, 1965).

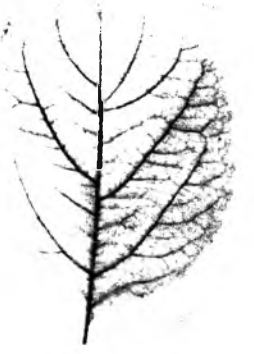
The injection of melanoproteins into inoculated plants had little effect on the subsequent development of lesions, although occasional stimulation was observed. However, the onset of necrosis in lesions was often delayed and inhibited. The marked difference in response of the fungus to sprayed application and to petiole injection of subfractions 2 and 4 (Fig. 2) may reflect the critical nature of the dose rate at the site of infection in relation to lesion development. It may also reflect the importance of the direction of travel of the melanoprotein in the vascular system: the directional effect of one melanoprotein fraction has already been mentioned, and penetration by the melanoprotein of the cuticle from the outer surface of the leaf to the site of infection is indicated by the marked lesion stimulation observed. The latter result must be due to interactions at the infection site since fungal products exerted no effects on germination or growth of isolated conidia in culture.

Observation of the more active subfraction 4 produced by freshly re-isolated *Venturia inaequalis* as compared with that produced from cultures after storage for 3 months under oil at 0° suggests that the differences might be related to the loss of pathogenicity during storage, which commonly occurs with this organism.

Type I



Type Ia



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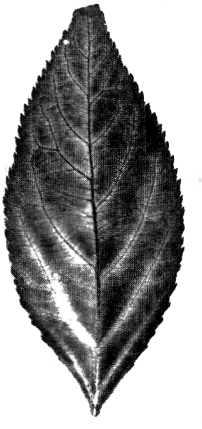

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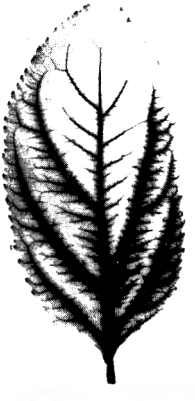

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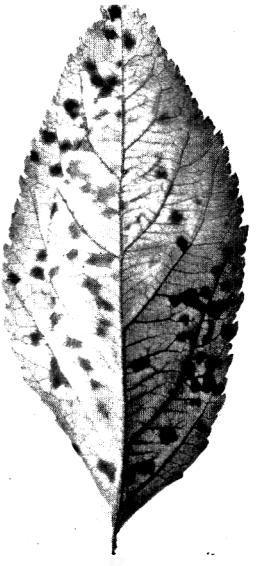

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4

R. C. HIGNETT AND D. S. KIRKHAM

(*Facing p.* 275)

The following mechanism is suggested to account for the observations described. After germination of the conidia and infection of a susceptible host leaf, fungal melanoproteins are produced at the site of infection and spread outwards within the leaf, partially confining metabolites to the vascular system. Thus a specialized transport system of preferred routes is set up leading directly back into the lesion. The export of nutrients from the leaf is progressively restricted, as the leaf matures, by melanoprotein fraction N50. Nutrients are therefore made more available to the developing lesion. Results of the present work suggest that the preferred routes are established long before lesions become visible, and that host metabolites accumulate in them. At the time of lesion eruption, a driving force directed towards the lesion site acts on the accumulated pool of host metabolites in the vascular system. This force is the result of a local increase in transpiration rate as the cuticle above a lesion is lifted and disrupted. Thus the accumulation of nutrients in the vascular system is transferred to the lesion, as indicated by the tracer experiments showing the disappearance of the vein pattern occurring simultaneously with the development of the final lesion pattern.

The technical assistance of Mrs G. F. Sewell, Miss J. Starkey and Mr H. Hutchins is acknowledged.

REFERENCES

Fox, D. L. & Kuchnow, K. P. (1965). The reversible light-screening pigment of elasmobranch eyes: chemical identity with melanin. *Science* **150**, 612.

Harvey, R. B. (1930). The relative transpiration rate at infection spots on leaves. *Phytopathology* **20**, 359.

Hignett, R. C. & Kirkham, D. S. (1965). Factors concerned in host specificity of *Venturia inaequalis* (Cke). Wint. *Biochem. J.* **97**, 34P.

Kirkham, D. S. (1957). Relationships between cultural characters and pathogenicity in *Venturia inaequalis* and *Venturia pirina*. *J. gen. Microbiol.* **16**, 360.

Kirkham, D. S. & Hignett, R. C. (1966). Control of host susceptibility and solute transport by metabolites of *Venturia inaequalis*. *Nature, Lond.* **212**, 211.

Kirkham, D. S. & Hunter, L. D. (1965). Studies of the *in vivo* activity of esters of *o*-coumaric and cinnamic acids against apple scab. *Ann. appl. Biol.* **55**, 359.

Preece, T. F. (1959). A staining method for the study of apple scab infections. *Plant Pathology* **8**, 127.

Preece, T. F. (1962). Removal of apple leaf cuticle by pectinase to reveal the mycelium of *Venturia inaequalis* (Cke.) Wint. *Nature, Lond.* **193**, 902.

Shaw, M. (1961). The physiology of host-parasite relations. IX. *Can. J. Bot.* **39**, 1393.

Shaw, M. & Samborski, D. J. (1956). The physiology of host-parasite relations I. *Can. J. Bot.* **34**, 389.

Yarwood, C. E. & Jacobson, L. (1955). Accumulation of chemicals in diseased areas of leaves. *Phytopathology* **45**, 43.

EXPLANATION OF PLATE

Autoradiographed effects of injected melanoproteins of *Venturia inaequalis* and lesion development, on solute transport in apple leaf tissues.

Top and centre rows. (Apple variety MM. 109). Samples taken 6 hr after injection. Type I. Vein pattern effect produced by injection of indicator substance ([^{14}C]D-glucose) with melanoprotein material N75, or subfractions 3 or 4. Type I a produced by appropriate dilution of injectant used for type I. Type II. Restricted intake of indicator when injected with melanoprotein fraction N50, or subfractions 1 or 2 isolated from fraction N75. Control. Indicator injected alone.

Centre and bottom rows. (Apple variety Cox's Orange Pippin). Samples taken 24 hr after injection of [^{14}C]D-glucose. 1. Control. Uninoculated plant. 2. Sample taken 7 days after inoculation with *Venturia inaequalis*. 3. Sample taken 12 days after inoculation. 4. Sample taken 14 days after inoculation, showng sporulating lesions.

J. gen. Microbiol. (1967), **48**, 277–281
Printed in Great Britain

Induction of the Petite Mutation in *Saccharomyces cerevisiae* by *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine

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(*Accepted for publication* 15 *March* 1967)

SUMMARY

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), which is known to be a very effective mutagen in many systems, induces petite mutations when cells of *Saccharomyces cerevisiae* are treated in acetate buffer. Fifty per cent or more of the survivors may be petite mutants. NTG is a real mutagen in the process since the number of mutants increases at short times of exposure to NTG although the total number of cells decreases. Growing cells are more susceptible to killing and mutation than are cells in buffer. The production of petites may cause difficulties when screening for rare auxotrophs.

INTRODUCTION

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) is highly cancerostatic in mammalian systems (Greene & Greenberg, 1960) and highly mutagenic in microbial cells (Adelberg, Mandel & Chen, 1965). It has been reported that NTG-resistant mutants of *Escherichia coli* are induced with high frequency by exposure to NTG (Mandell & Greenberg, 1960); the process was shown to be due to *de novo* mutations and not to the selection of preformed mutants. NTG is also an excellent agent for the induction of auxotrophs in bacteria (Adelberg *et al.* 1965) and in yeast (Megnet, 1965).

Auxotrophs with respect to a defined biosynthetic pathway are formed in rather low frequencies by various mutagenic treatments. Thus, successful isolation of them often requires the use of selection or screening. A number of selection methods have been adopted since Lederberg & Zinder (1948) and Davis (1948) introduced the penicillin method for selective killing of bacterial prototrophs in mixture with auxotrophs.

Several methods for the selection of yeast auxotrophs have been investigated. Megnet has successfully used inositol deficiency (1964) or 2-deoxyglucose (1965) as selective agents for *Schizosaccharomyces pombe* auxotrophs. Moat, Peters & Srb (1959) tried several antibiotics for the selection of *Saccharomyces cerevisiae* auxotrophs. It was found, however, that the recognition of the mutants after replica plating or delayed enrichment was obscured by the simultaneous induction of respiratory deficient mutants, the so-called petites. These grow more slowly aerobically than the wild type and may, thus, present a negative result on minimal medium if the plates are studied after a short incubation period. However, it is possible to identify petites by various methods; they remain white when the wild type is coloured red by an overlay of triphenyl tetrazolium chloride (TTC) agar (Nagai, 1959; Nagai, Yanagishima & Nagai, 1961; Ogur, St John & Nagai, 1957).

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Petites are induced by a great number of agents, the most notable being acriflavine (Nagai *et al.* 1961), The site of the mutation is extranuclear; it seems to be located within the mitochondria (Ephrussi, 1953). The latter have recently been shown to contain DNA (Schatz, Halsbrunner & Tuppy, 1964), which may explain their relative autonomy with respect to the nucleus. At higher concentrations of acriflavine, up to 100 % of the population is transformed to petites. Ephrussi (1953) has shown that petites do not arise by selection of pre-existing mutants but are induced *de novo* in all cells.

The aim of the present work was to find methods for the selection of auxotrophs of yeast, especially of Saccharomyces. Since NTG is such a good mutagenic agent in many organisms, even in the yeast species Schizosaccharomyces (Megnet, 1965), it was used to induce mutations. It was then necessary to study whether petite mutations were induced by NTG. This part of the work is reported in this paper.

METHODS

A strain of *Saccharomyces cerevisiae*, isolated from commercial baker's yeast, was used. It was grown without aeration in 100 ml. of 9 % (w/v) unhopped beer wort in 200 ml. Erlenmeyer flasks at 25°. All platings were made on wort agar (2 % (w/v) agar).

Actively growing cells were centrifuged and washed twice with 0·9 % (w/v) saline and suspended in 0·2 M-acetate buffer (pH 5·0) (Megnet, 1965) to a concentration of 2×10^7 cells/ml. NTG was dissolved without sterilization in the same buffer immediately before the mutation experiments (Adelberg *et al.* 1965). One ml. of yeast suspension and 2 ml. of NTG solution were mixed (Megnet, 1965) and incubated at 25°. Samples were diluted from time to time in saline and 0·1 ml. was plated on wort agar. If the NTG concentration of the solution to be spread was 0·1 mg./ml. or more 0·1 ml. was diluted in saline and filtered through a millipore filter. The filter was then placed on wort agar. Without this filtration, a considerable portion of the cells plated were killed on the plates (cf. Fig. 1).

In another series of experiments, yeast was exposed to NTG in a medium permitting growth, namely wort. 10 ml. medium was incubated in 25 ml. test tubes. After various times of incubation, aliquots were diluted in saline and spread on wort agar.

The plates were incubated at 25°. Colonies could be detected after 2 days, but petite colonies require a longer incubation period. After 5 days, the colonies in each Petri dish were overlayed (Nagai, 1959; Nagai *et al.* 1961; Ogur *et al.* 1957) with 20 ml. melted agar (1·5 % (w/v)) + phosphate buffer (0·067 M, pH 7·0), glucose (5 g./l.), and TTC (1 g./l.); the solution was melted and then kept at 45°. TTC was added immediately before the solution was poured on to the colonies since TTC is not very stable (Ogur *et al.* 1957). White and red colonies were counted after 3 hr, but the plates could even be scored the next day.

For comparison, acriflavine was added to growing yeast cells in 10 ml. wort in 25 ml. test tubes. Samples were diluted and spread after various times of incubation at 25° and treated in the same way as the NTG samples with TTC overlay.

To show the killing effect of NTG on the plates, the following experiment was performed. 0·1 ml. cell suspensions was spread on wort agar. When the plates were dry, 0·1 ml. NTG solutions of various concentrations was added. The plates were incubated and the colonies counted after TTC overlaying as described above.

RESULTS AND DISCUSSION

Killing on the plates. The lethal effect of NTG on cells plated on wort agar is shown in Fig. 1. It can be seen that the NTG concentration in the added solution may reach 0·1 mg./ml. without any appreciable lethal effect. At higher concentrations, however, the number of detectable colonies decreases considerably. Thus, it was necessary to dispose of NTG when the samples were not diluted to a concentration below 0·1 mg./ml. Indeed, NTG incorporated in the plating agar has been used to induce revertants *in situ* (Marquardt, Zimmermann & Schwaier, 1964).

Adelberg *et al.* (1965) recommend a mutation procedure in which the cells are collected on millipore filters and NTG is washed away with buffer. This procedure is not necessary in yeast if the concentration of NTG is low in the solution plated, since

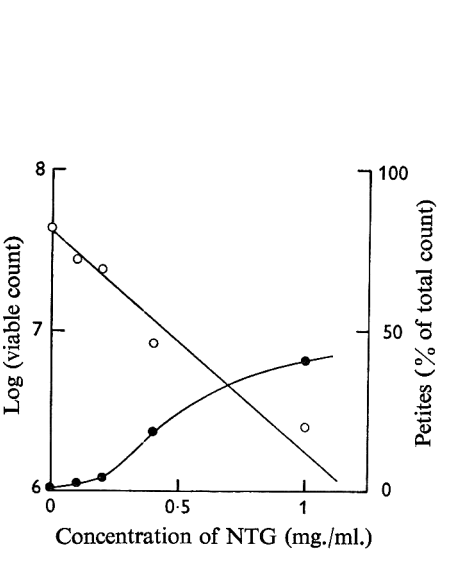

Fig. 1

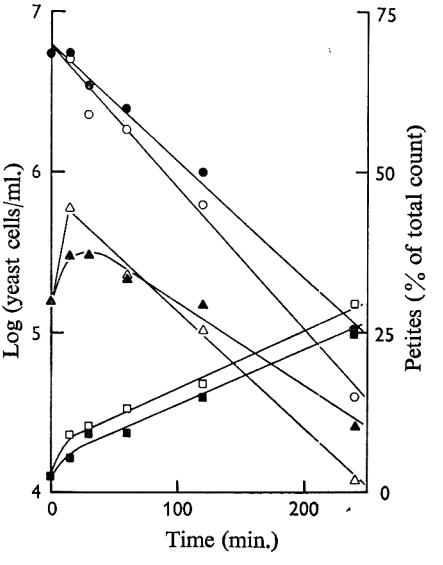

Fig. 2

Fig. 1. 0·1 ml. of yeast suspension was spread on wort agar and allowed to dry, then 0·1 ml. NTG solutions (concentration given on the abscissa) was spread on the agar surface. Viable count (○) and petite fraction (●) were determined.

Fig. 2. Effect of NTG treatment on viable count (circles), petite count (triangles), and petite fraction (squares). The yeast was treated with 2·0 (open symbols) or 1·0 (closed symbols) mg./ml. NTG in acetate buffer during the time indicated on the abscissa.

Table 1. *Effect of NTG on viable count*

The values have been taken from Figs. 2 and 3 and from additional experiments.

NTG concentration (mg./ml.)	Time required to reduce the viable count by a factor of 10 (min.)	
	In buffer	In wort
2·0	110	40
1·0	140	—
0·4	170	—
0·2	210	—

petites were induced to only a small extent at concentrations below 0·2 mg./ml. (Fig. 1).

Death curves of NTG-treated cells. The plate counts decreased with time when cells were suspended in NTG solutions (Fig. 2). Time zero means that the samples were diluted and plated immediately after the addition of NTG. Linear curves were obtained for log N_t/N_0, where N_t and N_0 are the viable counts at time t and zero, respectively. At 2 mg./ml. the viable count decreased by a factor of 10 every 110 min. The slopes of the survival curves obtained with various concentrations of NTG are given in Table 1. Still steeper slopes were obtained when cells were exposed to NTG in wort; the viable count decreased by a factor of 10 within 40 min. at a NTG concentration of 2 mg./ml. (Fig. 3). An increased lethal effect in media permitting growth has been

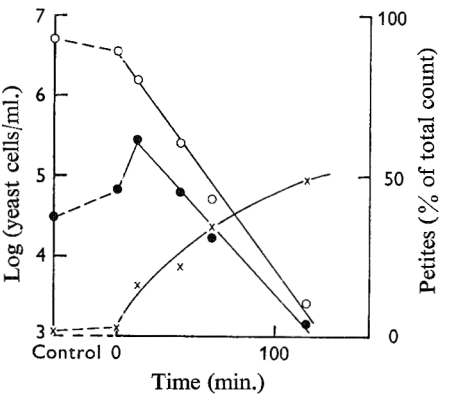

Fig. 3

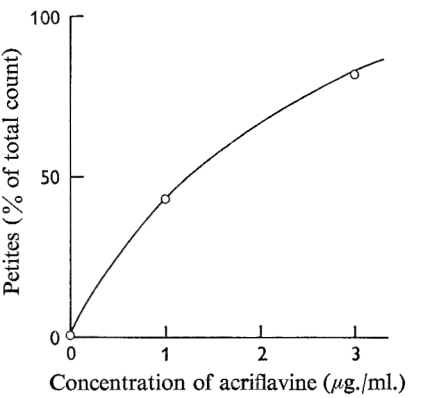

Fig. 4

Fig. 3. Effect of NTG treatment on viable count (○), petite count (●), and petite fraction (×). The yeast was treated with 2·0 mg./ml. NTG in wort during the time indicated on the abscissa.

Fig. 4. Effect of acriflavine treatment on petite fraction. The yeast was incubated for 48 hr in wort to which acriflavine was added. The wort was inoculated with 8×10^6 cells/ml., which was about 1/10 of the final count obtained.

obtained by Adelberg *et al.* (1965) in experiments with *Escherichia coli.* These authors also report that the fraction of mutants among the survivors was the same when cells were treated in buffer and in growth-promoting media. They thus recommend a procedure in which the mutagenic treatment is performed in buffer. They also report that 40 % of the survivors of *E. coli* were auxotrophs when treated with 1 mg. NTG/ml. Megnet (1965) used 2 mg. NTG/ml. to induce auxotrophs in *Schizosaccharomyces pombe*; he found 8 % auxotrophs without screening. If, however, a particular kind of auxotroph is required, the probability of finding it is much less than these figures may indicate. The figures are a measure of the induction of a great number of mutants with many kinds of deficiencies. Adelberg *et al.* (1965) assume that about 10 % of the total genome consists of loci capable of mutations giving rise to autotrophs. Screening procedures may be needed if more specified auxotrophs have to be isolated.

Induction of petites. The induction of petites in non-growing and in growing cells is illustrated in Figs 2 and 3. In the controls, the petite fraction was 0·7 %. The absolute number of petites increased by NTG treatment and a great number of the sur-

vivors were mutants. This result is comparable to the effects of acriflavine on growing cells (Fig. 4), where the majority of the cells are mutants. Several μg./ml. of acriflavine can be included in the medium without significant effects on the growth rate (Ephrussi, 1953).

The results of this paper show that a great portion of the survivors after NTG treatment consists of petite mutants. This may create some difficulty in the isolation of auxotrophs of Saccharomyces. Studies on the selection of auxotrophs of yeast are in progress in this laboratory.

This work was supported by the Swedish Technical Research Council. The skilful technical assistance of Mrs Kerstin Ekengren-Jansson is gratefully acknowledged.

REFERENCES

ADELBERG, E. A., MANDEL, M. & CHEN, G. C. C. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K 12. *Biochem. biophys. Res. Commun.* **18**, 788.

DAVIS, B. D. (1948). Isolation of biochemically deficient mutants of bacteria by penicillin. *J. Am. chem. Soc.* **70**, 4267.

EPHRUSSI, B. (1953). *Nucleo-Cytoplasmic Relations in Microorganisms. Their Bearing on Cell Heredity and Differentiation*, p. 13. London: Oxford University Press.

GREENE, M. O. & GREENBERG, J. (1960). The activity of nitrosoguanidines against ascites tumors in mice. *Cancer Res.* **20**, 1166.

LEDERBERG, J. & ZINDER, N. (1948). Concentration of biochemical mutants of bacteria with penicillin. *J. Am. chem. Soc.* **70**, 4267.

MANDELL, J. D. & GREENBERG, J. (1960). A new chemical for bacteria, 1-methyl-3-nitro-1-nitrosoguanidine. *Biochem. biophys. Res. Commun.* **3**, 575.

MARQUARDT, H., ZIMMERMANN, F. & SCHWAIER, R. (1964). Die Wirkung krebsauslösender Nitrosamine und Nitrosamide auf das Adenin-6-45-Rückmutatationssystem von *Saccharomyces cerevisiae*. *Z. Vererbungslehre* **95**, 82.

MEGNET, R. (1964). A method for the selection of auxotrophic mutants of the yeast *Schizosaccharomyces pombe*. *Experientia* **20**, 320.

MEGNET, R. (1965). Screening of auxotrophic mutants of *Schizosaccharomyces pombe* with 2-deoxyglucose. *Mutation Res.* **2**, 328.

MOAT, A. G., PETERS, JUN., N. & SRB, A. M. (1959). Selection and isolation of auxotrophic yeast mutants with the aid of antibiotics. *J. Bact.* **77**, 673.

NAGAI, S. (1959). Induction of the respiration-deficient mutation in yeast by various synthetic dyes. *Science* **130**, 1188.

NAGAI, S., YANAGISHIMA, N. & NAGAI, H. (1961). Advances in the study of respiration-deficient (RD) mutation in yeast and other microorganisms. *Bact. Revs.* **25**, 404.

OGUR, M., ST JOHN, R. & NAGAI, S. (1957). Tetrazolium overlay technique for population studies of respiration deficiency in yeast. *Science* **125**, 988.

SCHATZ, G., HALSBRUNNER, E. & TUPPY, H. (1964). Deoxyribonucleic acid associated with yeast mitochondria. *Biochem. biophys. Res. Commun.* **15**, 127.

J. gen. Microbiol. (1967), 48, 283–292
Printed in Great Britain

Chlorophyll Formation in *Euglena gracilis* var. *bacillaris*: Interference by Analogues of Purines, Pyrimidines and Amino Acids

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(*Accepted for publication* 16 *March* 1967)

SUMMARY

Chlorophyll formation in non-growing etiolated cells of *Euglena gracilis* var. *bacillaris* is inhibited by certain antimetabolite analogues of purines, pyrimidines and amino acids. The inhibitory effects of bromo- and nitro-uracils were annulled by uracil and thymine; those of fluoro- and thio-uracils were not. Ethionine inhibition was completely annulled by methionine. All the base analogues were more inhibitory for the dark growth of the alga than in the light and an adaptation to these antimetabolites was apparent during growth. The growth inhibitory effects of some of these base analogues was annulled by several Krebs's cycle intermediates; the latter in themselves stimulated growth of photosynthesizing euglenas although they were not utilized in the dark with any degree of efficiency.

Long-term exposure of non-proliferating euglenas, green or etiolated, to 5-fluoro-uracil had a profound effect on the chloroplast integrity of the organisms. Such euglenas on further subculture in an adequate growth medium were permanently bleached. The effect of 5-bromo-uracil, though similar, was less marked.

INTRODUCTION

Euglena gracilis, a green photosynthetic flagellate, loses its chlorophylls when grown heterotrophically in the dark. Exposure of such colourless euglenas to light brings about the re-synthesis of chlorophyll accompanied by a considerable increase in chloroplast proteins and the RNA of several subcellular fractions (Brawerman & Chargaff, 1959*a*; Brawerman, Pogo & Chargaff, 1962). An additional RNA species associated with the chloroplasts and which is specifically formed during their induced formation in light was demonstrated by Pogo, Brawerman & Chargaff (1962). These results suggest that light induces the associated synthesis of specific types of proteins concomitant with chlorophyll synthesis. The effect of certain antimetabolite analogues of purines, pyrimidines and amino acids was therefore tested to verify such an association.

Euglena gracilis is the only phytoflagellate which can be rendered permanently apochlorotic by an assortment of chemical agents, e.g. streptomycin (Provasoli, Hutner & Schatz, 1948; Jírovec, 1949), antihistamines (Gross, Jahn & Bernstein, 1955), *O*-methylthreonine (Aaronson & Bensky, 1962), furadantin (McCalla, 1962), erythromycin (Ebringer, 1962) and magnesium starvation (Dubash & Rege, 1967); and physical agents such as heat (Pringsheim & Pringsheim, 1952; Brawerman &

Chargaff, 1959*b*) and ultraviolet radiation (Pringsheim, 1958). This phenomenon of permanent apochlorosis is one of the few instances of a cytoplasmic mutation, with a consequent alteration of phenotype, being induced by environmental changes. In *E. gracilis* var. *bacillaris*, diverse agents operating in unknown ways affect finally the synthesis of chlorophyll or plastid or both. The mechanism of action of any of such agents is not yet fully understood. 5-fluoro-uracil, which inhibits growth and chlorophyll synthesis in *E. gracilis*, has been found to render organisms of this alga permanently apochlorotic. Organisms grown in the dark with sulphanilamide and 5-bromo-uracil and subsequently plated on a basal medium were shown by Scher & Collinge (1965) to be markedly mutated to the apochlorotic form, but although mere exposure to 5-fluoro-uracil can bring about a 100 % mutation, we were not able to show a similar effect with 5-bromo-uracil even on longer exposures.

METHODS

Organisms of the photosynthetic strain of *Euglena gracilis* var. *bacillaris* were grown in the defined medium of Hutner, Provasoli, Schatz & Haskins (1950) as described by Coelho & Rege (1963); the medium for dark-grown cultures was supplemented with 1 % glucose. Growth studies were made essentially by the method described earlier (Coelho & Rege, 1963). For studies on chlorophyll synthesis by non-growing cultures under illumination, etiolated euglenas were obtained by growth in the dark for 7 days; these forms were harvested, washed twice with sterile distilled water under aseptic conditions and then suspended in a measured volume of sterile distilled water. Samples of this suspension were then dispensed in 6 in. $\times \frac{1}{2}$ in. rimless test tubes containing double- (or greater) strength of the 'resting' medium suggested by Brawerman & Chargaff (1959*a*) composed of 1 % glucose, 0·01 M-KH_2PO_4, and 0·01 M-$MgCl_2 \cdot 6H_2O$ with additions where required. No rigorous sterility precautions needed to be observed as this medium did not favour bacterial growth. The euglenas were then illuminated with frequent shaking for 72 hr at 28° in a transparent glass tank by a pair of daylight fluorescent tubes placed 10 in. from the bottom of the tank.

Estimates of growth were made, after suitable dilution of cultures, by the method of Hutner, Bach & Ross (1956), by measuring the turbidity with a Klett–Summerson photoelectric colorimeter with a 66 (640–700 mμ) filter. Photosynthetic pigments were routinely determined by extraction of euglenas with 3 × 5·0 ml. methanol; these extracts were pooled, the volume made to 20 ml. with methanol and the colour intensity read with a Klett–Summerson colorimeter, using the same 66 filter. Absolute values, where given, were determined by the method described earlier (Dubash & Rege, 1967).

Counts of euglenas were made in a Levy blood counting chamber after immobilizing the organisms by adding a drop of chloroform.

RESULTS

Effect of antimetabolites on chlorophyll synthesis

It was observed that all the analogues of uracil tried and 2,6-diamino-purine inhibited the synthesis of chlorophyll in suspensions of the euglena. Benzimidazole was without effect; 6-mercaptopurine showed a slight stimulation; ethionine, at higher concentration, was also inhibitory (Table 1). The effects of 5-bromo- and 5-nitro-

uracil were annulled by uracil and thymine; those of 5-fluoro- and 5-thio-uracil were not (Table 2). The inhibition by ethionine was completely annulled by methionine. The incorporation of ethionine into the euglenas showed a lag period; short-term exposure to this analogue before illumination did not inhibit chlorophyll synthesis (Table 3).

Table 1. *Effect of analogues of purines, pyrimidines and amino acids on chlorophyll synthesis in resting etiolated Euglena gracilis var. bacillaris*

Addition to non-proliferation medium	Concentration of addition	Inhibition of chlorophyll synthesis (%)
None	—	0
5-Bromo-uracil	400 μg./ml.	37·4
5-Thio-uracil		47·5
5-Nitro-uracil		24·6
5-Fluoro-uracil		26·7
2,6-Diaminopurine	10^{-3} M	36·1
6-Mercaptopurine		−14·0
Benzimidazole		2·8
Ethionine	2 mg./ml.	69·2

Table 2. *Effect of uracil analogues on chlorophyll synthesis by etiolated Euglena gracilis var. bacillaris: annulment by uracil and thymine*

Addition to non-proliferation medium (400 μg./ml.)	Net synthesis of chlorophyll/mg. dry wt euglenas*/ 72 hr mμg.	Inhibition (%)	Annulment (%)
None	1812	—	—
5-Bromo-uracil (BU)	1101	37·4	—
5-Thio-uracil (TU)	964	47·5	—
5-Nitro-uracil (NU)	1376	24·6	—
5-Fluoro-uracil (FU)	1330	26·7	—
Uracil (U)	1796	1·0	—
Thymine (Th)	1536	15·5	—
BU+U	1697	6·5	85·0
BU+Th	1468	19·0	49·1
TU+U	1009	44·5	6·4
TU+Th	917	49·5	−4·2
NU+U	1812	0	100
NU+Th	1514	16·6	32·6
FU+U	1399	23·0	13·9
FU+Th	1005	44·5	−66·5

* Dry weights of euglenas were calculated from counts according to the relationship suggested by Kirk (1962).

Effect of antimetabolites on the growth of Euglena gracilis var. bacillaris

In view of the effect of these various antimetabolite analogues on chlorophyll synthesis in non-proliferation conditions, their influence on growth was studied. To verify whether the effect on growth was due only to interference with chlorophyll biosynthesis, the studies were extended to dark-grown cultures as well. Table 4 shows that all purine and pyrimidine analogues tested were more inhibitory in the dark than in the light. The organism thus appeared to use pathways for carbohydrate metabolism which were

different in the heterotrophic and photosynthetic phases. The possibility of light instability of some of the compounds cannot, however, be precluded. Whether such an action indicates that these analogues might hamper in some way the utilization of glucose as a source of energy was examined by experiments designed to test the effects of various breakdown products of glucose and the Krebs cycle intermediates in the presence of these antimetabolites. Table 5 shows the effect of the Krebs cycle intermediates on the growth of Euglena in the presence and absence of 8-azaguanine. The

Table 3. *Effect of ethionine on chlorophyll synthesis in non-proliferating etiolated Euglena gracilis var. bacillaris organisms: annulment by methionine*

Euglenas were grown heterotrophically in the dark for 7 days, harvested, washed and resuspended in water. Equal samples were used for the different treatments. Illumination was for 96 hr; the chlorophylls were extracted with methanol as described in the text. Klett readings were taken with the 66 filter. Pre-exposure was effected by suspending the etiolated euglenas in non-proliferation medium containing the additions at 2 mg./ml.; the same concentration was used during illumination.

Treatment	Chlorophyll index (Klett units)	Inhibition (%)	Annulment (%)
1. Blank	162	—	—
2. Non-proliferation medium + ethionine	50	69·2	—
3. Euglenas exposed to ethionine for 15 min., washed and taken up in non-proliferation medium	161	0	—
4. Euglenas exposed to ethionine for 15 min., centrifuged and taken up in non-proliferation medium without washing	93	42·5	—
5. Non-proliferation medium + ethionine + methionine	161	0	100
6. Euglenas exposed to ethionine + methionine for 15 min., washed and taken up in non-proliferation medium	165	0	—
7. Euglenas exposed to ethionine + methionine for 15 min., centrifuged and taken up in non-proliferation medium without washing	165	0	—
8. Euglenas exposed first to ethionine for 15 min., then to methionine for 15 min., washed and taken up in non-proliferation medium	165	0	—
9. Euglenas exposed to ethionine for 15 min., washed and taken up in non-proliferation medium containing methionine	167	0	—
10. Non-proliferation medium + methionine	167	0	—

Table 4. *Effect of purine and pyrimidine antimetabolite analogues on the light and dark growth of Euglena gracilis var. bacillaris*

Analogue added	Concentration ($\times 10^{3-}$ M)	% Inhibition of growth in Light	% Inhibition of growth in Dark
None	—	0	0
5-Bromo-uracil	1·048	4·0	43·6
5-Thio-uracil	1·560	2·8	8·5
5-Nitro-uracil	1·272	4·4	5·6
5-Fluoro-uracil	1·526	97·9	100
	0·153	27·2	71·1
8-Azaguanine	1·314	19·4	53·5
8-Azaxanthine	1·304	0	5·0
8-Azaadenine	1·470	19·4	49·3

inhibition due to 8-azaguanine was less evident in the dark in the presence of lactate or α-ketoglutarate and was completely overcome by citrate, although these compounds cannot replace glucose in the medium with growth stimulatory action. In the light, 8-azaguanine was completely ineffective in the presence of α-ketoglutarate and citrate. It was further observed that many of the carbon compounds stimulated growth of the organism in the light although some of these could not be utilized in the dark.

Table 5. *Effect of Krebs cycle intermediates on the 8-azaguanine inhibition of growth of Euglena gracilis var. bacillaris*

	Growth (Klett units at 660 mμ)					
	Without 8-azaguanine		With 8-azaguanine			
Krebs cycle acid added* (1 %)	Dark	Light	Dark	Inhibition (%)	Light	Inhibition (%)
None	79	286	42	46·9	264	7·6
Glucose	298	422	142	52·4	345	18·4
Pyruvate	48	126	23	52·0	114	9·5
Lactate	85	350	70	17·6	305	13·0
Succinate	115	422	53	54·0	252	40·3
Malate	95	422	48	49·5	320	24·1
α-Ketoglutarate	27	194	22	18·6	208	−7·0
β-Glycerophosphate	54	211	36	33·4	100	52·6
Fumarate	168	350	76	54·7	234	33·1
Citrate	65	325	64	0	348	−7·0

* Additions of Krebs intermediates were to the basal medium containing glutamate (1 %) and malate (0·2 %), but without glucose.

Adaptation to the presence of antimetabolites

When euglenas grown in presence of 8-azaguanine were used to study their chlorophyll-synthesizing capacity, it was observed that 8-azaguanine was not as inhibitory to the synthesis of chlorophyll as it was to euglenas grown in absence of this analogue. Tables 6 and 7 show that not only were the analogues ineffective in most of the cases tried, but 8-azaderivatives of guanine and xanthine, and *S*-methylcysteine actually stimulated the synthesis of chlorophyll when euglenas grown in the dark in the presence of the antimetabolite analogue were illuminated in a non-proliferation medium in the continued presence of the same (Table 6). The effect of 5-bromo-uracil addition to the growth medium in the dark was to enhance synthesis of chlorophyll when etiolated euglenas were illuminated in a non-proliferation medium with or without this analogue; 5-bromo-uracil, however, inhibited chlorophyll synthesis in normal etiolated euglenas (Table 1). With 5-nitro-uracil, with 8-azaderivatives of guanine and xanthine, and with *S*-methylcysteine there was only an increase in chlorophyll synthesis when the illumination in non-proliferation medium was also continued in the presence of the inhibitor. It was thought that this effect may have been due to some sort of adaptation of the euglenas to the inhibitor during the growth phase. After 7 days' growth in the dark in the presence of inhibitor a second transfer in the same medium brought about a decrease in the degree of inhibition in the case of 8-azaxanthine and *S*-methylcysteine, whereas the inhibition was increased in the case of 8-azaguanine. These results are given in Table 7.

Table 6. *Effect of continued presence of purine and pyrimidine antimetabolite analogues on chlorophyll synthesis in non-proliferating etiolated organisms of Euglena gracilis var. bacillaris grown in the presence of the analogue: adaptation to certain analogues*

Analogue	Concentration in the growth and non-proliferation medium ($\times 10^{-3}$ M)	Dark Growth (Klett)	% Chlorophyll synthesized per euglena in 72 hr in non-proliferation medium	
			Alone	+Analogue
None	—	142	100	100
5-Bromo-uracil	1·048	80	128·1	126·0
5-Thio-uracil	1·560	130	78·3	77·0
5-Nitro-uracil	1·272	134	65·8	72·0
5-Fluoro-uracil	1·526	0	0	0
8-Azaguanine	1·314	66	96·0	129·5
8-Azaxanthine	1·304	135	72·8	85·5
8-Azaadenine	1·470	72	91·3	78·3
S-Methylcysteine	1·480	124	77·3	90·4

Table 7. *Effect of continued presence of antimetabolite growth inhibitors on the growth of Euglena gracilis var. bacillaris*

Dark-grown euglenas in presence of the inhibitor were diluted to the same cell count and used as inocula for growth in presence or absence of the inhibitor as indicated.

Growth studies were done in the dark on a glucose-supplemented growth medium for 7 days.

Addition	Concentration in the growth medium ($\times 10^{-3}$ M)	Dark Growth (Klett)	Inhibition of growth (I) (%)	DG* euglenas inoculated in: Basal medium Growth (Klett)	Basal medium I (%)	Basal medium +addition Growth (Klett)	Basal medium +addition I (%)
None	—	156	0	298	0	298	0
8-Azaxanthine	1·304	143	8·5	315	−7	289	3
8-Azaguanine	1·314	116	25·6	286	4	134	55
S-Methylcysteine	1·480	135	13·5	284	5	269	10

* DG = dark-grown euglenas in presence of inhibitor.

Mutagenic effect of prolonged exposure to antimetabolite analogues of certain bases

Scher & Collinge (1965) demonstrated that 5-bromo-uracil gets incorporated into the euglenas during chloroplast replication leading to mutagenic changes and giving rise to bleached colonies. The effect of 5-fluoro- and 5-bromo- analogues of uracil was studied on non-proliferating suspensions of Euglena in the light. Long exposures to these base analogues brought about certain mutagenic changes in Euglena as evinced when these organisms were subcultured in a glucose-supplemented basal medium in the light. Exposure of green or etiolated euglenas for 144 hr to 5-fluoro-uracil gave pure white cultures when a small population of the euglenas undergoing treatment was inoculated into a glucose-containing growth medium; the cultures were 100 % bleached. The same effect was also observed with 5-bromo-uracil, but the bleaching was not 100 %. The 5-fluoro-uracil treated cultures were permanently bleached and never regained even a part of their chlorophyll when subsequently grown

in the light for many transfers. On the other hand, 5-bromo-uracil-treated cultures, or cultures treated with 5-fluoro-uracil for less than 144 hr were not completely or permanently bleached and returned to the original green state gradually after the analogue was withheld from the growth medium. The results of this experiment are summarized in Table 8.

Table 8. *Effect of 5-fluoro-uracil and 5-bromo-uracil on non-proliferating Euglena gracilis var. bacillaris: loss of the photosynthetic apparatus on prolonged exposure to 5-fluoro-uracil*

The euglena populations were exposed to the base antimetabolite analogues during the non-proliferation period whereafter 2 drops of a 1/50 diluted euglena suspension was used as inoculum.

Type of euglenas suspended in non-proliferation medium	Addition to non-proliferation medium	Concentration of addition (mg. %)	Colour of cultures after 7-day growth in light. Time of exposure to light (hr)* 0	24	72	144
			Colour†			
Dark-grown etiolated	None	—	G	G	G	LG
	5-Fluoro-uracil	200	G	G	G	W‡
		50	G	G	G	W
	5-Bromo-uracil	200	G	G	G	LG
		50	G	G	G	LG
Light-grown green	None	—	G	G	G	LG
	5-Fluoro-uracil	200	G	G	G	W
		50	G	G	G	W
	5-Bromo-uracil	200	G	G	G	PY
		50	G	G	G	PY

* Time of exposure to analogue in the light in non-proliferation medium before transferring a 1/50 diluted inoculum to a glucose-supplemented growth medium.

† G = green; LG = light green; PY = pale yellow; W = white.

‡ White cultures permanently bleached.

DISCUSSION

Lyman, Epstein & Schiff (1961) suggested the implication of self-reproducing cytoplasmic nucleoproteins during the formation of chloroplasts in *Euglena gracilis*. Ultraviolet radiation prevents the transmission of the chloroplasts of Euglena to their progeny; the process, which shows peaks of effectiveness at 260 and 280 mμ, can be photo-reactivated and these cytoplasmic entities are replicated at division and control the formation of chloroplasts (Schiff, Lyman & Epstein, 1961). This cytoplasmic factor was thought to be DNA (Leff, Mandel, Epstein & Schiff, 1963); subsequent work has definitely established the presence of chloroplastic DNA in Euglena (Edelman, Cowan, Epstein & Schiff, 1964; Brawerman & Eisenstadt, 1961; Ray & Hanawalt, 1964, 1965; Edelman, Schiff & Epstein, 1965; Ray, 1965). The inhibition of chlorophyll synthesis in resting Euglena by actinomycin-D suggests a DNA-dependant synthesis of RNA during chloroplast development (McCalla & Allan, 1964).

The inhibition of chlorophyll synthesis by 5-bromo-uracil and its annulment by uracil or thymine shows that there is possibly a *de novo* synthesis of nucleoproteins

occurring during the development of chloroplasts in resting etiolated euglenas and this in some manner is closely associated with the synthesis of chlorophyll. These nucleoproteins are presumably involved in the synthesis of specific proteins: either the enzymes required for photosynthesis, and/or the protein involved in the chlorophyll-protein complex. The inhibition of greening by ethionine and its annulment by methionine also shows that such proteins are possibly being formed *de novo* during illumination of dark-grown organisms. However, according to Gibson, Neuberger & Tait (1962*b*), the inhibition of chlorophyll synthesis by ethionine, in *Rhodopseudomonas spheroides*, is likely to be due to inhibition by competition with methionine of the formation of the methyl ester group of chlorophyll. The formation of NADP-requiring glyceraldehyde-3-phosphate dehydrogenase during chloroplast formation was shown by Fuller & Gibbs (1959) and Brawerman & Konigsberg (1960). This enzyme is characterized by its absence from dark-grown colourless euglenas and its synthesis is blocked by any agent, chemical or physical, which blocks chloroplast formation. The *de novo* synthesis of the protein involved in the chlorophyll-protein complex is also suggested since any blockage in the synthesis of this protein, as with ethionine, would prevent chlorophyll formation simply because the chlorophyll would have no protein to which to attach. A similar mechanism of inhibition by streptomycin was suggested by Kirk (1962). The breakdown of existing proteins and peptides to amino acids which are then utilized for synthesis of chloroplast protein was also suggested by Kirk (1962) in contrast to a hypothesis suggested by Brawerman & Chargaff (1959*a*) that there was an actual transfer of intact proteins from the soluble fraction to the chloroplast fraction. Gibson *et al.* (1962*a*, *b*) suggested that the porphyrin excretion by *Rhodopseudomonas spheroides* in the presence of ethionine was due to its interference with the utilization or synthesis of methionine.

All the antimetabolite analogues of bases tested here were more inhibitory to the growth of *Euglena gracilis* var. *bacillaris* in the dark than in the light. This suggests that the observed effect on growth was due more likely to the inability of the euglenas to utilize glucose rather than to their ability to photosynthesize it. It is apparent that the metabolic pattern of the organisms is markedly influenced by the mode of life, photosynthetic or otherwise. How far the stability of the antimetabolite compounds, or the permeability of the euglenas to these is affected in light is not known. In the light, ATP is generated by the process of photosynthetic phosphorylation and the organisms do not require to metabolize glucose to derive energy. In the dark, where ATP formation depends entirely on glucose utilization, this inhibition is more pronounced. The dark-inhibition by 8-azaguanine ws overcome to some extent by certain Krebs's cycle intermediates like lactate and α-ketoglutarate and completely by citrate. During the formation of chloroplasts by non-proliferating etiolated *Euglena gracilis* organisms, the chlorophyll-less euglenas in the initial stages have to rely on exogenous glucose as sole source of ATP until such time as they synthesize enough chlorophyll to derive energy from other pathways such as photosynthetic phosphorylation.

The work of Scher & Collinge (1965) which suggested the incorporation of 5-bromouracil into the plastid material during chloroplast replication provides another explanation to the mode of inhibition of greening by these base analogues. That such an incorporation takes place is manifested by the formation of bleached colonies, indicating mutagenesis. 5-Fluoro-uracil-treated euglenas also give rise to bleached cultures, although 5-fluoro-uracil does not totally inhibit the greening of resting etio-

lated euglenas at a concentration at which this analogue exerts a permanent bleaching effect on cultures derived even from green euglenas. This suggests that the site of action of 5-fluoro-uracil is at a stage before the accumulation of the 'dark-precursor' of chlorophyll. It is, however, not clear whether 5-fluoro-uracil interferes with the formation or functioning of the chloroplastic satellite DNA of Euglena or produces drastic metabolic changes by affecting the nuclear DNA. 5-Fluoro-uracil is known to inhibit DNA synthesis by being converted to the deoxyribotide which is a potent inhibitor of thymidylate synthetase activity (Heidelberger, 1963). Thymidine has been shown to annul the inhibition of growth of *Escherichia coli* by 5-fluoro-uracil (Mankodi, 1964). 5-Fluoro-uracil is also known to be incorporated into RNA, rendering it inefficient in directing normal protein synthesis (Nakada & Magasanik, 1964). 5-Bromo-uracil had a similar effect, though less pronounced with *Euglena gracilis* var. *bacillaris*, and further exposures up to 312 hr failed to bring about complete bleaching. The 5-fluoro-uracil bleaching of Euglena is analogous to the bleaching effects previously observed by exposures to streptomycin (Provasoli *et al.* 1948; Jírovec, 1949; Provasoli, Hutner & Pintner, 1951), antihistamines (Zahalsky, Hutner, Keane & Burger, 1962), furadantin (McCalla 1962), *O*-methylthreonine (Aaronson & Bensky, 1962), erythromycin (Ebringer, 1962), heat (Brawerman & Chargaff, 1959*b*), short-term ultraviolet radiation (Lyman *et al.* 1961; Schiff *et al.* 1961), and Mg^{2+} starvation (Dubash & Rege, 1967). Although the site of action of each of these mutagens may be different, in all these cases the permanent loss of photosynthetic ability is preceded by a reversible lesion.

Evidence has been presented for the operation of the Krebs cycle in Euglena by Danforth (1953); the reversal of 8-azaguanine inhibition by several Krebs intermediates, both in light as well as in dark, indicates the involvement of this cycle. However, the organism appears to employ pathways for carbohydrate metabolism which are fundamentally different in the dark and photosynthetic phases. A possibility of greater turnover of nucleic acids during the dark adaptive phase is also indicated. The increased formation of chlorophyll on the continued presence of the analogues during chloroplast formation after the cells are first grown in the dark in the presence of the analogue indicates a degree of adaptation of the cells during the growth phase towards the inhibitor.

REFERENCES

Aaronson, S. & Bensky, B. (1962). *O*-Methylthreonine, a new bleaching agent for *Euglena gracilis*. *J. gen. Microbiol.* **27**, 75.

Brawerman, G. & Chargaff, E. (1959*a*). Changes in protein and ribonucleic acid during the formation of chloroplasts in *Euglena gracilis*. *Biochim. biophys. Acta* **31**, 164.

Brawerman, G. & Chargaff, E. (1959*b*). Factors involved in the development of chloroplasts in *Euglena gracilis*. *Biochim. biophys. Acta* **31**, 178.

Brawerman, G. & Eisenstadt, J. M. (1964). Deoxyribonucleic acid from the chloroplasts of *Euglena gracilis*. *Biochim. biophys. Acta* **91**, 477.

Brawerman, G. & Konigsberg, N. (1960). On the formation of the TPN requiring glyceraldehyde-3-phosphate dehydrogenase during the production of chloroplasts in *Euglena gracilis*. *Biochim. biophys. Acta* **43**, 374.

Brawerman, G., Pogo, A. O. & Chargaff, E. (1962). Induced formation of ribonucleic acids and plastid protein in *Euglena gracilis* under the influence of light. *Biochim. biophys. Acta* **55**, 326.

Coelho, J. & Rege, D. V. (1963). On hemoglobin inhibition of the growth of *Euglena gracilis*. *J. Protozool.* **10**, 473.

Danforth, W. F. (1953). Oxidative metabolism of Euglena. *Arch. Biochem.* **46**, 164.

Dubash, P. J. & Rege, D. V. (1967). Permanent bleaching of Euglena by Mg^{2+} starvation. *Biochim. biophys. Acta* **136**, 185.

EBRINGER, L. (1962). Erythromycin induced bleaching of *Euglena gracilis*. *J. Protozool.* **9**, 373.

EDELMAN, M., COWAN, C. A., EPSTEIN, H. T. & SCHIFF, J. A. (1964). Chloroplast development in Euglena. VIII. Chloroplast associated DNA. *Proc. natn. Acad. Sci., U.S.A.* **52**, 1214.

EDELMAN, M., SCHIFF, J. A. & EPSTEIN, H. T. (1965). Chloroplast development in Euglena. XII. Two types of satellite DNA. *J. molec. Biol.* **11**, 769.

FULLER, R. C. & GIBBS, M. (1959). Intracellular and phylogenetic distribution of ribulose 1,5-diphosphate carboxylase and d-glyceraldehyde-3-phosphate dehydrogenase. *Plant Physiol.* **34**, 324.

GIBSON, K. D., NEUBERGER, A. & TAIT, G. H. (1962*a*). Studies on the biosynthesis of porphyrin and bacteriochlorophyll by *Rhodopseudomonas spheroides*. *Biochem. J.* **83**, 539.

GIBSON, K. D., NEUBERGER, A. & TAIT, G. H. (1962*b*). Studies on the biosynthesis of porphyrin and bacteriochlorophyll by *R. spheroides*. 2. The effects of ethionine and threonine. *Biochem. J.* **83**, 550.

GROSS, J. A., JAHN, T. L. & BERNSTEIN, E. (1955). The effect of antihistamines on the pigments of green protista. *J. Protozool.* **2**, 71.

HEIDELBERGER, C. (1963). Biochemical mechanisms of action of fluorinated pyrimidines. *Exp. cell Res. Suppl.* **9**, 462.

HUTNER, S. H., BACH, M. K. & ROSS, G. I. M. (1956). A sugar-containing basal medium for vitamin B_{12}-assay with Euglena: application to body fluids. *J. Protozool.* **3**, 101.

HUTNER, S. H., PROVASOLI, L., SCHATZ, A. & HASKINS, C. (1950). Some approaches to the study of the role of metals in the metabolism of micro-organisms. *Proc. Am. Phil. Soc.* **94**, 152.

JÍROVEC, O. (1949). Účinek antibiotik na některé prvoky. *Mém. Soc. zool. tchécosl.* **13**, 216.

KIRK, J. T. O. (1962). Effect of streptomycin on greening and biosynthesis in *Euglena gracilis*. *Biochim. biophys. Acta* **56**, 139.

LEFF, J., MANDEL, M., EPSTEIN, H. T. & SCHIFF, J. A. (1963). DNA satellites from cells of green and aplastidic algae. *Biochem. biophys. Res. Communs.* **13**, 126.

LYMAN, H., EPSTEIN, H. T. & SCHIFF, J. A. (1961). Studies of chloroplast development in Euglena. I. Inactivation of green colony formation by u.v.-light. *Biochim. biophys. Acta* **50**, 301.

MANKODI, B. S. (1964). *Studies in nucleotide metabolism in micro-organisms*. Ph.D. (Tech.) Thesis, University of Bombay.

MCCALLA, D. R. (1962). Chloroplasts of *Euglena gracilis* affected by furadantin. *Science* **137**, 225.

MCCALLA, D. R. & ALLAN, R. K. (1964). Effect of actinomycin D on Euglena chloroplast formation. *Nature, Lond.* **201**, 504.

NAKADA, D. & MAGASANIK, B. (1964). The roles of inducer and catabolite repressor in the synthesis of β-galactosidase by *Escherichia coli*. *J. molec. Biol.* **8**, 105.

POGO, A. O., BRAWERMAN, G. & CHARGAFF, E. (1962). New ribonucleic acid species associated with the formation of the photosynthetic apparatus in *Euglena gracilis*. *Biochemistry* **1**, 128.

PROVASOLI, L., HUTNER, S. H. & PINTNER, I. J. (1951). Destruction of chloroplasts by streptomycin. *Cold Spr. Harb. Symp. quant. Biol.* **16**, 113.

PROVASOLI, L., HUTNER, S. H. & SCHATZ, A. (1948). Streptomycin-induced chlorophyll-less races of Euglena. *Proc. Soc. exp. Biol. Med.* **69**, 279.

PRINGSHEIM, E. G. (1958). Die Apoplastidie bei Euglena. *Rev. Algol.* **4**, 41.

PRINGSHEIM, E. G. & PRINGSHEIM, O. (1952). Experimental elimination of chromatophores and eye-spot in *Eugiena gracilis*. *New Phytol.* **51**, 65.

RAY, D. S. (1965). Isolation and physical characterization of the principal and satellite DNAs of *Euglena gracilis*. *Disser. Abstr.* **25**, 6205.

RAY, D. S. & HANAWALT, P. C. (1964). Properties of the satellite DNA associated with the chloroplasts of *Euglena gracilis*. *J. molec. Biol.* **9**, 812.

RAY, D. S. & HANAWALT, P. C. (1965). Satellite DNA components in *Euglena gracilis* cells lacking chloroplasts. *J. molec. Biol.* **11**, 760.

SCHER, S. & COLLINGE, J. C. (1965). Chloroplast replication: Evidence for 5-bromo-uracil incorporation and plastid mutation in *Euglena gracilis*. *Nature, Lond.* **205**, 828.

SCHIFF, J. A., LYMAN, H. & EPSTEIN, H. T. (1961). Studies of chloroplast development in Euglena. II. Photoreversal of the UV inhibition of green colony formation. *Biochim. biophys. Acta* **50**, 310.

ZAHALSKY, A. C., HUTNER, S. H., KEANE, M. & BURGER, R. M. (1962). Bleaching *Euglena gracilis* with antihistamines and streptomycin-type antibiotics. *Arch. Mikrobiol.* **42**, 46.

J. gen. Microbiol. (1967), **48**, 293–298
Printed in Great Britain

The Identification of Two Antibacterial Products of the Marine Planktonic Alga *Isochrysis galbana*

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(*Accepted for publication* 18 *March* 1967)

SUMMARY

The acetone-extractable antibacterial components of *Isochrysis galbana* have been resolved by paper-chromatography and bioautography. Two pigmented products with similar antibacterial properties were obtained which were tentatively identified from their absorption spectra as chlorophyll *a* derivatives, probably pheophytin *a* and an atypical chlorophyllide *a*.

INTRODUCTION

A survey of marine planktonic algae for antibacterial activity (Duff, Bruce & Antia, 1966) revealed a wide range and degree of activity in several algal species, of which a diatom *Skeletonema costatum* and the chrysophyte *Isochrysis galbana* appeared most promising for further examination. The study of *I. galbana* lead to the characterization of the products responsible for the observed activity. The resolution of acetone extracts of this alga into two active components and their identification as derivatives of chlorophyll *a* are reported in this paper.

METHODS

Algal culture and extracts. Isochrysis galbana Parke was grown in axenic culture and the algal material was harvested and dried as described by Antia & Kalmakoff (1965).

Algal extracts were prepared by three methods. Method (*a*) involved sequential extractions with three solvent systems (acetone; chloroform; chloroform + methanol (1 + 1, v/v)) at room temperature as described by Duff *et al.* (1966). Method (*b*) was similar to method (*a*) but with the extraction with each solvent system repeated twice before using the next solvent in the sequence. Method (*c*) involved sequential Soxhlet extractions (6 hr each) of algal cell powder (100 mg.) with the same solvent systems (25 ml. samples); each solvent extract was concentrated *in vacuo* to a 2 ml. equivalent of the extracts obtained by the other methods. The ratio of the weight of algal material to final volume of extract used in activity tests was taken as a measure of this equivalence. All extracts were stored at $-30°$ and were protected from direct light at all times.

Activity tests. Seven strains of the test bacteria showing high sensitivity against *Isochrysis galbana* were chosen from the organisms previously surveyed (Duff *et al.* 1966) with a view to obtaining a fair representation of the activity spectrum; they are listed in Table 1. In addition, a freshly isolated organism (*Staphylococcus aureus* C from a patient with chronic furunculosis) was included. Activity test methods were as used in the earlier survey. (Duff *et al.* 1966).

Paper chromatography and bioautography of algal extracts. All solvents (mostly analytical reagent grade) were distilled before use. As far as possible, all operations were done in subdued light or in the dark. A cm.^{2} grid system was marked on a 15 cm.^{2} area of a Whatman no. 1 chromatography paper sheet (25 cm.^{2}) and a labelling system was devised to enable later identification of each square. A sample (0·05 ml.) of extract was applied to the point of chromatographic origin taken at the centre of a corner square of the grid. Two-dimensional descending chromatography was used with 4 % (v/v) propan-1-ol in light petroleum (b.p. 60–80°) as developing solvent in the first dimension and 30 % (v/v) chloroform in light petroleum (b.p. 60–80°) in the second dimension. The chromatograms were developed for 1 hr in each dimension, dried and transferred to a sterile container. Test plates were prepared for bioautography by the same methods used earlier for testing extract-charged discs (Duff *et al.* 1966). Only *Brevibacterium* sp. C–7b and *Micrococcus* sp. C–54b were used as test organisms. The developed chromatogram was cut aseptically into 1 × 5 cm. strips along the lines of the marked grid and placed on the surface of the seeded test plates, which were then incubated and examined as reported by Duff *et al.* (1966). Areas of inhibition were related to chromatographic R_F values from the grid identification marks.

The areas known to be active were cut from subsequent chromatograms and extracted with diethyl ether. These eluates were concentrated to a volume equivalent to that of the original algal extract, for quantitative comparison of activity by disc tests (Table 1). Absorption spectra of the eluates were measured on a Beckman DB-G grating spectrophotometer fitted with a linearlog 10 in. potentiometric recorder.

RESULTS

A comparison of the three methods of extraction of the alga showed that exhaustive acetone extraction by either method (*b* or *c*) was sufficient to remove almost all the active components of the alga and that subsequential extraction with the other solvents was unnecessary. Since the higher temperature (70–75°) of the Soxhlet extraction did not appear to be deleterious to the active components, method (*c*) was chosen for all subsequent extract preparations because of its convenience.

The bioautography of acetone extracts of the alga showed that all the antibacterial activity was associated with two pigmented components which appeared to be easily separable by adequate development of the chromatogram in the first dimension only. Subsequent unidimensional chromatography of the extracts gave good separation of the slower-moving component (*A*, R_F 0·35) in about 2·5 hr, but required 4 hr for complete separation of the faster-moving component (*B*, R_f 0·9). Since component *A* was unstable on prolonged chromatography, it was preferred to run separate chromatograms for isolation of these compounds by elution and to use the minimum period of development required for complete separation of each.

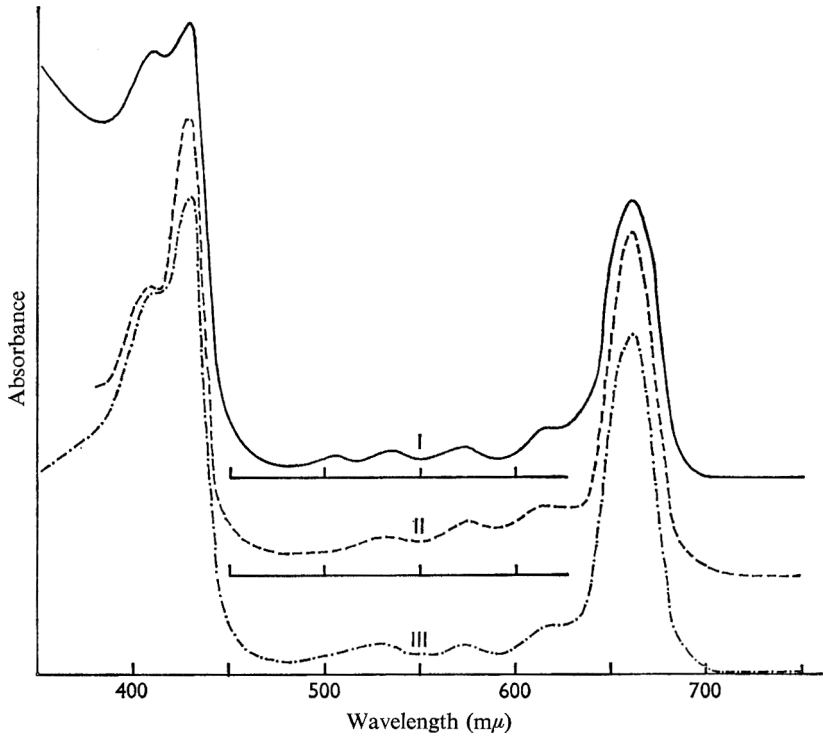

Fig. 1. Absorption spectra of compound *A*, ethyl chlorophyllide *a* (from Holt & Jacobs, 1954), and atypical chlorophyllide *a* (from Barrett & Jeffrey, 1964) in diethyl ether represented by curves I, II, and III respectively.

Table 1. *Growth inhibition of bacteria from whole extracts of Isochrysis galbana and their active components*

Test concentration of the active components was equivalent to that present in the whole extract.

Test bacteria	Degrees of inhibition†		
	Acetone extract*	Compound *A*	Compound *B*
Terrestrial strains:			
Streptococcus faecalis	—	s	s
Staphylococcus aureus C	s	s	s
S. aureus E	s	s	s
S. aureus M	s	s	s
Marine strains:			
Flavobacterium sp. P-4b	m	m	m
Brevibacterium sp. C-7b	v	m	m
Micrococcus sp. C-54b	v	m	m
Micrococcus sp. P-59	m	m	m

* Prepared by extraction method *c* (see text).

† Growth inhibition was classified into three categories based on diameter of inhibition zones as follows: v, very active (> 1·5 cm.); m, moderately active (> 1·0 < 1·5 cm.); s, slightly active (< 1·0 cm.).

The activities observed from the isolated compounds *A* and *B* and from the original acetone extracts are summarized in Table 1. The results show that little loss or change of activity was incurred during the resolution of extracts and that the isolated compounds possess the same range and similar degrees of activity. Compounds *A* and *B* showed blue-green and grey-green hues, respectively, on paper-chromatograms and their locations corresponded closely to those of chlorophyllide *a* and pheophytin *a* observed by Jeffrey (1961) on similarly developed two-dimensional chromatograms of

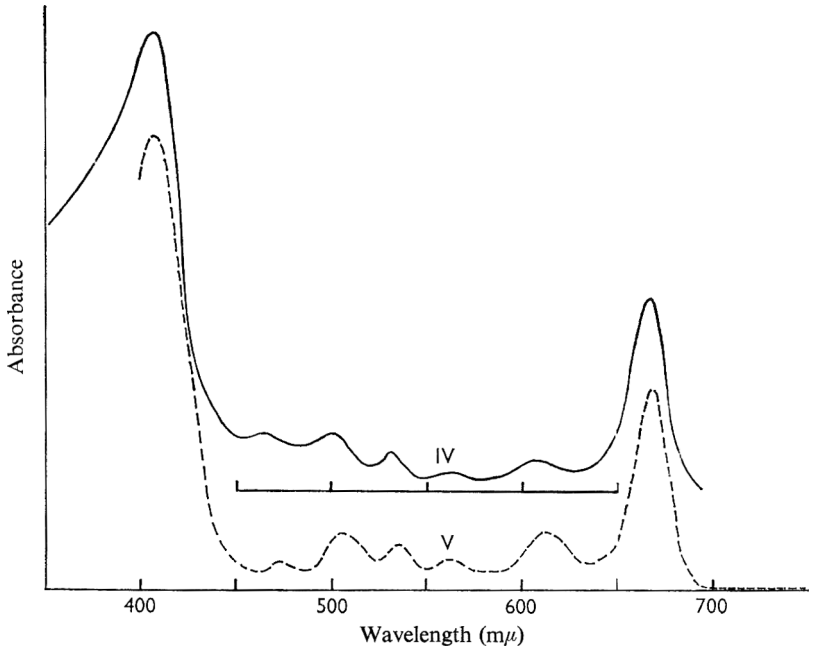

Fig. 2. Absorption spectra of compound *B* and pheophytin *a* (from Smith & Benitez, 1955) in diethyl ether, represented by curves IV and V respectively.

Table 2. *Absorption maxima of active components of Isochrysis galbana and some chlorophyll a derivatives*

All maxima (mμ) are reported for diethyl ether solutions.

	Absorption maxima (mμ)						
Compound *A*	660	613	573	536	506	428	410
Ethyl chlorophyllide *a**	659–660	613–614	574–576	530–532	—	428–429	410
Atypical chlorophyllide *a*†	660	615	573	530	—	429	~415
Compound *B*	668	612	562	536	504	468	408
Pheophytin *a*‡	667	609·5	560	534	505	471	408·5

* Holt & Jacobs (1954). † Barrett & Jeffrey (1964). ‡ Smith & Benitez (1955).

marine algal pigments. The first-dimension R_F values of 0·38 and 0·87 reported by this author for chlorophyllide *a* and pheophytin *a* are also in close agreement with those obtained by us for compounds *A* (R_F, 0·35) and *B* (R_F, 0·9). These clues to the identity of the active compounds were verified by their absorption spectra (Figs 1, 2). The close correspondence in the absorption of compound *B* with that previously reported for

pheophytin *a* (Fig. 2, Table 2) supports the chromatographic evidence that these two compounds may be identical. The absorption of compound *A* is very like that of ethyl chlorophyllide *a* but shows some significant differences (Fig. 1 and Table 2). Recently, an atypical chlorophyllide *a* was reported to be produced by some planktonic algae, which differed spectroscopically from the standard chlorophyllide *a* in the Soret band region and in the ratio of the absorbance of the blue maxima to the red maxima (Barrett & Jeffrey, 1964). The spectrum of compound *A* resembles that of the atypical chlorophyllide *a* in the ratios of the absorbance of the blue maxima (428 and 410 mμ) to the red maximum (660 mμ) but differs in the location of the minor blue maximum (410 mμ) which is in closer agreement with that of the standard chlorophyllide (see Fig. 2, Table 2). Compound *A* shows a minor maximum at 506 mμ which is absent from the spectra of both chlorophyllides. Although not identical with either chlorophyllide, the overall spectral resemblance and the chromatographic evidence indicate that compound *A* may be a hitherto uncharacterized chlorophyllide *a*. The recent finding of several 'isomers' of chlorophyll *a* in Chlorella (Michel-Wolwertz & Sironval, 1965) anticipates the occurrence of a number of atypical chlorophyllides in algae.

DISCUSSION

The antibacterial activity of chlorophyll derivatives from algae has been previously reported. Jørgensen (1962) observed growth-inhibition of *Bacillus subtilis* from photo-oxidized chlorophyllides and pheophytin-like substances produced by three species of unicellular Chlorophyceae; the active substances were not clearly identified and pheophytin *a* was stated to be inactive. Sieburth (1965) reported some evidence which suggested that part of the wide-spectrum activity of the diatom *Skeletonema costatum* might be due to chlorophyll derivatives (presumably a chlorophyllide and its photo-oxidation product). The present finding of active chlorophyll *a* derivatives in *Isochrysis galbana* extends the list of algal classes which produce such antibiotics to the Chrysophyceae and adds weight to the suggestions of Jørgensen (1962) and Sieburth (1965) that active 'chlorophyllides' may play a significant antibacterial role in the microbial ecology of natural waters.

The earlier investigators have generally considered the active 'chlorophyllides' to be degradation products of chlorophyll produced by senescent or autolysed organisms. The culture of *Isochrysis galbana* used in the present work was harvested towards the end of exponential growth and could not be considered to be senescent, although the possibility that a small proportion of organisms were undergoing premature ageing and autolysis is not excluded. It does seem that bioactive chlorophyll derivatives may also be produced by algae during active growth. That chlorophyllides may not necessarily be products of chlorophyll catabolism is suggested by inspection of the scattered evidence such as the finding of highly active chlorophyllase systems in marine algae (Barrett & Jeffrey, 1964) and the observation that chlorophyllide *a* may be an integral part of a major form of *in vivo* chlorophyll (Sironval, Michel-Wolwertz & Madsen, 1965).

Barrett & Jeffrey (1964) did not find in *Isochrysis galbana* the chlorophyllase system which was shown in other algae to produce the atypical chlorophyllide *a* mentioned earlier (see Results). This confirms our conclusion, based on spectral differences, that the antibacterial chlorophyllide *a* (compound *A*) is not identical with the Barrett–Jeffrey chlorophyllide. Our finding of the antibacterial activity of pheophytin *a* from *I. galbana*

is surprising and requires verification by tests on the compound isolated from other sources, since Jørgensen (1962) found it to be inactive against *Bacillus subtilis* and yet he obtained pheophytin-like substances from Chlorella, Scenedesmus, and Chlamydomonas which inhibited growth of this bacterium under illumination but not in the dark.

The financial assistance received from the National Research Council of Canada by two of the authors (D. L. B. and D. C. B. D.) in support of this study is gratefully acknowledged.

REFERENCES

ANTIA, N. J. & KALMAKOFF, J. (1965). Growth rates and cell yields from axenic mass culture of fourteen species of marine phytoplankters. *Fisheries Res. Board Can. Manuscript Rept. Ser.* (*Oceanog. and Limnol.*) no. 203.

BARRETT, J. & JEFFREY, S. W. (1964). Chlorophyllase and formation of an atypical chlorophyllide in marine algae. *Plant Physiol.* **39**, 44.

DUFF, D. C. B., BRUCE, D. L. & ANTIA, N. J. (1966). The antibacterial activity of marine planktonic algae. *Can. J. Microbiol.* **12**, 877.

HOLT, A. S. & JACOBS, E. E. (1954). Spectroscopy of plant pigments. I. Ethyl chlorophyllides *a* and *b* and their pheophorbides. *Am. J. Bot.* **41**, 710.

JEFFREY, S. W. (1961). Paper-chromatographic separation of chlorophylls and carotenoids from marine algae. *Biochem. J.* **80**, 336.

JØRGENSEN, E. G. (1962). Antibiotic substances from cells and culture solutions of unicellular algae with special reference to some chlorophyll derivatives. *Physiol. Plant.* **15**, 530.

MICHEL-WOLWERTZ, M.-R. & SIRONVAL, C. (1965). On the chlorophylls separated by paper chromatography from Chlorella extracts. *Biochim. biophys. Acta* **94**, 330.

SIEBURTH, J. MCN. (1965). Role of algae in controlling bacterial populations in estuarine waters. In *Pollutions marines par les Microorganismes et les Produits pétroliers, Symposium de Monaco* (*avril* 1964), p. 217. Publ. by Secrétariat général de la Commission Internationale pour l'Exploration scientifique de la Mer Méditerranée, Paris.

SIRONVAL, C., MICHEL-WOLWERTZ, M. R. & MADSEN, A. (1965). On the nature and possible functions of the 673- and 684-mμ forms *in vivo* of chlorophyll. *Biochim. biophys. Acta* **94**, 344.

SMITH, J. H. C. & BENITEZ, A. (1955). Chlorophylls: Analysis in plant materials. In *Modern Methods of Plant Analysis*. Ed. by K. Paech and M. V. Tracey, **4**, 142. Berlin: Springer-Verlag.

J. gen. Microbiol. (1967), **48**, 299–304
Printed in Great Britain

Isolation and Some Properties of the Competence Factor from Group H Streptococcus Strain CHALLIS

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(*Accepted for publication* 11 *October* 1966)

SUMMARY

In several bacterial species the occurrence of competence in transformation is strictly related to the production of a competence factor (c.f.). In the present work this factor was isolated from the cell surface of Streptococcus group H strain CHALLIS by the centrifugation of competent bacteria at pH 2·0, followed by thermal shock. Streptococci of strain WICKY adsorb c.f. isolated from the CHALLIS organisms because supernatant fluids containing c.f. lose their activity after having been in contact with organisms. It was possible to 'tear off' again from such organisms of strain WICKY the c.f. they had adsorbed. The re-isolated c.f. was still active in the transformation systems used.

Some biochemical properties of the c.f. from strain CHALLIS are described. The factor was relatively heat resistant and sensitive to proteolytic enzymes. Streptococcal c.f. or a fragment of it seems to be a protein or polypeptide of fairly low molecular weight. This streptococcal c.f. is probably different from the pneumococcal c.f.

INTRODUCTION

The incorporation of DNA in bacterial transformation depends on the occurrence in recipient cells of a transient competence state. The elucidation of the mechanism of competence—that is to say, the mechanism of incorporation of DNA—is a biologically important problem.

Recently it has been shown that the appearance of competence is strictly related to the production by the cells of a special competence factor (c.f.) (Pakula & Walczak, 1963; Felkner & Wyss, 1964; Tomasz & Hotchkiss, 1964; Charpak & Dedonder, 1965; Tomasz & Mosser, 1966).

In Streptococcus group H strain CHALLIS Pakula & Walczak (1963) discovered and observed c.f. in the supernatants of cultures after the cells had excreted it into a rich, undefined medium, already containing several different polypeptides. Under conditions which produce competence and transformation of other streptococci and of pneumococci it is impossible to get a competent population and thus transformation in Streptococcus strain WICKY (Pakula, 1965). This strain can be transformed only in the presence of the c.f. produced by strain CHALLIS.

It was recently reported that it is possible 'to tear off' the c.f. from the cell surface of *Bacillus subtilis* and pneumococci. Some biochemical properties of the both factors have been described (Charpak & Dedonder, 1965; Tomasz & Mosser, 1966).

In the present work, the c.f. from the surface of cocci of strain CHALLIS was isolated and a preliminary biochemical characterization made.

METHODS

Bacterial strains. The transformable strain CHALLIS of Streptococcus group H, sensitive to streptomycin, was used to obtain the competence factor. A mutant of strain CHALLIS resistant to 2 mg. streptomycin/ml. served as donor of transforming DNA. Streptococcus group H strain WICKY was used as recipient. Both these strains originated from Dr Pakula's collection.

DNA preparation. DNA was extracted from strain CHALLIS cultures by a method described in Pakula, Piechowska, Bankowska & Walczak (1962). This method involved lysis of cells by the lytic factor from a culture of *Streptomyces albus* and isolation and purification of the DNA according to the procedure elaborated by Marmur (1961).

Media. For the production of c.f. by strain CHALLIS and for the transformation of strain WICKY, two media employed by Pakula & Walczak (1963) and Pakula (1965) were used: ET 3 (1 % Difco Neopeptone, 0·5 % Difco yeast extract, 0·5 % NaCl, 0·17 % K_2HPO_4 and 0·2 % glucose in distilled water) and HE, which differed from ET 3 by the replacement of water with beef heart extract. The pH of both media was adjusted to 7·6. The media were sterilized by filtration through a glass filter G-5 or through a Seitz filter. Albumin (0·2 %) was always added to both media to obtain c.f. from strain CHALLIS and transformation in strain WICKY.

Transformation procedure and measurement of the activity of the competence factor An overnight culture of strain WICKY in horse blood broth was inoculated at 4×10^6 cocci/ml. into ET 3 or HE medium and incubated for 90 min. at 37° in a water bath. After this period, 1·75 ml. of the culture was supplemented with 0·5 ml. of a suitable dilution of treated and untreated c.f. (first positive control) and incubated for 30 min. at 37°. At this time, 0·25 ml. of transforming DNA (10 μg./ml.) was added to the mixture and incubated 15 min., then deoxyribonuclease ('Distreptaza'—about 20 u./ml.) was added to stop the reaction. Afterwards the culture was incubated for an additional 120 min. for phenotypic expression of the transformed marker.

Immediately after the addition of DNA, a count was made of the number of viable colony-forming units. After phenotypic expression, the number of streptomycin-resistant transformants was determined on blood agar plates containing 250 μg./ml. of streptomycin.

As a second positive control, the transformation of strain WICKY provoked by Pakula's type of supernatant containing c.f. (Pakula & Walczak, 1963) was performed in each experiment. As a negative control in each experiment, physiological saline was added to the reaction mixture in place of c.f.

The method of 'tearing off' c.f. is described below. Each sample of c.f., before being used in the experiments, was characterized by the determination of the yield of a transformation of strain WICKY performed in the presence of controlled c.f. in 10 % (v/v) concentration.

Reagents. Dihydrostreptomycin sulphate ('Polfa'), deoxyribonuclease ('Distreptaza'–'Biomed'), pronase ('Calbiochem'), papain ('Dembach'), trypsin ('Merck'), pancreatin ('Calbiochem'), lysozyme ('Reanal') and ribonuclease ('BDH') were used.

RESULTS

Preparation of competence factor.

An overnight culture of strain CHALLIS in horse blood broth was inoculated at $4{\cdot}3 \times 10^6$ cocci/ml. into a quantity (usually 2 l.) of ET 3 or HE medium with albumin. The culture was incubated, usually for 100 min., in a water bath at 37°. Each batch of

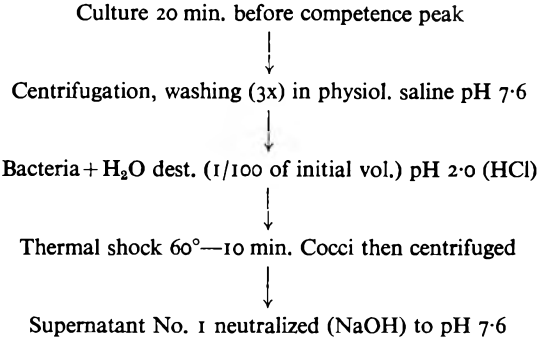

Fig. 1. Method of 'tearing off' c.f. from Streptococcus strain CHALLIS. An amount of ET 3 or HE medium containing 0·2% albumin was inoculated with an overnight culture of the strain CHALLIS in horse blood broth. At the beginning of the incubation the medium contained about $4{\cdot}3 \times 10^6$ cocci/ml. The culture was incubated for a suitable period of time (usually 100 min.) in a water bath at 37°.

ET 3 or HE medium used for the preparation of c.f. was thoroughly tested, by determining the time at which the peak of the competence occurred. Further details of the procedure for 'tearing off' c.f. from strain CHALLIS devised during the course of the present study are shown in Fig. 1.

We did not succeed in isolating the strain CHALLIS c.f. by the method used for pneumococci by centrifugation in physiological saline + phosphate buffer + 2-mercaptoethanol (pH 7·5) (Tomasz & Mosser, 1966).

Optimal period of contact.

The supernatant fraction of media containing streptococcal c.f. obtained by Pakula & Walczak (1963) requires about 30 min. of contact with strain WICKY cells to produce the maximum yield of transformation. To find the optimum time of contact for the c.f. prepared according to the method described above, 10 % of 'torn off' c.f. was added to samples taken from the same culture of strain WICKY. After incubation for the times as indicated in Fig. 2, the colony count and the number of transformants were determined.

Figure 2 shows that the optimal time of contact between the c.f. and strain WICKY is also about 30 min.

Effect of competence factor concentration on yield of transformants.

To test the relationship between transformation yield and the concentration of c.f. added to the recipient cells in a transforming medium, 0·5 ml. of phosphate buffer containing various concentrations of c.f. was added to samples of the same culture of strain WICKY, and after 30 min. incubation the number of transformants was determined.

Figure 3 presents the results of such an experiment with the use of one of our

supernatants no. 1 (see Fig. 1) as c.f. Using this c.f. 10 % (v/v) concentration of the factor was saturating. This concentration was saturating in the majority of our preparations, although in some preparations 15 or 20 % (v/v) of c.f. was required to reach saturation.

Effect of proteolytic enzymes.

Sensitivity of the competence factor to various enzymes was studied to obtain some information about the chemical nature of the factor.

In these experiments, presented in Table 1, 0·4 ml. of c.f. was incubated at 37° with 0·1 ml. of the given enzyme at the desired concentration for the period of time indicated. Then 1·75 ml. of strain WICKY culture prepared in the usual manner was added to this sample and incubated for 30 min. at 37°. The remaining c.f. activity was assayed

Table 1. *Enzyme sensitivity of competence factor from Streptococcus strain* CHALLIS

0·4 ml. of c.f. was incubated with 0·1 ml. of the given enzyme in desired concentration at 37° for the indicated period of time. Then 1·75 ml. of strain WICKY culture prepared in the usual manner was added to this sample and incubated for 30 min. at 37°. Afterwards a transformation was performed as described under Methods.

Enzyme	Concentration (μg./ml.)	Percentage of residual c.f. activity after treatment		
		10 min.	90 min.	180 min
Pronase	1	1·0	—	—
	10	—	0	—
	100	—	—	0
Trypsin	1	63	—	—
	10	—	5·0	—
	100	—	1·7	0
Pancreatin	1	100	—	—
	10	—	14·7	—
	100	—	0·25	0·4
Papain	1	100	—	—
	10	—	3·0	—
	100	—	0	0·4
		10 min.		30 min.
Lysozyme	1	100		—
	5	100		—
	10	—		100
RNAse	1	100		—
	5	100		—
	10	—		100

by transformation of strain WICKY as described previously. Thus, during the period of treatment with c.f. and transformation, an enzyme was present, but in a concentration five times lower than that at the beginning of the experiment. To test if the decrease in the yield of transformants demonstrated in Table 1 was indeed due to enzymatic destruction of c.f. during incubation of the factor with the enzyme before treatment and transformation, special control experiments were carried out. In these experiments, a 30 min. exposure of strain WICKY to c.f. was performed in the presence of 10 μg./ml of trypsin, papain, or pancreatin. After this period, the cells were centrifuged, resuspended in fresh transformation medium and transformation was

carried out. The yield of transformants in these experiments was normal or only slightly lower (about 10 %) than in positive controls treated in the same manner. The results of these experiments justify the conclusion that the streptococcal c.f. is sensitive to proteolytic enzymes. Termination of trypsin treatment of c.f. by the addition of soybean trypsin inhibitor does not prevent inhibition of activation in the case of similar observations on pneumococcal competence factor (Tomasz & Mosser, 1966).

After 10 min. of contact between c.f. and the enzymes at a concentration of 1 μg./ml., only pronase destroyed the activity of the factor. Trypsin decreased its activity up to about 35 %, while pancreatin and papain were not active in these conditions. Ninety minutes contact between c.f. and the enzymes at a concentration of 10 μg./ml. caused almost complete destruction of c.f. activity. The pneumococcal factor is also very sensitive to proteolytic enzymes (Tomasz & Mosser, 1966). Subtilisin, chymotrypsin, and trypsin at a concentration of 1 μg./ml. inactivate it completely after 10 min. This difference indicates that the two factors differ in enzyme sensitivity. Lysozyme and ribonuclease do not inactivate either of the competence factors.

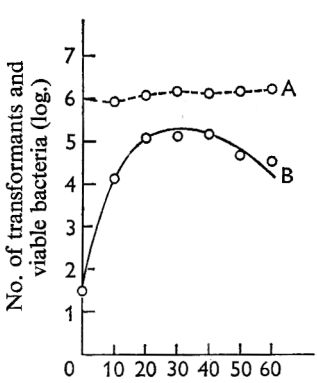

Fig. 2

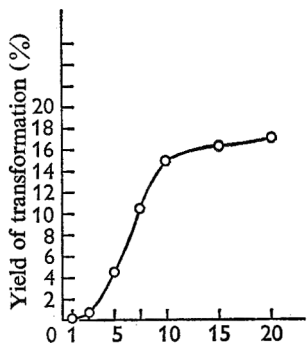

Fig. 3

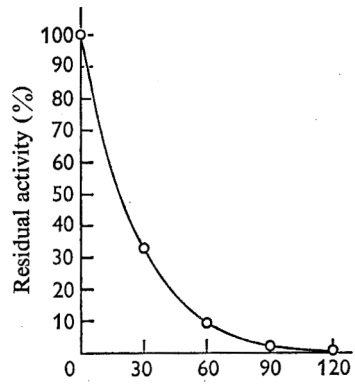

Fig. 4

Fig. 2. Optimal period of contact (before DNA addition) of strain WICKY with competence factor 'torn off' from cell surface: A, number of viable bacteria (log.); B, number of transformants (log.). To the samples taken from the same culture of strain WICKY, the c.f. 10% (v/v) was added. Then, all samples were incubated for different periods of time. After the periods indicated in the figure, the numbers of viable centres and transformants were determined.

Fig. 3. Dependence of yield of reaction (%) in strain WICKY on c.f. concentration in HE transforming medium. To the samples of the same culture of strain WICKY, 0·5 ml. of phosphate buffer containing various concentrations of c.f. was added and after 30 min. incubation and transformation the yield of reaction was determined.

Fig. 4. Sensitivity to temperature (100°) of c.f. from strain CHALLIS. An amount of non-diluted c.f. was heated to 100°. After the periods indicated in the figure the samples of c.f. were taken, immediately diluted to 7·5% (v/v) and added to the recipient cells. Then, a transformation reaction was performed. The concentration of c.f. used in these experiments was a non-saturating one.

Heat-inactivation of the competence factor

The purpose of this experiment was to compare the heat-sensitivity of our c.f. with the sensitivity of streptococcal c.f. reported by Pakula & Walczak (1963), pneumococcal c.f. (Tomasz & Mosser, 1966) and *Bacillus subtilis* c.f. (Charpak & Dedonder, 1965).

In this experiment, a non-saturating concentration (i.e. 7·5 %, v/v) of heated c.f. was used to evoke transformation. Undiluted c.f. was heated to 100° and after the times indicated in Fig. 4 samples were taken, immediately diluted to the above-mentioned non-saturating concentration and added to the recipient cells and its activity assayed by the transformation method. The results were compared with the result of a control experiment performed in parallel in which an unheated, suitably diluted sample of the same c.f. was used.

The results in Fig. 4 show that the factor was relatively heat-resistant: 10 min. exposure to 100° resulted in the loss of only about 22 % of its activity. Complete loss of activity only occurred after 90 min. heating at 100°. The c.f. from strain CHALLIS is much more heat-resistant than pneumococcal c.f., which lost all its activity after 20 min. at 100° (Tomasz & Mosser, 1966). The c.f. from the Marburg strain of *Bacillus subtilis* is also much more heat-sensitive. It lost complete activity after 5 min. at 100° (Charpak & Dedonder, 1965).

At −15°, the streptococcal factor conserved activity for 5–7 days. Alkalization and storage in alkali at pH 11 prolongs activity up to about 20 days.

From these experiments we may conclude, from its relative heat resistance, that (1) streptococcal c.f. is or contains a protein or polypeptide of low molecular weight, and (2) that streptococcal c.f. is probably different from pneumococcal c.f.

We would like to thank Professor D. Shugar and Professor P. Szafranski for valuable discussion during the preparation of this manuscript, and J. Nalecz for excellent technical assistance.

REFERENCES

CHARPAK, M. & DEDONDER, R. (1965). Production d'un 'facteur de competence' soluble par *Bacillus subtilis* Marburg *ind* 168. *C. r. hebd. Séanc. Acad. Sci., Paris.* **260**, 5638.

FELKNER, I. C. & WYSS, O. (1964). A substance produced by competent *Bacillus cereus* 569 cells that affects transformability. *Biochim. biophys. Res. Comm.* **16**, 94.

MARMUR, J. (1961). A procedure for isolation of deoxyribonucleic acid from micro-organisms. *J. molec. Biol.* **3**, 208.

PAKULA, R. (1965). Production of competence-provoking factor and development of competence of a transformable Streptococcus in serum-free media. *Can. J. Microbiol.* **11**. 811.

PAKULA, R. & WALCZAK, W. (1963). On the nature of competence of transformable streptococci. *J. gen. Microbiol.* **31**, 125.

PAKULA, R., PIECHOWSKA, M., BANKOWSKA, E. & WALCZAK, W. (1962). A characteristic of DNA mediated transformation system of two streptococcal strains. *Acta microbiol. pol.* **11**, 205.

TOMASZ, A. & HOTCHKISS, R. D. (1964). Regulation of the transformability of pneumococcal cultures by macromolecular cell products. *Proc. natn. Acad. Sci. U.S.A.* **51**, 480.

TOMASZ, A. & MOSSER, J. L. (1966). On the nature of the pneumococcal activator substance. *Proc. natn. Acad. Sci., U.S.A.* **55**, 58.

J. gen. Microbiol. (1967), **48**, 305–308
Printed in Great Britain

Lack of Swelling and Shrinking of *Pityrosporum ovale* in Media of Different Osmotic Pressures and its Relationship with Survival in the Relatively Dry Conditions of the Scalp

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(*Accepted for publication 21 March 1967*)

SUMMARY

Pityrosporum ovale did not show the normal swelling and shrinking such as is undergone by *Saccharomyces cerevisiae* in media of different osmotic pressures. *Pityrosporum ovale* shrank slightly in media below 0·33 osmolar and remained at constant volume between 0·33 and 3·4 osmolar, while *S. cerevisiae* swelled below 0·10 osmolar and shrank markedly and progressively from 0·33 to 3·4 osmolar. The resistance of *P. ovale* to this effect is believed to be associated with its survival on the scalp in an environment low in water and high in salt from sweat, and to be a property of the remarkable construction of its cell wall as revealed by electron microscopy.

INTRODUCTION

When swabs are taken of human skin and scalp, large numbers of bacteria, mainly micrococci, can be cultivated (Pachtman, Vicker & Brunner, 1954). There are two main mechanisms whereby bacteria are eliminated from the skin surface, namely dehydration and the germicidal activity of unsaturated fatty acids secreted in the sebum. The two mechanisms have been found to be effective separately, according to the species of bacteria (Ricketts, Squire & Topley, 1951). However, the scalps of a significant proportion of the population also support large numbers of the lipophilic yeast, *Pityrosporum ovale*, which has often been associated with dandruff (Benham, 1945; Spoor, Traub & Bell, 1954). This organism was originally grown on commercial oleic acid and is resistant to the fungicidal properties of unsaturated fatty acids. Its survival in the almost complete absence of water is more difficult to understand. On the scalp this must mean growth and metabolism in a medium of very high osmotic pressure, as the water, but not the salts, in the sweat evaporates off. Yeast cells in common with Gram-positive bacteria cannot be plasmolysed but swell and shrink as a whole in solutions of different osmotic pressures (Mitchell & Moyle, 1956; Ørskov, 1945).

METHODS

Pityrosporum ovale 9265/3, which had originally been isolated from a human scalp in this laboratory and which is indistinguishable morphologically and biochemically from *P. ovale* type cultures 3073 and 3074 obtained from the London School of Hygiene and Tropical Medicine, was maintained on Littman ox-gall agar slopes (Oxoid). Bulk

cultures were prepared at 37° with shaking in a medium made up from: 3 mg. palmitic acid, 8 ml. 0·0224 M-ammonium chloride, 15 ml. salts solution and 2 ml. growth-factor solution of Schultz & McManus (1950), 0·2 ml. trace element solution of Skerman & Singleton (1964), 3 ml. phosphate buffer (pH 5·0; Schultz, McManus & Pomber, 1949), 1 ml. methionine solution containing equiv. 1 mg. sulphur/ml. which had been sterilized by filtration; total volume 19·2 ml. To this was added 1 g. egg-yolk lecithin (95–100 % pure, British Drug Houses Ltd.) which served to emulsify the palmitic acid and to provide further unidentified water-soluble growth factors. The

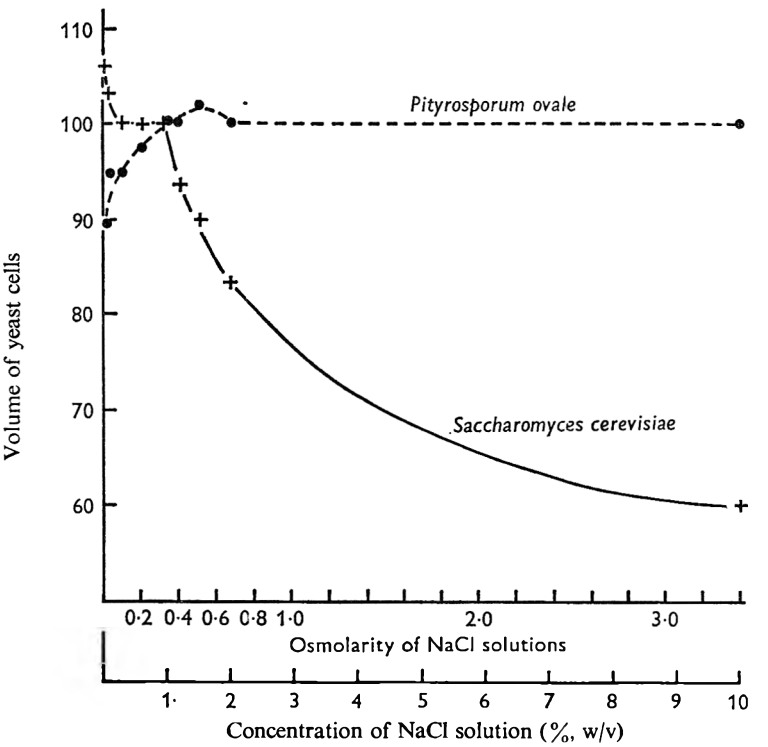

Fig. 1. Swelling and shrinking of cells in media of different osmotic pressure.

same effect can be produced by sodium taurocholate (67 % pure, Difco). By the addition of 1 g. egg-yolk lecithin, 62 mg. dry weight of organism was produced in 48 hr as compared with 170 mg. dry weight obtained by Benham (1945) in 4–6 weeks in a salts medium. *Saccharomyces cerevisiae* 456 (Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire) was used as a control organism. Bulk cultures were grown in malt extract broth (Oxoid) at 29°.

The swelling and shrinking of the organisms in saline of different osmolarities was determined by the method of Ørskov (1945). Organisms were harvested, washed three times with 15 ml. 0·9 % NaCl solution and resuspended in 0·5 ml. 0·9 % NaCl solution. This suspension was then placed in a standard haematocrit tube and centrifuged to constant packed-cell volume in an M.S.E. Super-Minor centrifuge with a swing-out head, at maximum speed. One minute was all that was necessary to obtain a constant packed-cell volume. The haematocrit reading was then taken immediately, since yeast

cells are elastic. The supernatant liquid was aspirated off and the same organisms suspended in 0·5 ml. lots of NaCl solutions of different osmolarities and the packed cell volumes again determined.

RESULTS

The swelling and shrinking of the cells in media of different osmolarities is shown in Fig. 1, for one experiment with each species of yeast. To calculate the true volume of the yeast cells by subtracting the volume of NaCl solution round the cells, it was assumed as an approximation that all the cells were spherical. If r is the radius of the haematocrit tube (1·5 mm.), the volume of cells + interstitical salt solution is $\pi r^2 h$, where h is the haematocrit reading in mm. At maximum packing, the volume taken up by n spheres of radius R is $6R^3n = \pi r^2 h$. Thus R^3n can be calculated as $\pi r^2 h/6$. The true volumes of the cells can then be calculated as $4/3\pi R^3 n$ and expressed as a percentage of the original volume of the cells in 0·9 % NaCl solution.

DISCUSSION

It is seen in Fig. 1 that *Saccharomyces cerevisiae* swelled in NaCl solutions of very low osmotic pressure and shrank in solutions of high osmotic pressure. Between 0·1 and 0·3 osmolar no swelling or shrinking occurred. This curve is very similar to that obtained by Ørskov (1945) who used malonamide, a solute which did not permeate into the cells. In contrast, *Pityrosporum ovale* shrank in NaCl solutions of low osmotic pressure and maintained a constant cell volume in solutions of high osmotic pressure. Thus *P. ovale* does not exhibit the normal shrinking of yeast cells in solutions of high osmotic pressure, which is the condition that occurs on the scalp. The internal osmotic pressure of Gram-negative bacteria is about 5 atmospheres and that of Gram-positive bacteria about 20 atmospheres; that of yeast cells is also known to be high (Mitchell & Moyle, 1956). This high internal osmotic pressure is maintained by the cell wall whose function is to strengthen the cell, permeability being regulated by the lipoprotein cytoplasmic membrane just inside the cell wall. Electron microscopy has shown that the cell walls of *P. orbiculare* (Barfatani, Munn & Schjeide, 1964) and *P. ovale* (Swift & Dunbar, 1965; Swift, 1966) are remarkable, being very thick and corrugated on the inside only in cross-section. Examination of the surface of fragmented cells has shown that these corrugations of *P. ovale* are a series of ridges which form a spiral net-work over the surface of the cell from one end to the other. Between 8 and 14 corrugations converge on the single bud scar and on the other pole of the organism. Each corrugation appears to spiral through one complete turn, i.e. through 360° in traversing the length of the organism. It is concluded that these structures appear to account for the extreme rigidity of the cell wall of *P. ovale* and its survival in media of high osmotic pressure.

REFERENCES

BARFATANI, M., MUNN, R. J. & SCHJEIDE, A. O. (1964). An ultrastructure study of *Pityrosporum orbiculare*. *J. invest. Derm.* **43**, 231.

BENHAM, R. W. (1945). *Pityrosporum ovale*. A lipophilic fungus. Thiamin and oxalo-acetic acid as growth factors. *Proc. Soc. exp. Biol. Med.* **58**, 199.

MITCHELL, P. & MOYLE, J. (1956). Osmotic function and structure in bacteria, in *Bacterial Anatomy*, *Symp. Soc. gen. Microbiol.* **6**, 150.

ØRSKOV, S. L. (1945). Investigations on the permeability of yeast cells. *Acta path. microbiol. scand.* **22**, 523.

PACHTMAN, E. A., VICKER, E. E. & BRUNNER, M. J. (1954). The bacteriologic flora in seborrheic dermatitis. *J. invest. Derm.* **22**, 389.

RICKETTS, C. R., SQUIRE, J. R. & TOPLEY, E. (1951). Human skin lipids with particular reference to the self-sterilising power of the skin. *Clin. Sci.* **10**, 89.

SCHULTZ, A. S. & MCMANUS, D. K. (1950). Amino acids and inorganic sulphur as sulphur source for the growth of yeasts. *Archs. Biochem.* **25**, 401.

SCHULTZ, A. S., MCMANUS, D. K. & POMBER, S. (1949). Amino acids as carbon source for the growth of yeasts. *Archs. Biochem.* **22**, 412.

SKERMAN, T. M. & SINGLETON, R. J. (1964). Sulphur nutrition of two strains of the yeast, *Debaromyces kloeckeri*, isolated from subantarctic soil. *Can. J. Microbiol.* **10**, 397.

SPOOR, H. J., TRAUB, E. T. & BELL, M. (1954). *Pityrosporum ovale* types cultured from normal and seborrheic subjects. *Archs. Derm. Syph., Chicago* **69**, 323.

SWIFT, J. A. (1966). The electron microscopy of yeasts from the family Cryptococcaceae with particular reference to those of the Pityrosporum genus. *Proc. 6th int. Congress Elect. Micro., Kyoto, Japan.*

SWIFT, J. A. & DUNBAR, S. F. (1965). Ultrastructure of *Pityrosporum ovale* and *Pityrosporum canis*. *Nature, Lond.* **206**, 174.

J. gen. Microbiol. (1967), **48**, 309–316
Printed in Great Britain

Properties of a *Lactobacillus fermenti* bacteriocin

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(*Accepted for publication 22 March* 1967)

SUMMARY

The properties of a bacteriocin derived from *Lactobacillus fermenti* strain 466 were investigated. The bacteriocin was present in low titre in supernatant fluids from overnight broth cultures and was not inducible by ultraviolet radiation. It was purified and concentrated to a titre of 1/1000 by dialysis, chromatography on Sephadex G100 and calcium phosphate gel columns. The bacteriocin is heat stable, and sensitive to trypsin and pepsin but not to lysozyme. No migration was demonstrated in electrophoretic fields in agar gel. Electron microscopy of the bacteriocin did not show any phage components. The bacteriocin is a macromolecular lipocarbohydrate protein which consists of 16 amino acids, four sugars, hexosamine and phosphorus. The biological activity of this complex is dependent on its structural integrity.

INTRODUCTION

Bacteriocins as defined by Reeves (1965) are produced by homofermentative and heterofermentative species of the genus *Lactobacillus* (de Klerk & Coetzee, 1961). The incidence of bacteriocinogeny in *Lactobacillus fermenti* is 15·5 % (de Klerk, 1967). All the *L. fermenti* bacteriocins have an identical host range and attempts to isolate resistant mutants have been unsuccessful. This has precluded the classification of these bacteriocins by cross-resistance tests (Fredericq, 1948). Colicins K (Goebel & Barry, 1958), V (Hutton & Goebel, 1961, 1962), F (E_2) (Reeves, 1963), A (Barry, Everhart, Abbot & Graham, 1965), I (Keene, 1966) and the unclassified colicin SG710 (Nüske, Hösel, Venner & Zinner, 1957) are complexes of lipid, carbohydrate and protein. Megacin 216 (Holland, 1961) and a pyocin (Homma & Suzuki, 1964) are proteins. The specific chemical nature of all other bacteriocins is unknown (Hinsdill & Goebel, 1964; Reeves, 1965). The purpose of the present investigation was the purification and chemical analysis of one of the apparently homogeneous group of *L. fermenti* bacteriocins.

METHODS

Media. The liquid medium used was the MRS broth of de Man, Rogosa & Sharpe (1960) freed from high molecular weight constituents by dialysis (tubing size 18/32, Visking Co., Chicago, Ill., U.S.A.) under negative pressure. Solid medium was MRS broth diffusate + 2 % (w/v) agar.

Bacterial strains. The bacteriocinogenic *Lactobacillus fermenti* strain 466, the indicator *L. fermenti* strain F1 and other indicator strains were described by de Klerk & Coetzee (1961). Cultures were incubated as previously described (de Klerk, 1967).

Preparation of bacteriocin. Crude preparations of bacteriocin were obtained by overnight growth of the bacteriocinogenic strain at 37° in broth diffusate medium. The culture was centrifuged, the supernatant fluid sterilized with chloroform and concentrated in a rotary evaporator at 40°. The concentrate was dialysed against deionized water to remove nutrients and small molecular weight bacterial products in the diffusate. The volume of the dialysis residue was further decreased by evaporation and then dialysed against 0·1 M-KCl + 0·05 M-tris + HCl buffer (pH 7·5).

Purification of bacteriocin. Crude bacteriocin was purified by gel filtration on Sephadex G 100. Samples (2 ml.) were applied to columns (50 cm. × 2·5 cm.) and eluted with 0·1 M-KCl + 0·05 M-tris + HCl buffer (pH 7·5). Column effluents were collected in 5 ml. fractions and assayed for activity and the extinction at 280 mμ determined. High titre fractions were pooled and centrifuged at 200,000 *g* for 15 min. to remove bacterial debris. The supernatant fluid concentrated, dialysed against buffer and the dialysis residue filtered through Sephadex or chromatographed on calcium phosphate gel columns.

Adsorption chromatography of bacteriocin on calcium phosphate gel. Precipitated calcium phosphate was prepared according to Tiselius, Hjertén & Levin (1956), suspended in 1 mM-sodium phosphate buffer (pH 6·8) and applied to a chromatography tube (0·8 cm. × 12 cm.). A sample (2 ml. equiv., 10 mg. dry wt) of partly purified bacteriocin was dialysed against 1 mM-sodium phosphate (pH 6·8) and applied to the column. The bacteriocin was eluted with 1 mM-sodium phosphate buffer (pH 6·8) and step-wise elution with 10, 50 and 200 mM-sodium phosphate buffer solutions (pH 6·8; 50 ml. of each concentration) was continued. Fractions (5 ml.) were collected and assayed for activity and the extinction at 280 mμ determined.

Ultraviolet induction of bacteriocin. Lactobacillus fermenti strain 466 organisms from a 20 ml. overnight broth culture were deposited by centrifugation and resuspended in an equal volume of sterile distilled water in a Petri dish. The suspension was irradiated with a 30 W. Hanovia Sterilamp (wavelength 2537 Å) from a height of 25 cm. At intervals of 5, 10, 15, 25, 40 sec., samples of 4 ml. were pipetted into 20 ml. warmed broth and incubated overnight in the dark. The supernatant fluids of these cultures were sterilized with chloroform and assayed for bacteriocin activity.

Assay of the bacteriocin. This was done by spotting drops of two-fold serial dilutions of the material in MRS broth on to agar plates seeded with the indicator strain and incubated overnight at 37°. The highest inhibitory dilution was the titre.

Analysis of bacteriocin

Purified bacteriocin was dialysed against repeated changes of distilled water and evaporated to constant dry weight under reduced pressure over P_2O_5. The bacteriocin (84·1 mg.) was then dissolved in distilled water (25 ml.).

Samples (0·2 ml.) of bacteriocin preparation were treated with 3 N-NaOH (0·1 ml.) and the protein content measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine albumin (Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.) as standard.

Total nitrogen was determined by a micro-Kjeldahl technique (Ballantine, 1957).

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were determined by the methods of Burton (1956) and Dische & Borenfreund (1957), respectively.

Bacteriocin (6·728 mg.) was hydrolysed in a sealed tube for 24 hr with 6 N-HCl

(5 ml.) at 105°. The acid was evaporated *in vacuo* over solid P_2O_5 and NaOH, and the amino acid composition determined with a Beckman automatic amino acid analyser, Model 120B.

Bacteriocin (3 ml.) was treated with 12 N-perchloric acid (0·36 ml.) and left at 0° for 16 hr. The precipitated protein was removed by centrifugation and total phosphate in the supernatant fluid estimated by the method of Gomori (1942).

Estimation of lipids. The total lipid content of bacteriocin solution (10 ml.) was determined according to Salton (1953). The procedure consisted of hydrolysis with 6 N-HCl for 8 hr at 100°, followed by ether extraction of the hydrolysate.

Hexosamine estimation. Samples (2 ml.) of bacteriocin solution were hydrolysed in sealed tubes with 4 N-HCl for 15 hr at 100°. The acid was evaporated *in vacuo* over solid P_2O_5 and NaOH, and the liberated hexosamine separated from interfering substances (sugars and lipids) by cation exchange chromatography on columns (1 cm. × 10 cm.) of Amberlite IR 120 (H^+-form) resin. The hexosamine was eluted with 2 N-HCl and estimated according to Boas (1953), with glucosamine hydrochloride (British Drug Houses Ltd.; BDH) as standard.

Liberation of carbohydrates. The bacteriocin solution (2·0 ml.) was hydrolysed for 2 hr at 100° in a final concentration of 2 N-H_2SO_4 and the hydrolysate applied to a column (1 cm. × 10 cm.) of Amberlite IR 120 (H^+-form) cation exchange resin (Weibull & Bergström, 1958). Neutral sugars were eluted with water and concentrated under reduced pressure below 40° to a small volume. The sugar solution was transferred quantitatively to a 5 ml. standard flask and made to volume.

Paper chromatography of carbohydrates. One-dimensional descending chromatography was done on Whatman no. 1 filter paper with 2,4,6-trimethylpyridine as solvent (Partridge, 1948). D(+)-Galactose, D(+)-glucose, D(+)-mannose and L(+)-rhamnose (British Drug Houses Ltd.) were used as references. After developing the chromatograms for 40 hr, the solvent was removed by evaporation and the chromatograms sprayed with *p*-anisidine phosphate in ethanol (Mukherjee & Srivastava, 1952).

Quantitative estimation of carbohydrate components. A sample (0·5 ml.) of the sugar extract was applied as a band (10 cm. wide) in the centre and 8 cm. from the top of Whatman no. 1 filter paper (30 cm. × 57 cm.) together with two guide spots, 5 cm. from each edge, to which 50 µl. of sugar extract were applied. This chromatogram and a blank were developed for 40 hr in 2,4,6-trimethylpyridine and dried for 16 hr at room temperature. The positions of the separated sugars on the guide strips were revealed by the reagent and the corresponding areas excised from the experimental and blank chromatogram. Sugars and blanks were quantitatively eluted from each strip with distilled water into measuring cylinders (5 ml.) according to Dent (1947). Elution was stopped when 2 ml. of solvent had been collected and samples (1 ml.) were assayed for sugar concentration by the anthrone method of Scott & Melvin (1953).

Dissociation of protein and carbohydrate fractions. The phenol method of Goebel & Barry (1958) and hydrolytic techniques (Knox & Hall, 1965) were used in attempts to dissociate protein and carbohydrate components of the bacteriocin respectively.

Sedimentation of bacteriocin. This was done at 59780 rev./min. at 20° in the AN-D rotor of a Spinco model E ultra-centrifuge. A solution of about 1 % purified

bacteriocin was used in a 12 mm. standard cell and photographs were taken at 8 min. intervals, with schlieren optics.

Action of enzymes on bacteriocin

Lysozyme. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of lysozyme (BDH; 100 μg./ml.) in 0·1 M-ammonium acetate for 5 hr.

Pepsin. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of crystalline pepsin (BDH; 1 mg./ml.) in 0·02 N-HCl for 24 hr. After digestion the mixture was adjusted to pH 7·0 with 0·1 N-NaOH.

Trypsin. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of crystalline trypsin (BDH; 0·5 mg./ml.) in 200 mM-sodium phosphate buffer (pH 7·5) for 3 hr. Control experiments were done without enzymes and bacteriocin titres determined.

Other investigations

Action of urea on bacteriocin. Bacteriocin was dissolved in a final concentration of 6 M-urea. Controls consisted of bacteriocin in water and a 6 M-urea solution. After 16 hr at 37° the solutions were assayed.

Chromatography on ion-exchange resins. This was done on Amberlite IR 410 (Cl^--form) anion and Amberlite IR 120 (H^+-form) cation exchange resins.

Agar electrophoresis. Agar electrophoresis of purified bacteriocin at pH 5·2 and pH 8·5 was done by the method of Maré, Coetzee & de Klerk (1964).

Electron microscopy. Purified bacteriocin dissolved in distilled water was negatively stained with neutral potassium phosphotungstate (Brenner & Horne, 1959). Samples were mounted on carbon support films by a spreading technique (Bradley, 1962) and examined with a Philips EM 200 electron microscope.

RESULTS

Isolation of pure bacteriocin

Supernatant fluids of overnight cultures of *Lactobacillus fermenti* strain 466 in the MRS diffusate medium had inhibitory titres of 1/4. This medium did not contain molecules with a weight exceeding 10,000 (Leggett Bailey, 1962) and facilitated the separation of the bacteriocin which was not diffusible through the Visking tubing (de Klerk & Coetzee, 1961). Bacteriocin production was not enhanced by ultraviolet irradiation. One litre of crude bacteriocin was purified and concentrated to 2 ml. with a titre of 1/1000, this represented a recovery of 40–60 %. The primary separation of fractions on Sephadex (Fig. 1) and further purification on calcium phosphate gel (Fig. 2) are shown. In both graphs two peaks are present. The first had an absorption maximum of 277 mμ and contained all the activity. The second peak represented bacterial protein and broth constituents, and was virtually absent after repassage through either column. Ultracentrifugation of the purified bacteriocin revealed a single peak with an $S_{20,w}$ value of 2·8.

Chemical composition

The chemical composition of the bacteriocin is shown in Table 1. It consisted mainly of carbohydrate, protein and lipid, with lesser amounts of hexosamine and phosphorus, and was free from nucleic acids. The carbohydrate fraction (Table 2) contained four sugars; the mannose content being 56·7 %. The protein fraction con-

tained 16 amino acids (Table 3). Alanine, glycine, serine and the acidic amino acids were present in the highest concentrations.

Properties of the bacteriocin

Purified bacteriocin was a fluffy, white amorphous powder freely soluble in water. Aqueous solutions kept at room temperature for several days showed no loss of activity and neutral solutions withstood heating at 96° for 30 min. Activity was completely destroyed by trypsin and was decreased to half when treated with pepsin for 24 hr. The bacteriocin was unaffected by lysozyme. Phenol did not split a protein with bacteriocin activity from the complex; this corresponds to the findings of Barry *et al.* (1965) with colicin A. Attempts to separate the carbohydrate moiety by hydrolysis with hot 0·1 N-sulphuric acid or warm 10 % (w/v) trichloroacetic acid resulted in total

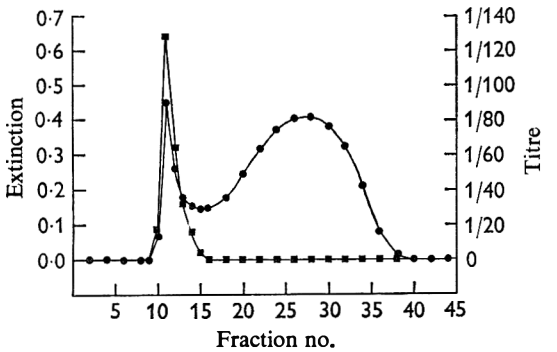

Fig. 1

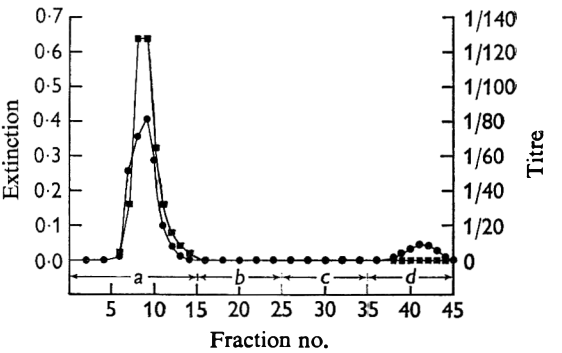

Fig. 2

Fig. 1. Chromatography of crude bacteriocin of *Lactobacillus fermenti* 466 on Sephadex G-100. A 2 ml. sample (titre: 1/1000) was applied to the column and eluted with 0·1 M-KCl + 0·05 M-tris + HCl buffer (pH 7·5). Fractions (5 ml.) were collected and assayed for: ●——●, extinction; and ■——■, inhibitory activity.

Fig. 2. Chromatography of bacteriocin of *Lactobacillus fermenti* 466 on calcium phosphate gel. Partially purified bacteriocin was concentrated and dialysed against 1 mM-sodium phosphate buffer (pH 6·8). A 2 ml. sample (titre: 1/1000) was applied to the column and stepwise elution done with sodium phosphate buffer (pH 6·8) at concentrations of 1, 10, 50 and 200 mM. Fractions (5 ml.) were collected and assayed for: ●——●, extinction; and ■——■, inhibitory activity. (*a*) 1 mM, (*b*) 10 mM, (*c*) 50 mM, (*d*) 200 mM-sodium phosphate.

Table 1. *Chemical composition of bacteriocin of Lactobacillus fermenti strain 466*

	(%)		(%)
Nitrogen	4·96	Carbohydrate	53·20
Protein	23·80	Hexosamine	0·80
Lipid	20·80	Phosphorus	0·30

Table 2. *Carbohydrate composition of bacteriocin of Lactobacillus fermenti strain 466*

	(%)		(%)
Galactose	19·4	Mannose	56·7
Glucose	16·7	Rhamnose	7·2

inactivation. Extraction of a bacteriocin solution with chloroform or ether removed no lipid material and did not affect activity. The bacteriocin activity was also unaffected by treatment with urea. Agar electrophoresis at different pH values showed no migration of the bacteriocin when several different *Lactobacillus fermenti* strains were used as indicators. Anion and cation exchange resins did not retain the bacteriocin. Electron microscopic examination showed only amorphous material.

Table 3. *Amino acid composition of the protein fraction of bacteriocin of Lactobacillus fermenti strain 466*

Amino acid	Residues/1000 total amino acid residues	Amino acid	Residues/1000 total amino acid residues
Lysine	57·3	Glycine	111·2
Histidine	18·9	Alanine	134·3
Arginine	29·0	Valine	56·0
Aspartate	100·1	Methionine	4·5
Threonine*	87·6	Isoleucine	37·6
Serine*	101·0	Leucine	64·0
Glutamate	92·3	Tyrosine	30·7
Proline	47·2	Phenylalanine	28·3

* Corrected for loss during hydrolysis.

DISCUSSION

Lactobacillus fermenti strain 466 produces a single bacteriocin, which is non-inducible by ultraviolet radiation. The bacteriocin differs in the latter respect from megacin 216 (Holland, 1961), colicin F (Reeves, 1963) and pyocin (Homma & Suzuki, 1964). It is a lipocarbohydrate protein similar in general composition to colicins (Hinsdill & Goebel, 1964). Attempts to isolate an active protein fraction from the complex were unsuccessful although the phenol method ruptures covalent bonds (Holland, 1961) and an active protein was split from the colicin K complex in this way (Goebel & Barry, 1958). Mild techniques of hydrolysis which split sugar phosphate bonds to release a carbohydrate fraction (Knox & Hall, 1965) destroyed the biological activity of the bacteriocin. These findings support the conclusion that activity depends on the integrity of the lipocarbohydrate protein complex. The chemical composition of only one of the bacteriocins produced by Gram-positive organisms, megacin 216 (Holland, 1961), is known; this is a protein with traces of phosphorus and carbohydrate. The enterocin of *Streptococcus zymogenes* (Brock & Davie, 1963) is possibly a protein (Hamon, 1964) with a lipid component. The 16 amino acids of the *Lactobacillus fermenti* strain 466 bacteriocin correspond to those of megacin 216 (Holland, 1961), but the concentration of acidic amino acids in the latter bacteriocin is higher. The lactobacillus bacteriocin has a trace of methionine whilst cysteine/cystine are absent. This suggests that the three-dimensional structure of the macromolecule does not depend on the presence of disulphide linkages. The activity of this bacteriocin is probably independent of hydrogen bonds since it is not affected by high concentrations of urea.

The absence of migration in an electric field at different pH values was unexpected since the dicarboxylic amino acids, aspartate and glutamate, predominate. The bacteriocin diffused through 0·8 % (w/v) agar (de Klerk, 1967) and its electrophoretic immobility did not result from inability to penetrate the agar lattice. The failure of

ion-exchange resins to retain the bacteriocin confirms its lack of net ionic charge: its ionic groups may be masked by lipid.

Homma & Suzuki (1964) isolated a cell-wall protein with bacteriocin activity from *Pseudomonas aeruginosa*. Cell walls of *Lactobacillus fermenti* contain galactose, glucose, glucosamine and aspartate, glutamate, alanine and lysine (Ikawa & Snell, 1960). The bacteriocin of *L. fermenti* strain 466 differs in composition from its cell wall and is much more complex.

This work was supported by grants to Professor J. N. Coetzee from the South African Council for Scientific and Industrial Research.

REFERENCES

BALLANTINE, R. (1957). Determination of total nitrogen and ammonia. *Meth. Enzymol.* **3**, 984.

BARRY, G. T., EVERHART, D. L., ABBOTT, V. & GRAHAM, M. G. (1965). Preparation, properties and relationship of substances possessing colicine A activity obtained from Enterobacteriaceae. *Zentbl. Bakt.* (*Orig. Abt.* 1), **196**, 248.

BOAS, N. F. (1953). Method for the determination of hexosamines in tissues. *J. biol. Chem.* **204**, 553.

BRADLEY, D. E. (1962). A study of the negative staining process. *J. gen. Microbiol.* **29**, 503.

BRENNER, S. & HORNE. R. W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochim. biophys. Acta* **34**, 103.

BROCK, T. D. & DAVIE, J. M. (1963). Probable identity of a group D hemolysin with a bacteriocine. *J. Bact.* **86**, 708.

BURTON, K. (1956). Diphenylamine reaction for the colorimetric estimation of DNA. *Biochem. J.* **62**, 315.

DE KLERK, H. C. (1967). Bacteriocinogeny in *Lactobacillus fermenti*. *Nature, Lond.* **214**, 609.

DE KLERK, H. C. & COETZEE, J. N. (1961). Antibiotics among Lactobacilli. *Nature, Lond.* **192**, 340.

DE MAN, J. C., ROGOSA, M. & SHARPE, M. E. (1960). A medium for the cultivation of Lactobacilli. *J. appl. Bact.* **23**, 130.

DENT, C. E. (1947). The amino-aciduria in Fanconi syndrome. A study making extensive use of techniques based on paper partition chromatography. *Biochem. J.* **41**, 240.

DISCHE, Z. & BORENFREUND, E. (1957). A new colour reaction for the determination of aldopentose in presence of other saccharides. *Biochim. biophys. Acta* **23**, 639.

FREDERICQ, P. (1948). Actions antibiotiques réciproques chez les Enterobacteriaceae. *Rev. Belge Pathol. Med. Exp.* **29** (Suppl. 4), 1.

GOEBEL, W. F. & BARRY, G. T. (1958). Colicine K. II. The preparation and properties of a substance having colicine K activity. *J. Exp. Med.* **107**, 185.

GOMORI, G. (1942). A modification of the colorimetric phosphorus determination for use with the photo-electric colorimeter. *J. lab. clin. Med.* **27**, 955.

HAMON, Y. (1964). Les bactériocines. *Annls Inst. Pasteur, Paris*, Suppl. 5, 18.

HINSDILL, R. D. & GOEBEL, W. F. (1964). The chemical nature of bacteriocins. *Annls Inst. Pasteur, Paris*, Suppl. 5, 54.

HOLLAND, I. B. (1961). The purification and properties of megacin, a bacteriocin from *Bacillus megaterium*. *Biochem. J.* **78**, 641.

HOMMA, J. Y. & SUZUKI, N. (1964). 'Cell-wall protein A' of *Pseudomonas aeruginosa* and its relationship to 'original endotoxin protein'. *J. Bact.* **87**, 630.

HUTTON, J. J. & GOEBEL, W. F. (1961). Colicine V. *Proc. natn. Acad. Sci., U.S.A.* **47**, 1498.

HUTTON, J. J. & GOEBEL, W. F. (1962). The isolation of colicine V and a study of its immunological properties. *J. gen. Physiol.* **45** (suppl.), 125.

IKAWA, M. & SNELL, E. (1960). Cell wall composition of lactic acid bacteria. *J. biol. Chem.* **235**, 1376.

KEENE, J. H. (1966). Preparation and chemical properties of colicine I. *Can. J. Microbiol.* **12**, 425.

KNOX, K. W. & HALL, E. A. (1965). The linkage between the polysaccharide and mucopeptide components of the cell wall of *Lactobacillus casei*. *Biochem. J.* **96**, 302.

LEGGETT BAILEY, J. (1962). *Techniques in Protein Chemistry*. London: Elsevier Publishing Company.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MARÉ, I. J., COETZEE, J. N. & DE KLERK, H. C. (1964). Agar electrophoresis of colicines with an *Alcaligenes faecalis* indicator strain. *Nature, Lond.* **202**, 213.

MUKHERJEE, S. & SHRIVASTAVA, H. C. (1952). Improved spray reagent for the detection of sugars. *Nature, Lond.* **169**, 330.

NÜSKE, R., HÖSEL, H., VENNER, H. & ZINNER, H. (1957). Über ein Colicin aus *Escherichia coli* SG 710. *Biochem. Z.* **329**, 346.

PARTRIDGE, S. M. (1948). Filter-paper partition chromatography of sugars. *Biochem. J.* **42**, 238.

REEVES, P. (1965). The bacteriocins. *Bact. Rev.* **29**, 24.

REEVES, P. R. (1963). Preparation of a substance having colicin F activity from *Escherichia coli* C.A. 42. *Aust. J. exp. Biol.* **41**, 163.

SALTON, M. R. J. (1953). Studies of the bacterial cell wall. IV. The composition of the cell walls of some Gram-positive and Gram-negative bacteria. *Biochim. biophys. Acta* **10**, 512.

SCOTT, T. A. & MELVIN, E. H. (1953). Determination of dextran with anthrone. *Analyt Chem.* **25**, 1656.

TISELIUS, A., HJERTÉN, S. & LEVIN, Ö. (1956). Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys.* **65**, 132.

WEIBULL, C. & BERGSTRÖM, L. (1958). The chemical nature of the cytoplasmic membrane and cell wall of *Bacillus megaterium*, strain M. *Biochim. biophys. Acta* **30**, 340.