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Release of Influenza Virus Neuraminidase by Caseinase C of *Streptomyces albus* G

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SUMMARY

When concentrated purified influenza virus PR 8 was incubated at 37° with caseinase C in phosphate buffer at pH 7 and ionic strength 0·01 or 0·1, the neuraminidase activity was released from the virus particle and progressively destroyed. Under these conditions a material was released from virus which neutralized the haemagglutination inhibiting antibodies of anti-PR 8 rabbit serum. This material and neuraminidase, released by caseinase C, were both sedimented by centrifugation at 125,000 *g*. Neither was adsorbed to fowl red cells. After 4-hr incubation with caseinase C in phosphate buffer at pH 7 and ionic strength 0·1, the infectivity of influenza virus PR 8 suspension was considerably decreased, but its haemagglutinating activity was unchanged and remained bound to the virus particles. Under these conditions, most of the surface projections of the virus particles were removed.

INTRODUCTION

We reported (Reginster, 1965*a*) that when influenza virus PR 8 was incubated for 4 hr at 37° with caseinase C in ionic strength 0·01 pH 7 phosphate buffer, the infectivity, the haemagglutinating activity and the neuraminidase activity were destroyed and the serological properties of the virus suspension were modified. When the time of incubation was decreased to 1 hr, the infectivity and the haemagglutinating activity decreased significantly, but the neuraminidase was unchanged (Reginster, 1965*a*). Caseinase C alters the surface of virus particle, removing its typical projections (Reginster, 1965*b*) and liberating at least two fragments distinguishable by their sedimentation constants and biological properties (Reginster, 1965*c*). In the present paper, we describe the effect of caseinase C on influenza virus PR 8 neuraminidase and discuss the relationship between neuraminidase, haemagglutinating activity, serological properties and infectivity of virus PR 8.

METHODS

Concentration and purification of virus PR 8, production of anti-PR 8 rabbit serum and techniques for measuring haemagglutinating activity, infectivity, neuraminidase activity and ability to neutralize haemagglutination inhibiting antibodies were as described by Reginster (1965*a*) Two preparations of caseinase C, kindly supplied by Dr J. M. Ghuysen, were used; they behaved identically. Incubations of virus PR 8 with caseinase C were done in pH 7 phosphate buffer, at either 0·01 or 0·1 ionic strength (μ). Caseinase C was used at a final concentration

of 35 caseinolytic units. One caseinolytic unit was defined as the amount of enzymic preparations which digests 50 % of the casein present in 0·5 ml. of standard solution (0·2 % soluble casein in 0·05 M-K_2PO_4) within 15 min. of incubation at 37°. The casein left after enzymic digestion was measured by trichloroacetic acid precipitation (4·5 ml. of 1 % trichloroacetic acid solution added to the 0·5 ml. assay). Untreated control suspensions of virus PR 8 were obtained by incubating the virus in buffer without caseinase C. The neuraminidase activity of material obtained after incubation of virus PR 8 with caseinase C or after high speed centrifugation of untreated suspensions was expressed as the concentration of control suspension which would have the same neuraminidase activity. This concentration, expressed as a percentage of the undiluted control suspension, was graphically determined in each experiment from the curve showing the neuraminidase activity of the corresponding control suspension as a function of virus concentration (Reginster, 1965*a*).

Pellets and supernatant fluids from virus suspensions were obtained by centrifugation for 1 hr at 31,000*g* in the swinging bucket rotor SW 39 of a Spinco model L centrifuge (20,000 rev./min.) The 5 ml. tubes were filled with undiluted suspensions adjusted, when necessary to μ 0·1 by adding concentrated phosphate buffer (pH 7) or with virus suspensions diluted 1/5 in saline. The supernatant fluids were collected with a syringe and needle. The activities of the supernatant fluids were measured in the 4 ml. first collected, the bottom 1 ml. being discarded. The pellets were resuspended in a volume of diluent suitable for comparison with the corresponding supernatant and with the uncentrifuged suspension of virus.

Adsorption to red cells was done as follows. Samples of virus suspensions, untreated by caseinase C, or samples of supernatant fluids and pellets from these suspensions, were diluted 1/5 in saline and mixed with 20 % (v/v) packed fowl red cells. After 15 min. at room temperature, mixtures were centrifuged (15 min. at 1500*g*) and the supernatant fluids collected.

RESULTS

Release of neuraminidase

Concentrated purified virus PR 8 was incubated at 37° for various times with caseinase C at μ 0·01 or μ 0·1; corresponding control suspensions were prepared. The neuraminidase activity was measured, either in the uncentrifuged caseinase C treated virus suspensions, in the supernatant fluids or in the pellets of caseinase C treated and control untreated suspensions (Table 1). When collecting supernatant fluids and pellets of control virus suspensions, about 60 % of the neuraminidase activity of the uncentrifuged suspensions was recovered; about 90 % of this activity was associated with the pellet.

The neuraminidase activity of virus suspensions previously incubated for 1 hr or less at μ 0·01 or μ 0·1 with caseinase C was equal to, or higher than, the neuraminidase activity of untreated suspensions. When these suspensions were centrifuged, all the neuraminidase activity was recovered and was associated up to 90 %, with the supernatant fluid. After incubation for 2 hr or more with caseinase C at μ 0·01, the neuraminidase activity decreased significantly. When the incubation with caseinase C lasted 90 min. or more at μ 0·1, the neuraminidase activity (whole

Table 1. *Neuraminidase activity of PR 8 virus suspension incubated at 37° in phosphate buffer (pH 7; μ 0·01 or μ 0·1) with or without caseinase C; and of supernatant fluids and pellets of these suspensions, unadsorbed or adsorbed by fowl red cells*

			Neuraminidase activity* after incubation								
			Without caseinase C				With caseinase C				
			Supernatant		Pellet			Supernatant		Pellet	
Expt. (no.)	Time of incubation (min.)	Ionic strength (μ)	U†	A‡	U†	A‡	Suspension	U†	A‡	U†	A‡
6	30	0·01	.	.	.	.	108	122	.	15	.
2	60	0·01	.	.	.	.	150	.	.	.	.
3	60	0·01	.	.	.	.	155	.	.	.	.
4	60	0·01	.	.	.	.	110	100	.	< 1	.
1	60	0·01	.	.	56	.	90	.	.	3	.
5	60	0·01	.	.	.	.	140	145	.	1	< 1
2	120	0·01	.	.	.	.	46	.	.	.	.
3	120	0·01	.	.	.	.	52	.	.	.	.
5	120	0·01	.	.	.	.	70	.	.	.	.
2	240	0·01	.	.	.	.	3	.	.	.	.
3	240	0·01	.	.	.	.	36	.	.	.	.
4	240	0·01	.	.	.	.	36	.	.	.	.
5	240	0·01	.	.	.	.	23	.	.	.	.
7	240	0·01	4	.	64	.	.	.	.	.	.
4	360	0·01	.	.	.	.	12	.	.	.	.
8	60	0·1	.	.	.	.	.	140	140	19	1
12	60	0·1	.	.	.	.	.	62	55	30	9
6	90	0·1	13	< 1	.	.	119	76	.	32	< 1
8	120	0·1	.	.	.	.	.	100	145	7	< 1
8	240	0·1	1	< 1	50	1	.	100	84	4	< 1
9	240	0·1	4	< 1	60	10	.	.	.	.	.
11	240	0·1	.	.	.	.	.	25	.	< 1	.

* As concentration (%) of virus suspension pre-incubated without caseinase C which would have the same neuraminidase activity; † Unadsorbed by fowl red cells; ‡ Adsorbed by fowl red cells.

Table 2. *Haemagglutinating activity of PR 8 virus suspension incubated at 37° in phosphate buffer (pH 7; μ 0·01 or μ 0·1) with or without caseinase C; and of supernatant fluids and pellets of these suspensions, unadsorbed or adsorbed by fowl red cells*

			Haemagglutinating activity* after incubation									
				Without caseinase C					With caseinase C			
				Supernatant		Pellet			Supernatant		Pellet	
Expt. (no.)	Time of incubation (min.)	Ionic strength (μ)	Suspension A†	U‡	A†	U‡	A†	Suspension U‡	U‡	A†	U‡	A†
6	30	0·01	.	.	.	.	.	100	8	.	100	.
6	90	0·01	< 1	12	.	.	.	.	.	.	.	.
7	240	0·01	.	4	.	100	.	.	.	.	.	.
8	60	0·1	.	.	.	.	.	.	4	< 1	100	< 1
12	60	0·1	.	.	.	.	.	100	3	< 1	100	< 1
6	90	0·1	< 1	.	.	.	.	100	11	.	.	.
8	120	0·1	.	–	–	.	.	.	4	< 1	100	< 1
10	240	0·1	.	.	.	.	.	100	.	.	.	.
8	240	0·1	.	.	.	.	.	.	6	< 1	100	< 1
9	240	0·1	.	2	< 1	100	< 1	.	.	.	.	.
7	240	0·1	.	4	.	100	.	.	9	.	100	.
11	240	0·1	.	.	.	.	.	.	.	.	.	.

* As % of the haemagglutinating activity of the virus suspension incubated for the same time in the same buffer without caseinase C; † adsorbed by fowl red cells; ‡ unadsorbed by fowl red cells.

virus suspension, or supernatant plus pellet) was unchanged in 5 out of 6 experiments. The neuraminidase activity bound to the virus particles diminished as the length of incubation with caseinase C increased (Table 1, Expt. 8). After 60 min. with caseinase C at μ 0·01, there was very little neuraminidase bound to the virus particles (Table 1, Expts. 1, 4, 5). Nevertheless, after 60 or 90 min. with caseinase C at μ 0·1, the neuraminidase activity associated with the virus particles corresponded to the activity of the untreated control suspension diluted from about 1/5 to 1/3 (Table 1, Expts. 6, 8, 12).

Ability of neuraminidase to adsorb to red cells

Concentrated purified virus PR 8 was incubated at 37° with or without caseinase C at μ 0·1. These suspensions were centrifuged and the neuraminidase activity of supernatant fluid or pellet, or of both, was measured before and after red cells adsorption. The neuraminidase activity in the pellets of untreated control suspensions was decreased about 10-fold by red-cell adsorption and the low activity found in the supernatant fluid of the same suspensions was completely removed (Table 1, Expt. 8, 9). Red-cell adsorption did not modify significantly the neuraminidase activity of the supernatant fluids of virus suspensions pre-incubated with caseinase C, but it abolished or decreased considerably the neuraminidase activity found in the pellets of the corresponding control suspensions (Table 1, Expts. 6, 8, 12).

Table 3. *Infectivity of PR 8 virus incubated at 37° for 4 hr in phosphate buffer (pH 7; μ 0·1) with or without caseinase C*

	Infectivity after incubation			
	Without caseinase C		With caseinase C	
Expt. (no.)	log. ID 50 in 1 ml.	Standard error	log. ID 50 in 1 ml.	Standard error
10	6·15	±0·11	4·55	±0·15
11	8·48	±0·11	5·06	±0·13

Properties of PR 8 virus suspensions after release of neuraminidase

Haemagglutinating activity. Concentrated purified virus PR 8 was incubated at 37° with or without caseinase C, at μ 0·01 and at μ 0·1. The haemagglutinating activity was measured in these suspensions, in their supernatant fluids and pellets, adsorbed and unadsorbed by fowl red cells (Table 2). After 30 min. at μ 0·01, and after 1, 2 or 4 hr at μ 0·1, the haemagglutinating activity of the virus suspensions incubated with caseinase C was the same as that of the control suspensions. The haemagglutinating activity of all these suspensions was bound to material in the pellets. The supernatant fluids contained less than 10% of the haemagglutinating activity of the corresponding uncentrifuged suspensions. Red-cell adsorption decreased considerably the haemagglutinating activity of any virus suspension, supernatant fluid or pellet material as well.

Infectivity. Concentrated purified virus PR 8 was incubated for 4 hr at 37° with or without caseinase C at μ 0·1 (Table 3). The infectivity of the virus incubated with caseinase C was $<5\%$ the infectivity of the virus incubated in buffer alone.

Release of material neutralizing haemagglutination inhibition. Concentrated purified virus PR 8 was incubated at 37° in μ 0·1 buffer for 1, 2 or 4 hr with caseinase C, and for 4 hr without caseinase C. Haemagglutination inhibition neutralizing, haemagglutinating and neuraminidase activities were titrated in the supernatant fluids of these suspensions (Table 1, 2 Expt. 8). There was twice as much haemagglutination inhibition neutralizing material in the supernatant fluid of the virus suspension pre-incubated for 4 hr with caseinase C as in the supernatant of the same suspension incubated for only 1 hr with caseinase C, and 4 times as much as in the supernatant

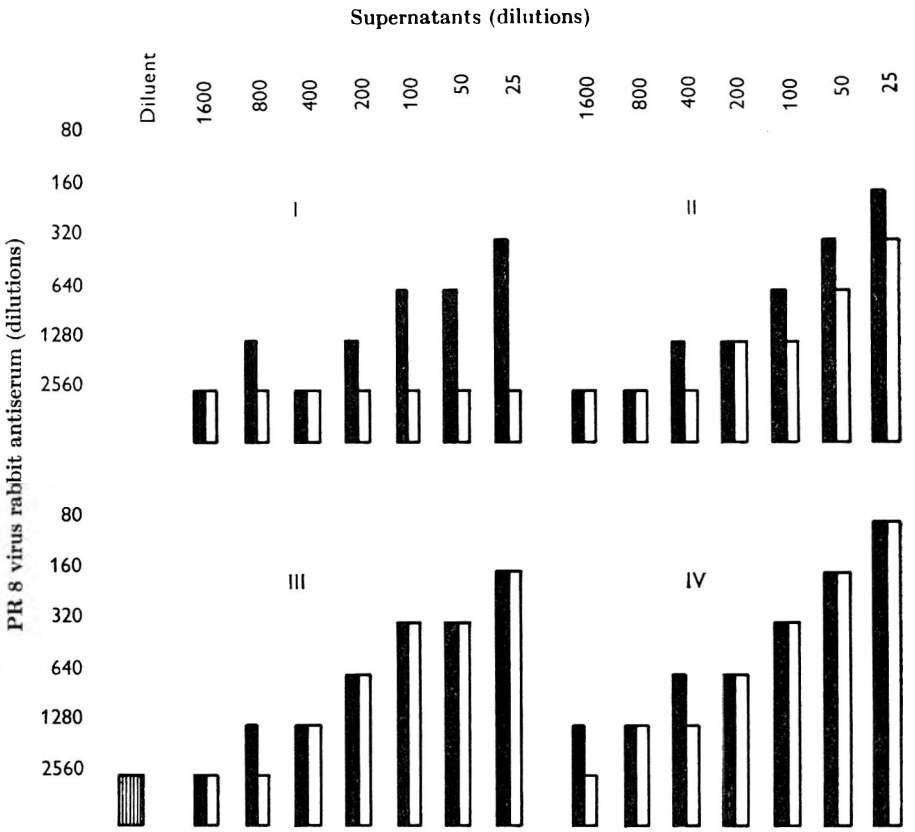


Fig. 1. Neutralization of haemagglutination inhibiting antibodies of PR 8 rabbit antiserum by the supernatant fluid from virus PR 8 suspension pre-incubated at 37° for 4 hr. in μ 0·1, pH 7 phosphate buffer (I); or for 1 hr. (II), 2 hr. (III), 4 hr. (IV) in μ 0·1, pH 7 phosphate buffer + caseinase C (see Expt. no. 8). ▥, Haemagglutination in the presence of diluent; □, in the presence of supernatant fluid adsorbed; ■, in the presence of supernatant fluid unadsorbed.

fluid of the untreated suspension (Fig. 1). Red-cell adsorption abolished the activity of the supernatant fluid of the control suspension, decreased by 50 % the activity of the supernatant fluid of the virus suspension pre-incubated for 1 hr with caseinase C, but did not decrease the activity of the supernatant fluid of virus suspensions treated for 2 or 4 hr with caseinase C (Fig. 1).

Sedimentation of neuraminidase and haemagglutination inhibition neutralizing activities from supernatant of caseinase C treated virus suspension

The sedimentation was done twice, by centrifugation at 125,000*g* (39·000 rev./min., swinging bucket rotor S.W. 39, Spinco Model L centrifuge) of 5 ml. supernatant fluid from concentrated purified virus PR 8 pre-incubated for 1 hr with caseinase C. In one experiment (Tables 1, 2, Expt. 5), the supernatant fluid was obtained after caseinase C treatment at μ 0·01; it was centrifuged for 10 hr at 125,000*g*. In the other experiment (Tables 1, 2, Expt. 12), the supernatant fluid was obtained after caseinase C treatment at 0·1; it was centrifuged for 12 hr at 125,000*g*. In both cases, a thin pellet was obtained. The supernatant fluids were collected with a syringe and needle. In experiment number 5, 4 ml. of supernatant were removed and the pellet was resuspended in the 1 ml. fluid left in the tube. In experiment number 12, 4·5 ml. of supernatant were removed and the pellet resuspended in 0·5 ml. bottom fluid. The haemagglutination inhibition neutralizing and neuraminidase activities of these fractions were measured and compared to the activities of samples of the corresponding preparations kept at 4° during the prolonged high-speed centrifugation (Table 4). In experiment number 5, 100% of the neuraminidase and 80% of the haemagglutination inhibition neutralizing activities were recovered; bottom fractions contained 100% of the neuraminidase activity and 72% of the haemagglutination inhibition neutralizing activity. In experiment number 12, 100% of the neuraminidase and 90% of the haemagglutination inhibition neutralizing activities were recovered; bottom fraction contained 80% of the neuraminidase activity and 75% of the haemagglutination inhibition neutralizing activity.

Table 4. *Sedimentation of neuraminidase and haemagglutination inhibition neutralizing activities released by caseinase C treatment of PR 8 virus*

Fraction collected after 125,00*g* centrifugation	Neuraminidase activity*		Haemagglutination inhibition neutralizing activity*	
	Expt. 5†	Expt. 12‡	Expt. 5†	Expt. 12‡
Supernatant fluid	2	10	35	15
Bottom fraction	102	72	80	75

* As % of the activity of the material not centrifuged at 125,000*g*; † 10-hr centrifugation at 125,000*g*; ‡ 12-hr centrifugation at 125,000*g*.

Morphology of the virus particles treated by caseinase C at μ 0·1

Concentrated purified PR 8 virus was incubated for 1 or 4 hr at 37° with caseinase C at μ 0·1. After this treatment, the virus particles were sedimented by centrifugation for 1 hr at 31,000*g*. They were then resuspended in water, mixed with an equal volume of potassium phosphotungstate solution (2%, pH 7) and examined with a Siemens Elmiskop I electron microscope. Controls for infectivity, neuraminidase activity and haemagglutinating activity were done in parallel in the 4 hr experiment (Tables 1–3, Expt. 11). Haemagglutinating activity and neuraminidase activity only were checked in the 1 hr incubation experiment (Tables 1 and 2, Expt. 12) but the neuraminidase and the haemagglutination inhibition neutralizing

material released by the virus particles were sedimented by 12 hr centrifugation at 125,000*g* (Table 4).

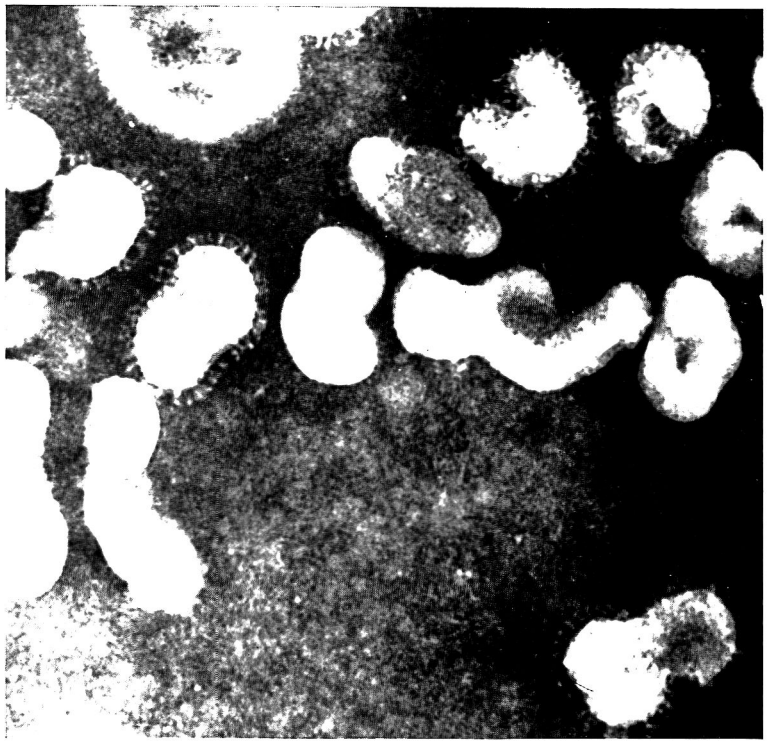
After 4 hr incubation, some virus particles looked unaltered, some had lost almost all their surface projections. The majority had fewer projections than normal virus particles (Pl. 1A). After 1 hr incubation, most virus particles had no obvious alteration of their surface, although some had lost a few projections (Pl. 1B).

DISCUSSION

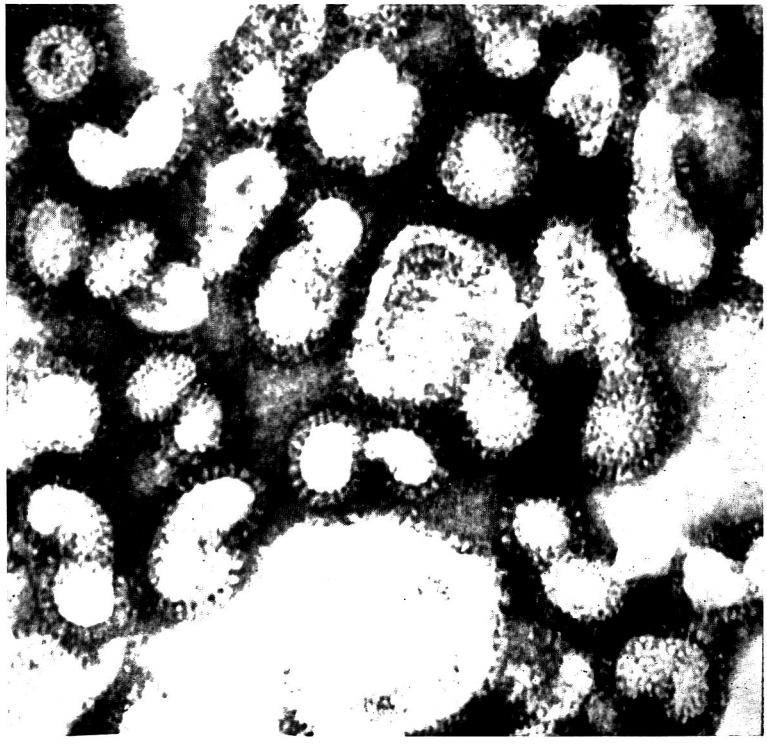
By incubating influenza virus PR 8 with caseinase C at two ionic strengths (Reginster 1965*a*, and this paper) we found quantitative but not qualitative differences in the modifications of the virus properties. This does not imply that all the effects of the caseinase C preparations are due to one enzyme. Haemagglutination is obviously related to the surface projections of the virus. Indeed, when electron micrographs showed virus particles completely lacking their normal surface projections (Reginster, 1965*b*) the haemagglutinating activity was considerably decreased. Nevertheless, the surface of most of the virus particles can be considerably freed from surface projections without modification of haemagglutinating ability as measured by the standard technique. This fits in well with the idea that the surface of the virus is made up of repeating units, two of which, when suitably located, would suffice to link two red cells together.

By high speed centrifugation we succeeded in concentrating at the same rate free neuraminidase and haemagglutination inhibition neutralizing material. Thus caseinase C split up superficial layers of influenza virus either into subunits differing in their biological properties but sedimenting under the same conditions, or more likely into subunits carrying at least neuraminidase and haemagglutination inhibition neutralizing activities. Sedimentation analysis revealed the presence of 6 S material in virus PR 8 suspension treated for 1 hr by caseinase C at μ 0·01 (Reginster, 1965*c*). The weight of one surface projection, calculated on the basis of Horne & Wildy's mensurations (1963) and by assuming that it is made of protein, is 90,000. It appears from Greenberg's table (1951) that this weight is not inconsistent with a sedimentation constant of 6 S. Haemagglutination, although related to the surface projections, does not seem to be located on the site responsible for neutralization of haemagglutination inhibition or for neuraminidase activity since adsorption to red cells did not modify these activities when they were released from the virus particles by caseinase C. This would mean that the inhibition of haemagglutination by antibodies is a 'steric' one (Fazekas de St Groth, 1963) since the antigenic sites responsible for fixation of antibodies do not adsorb to red cells. The question of identity or non-identity of the sites responsible for neuraminidase activity and neutralization of haemagglutination inhibition still remains unanswered. It has been, so far, impossible to determine what degree of integrity of the virus surface is necessary for full infectivity of the virus particle because accurate quantitative data are lacking.

Mayron, Robert, Winzler & Rafelson (1961) obtained separation of neuraminidase from virus PR 8 by trypsin treatment. This neuraminidase did not haemaglutinate nor block agglutination of red cells by 'indicator' Lee virus. It has been, so far, impossible to establish complete comparison between the effects of trypsin and



a



b

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of caseinase C. Nevertheless, trypsin is able to release surface projections of an influenza virus strain highly sensitive to its action (Borecký, Lackovič & Mrena, 1964), as is caseinase C action on PR 8 strain.

We thank Dr C. M. Calberg-Bacq for electron microscope examination, Mr L. Dierickx for determining the caseinolytic activity of enzymic preparations. Mr J. L. Renson and Miss D. Marique for technical assistance. We are indebted to the Fonds de la Recherche Scientifique Médicale for a grant in aid.

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EXPLANATION OF PLATE

Influenza virus PR 8 after incubation at 37° in pH 7, μ 0·1 phosphate buffer with caseinase C. Magnification: 4,4 × 40,000.

A. 4-hr incubation. B. 1-hr incubation.

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The Intracellular Accumulation of ^{14}C-streptomycin by *Escherichia coli* strain B in Relation to its Growth-Inhibitory Effect

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SUMMARY

The intracellular concentrations of ^{14}C-streptomycin accumulated by *Escherichia coli* strain B during growth in the presence of different extracellular concentrations of the compound have been measured. They increased logarithmically with time at rates proportional to the logarithm of the extracellular concentration; the growth rates declined linearly with time at rates which were also proportional to the extracellular concentrations. Thus the same decrease in growth rate resulted from different intracellular concentrations of streptomycin, according to the conditions of uptake. After removal of extracellular streptomycin, the growth rates remained constant for several hours, during which time 40–60% of the total cell-bound radioactivity was lost. Of the radioactivity lost from the organisms during growth after removal of extracellular streptomycin, 50–80% was recovered from culture filtrates but did not behave as streptomycin with respect to adsorption to charcoal.

INTRODUCTION

In two papers we reported (Kogut, Lightbown & Isaacson, 1965*a*, *b*) that when aerobic cultures of *Escherichia coli* strain B were taken after addition of dihydrostreptomycin in the early logarithmic phase, and treated to remove extracellular antibiotic before growth has ceased (Hancock, 1962*a*) they continued to grow at exponential but decreased rates for several hours, after which the growth rates increased and gradually returned to those of controls. From the results of study of this inhibited growth in the absence of extracellular antibiotic (by extinction measurements, viable counts, direct microscopic observations) we concluded that the extent of diminution in growth rates of treated populations should reflect the mean dihydrostreptomycin concentration at the sites of inhibition at the time of removal of extracellular antibiotic. We further inferred that this might be only a fraction of the total intracellular dihydrostreptomycin concentration, which might vary within the treated populations. Thus, we postulated that intracellular streptomycin can be in two phases, namely, an inhibitory fraction—presumably bound to the ribosomes, (Flaks, Cox & White, 1962; Spotts & Stanier, 1961; Davies, 1964; Cox, White & Flaks, 1964), and a non-inhibitory 'pool' fraction whose size would differ between different fractions of the population, and which could be transferred to the inhibitory sites according to the availability of these and to the size of the

'pool'. This view implied that the concentrations of streptomycin in these two phases must be governed, at least in part, by independent factors.

In a detailed study of the intracellular accumulation of ^{14}C-streptomycin by a number of micro-organisms, Hancock (1962*a*, *b*) described some of the factors which govern this, and stated (Hancock, 1962*b*) that 'death of *Bacillus megaterium* does not appear to follow inevitably the attainment of a certain intracellular concentration of streptomycin, since at lower (extracellular) streptomycin concentrations the organisms were killed after a smaller amount of streptomycin had been taken up, and vice versa'. The intracellular concentrations of ^{14}C-streptomycin measured in his experiments, at the time when growth ceased, would have comprised both the phases postulated above. Hancock also reported that in lysed protoplast preparations from *B. megaterium* grown in the presence of ^{14}C-streptomycin, the proportion of radioactivity which sedimented with the ribosomal fraction constituted from 50 to 99% of the total radioactivity in different preparations. However, various extraction procedures designed to break the osmotic barrier and to disrupt different kinds of bonds, did not produce any differential or sequential release of intracellular streptomycin from the organisms. Hancock concluded that 'breakage of the osmotic barrier is necessary but not sufficient to release bound streptomycin'.

In the present work, by using a small amount of ^{14}C-streptomycin of low specific activity, available to us, we have measured its intracellular accumulation from different external concentrations and examined the quantitative relationships between such intracellular concentrations of streptomycin and decreases in growth rates after removal of extracellular antibiotic (Kogut, Lightbown & Isaacson, 1965*a*, *b*). Parts of this study have been published (Kogut & Lightbown, 1964*a*).

METHODS

Cultures. The organism, *Escherichia coli* strain B, its maintenance, growth media and conditions, the preparation of inocula, procedure for removing extracellular streptomycin and measurements of growth and growth rates were as described by Kogut, Lightbown & Isaacson (1965*a*, *b*).

Labelled streptomycin and measurement of radioactivity. ^{14}C-streptomycin was the $CaCl_2$ complex, kindly provided by Dr C. Rosenblum (Merck, Sharpe and Dohme, Rahway, New Jersey, U.S.A.) with a specific activity of 0·054 μC/mg. The material, dissolved in 25 ml. of water, was assayed by the plate diffusion technique (Humphrey & Lightbown, 1952) against the International Standard of Streptomycin, and found to contain 700 i.u./ml. Radioactivity was determined in a Nuclear Chicago, Model D 47 ultrathin-window counter, giving background activity of less than 2 c.p.m. The ^{14}C-streptomycin preparation gave 17 c.p.m./i.u. = 17 c.p.m./μg. streptomycin base (Humphrey, Lightbown & Mussett, 1959). Non-radioactive streptomycin $CaCl_2$ complex was obtained from Glaxo Laboratories Ltd. and contained 673 μg. base/mg.

Intracellular concentrations of ^{14}C-streptomycin were defined and measured as follows. After addition of labelled streptomycin to aerobically growing cultures of *Escherichia coli* B in the early logarithmic phase, samples containing equiv. 1–3 mg. dry wt. organisms were removed at the appropriate times and filtered on a Millipore membrane filter of 0·22 μ pore size and 2·9 cm. diameter. As it is known that

streptomycin can be strongly, but non-specifically, adsorbed to bacteria, but can in this form be readily removed by washing in media of high ionic strength (Hancock, 1962*a*; Plotz, Dubin & Davis, 1961), the organisms were washed three times on the filter with a total of one half their original volume of growth medium containing unlabelled streptomycin 50 μg./ml. to remove and exchange any adsorbed radioactivity. We determined in preliminary experiments that no further radioactive material was removed from the organisms by repeating such washing. The retained radioactivity is what we here define as 'intracellular'. The membranes with the organisms were dried in air and fixed to aluminium planchet. A minimum of 1000 counts were obtained from each sample, and the values corrected for background counts. When necessary, adjustment for infinite thinness was made by means of a calibration graph prepared from a culture of *E. coli* B, labelled with ^{14}C-glycine.

RESULTS

The intracellular accumulation of streptomycin and decrease in growth rates

As already reported (Kogut, Lightbown & Isaacson, 1965*a*), the extent of growth inhibition (as % of control rates) produced by dihydrostreptomycin treatment of *Escherichia coli* B under conditions where intracellular accumulation is known to occur (Hancock, 1962*a*) and measured after removal of extracellular antibiotic, is a linear function of the duration of previous aerobic growth in a given concentration of antibiotic. We had suggested that this might directly reflect the intracellular antibiotic accumulation if this were also linear with time. However, intracellular accumulation (in the sense defined above) of radioactive label by aerobically growing cultures of *Escherichia coli* B, after addition of ^{14}C-streptomycin during the early exponential phase of growth was not linear with time. Figure 1 shows the time courses of decrease in growth rates and of intracellular concentrations of labelled streptomycin (c.p.m./mg. dry wt. organisms) after treatment with different external concentrations of the antibiotic. The rate of accumulation apparently increased with duration of growth in the presence of streptomycin, but it also seemed to vary with the external concentration of antibiotic in the medium, and to increase more rapidly with time at the higher external concentrations. Figure 2 shows the relationship between intracellular concentrations of ^{14}C-streptomycin and the % inhibition of growth rates for four different initial concentrations of streptomycin in the medium. Because of the low specific activity of the streptomycin preparation, and the apparently very low intracellular concentrations which were effectively growth-inhibitory under these conditions, it was not possible to measure intracellular concentrations associated with slight decreases in growth rate (by less than 40 %). With the higher external concentrations of antibiotic, there was the additional difficulty of the rapid progress of growth inhibition. However, in all four cases, the relationship between inhibition of growth rates and intracellular concentrations resembled a saturation curve; the intracellular concentration approached asymptotically the value for complete growth inhibition. The shapes of the four curves, for the four external concentrations of streptomycin used, also appear to differ, suggesting that the intracellular concentrations associated with a given decrease in growth rate varied with the extracellular concentrations from which they were accumulated, as suggested by Hancock (1962*b*) for *Bacillus*

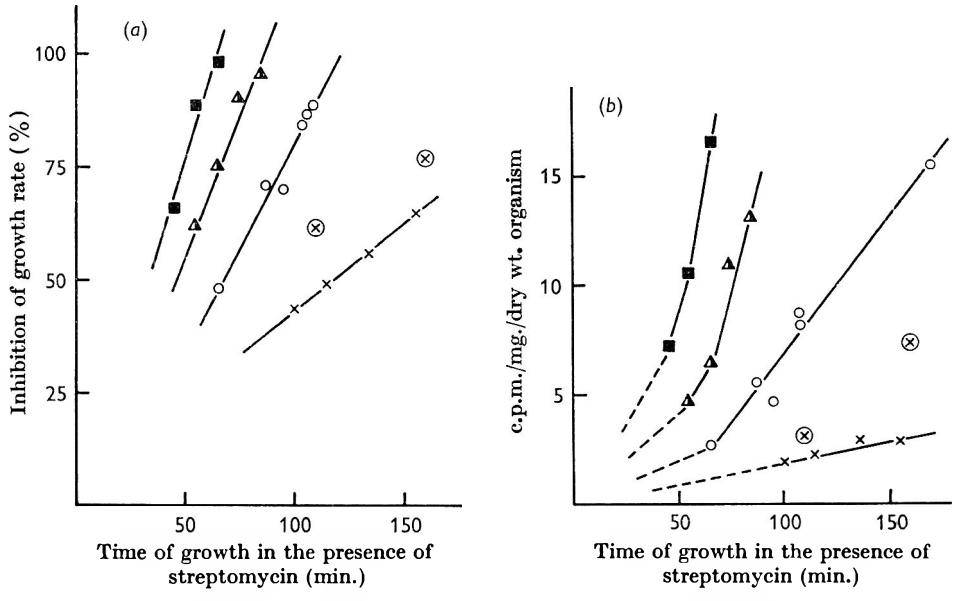

Fig. 1. Time course of inhibition of growth rate and of intracellular accumulation of radioactive label, during growth in different concentrations of ^{14}C-streptomycin. (*a*) % inhibition of growth rate. (*b*) Cell-bound radioactivity. ^{14}C-streptomycin concentrations in the medium: ×——×, ⊗——⊗, 15 μg./ml., two separate experiments; ○——○, 20 μg./ml.; ▲——▲ = 30 μg./ml.; ■——■, 40 μg./ml.

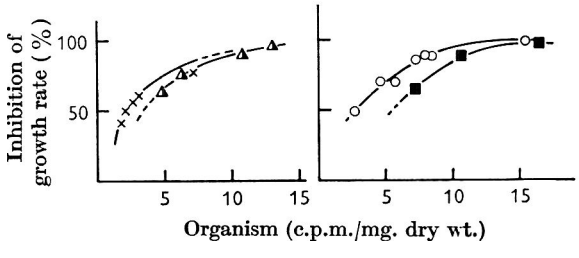

Fig. 2

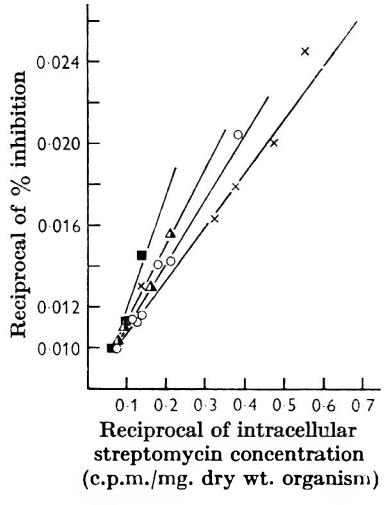

Fig. 3

Fig. 2. Reduction in growth rate as a function of intracellular streptomycin concentrations. ^{14}C-streptomycin concentrations in the medium from which intracellular accumulation took place: ×——×, 15 μg./ml.; ○——○, 20 μg./ml.; ▲——▲, 30 μg./ml.; ■——■, 40 μg./ml.

Fig. 3. Double reciprocal plot of data in Fig. 2.

megaterium. These differences are more clearly shown by transforming the data in Fig. 2 to double reciprocal plots, as in Fig. 3. Although it is not possible to evaluate the slopes precisely, because of the difficulties in obtaining a full range of measurements, the figure indicates four different slopes. This would confirm that a given reduction in growth rate can result from different intracellular concentrations of streptomycin, when the extracellular concentrations from which they are accumulated vary.

Loss of intracellular streptomycin during inhibited growth. We have also shown previously (Kogut *et al.* 1965*b*) that after cultures of *Escherichia coli* B were treated as described, periods of exponential growth at decreased rates were followed by recovery of most individuals in such cultures. Such recovery of growth rates was not due to continued uninhibited growth of portions of such treated populations. Further, we inferred from the relationship between time of onset of recovery and inhibition of growth rates that recovery could not be the consequence of a given amount of cell synthesis. At its simplest, it might be due to a time-dependent loss of dihydrostreptomycin from the cells, either by degradation or 'leakage'. In the present study, therefore, we have followed the intracellular concentrations of ^{14}C-streptomycin during the periods of exponential growth at decreased rates, as illustrated in Fig. 4. The cell-bound radioactivity (expressed as c.p.m./mg. dry wt. organisms of original culture at the time of removal of extracellular streptomycin)

Table 1. *Loss of cell-bound radioactivity during exponential growth of Escherichia coli* B *at decreased rates, after removal of extracellular streptomycin*

Treatment with streptomycin				After removal of extracellular streptomycin			
				Intracellular concentrations: c.p.m./mg. dry wt. organism at filtration time			
Expt.	Concentration (μg./ml.)	Time (min.)	% decrease of growth rate	At filtration	At onset of recovery	Lost during inhibited growth	% lost
1	15	100	41	1·82	0·91	0·91	50
1	15	115	50	2·11	1·07	1·04	49
1	15	135	56	2·66	1·60	1·06	40
2	15	110	61	3·09	1·47	1·62	52
1	15	155	64	2·64	1·42	1·22	46
2	15	160	77	7·21	—	—	—
3	20	65	49	2·60	1·54	1·06	41
4	20	95	70	4·57	2·17	2·40	51
3	20	87	71	5·54	2·99	2·55	46
3	20	107	87	8·45	3·72	4·73	56
5	20	108	88	8·05	—	—	—
3	20	170	100	15·4	—	—	—
6	30	55	64	4·77	—	—	—
7	30	65	76	6·31	3·41	2·90	46
6	30	75	90	10·90	4·23	6·67	62
7	30	89	96	13·04	5·15	7·89	60
8	40	45	66	7·22	2·89	4·33	60
8	40	55	89	10·60	4·37	6·23	59
8	40	65	98	16·5	—	—	—

decreased at an approximately exponential rate with time. Table 1 shows the intracellular concentrations of streptomycin at the time of removal of extracellular streptomycin and those at the beginning of the recovery phase, in cultures treated with different external concentrations of ^{14}C-streptomycin for various periods, and the resulting decreases in growth rates. The intracellular streptomycin concentrations are referred to dry wt. organisms present at the time of removal of extracellular streptomycin as above, so that the apparent loss of cell-bound antibiotic during growth is genuine and not an artifact due to continued cell synthesis. Although the growth rates and initial intracellular streptomycin concentrations varied widely in the different cultures (over an approximately 9 to 12-fold range), the percentage of the initially-fixed streptomycin which was 'lost' during the periods of inhibited growth by the various cultures varied only between 40 and 60 %.

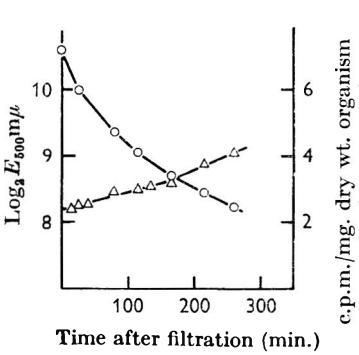

Fig. 4

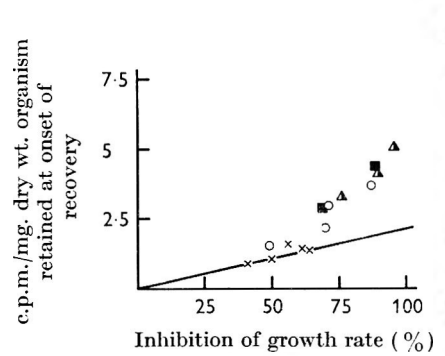

Fig. 5

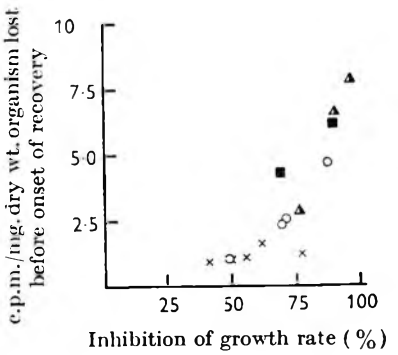

Fig. 6

Fig. 4. Intracellular streptomycin concentrations during growth of *E. coli* B, after removal of streptomycin from the medium. △——△, $\log_2 E_{500}$; ○——○, intracellular streptomycin as c.p.m./mg. dry wt. organism at the time of removal of extracellular streptomycin.

Fig. 5. Extent of decrease in growth rate of *Escherichia coli* B and cell-bound ^{14}C-streptomycin retained at onset of recovery phase. Symbols refer to ^{14}C-streptomycin concentrations from which accumulation took place: ×——×, 15 μg./ml.; ○——○, 20 μg./ml.; ▲——▲, 30 μg./ml.; ■——■, 40 μg./ml.

Fig. 6. Extent of decrease in growth rate and cell-bound ^{14}C-streptomycin lost during inhibited growth, before onset of recovery. Symbols refer to ^{14}C-streptomycin concentrations from which accumulation took place: ×——×, 15 μg./ml.; ○——○, 20 μg./ml.; ▲——▲, 30 μg./ml.; ■——■, 40 μg./ml.

Such losses of cell-bound radioactivity during continued growth in absence of extracellular streptomycin might have occurred by several mechanisms. In the first place, they might have been due to lysis of some individuals in treated populations, more probably those with the greatest intracellular concentrations of antibiotic. A small degree of lysis, say less than 10–15 % of the population, would not necessarily be detected by the extinction measurements. However, if such small proportions of the population were to account for 40–60 % of the total initial intracellular streptomycin concentrations of the population it would mean that the intracellular concentrations of these organisms would have to be many times higher than the population mean. It would also suggest that the actual concentrations which produced lysis would vary (over a 9 to 12-fold range; see above) with the

total intracellular concentrations. Further, it is difficult to see why such losses, if they occurred by lysis of some individuals with exceptionally high intracellular streptomycin concentrations, should proceed for several hours when the extracellular streptomycin concentrations must be negligible, as compared to those from which accumulation originally took place.

The intracellular streptomycin concentrations, associated with a given decrease in growth rate, vary with the extracellular concentration from which accumulation took place. This suggests that increasing portions of intracellular streptomycin are non-inhibitory when the extracellular concentrations are increased; in other words that intracellular streptomycin contains a non-inhibitory 'pool' fraction. The subsequent loss of cell-bound radioactivity during inhibited growth could, therefore, be from such 'non-inhibitory' antibiotic or from the 'inhibitory' fraction. In the former case, were the losses to represent all of the 'pools' then the streptomycin retained should represent the inhibitory fraction, tightly bound to the sites of inhibition. This reasoning demands that the lowest values for streptomycin retained must represent the maximum values for the 'inhibitory' fraction. Figure 5 shows the cell-bound streptomycin, retained at the onset of recovery (again expressed as c.p.m./mg. dry wt. organism in the original culture) against the % decrease in growth rates. The lowest values, mainly those from experiments where accumulation took place from 15 μg. streptomycin/ml. culture, give a straight line which goes through the origin and extrapolates to approximately 2·2 c.p.m./mg. dry wt. organism for complete growth inhibition. It could be argued, therefore, that this line represents the minimun 'inhibitory' concentration of intracellular streptomycin. The fact that the values obtained in some of the experiments, where intracellular accumulation occurred from higher external concentrations of streptomycin, diverge from this line, might mean that in these cultures parts of the 'pools' were retained at the times when the final measurements were made (approximately at the time of onset of recovery).

If, on the other hand, the losses of intracellular streptomycin during inhibited growth occurred by breakdown or degradation of the 'inhibitory' fraction of antibiotic, then the maximum amounts lost at onset of recovery would represent minimum values for the concentration of streptomycin initially at the inhibition sites. In general, one would expect that if the 'inhibitory' fraction were gradually lost during growth, the growth rates should show a continuous recovery. Since they do not, but remain exponential for several hours, one might assume that combination with inhibition sites amounts to irreversible inactivation of these, in which case the loss of such an 'inhibitory' fraction need not affect the growth rates. Figure 6 relates the amounts of cell-bound radioactivity lost during inhibited growth until the onset of recovery, to the degree of decrease in growth rates. It indicates that in this case the size of the 'inhibitory' fraction would increase exponentially with increasing inhibitory effect.

Recovery of radioactivity from culture filtrates at the end of inhibited growth

We tried to recover the radioactivity lost from the organisms in the culture filtrates collected at the end of the phase of exponential inhibited growth. Since the total amounts of radioactivity involved were very small, and to gain some idea whether the lost radioactivity still behaved as streptomycin, we treated the culture

filtrates with activated charcoal, and counted the radioactivity adsorbed to this. In a preliminary experiment to test the adsorption of streptomycin to charcoal, a culture of *Escherichia coli* B was grown in the usual medium to the middle of the logarithmic phase (about equiv. 0·3 mg. dry wt. organism/ml.) and after removal of the organisms, streptomycin calcium chloride complex (to 200 μg. base/ml.) was dissolved in the filtrate. Three 10 ml. portions, each containing 2·0 mg. streptomycin, were treated with 100, 150 and 200 mg. charcoal, respectively. After filtration, the streptomycin in the filtrate was estimated, after appropriate dilution, by the plate diffusion assay (Humphrey & Lightbown, 1952). Table 2 shows the results. Thus, 99 % of the streptomycin in the culture filtrate was adsorbed by 100 times its weight of charcoal.

Table 2. *Adsorption of streptomycin to charcoal from culture filtrate of Escherichia coli* B

Culture filtrate (ml.)	10	10	10
Streptomycin $CaCl_2$ added (mg. base)	2	2	2
Charcoal added (mg.)	100	150	200
Weight ratio, charcoal: streptomycin	50	75	100
Streptomycin (μg.) left in filtrate after removal of charcoal	59·2	24·7	17·9
Streptomycin adsorbed on charcoal (%)	97·1	98·8	99·1

Table 3. *Radioactivity adsorbed to charcoal from filtrates of Escherichia coli* B *cultures which had been treated with* ^{14}C*-streptomycin and continued to grow after removal of extracellular streptomycin*

Filtrate 1		Filtrate 2	
Volume of filtrate (ml.)	19	Volume of filtrate (ml.)	20·5
Maximum expected radioactivity from amounts of cell-bound radioactivity lost		Maximum expected radioactivity from amounts of cell-bound radioactivity lost	
As c.p.m.	20	As c.p.m.	6·1
As μg. streptomycin	1·18	As μg. streptomycin	0·36
Charcoal treatment			
	c.p.m. on charcoal		c.p.m. on charcoal
0·2 mg. charcoal, left 20 min. at 20°	1·3	1·0 mg. charcoal, occasional shaking 20 min. at 20°	2·3
+1·5 mg. charcoal occasional shaking, 30 min. at 20°	4·63	+20 μg. cold streptomycin, left 18 hr. occasional shaking, +2·1 mg. charcoal, shaken for 30 min. at 20°	2·35
+2·1 mg. charcoal, left with shaking 50 min at 20°	3·08		
Filtrate was lost			
Totals recovered	9·1		4·95

The filtrates from two of the streptomycin-treated cultures, after re-suspension in streptomycin-free media and inhibited growth during which radioactivity was lost from the organisms, were assayed for radicactivity. After storage at $-40°$ they were treated with charcoal and the radioactivity adsorbed to the charcoal counted (Table 3). In this case, up to 80% of the radioactivity lost from the organisms

during inhibited growth after treatment with ^{14}C-streptomycin, was recovered from the filtrates. However, comparison of the data in Tables 2 and 3 shows that this radioactive material did not behave like streptomycin with respect to adsorption to charcoal. In filtrate 1, a weight ratio of charcoal/streptomycin (estimated on the basis of streptomycin lost from the organisms) of 165, adsorbed only 6·5% of the calculated maximum, and 14% of the total radioactivity which was finally recovered, for which a weight ratio (charcoal/streptomycin) of about 7600 was used. In filtrate 2, the weight ratio of charcoal/streptomycin lost from the organisms was about 8000 and adsorbed 80% of the calculated maximum radioactivity which could have been present. In this experiment unlabelled streptomycin, 20 μg. base, was added to the filtrate after the first charcoal treatment and allowed to stand overnight to facilitate desorption of radioactive material which might have been adsorbed to the glass of the vessel. The possibility that this difference in adsorption to charcoal between the control experiment and the treated samples might have been due to differences in the culture filtrates from which adsorption took place cannot be excluded. Although in the control experiment a filtrate from *Escherichia coli* B which had grown in the same medium for about the same number of generations was used, this culture had not grown in presence of streptomycin. It is possible that there was a difference in the metabolism and/or excreted products as between streptomycin-treated and control cultures which affected the adsorption of streptomycin from such filtrates to charcoal. The alternative view that the radioactive material lost from the organisms was not in the form of free streptomycin, but some degradation product or complex with other cell-constituents seems equally likely. The concentrations were too low to allow biological assay.

DISCUSSION

This work was undertaken to test the inference from other observations (Kogut *et al.* 1965*a*, *b*) and from a review of the literature (Kogut & Lightbown, 1964*b*) that intracellular streptomycin and dihydrostreptomycin can be in two phases, an 'inhibitory fraction' tightly bound to the sites of inhibition and a 'non-inhibitory pool fraction', and that the uptake into these two fractions must be governed, at least in part, by independent factors. None of the earlier studies on the intracellular accumulation of streptomycin by several bacterial species, as well as algae (Pramer 1956; Litwak & Pramer, 1957; Szybalski & Mashima, 1959; Hancock, 1962*a*, *b*; Hurwitz & Rosano, 1962) dealt with rates of uptake from different external concentrations and quantitative comparisons between intracellular concentrations and antibiotic effects. However, Hurwitz & Rosano (1962) proposed that 'binding' or uptake of streptomycin by *Escherichia coli* B can be in three phases; (1) adsorption which is rapid, reversible and not related to the antibiotic action; (2) a second phase, claimed to occur 'prior to killing' and presumably before cessation of growth (see Kogut *et al.*, 1965*b*, for discussion of the relation between so-called 'killing' and growth inhibition) which was thought to be the 'effective' intracellular concentration; (3) a third phase which was thought to occur after the organisms were rendered non-viable, but which was shown to be different in character from the uptake of streptomycin exhibited by organisms previously killed by heat or toluene treatment.

We have sought to exclude the first, adsorption, phase by washing procedures,

and have defined as 'intracellular' the radioactivity accumulated during growth in presence of ^{14}C-streptomycin which cannot be removed from the organisms by washing in growth medium containing excess of unlabelled streptomycin, as first proposed by Hancock (1962*a*). Whilst the present paper was in preparation, Dr R. Hancock (personal communication) reported on a 'non-exchangeable fraction of bound streptomycin in *Escherichia coli*'. His use of ditritiostreptomycin of much higher specific activity than the ^{14}C-streptomycin used by us, provided a more sensitive system for the study of the early stages of streptomycin uptake. Dr Hancock's uptake curve for *Escherichia coli* ML 35, from ^{3}H-streptomycin 25 μg./ml. extending over 25–30 min. is very different from ours. Although his washing experiment showed that 50 mμg. ^{3}H-streptomycin/mg. dry wt. organism was retained after two washings, (hence defined as the 'non-exchangeable fraction of bound streptomycin') the uptake curve showed that this amount was found within the first few minutes, after which the curve became approximately linear. In our experiments, the cultures might also have contained such a relatively small rapidly bound fraction (presumably residual adsorbed streptomycin) and Hancock's uptake curve would approximately fit ours for 30 μg. streptomycin/ml. external concentration (Fig. 1).

Most of our 'intracellular accumulation', however, overlaps the phases 2 and 3 of Hurwitz & Rosano (1962). Like these authors, we found that intracellular accumulation was not linear with duration of exposure to streptomycin, but we also found that it was not a linear function of the degree of decrease in growth rate. This degree of decrease in growth rate (i.e. the inhibitory effect) was, however, linearly related to the duration of growth in a given streptomycin concentration and the slopes of these linear rates were proportional to the logarithms of the external streptomycin concentrations. We previously inferred that the 'inhibitory effect' must be a direct result of the 'effective concentration' of inhibitor, namely, its concentration at inhibition sites. This inference involves the assumption that the number or concentration of inhibition sites is the same as in controls. It would perhaps be more legitimate to say that the residual growth rates, which must of course also be a linear function of the duration of treatment, reflect the lack of combination between inhibitory sites and streptomycin. The fact that the same residual growth rate can exist with different intracellular streptomycin concentrations, and the fact that, after removal of extracellular streptomycin, the residual growth rates remain constant for several hours, must mean that under all these conditions the concentration of uncombined sites is constant. We have inferred that a non-inhibitory 'pool' fraction (possibly phase 3 of Hurwitz & Rosano, 1962) is accumulated more or less simultaneously with an 'inhibitory' streptomycin fraction which combines with the inhibition sites (presumably the ribosomes), but at different rates. By equating 'non-inhibitory' with 'pool' material, we are alluding to its possible function; the term 'pool' does not exclude combination with some cellular constituents. The proportion of intracellular streptomycin in these two fractions would accordingly vary with the duration of exposure and with the extracellular streptomycin concentrations from which accumulation takes place. After removal of extracellular streptomycin, transfer from the 'pool' to newly synthesized sites (ribosomes) must proceed at rates which would keep the concentration of uncombined sites constant until the onset of recovery. If the total con-

centration of inhibition or combining sites were constant and the same as in controls, this would mean that such transfer was not a simple equilibrium process. However, the total concentration of inhibitory or combining sites need not be constant and the same as in controls, when cultures are exposed to streptomycin. Several workers (Erdös & Ullmann, 1959; Flaks *et al.* 1962; Hancock, personal communication; Dubin, Hancock & Davis, 1963; Hahn *et al.* 1962) have reported that synthesis of some RNA fractions is stimulated on addition of streptomycin to growing cultures; the evidence is conflicting as to which RNA components are affected. Our observations would fit the hypothesis that on intracellular uptake of streptomycin, the synthesis of RNA material, including ribosomes or precursors of ribosomes, is stimulated, so that the concentrations of streptomycin in combination with this material increase logarithmically with time, whilst the concentration of uncombined active ribosomes would decrease linearly with time during growth in streptomycin-containing medium. In this case, after removal of extracellular streptomycin, the further synthesis of ribosomes might be at rates governed by the combined streptomycin fraction, with transfer occurring from this to the new sites, but without liberation of active sites. The rates of transfer could then be proportional to the concentration of streptomycin in the 'pool' or combined fraction. The constancy of growth rates after removal of extracellular streptomycin might also be explained if the synthesis of 'active' ribosomes were strictly dependent on the presence or functioning of active ribosomes. In this case, no transfer from any 'pool' or other sites would be involved.

The loss of cell-bound radioactivity after removal of extracellular streptomycin could provide a reasonable explanation of the recovery phenomenon. If only active ribosomes were replicated and no transfer from 'pool' fractions took place, recovery might occur if some ribosomes were re-activated or synthesized from liberated precursors, by loss of cell-bound streptomycin. Such 're-activation' would have to occur only occasionally, since about 50% of the intracellular radioactivity can be lost from the organisms before the recovery of growth rates becomes apparent. However, if the constancy of growth rates after removal of extracellular streptomycin involves transfer of intracellular antibiotic from 'pool' or 'combined' fractions to newly synthesized ribosomes, recovery could start when rate of transfer, due to depletion of this fraction, became slower than synthesis of new sites.

Several workers have estimated the 'minimum lethal' or 'growth-inhibitory' concentration of streptomycin inside organisms. This was reported to be 3×10^5 molecules/organism for *Escherichia coli* B (Hurwitz & Rosano, 1962), 5×10^4 molecules/organism for *Bacillus megaterium* (Hancock, 1962*b*) and 2×10^4 molecules/organism for *E. coli*, ML 35 (Dr R. Hancock, personal communication). We have obtained minimum values of streptomycin retained by *E. coli* B at the beginning of recovery, which extrapolate to 3×10^4 molecules/organism at complete growth inhibition (2·2 c.p.m./mg. dry wt. = 0·13 μg. streptomycin/3×10^9 organisms). These values could amount to a few molecules/ribosome; 1–2 molecules of streptomycin/ribosome will inhibit polypeptide synthesis *in vitro* by ribosomal preparations from a sensitive strain of *E. coli* (Flaks *et al.* 1962). Since the ribosome content of bacteria has been shown to vary with growth rate (Kjeldgaard & Kurland, 1963; Rossett, Monier & Julien, 1964), the 'effective inhibitory' concentrations of streptomycin/organisms, namely those required to inactivate enough ribosomes to

stop growth, might well vary with growth conditions. We suggest that they may also vary because the synthesis of ribosomes might vary with streptomycin treatment.

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The Role of Penicillin Acylase in the Resistance of Gram-Negative Bacteria to Penicillins

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SUMMARY

The resistance of 148 clinical isolates of Gram-negative bacteria to ampicillin and benzylpenicillin was determined in serial-dilution sensitivity tests, together with the extent of inactivation of the penicillins, and the production of 6-aminopenicillanic acid (6-APA). Many of the cultures were resistant to the penicillins and inactivated the compounds, but only one culture, a strain of *Escherichia coli*, showed penicillin acylase activity, as indicated by the production of 6-APA. However, the cultural conditions prevailing in serial-dilution tests, namely, stationary culture at 37°, although favourable for the functioning of penicillin acylases, were shown to be highly unfavourable for production of the enzyme by coliform bacteria.

INTRODUCTION

The resistance of certain Gram-negative bacteria to ampicillin and benzylpenicillin has been shown to be associated with the destruction of the penicillins by the β-lactamase (penicillinase, Fig. 1) activity of these organisms (Ayliffe, 1963; Percival, Brumfitt & de Louvois, 1963; Sutherland, 1964). However, the occurrence of penicillin acylase activity, leading to the hydrolysis of penicillins with the production of the relatively inactive 6-aminopenicillanic acid (6-APA) (Fig. 1) has been reported in many species of Gram-negative bacteria (Kaufmann & Bauer, 1960; Rolinson *et al.* 1960; Claridge, Gourevitch & Lein, 1960; Huang, Seto & Shull, 1963), and resistance to penicillins as a result of penicillin acylase activity has been described by English, McBride & Huang (1960) and by Holt & Stewart (1964*a*). The latter authors reported that 40% of 310 clinical strains of *Escherichia coli* examined produced penicillin amidase (penicillin acylase), but the properties of the enzyme extracted from one of these cultures, *E. coli* NCIB 9465, (Holt & Stewart, 1964*b*) differed very markedly from those of the penicillin acylases produced by other strains of *E. coli* (Rolinson *et al.* 1960; Kaufmann, Bauer & Offe, 1960; Kaufmann & Bauer, 1964).

The experiments described here were designed to investigate the occurrence of penicillin acylase activity among Gram-negative bacteria of clinical origin and to ascertain the role of this enzyme in the resistance of bacteria to penicillins. Because optimum cultural conditions for the formation of penicillin acylase by *Escherichia coli* (Kaufmann & Bauer, 1964) are different from those prevailing in antibiotic sensitivity tests, a lack of activity in these tests might be due either to intrinsic inability of the organism to form the enzyme or to the cultural conditions being unfavourable for production of penicillin acylase. Accordingly, the Gram-negative

bacteria used in these experiments were examined for penicillin acylase activity, not only in the serial-dilution tests employed routinely for the determination of bacterial sensitivity to antibiotics, but also in experiments using cultural conditions found to be especially favourable for the formation of penicillin acylase by Entero-

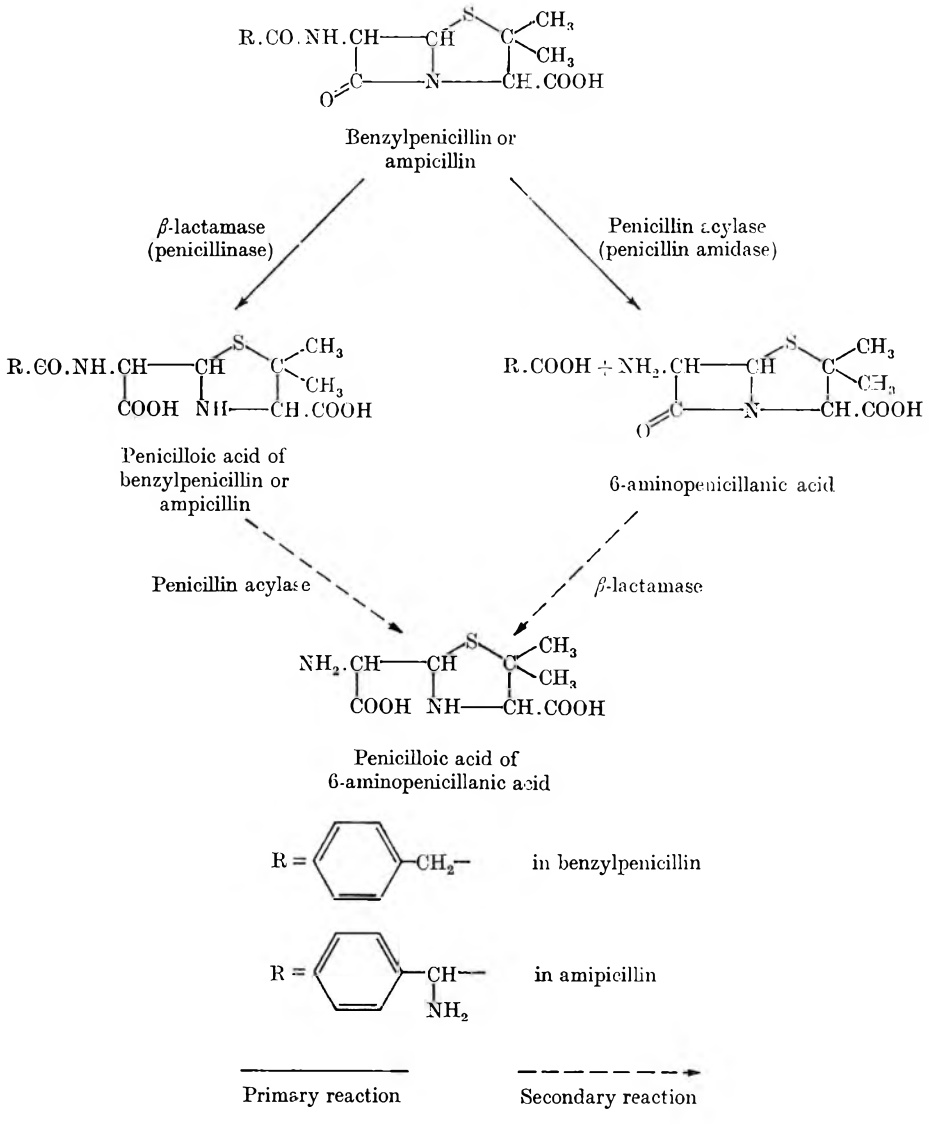

Fig. 1. Degradation of penicillins by Gram-negative bacteria.

bacteriaceae. It was hoped by this means to obtain a true measure of the occurrence of penicillin acylase among Gram-negative bacteria. Three strains of *E. coli* reported by Holt & Stewart (1964*a*, *b*) to be penicillin acylase-producing cultures were also included in the studies reported here.

METHODS

Bacterial cultures. A total of 148 strains of Gram-negative bacteria which were predominantly resistant to ampicillin and benzylpenicillin were obtained from several hospitals. In addition, ten strains of bacteria of non-clinical origin known to produce penicillin acylase were included in these experiments.

Effects of cultural conditions on penicillin acylase formation in Escherichia coli, strain c 975. Conical flasks (500 ml.) containing 50 ml. of medium were inoculated with 0·5 ml. of an overnight nutrient broth culture of a clinical strain of *E. coli*, c 975. The cultures were incubated stationary or on a rotary shaker, at 26° or 37° (see Table 1 for details). After 18 hr sodium benzylpenicillin was added to all flasks to a final concentration of 1 mg. penicillin/ml. The penicillin was added in M/4 phosphate buffer (pH 8·5) as penicillin acylase is known to be most active under alkaline conditions (Rolinson *et al.* 1960). All flasks were shaken for 4 hr at 30° after which samples were assayed for 6-APA as described below. The following media were employed:

(*a*) Yeatex medium consisting of 2·5% Yeatex paste (Standard grade, English Grains Ltd., Shobnall Road, Burton-on-Trent, Notts.) in water, adjusted to pH 7·0.

(*b*) Yeatex medium + 0·15% phenylacetic acid. This acid was dissolved in NaOH to give a 5% solution at pH 7·0 which was added to the Yeatex medium to the desired concentration, and the whole was adjusted to pH 7·0 before sterilization at 15 lb./sq. in. for 15 min.

(*c*) Nutrient broth (Oxoid no. 2) adjusted to pH 7·0.

(*d*) Nutrient broth + 0·15% phenylacetic acid as in (*b*).

Measurement of antibacterial activity. Minimal inhibitory concentrations of ampicillin and benzylpenicillin required to inhibit growth of the test bacteria were measured by two-fold serial dilution in nutrient broth (Oxoid no. 2). The penicillins were incorporated in graded concentrations in 5 ml. volumes of nutrient broth which were inoculated with one drop (0·03 ml.) of an overnight culture of the test organism. Inhibitory concentrations were determined after overnight incubation at 37°.

Measurement of penicillin destruction in penicillin sensitivity tests. The destruction of penicillins in the antibacterial tests described above was estimated by microbiological assay of residual penicillin with an agar diffusion method (Sutherland, 1964). Certain samples were also examined for β-lactamase activity by chromatographic examination for penicilloic acids.

Penicillin acylase activity in penicillin sensitivity tests. Samples were taken from cultures containing the highest concentration of penicillin allowing growth after overnight incubation and these were examined for 6-APA.

Penicillin acylase in shaken flask cultures. Two 500 ml. conical flasks containing 50 ml. of a solution consisting of 2·5% Yeatex paste and sodium phenylacetate at a final concentration equivalent to 0·1% phenylacetic acid were inoculated with 0·5 ml. of an overnight nutrient broth culture of the test organism. The flasks were shaken on a rotary shaker (1 in. throw) at 24° for 24 hr Ten ml. of a 6 mg./ml. solution of sodium benzylpenicillin in M/4 phosphate buffer (pH 8·5) were added to one of the flasks to give a concentration of 1 mg./ml. penicillin. A similar quantity of ampicillin was added to the second flask. The flasks were transferred to a rotary shaker and shaken at 30°. After 4 hr 0·5 ml. of *n*-butyl acetate was added to each

flask and the shaking was continued for a further hour by which time the cultures were non-viable. The flasks were assayed for 6–APA content by paper chromatography as described below. Solutions of benzylpenicillin and ampicillin were also chromatographed to provide standards for the estimation of the amount of residual penicillin in the reaction mixtures.

Analytical methods. Detection and estimation of 6-APA. Filter paper strips, Whatman no. 1, 1 cm. wide, were spotted in duplicate with 6 μl. of the samples under test. The benzylpenicillin reaction mixtures were chromatographed with a butanol + ethanol solvent system (*n*-butanol + ethanol + water 4 + 1 + 5 v/v top phase). Ampicillin reaction mixtures were chromatographed in a butanol + acetic acid solvent system (*n*-butanol + acetic acid + water, 12 + 3 + 5 v/v). In both cases chromatography was carried out overnight at 5°. The chromatograms were dried at 40° and one of each pair was sprayed with 5% aqueous sodium bicarbonate, 5% phenylacetyl chloride in acetone and 5% aqueous sodium bicarbonate, consecutively, to convert 6-APA into benzylpenicillin (Batchelor, Doyle, Naylor & Rolinson, 1959). The zones of ampicillin, benzylpenicillin and phenylacetylated 6-APA were located by placing dried chromatograms on nutrient agar seeded with a spore suspension of *B. subtilis* ATCC 6633 and incubated at 37°. The appearance on only the phenylacetylated chromatogram of an antibacterial zone at the R_F given below was taken to indicate the presence of 6-APA. Solutions of 6-APA in M/20, pH 7, phosphate buffer were also chromatographed alongside samples of reaction mixtures so as to provide standards for the estimation of 6-APA. On the phenylacetylated chromatograms a solution of 6-APA 5 μg./ml. yielded an inhibition zone 14–15 mm. wide at an R_F of about 0·44 in butanol + acetic acid and 16–18 mm. wide at an R_F of about 0·12 in butanol + ethanol. Under these conditions 6-APA 1 μg./ml. could be detected. Phenylacetylated chromatograms of the benzylpenicillin and ampicillin solutions showed no zones of 6-APA. Benzylpenicillin had an R_F of 0·39 in butanol + ethanol and ampicillin an R_F of 0·74 in butanol + acetic acid.

Detection of penicilloic acids. Test samples (6–30 μl.), containing about 3 μg. penicilloic acid were applied to sheets of Whatman No. 1 chromatography paper and 3 μg. of the penicilloic acids of ampicillin, benzylpenicillin and 6-APA were also treated in the same way, as standards. The penicilloic acids were prepared in two ways. In the first method 5 mg. of penicillin or 6-APA in 5 ml. of water was treated with 0·75 ml. of 5 N-NaOH for 30 min. at 37°, after which the solution was neutralized with 0·75 ml. of 5 N-HCl and the volume made up to 10 ml. with water. In the second method 5 mg. of penicillin or 6-APA in 5 ml. M/20 phosphate buffer (pH 7) was treated for 30 min. at 37° with 1 ml. of the supernatant fluid of a penicillinase (β-lactamase)-producing culture of *Bacillus cereus*, after which the volume was adjusted to 10 ml. The chromatograms were developed with a butanol + pyridine solvent (*n*-butanol + pyridine + water, 1 + 1 + 1 v/v) overnight at 5° after which they were dried at 40° and sprayed with the starch + iodine + acetic acid spray described by Thomas (1961), to reveal white penicilloic acid zones on a dark background. The approximate R_F values of the various compounds in the above solvent system were:

Benzylpenicillin	0·75	Ampicillin penicilloic acid	0·37
Ampicillin	0·62	6-APA penicilloic acid	0·28
Benzylpenicilloic acid	0·51		

RESULTS

Factors affecting penicillin acylase formation by Escherichia coli c 975

The results given in Table 1 illustrate the effect of cultural conditions on the formation of penicillin acylase in *Escherichia coli* c 975, one of the clinical strains of bacteria examined. Under the most favourable conditions, namely, growth at 26° in a shaken flask of Yeatex medium containing phenylacetic acid, all the available benzylpenicillin was converted to 6-APA. It can be seen that the cultural conditions had a significant effect on the amount of growth, but enzyme formation was not necessarily proportional to bacterial growth. For example, the shaken cultures grown in nutrient broth at 26° and at 37° produced similar quantities of bacteria, but the penicillin acylase activity of the culture grown at 26° was at least a hundred-fold greater than that of the culture grown at 37°. Likewise, the growth-promoting effect of phenylacetic acid did not account entirely for the observed increase in enzyme activity.

Table 1. *Effect of cultural conditions on the production of penicillin acylase by a clinical isolate of Escherichia coli c 975*

E. coli c 975 was grown for 18 hr under the conditions shown below, after which benzylpenicillin to a final concentration of 1 mg./ml. was added to each flask. The cultures were incubated at 30° for 4 hr, when the concentration of 6-APA was measured.

	Cultural conditions			Growth: dry wt. bacteria (mg./ml.)	Enzyme activity: 6-APA (μg./ml.)
Basal medium	Temperature	Aeration	0·15 % phenylacetic acid		
2·5 % nutrient broth pH 7·0	37°	Static	Absent	0·38	Trace
	37°	Shaken	Absent	1·13	Trace
	26°	Static	Absent	0·33	18
	26°	Shaken	Absent	1·39	100
	26°	Shaken	Present	2·14	515
2·5 % Yeatex pH 7·0	26°	Shaken	Present	3·6	635

Distribution of minimal inhibitory concentrations

The organisms tested for sensitivity to ampicillin and benzylpenicillin, together with the respective minimal inhibitory concentrations, are shown in Table 2. Because the organisms used in this work were predominantly resistant to the penicillins the proportion sensitive to the penicillins in these tests was smaller than is typical of strains encountered routinely in clinical practice (Sutherland & Rolinson, 1964; Sutherland & Batchelor, 1964).

Penicillin acylase activity in penicillin sensitivity tests

In the sensitivity tests with 148 clinical cultures of bacteria, 133 strains (90 %) destroyed benzylpenicillin and 91 strains (61 %) caused some destruction of ampicillin. However, only one culture, *Escherichia coli* 1 187, showed any penicillin acylase in these tests. With this culture a trace of 6-APA (< 1 μg./ml.) was detected in the test with benzylpenicillin but none was found with ampicillin. The conditions of pH (alkaline) and temperature (37°) prevailing in these sensitivity tests were favourable for penicillin acylase activity (Rolinson *et al.* 1960), hence lack of enzyme activity in these tests was due to absence of the enzyme.

Table 2. *Distribution of minimal inhibitory concentrations of ampicillin and benzylpenicillin against 148 strains of Gram-negative bacteria*

Organism	No. of strains	Penicillin	Minimal inhibitory concentrations (μg./ml.) and number of strains inhibited									
			> 500	500	250	125	50	25	12·5	5·0	2·5	1·25
Escherichia coli	51	Ampicillin	4	2	3	1	4	3	2	14	15	3
		Benzylpenicillin	10	1	2	3	22	9	2	2	.	.
Aerobacter aerogenes	17	Ampicillin	5	3	2	.	3	.	2	.	1	1
		Benzylpenicillin	8	2	2	3	.	1	1	.	.	.
E. coli (intermediate) and paracolon	18	Ampicillin	8	2	.	1	.	.	5	.	1	1
		Benzylpenicillin	10	1	.	.	3	3	.	1	.	.
Proteus mirabilis	16	Ampicillin	8	.	.	.	.	.	.	1	.	7
		Benzylpenicillin	8	.	.	.	.	.	.	1	6	1
P. morganii	7	Ampicillin	.	.	2	5	.	.	.	.	.	.
		Benzylpenicillin	6	.	.	1	.	.	.	.	.	.
P. rettgeri	8	Ampicillin	2	1	3	1	1	.	1	.	.	.
		Benzylpenicillin	8	.	.	.	.	.	.	.	.	.
P. vulgaris	11	Ampicillin	4	4	.	.	2	.	1	.	.	.
		Benzylpenicillin	7	1	.	3	.	.	.	.	.	.
Pseudomonas pyocyanea	13	Ampicillin	13	.	.	.	.	.	.	.	.	.
		Benzylpenicillin	13	.	.	.	.	.	.	.	.	.
Salmonella sp.	5	Ampicillin	.	.	.	.	.	.	.	3	2	.
		Benzylpenicillin	.	.	.	.	.	.	5	.	.	.
Shigella sp.	2	Ampicillin	.	.	.	.	.	.	.	2	.	.
		Benzylpenicillin	.	.	.	.	2	.	.	.	.	.
Total	148	Ampicillin	44	12	9	8	10	3	11	20	19	12
		Benzylpenicillin	70	5	4	10	27	13	8	4	6	1

Table 3. *Effect of cultural conditions on penicillin acylase activity of Gram-negative bacteria*

The minimal inhibitory concentrations of ampicillin and benzylpenicillin were determined after incubation overnight at 37°. Penicillin destruction was measured by microbiological assay of residual penicillin content and penicillin acylase activity was determined by measurement of 6-APA. In the shaken culture test for production of penicillin acylase activity under favourable conditions the cultures were shaken overnight at 24° in Yeatex medium containing 0·1 % phenylacetic acid. Penicillin (1 mg./ml.) was added and the cultures were shaken at 30° for 4 hr after which the 6-APA content was measured.

	Benzylpenicillin					Ampicillin				
	Shaken culture test for penicillin acylase		Serial-dilution test			Shaken culture test for penicillin acylase		Serial-dilution test		
Strain	6-APA (μg./ml.)	Residual penicillin (μg./ml.)	Minimal inhibitory concentration (μg./ml.)	Penicillin destruction	Penicillin acylase activity 6-APA (μg./ml.)	6-APA (μg./ml.)	Residual penicillin (μg./ml.)	Minimal inhibitory concentration (μg./ml.)	Penicillin destruction	Penicillin acylase activity 6-APA (μg./ml.)
E. coli I 187	105	0	50	±	Trace*	> 200	> 200	5	±	0
E. coli C 975	90	0	50	+	0	> 200	> 200	5	−	0
E. coli T 432	> 200	0	12·5	−	0	> 200	190	1·25	−	0
E. coli I 199	> 200	> 200	25	+	0	180	> 200	5	±	0
P. rettgeri A	> 200	68	> 500	+	0	32	> 200	50	±	0
P. rettgeri C	> 200	> 200	> 500	+	0	24	> 200	> 500	±	0
P. rettgeri D	> 200	0	> 500	+	0	32	> 200	250	±	0
P. rettgeri F	Trace	> 200	> 500	+	0	3	> 200	12·5	±	0
P. rettgeri G	> 200	68	> 500	+	0	37	> 200	500	±	0
P. rettgeri I	> 200	0	> 500	+	0	100	> 200	> 500	±	0
E. coli NCIB 8742†	> 200	0	50	±	0	> 200	170	5	±	0
E. coli NCIB 8744†	80	> 200	25	±	0	25	> 200	5	−	0
E. coli BRL 1009†	> 200	1	12·5	±	0	> 200	42	5	−	0
E. coli NCIB 8741†	> 200	0	50	±	0	> 200	70	5	±	0
A. faecalis BRL 1238†	> 200	> 200	12·5	±	Trace	160	> 200	12·5	±	0
A. faecalis BRL 1237†	> 200	130	25	±	1	200	> 200	25	−	0
A. faecalis NCTC 415†	28	200	50	±	2	32	> 200	25	±	0
A. faecalis NCIB 8156†	3	0	125	±	2	5	0	250	±	Trace
Achr. arsenoxydans NCIB 8687†	12	190	12·5	−	0	0	150	12·5	−	0
Ps. pyocyanea BRL 1058†	Trace	> 200	> 500	+	0	3	> 200	> 500	±	Trace

\+ = complete destruction; ± = partial destruction; − = no destruction.

† Known penicillin-acylase producing bacteria of non-clinical origin.

* In a repeat test no 6-APA was detected and no residual benzylpenicillin was found.

Formation of penicillin acylase by clinical strains of Gram-negative bacteria in shaken flask cultures

Of the 148 clinical strains of Gram-negative bacteria tested in the shaken flask test with Yeatex medium containing phenylacetic acid, ten strains were found to show penicillin acylase activity (see Table 3). Most of these strains formed large amounts of 6-APA from benzylpenicillin and ampicillin but the ratio of penicillin acylase activities against the two substrates varied from culture to culture. One of the reasons for this is the simultaneous production of penicillin acylase and β-lactamase in some cultures. For example, with benzylpenicillin as substrate, far less than the theoretical maximum yield of 6-APA (viz. 608 μg./ml.) was obtained from cultures of *Escherichia coli* I 187 and C 975, although the amount of residual benzylpenicillin in the reaction mixture had fallen to zero, suggesting that some other penicillin-destroying factor, such as β-lactamase, was operating. However, when ampicillin was used as substrate for these two cultures, the yields of 6-APA were greater and residual ampicillin was present presumably because it was not destroyed by β-lactamase. These results are consistent with the greater stability of ampicillin to penicillinase (β-lactamase) activities of certain Gram-negative bacteria (Ayliffe, 1963; Percival, Brumfitt & de Louvois, 1963; Smith, 1963; Sutherland, 1964). Where the penicillin acylase activity of cultures was not complicated by the presence of β-lactamase, as evidenced by the presence of substantial quantities of residual benzylpenicillin, viz. *Escherichia coli* I 199 and *Proteus rettgeri* C, benzylpenicillin was a better substrate for penicillin acylase than ampicillin. Cultures of *E. coli* T 432 and *P. rettgeri* A, D, G and I, incubated with benzylpenicillin contained more than 200 μg./ml. 6-APA, and low levels of residual penicillin, suggesting that penicillin acylase was the predominant enzyme, although the presence of some β-lactamase activity cannot be ruled out. Of these cultures, *E. coli* T 432 would appear to be the most active penicillin acylase producer judging by the high yield of 6-APA obtained from ampicillin.

The results in Table 3 also include data obtained with these cultures in the serial-dilution sensitivity tests. Although all ten strains produced penicillin acylase in the shaken culture test, this enzyme was absent in serial-dilution tests except in the case of *Escherichia coli* I 187 for which a trace of 6-APA was detected. These cultures were also examined in the serial-dilution tests for the presence of penicilloic acids. In all cultures where there was significant destruction of the penicillin, the cognate penicilloic acid was detected signifying β-lactamase activity. In no case was the penicilloic acid of 6-APA observed.

Comparative tests with known penicillin acylase-producing bacteria

The yields of 6-APA produced by ten strains of saprophytic bacteria in penicillin acylase-producing shaken culture tests are shown in Table 3. Comparison of the yields of 6-APA with the amount of residual benzyl penicillin or ampicillin showed that two strains of *Alcaligenes faecalis* (BRL 1237 and BRL 1238) produced high penicillin acylase activity and in view of the high residual penicillin values, little or no β-lactamase activity. Three strains of *Escherichia coli* (NCIB 8742, NCIB 8743 and BRL 1009) also produced high amounts of penicillin acylase but as the residual benzyl-

penicillin values were of a low order it is possible that β-lactamase was also present. The remaining bacteria demonstrated lower penicillin acylase activity and with *A. faecalis* NCIB 8156, which produced only 6-APA 3–5 μg./ml., substantial β-lactamase activity was present.

In serial-dilution tests with these cultures, penicillin acylase activity was not observed with any of the strains of *Escherichia coli*. Small amounts of 6-APA were detected with *Alcaligenes faecalis* in the tests with benzylpenicillin and only one of these strains produced 6-APA in the test with ampicillin. A trace of 6-APA was found in the culture of *Pseudomonas pyocyanea* BRL 1058 incubated with ampicillin. In no instance did the 6-APA measured account for all of the penicillin destruction observed in the sensitivity tests indicating that β-lactamase activity was also present.

Destruction of penicillins by Escherichia coli NCIB *9464, NCIB 9465 and* NCIB *9466*

Cultures of *Escherichia coli* NCIB 9464 NCIB 9465 and NCIB 9466, reported by Holt & Stewart (1964*a*, *b*) to produce 6-APA from penicillins, were obtained from the National Collection of Industrial Bacteria. All three cultures were resistant to

Table 4. *Destruction of penicillins by Escherichia coli* NCIB *9464, NCIB 9465 and* NCIB *9466 in sensitivity tests*

Minimal inhibitory concentrations of benzylpenicillin and 6-APA required to inhibit the growth of *E. coli* NCIB 9464, NCIB 9465, and NCIB 9466 for 18 hr at 37° were determined by serial dilution in nutrient broth. After overnight incubation, residual antibiotic activity was measured by microbiological assay, and 6-APA and penicilloic acid production in the benzylpenicillin sensitivity tests was determined by chromatography

Strain	Penicillin	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.						
		500*	250	125	50	25	12·5	5·0
Escherichia coli NCIB 9464	Benzylpenicillin	+	+	+	+	+	+	+
		0†	0	0	.	.	.	.
	6-APA	−	−	−	+	+	+	+
		.	.	.	36	16	10	4·2
E. coli NCIB 9465	Benzylpenicillin	+	+	+	+	+	+	+
		0†	0	0	.	.	.	.
	6-APA	−	−	−	+	+	+	+
		.	.	.	33	18	7·6	3·8
E. coli NCIB 9466	Benzylpenicillin	−	+	+	+	+	+	+
		360	0†	0	.	.	.	.
	6-APA	−	−	−	+	+	+	+
		.	.	.	38	19	6·1	3·8

+ = growth; − = no growth.

* The concentrations represent the initial concentrations (μg./ml.) of penicillins prior to incubation with the cultures.

† Chromatographic examination of these cultures showed large zones of benzylpenicilloic acid and no trace of 6-APA.

benzylpenicillin in serial-dilution tests and brought about complete destruction of the penicillin in these tests (Table 4). Chromatographic examination of these cultures failed to show the presence of 6-APA but, did show benzylpenicilloic acid.

Destruction of benzylpenicillin in these serial-dilution tests was due therefore to penicillinase (β-lactamase) activity. In contrast, 6-APA was highly stable to these cultures of *E. coli*, there being little or no destruction of the compound in the serial-dilution tests (Table 4). Moreover, when these strains of *E. coli* were grown under conditions favourable for penicillin acylase production as described in the Methods, no trace of penicillin acylase activity was detected in reactions with benzylpenicillin. However, chromatographic examination of the reaction mixtures showed the presence of benzylpenicilloic acid, indicating β-lactamase activity.

As the three strains of *Escherichia coli* failed to show penicillin acylase activity in the tests described above, *E. coli* NCIB 9465 was cultured and tested under the conditions reported by Holt & Stewart (1964*b*). Thus, the organism was grown for 48 hr at 37° in nutrient broth containing benzylpenicillin 100 μg./ml., but after incubation of the 48-hr old culture with benzylpenicillin at 40°, pH 5·5, no trace of 6-APA was detected. Instead, chromatographic examination of the reaction mixtures at pH 5·5 showed the presence of large amounts of benzylpenicilloic acid and a small amount of benzylpenilloic acid, the the decarboxylation product of benzylpenicilloic acid (Pl. 1). Similarly, a dialysed, freeze-dried specimen of enzyme prepared from *E. coli* NCIB 9465 as described by Holt & Stewart (1964*b*) failed to show penicillin acylase activity, but destroyed benzylpenicillin as a result of β-lactamase activity.

DISCUSSION

In the experiments described here many of the strains of Gram-negative bacteria used were resistant to ampicillin and benzylpenicillin and were capable of destroying these penicillins in the serial-dilution sensitivity tests, but in only one case, a strain of *Escherichia coli* I 187, was there any evidence of penicillin acylase activity, namely the production of 6-APA from benzylpenicillin. In this series of tests, therefore, penicillin acylase activity was not a significant factor in the penicillin resistance shown by these cultures. Even with *E. coli* I 187, in which penicillin acylase was present, penicillin destruction was due primarily to β-lactamase activity.

It has been our previous experience in this laboratory that production of penicillin acylase by non-clinical strains of *Escherichia coli* is favoured by low temperature, vigorous aeration and the addition of phenylacetic acid to the medium. The results reported here indicate that these cultural conditions are also favourable for the production of penicillin acylase by bacteria of clinical origin. Even so, out of 148 cultures of clinical bacteria tested, only ten cultures, four strains of *E. coli* and six strains of *Proteus rettgeri*, converted ampicillin and benzylpenicillin to 6-APA under these conditions. It is evident, therefore, that only a relatively small number of Gram-negative bacteria of clinical origin are capable of producing penicillin acylase. Moreover, these results confirm that the conditions prevailing in sensitivity tests where the bacteria are cultured at alkaline pH at 37° in stationary culture, although favourable for the functioning of the enzyme (Rolinson *et al.* 1960), are most unfavourable for penicillin acylase formation. Hence, most strains of penicillin acylase-producing bacteria are unable to produce the enzyme in serial-dilution tests, but may instead bring about destruction of penicillins by β-lactamase activity.

The resistance of bacteria to penicillins as a result of penicillin acylase activity

has been described by English *et al.* (1960) but few details of experimental conditions or extent of occurrence were given. More recently, Holt & Stewart (1964*a*, *b*) have reported penicillin acylase activity to play a significant role in bacterial resistance to penicillins and have described the occurrence of penicillin acylase activity in 40% of clinical strains of coliform bacteria grown in normal nutrient media at 37°. However, we have been unable to demonstrate penicillin acylase activity with cultures of *Escherichia coli* NCIB 9464, NCIB 9465 and NCIB 9466 reported by Holt & Stewart (1964*a*, *b*) to be active penicillin acylase-producing cultures. On the contrary, we have found that the inactivation of penicillin by these strains was due solely to β-lactamase activity with the formation of the cognate penicilloic acid.

The reason for the striking discrepancy between the results reported here with *Escherichia coli* NCIB 9464, NCIB 9465 and NCIB 9466 and those reported by Holt & Stewart (1964*a*, *b*) would appear to be due to the fact that Holt & Stewart classified as penicillin acylase-producing bacteria, cultures which inactivated benzylpenicillin but not 6-APA. However, it is known that 6-APA is usually more stable to the penicillinases (β-lactamases) of bacteria than is benzylpenicillin (Percival, Brumfitt & de Louvois, 1963; Smith & Hamilton-Miller, 1963). Hence, β-lactamase producing strains of *E. coli* might well destroy benzylpenicillin much more rapidly than 6-APA and this is the case with *E. coli* NCIB 9464, NCIB 9465 and NCIB 9466. The most satisfactory way of detecting the presence of penicillin acylase is by demonstrating the product of the reaction, 6-APA. This is most conveniently done by detecting the 6-APA on chromatograms of reaction mixtures by making use of the unique property of conversion of 6-APA to a biologically-active substance after treatment with phenylacetyl chloride. The incidence of penicillin acylase among clinical strains of *E. coli* reported by Holt & Stewart (1964*a*, *b*) must be regarded as being erroneously high.

There seems little doubt that in typical antibiotic sensitivity tests with clinical strains of Gram-negative bacteria, penicillin acylase activity is seldom seen, not only because of the limited number of cultures capable of producing 6-APA from ampicillin or benzylpenicillin, but also because of the unfavourable cultural conditions in serial-dilution tests for the production of penicillin acylase by bacteria. Penicillin acylase activity is thus not a factor normally responsible for resistance of Gram-negative bacteria to ampicillin and benzylpenicillin.

We are grateful to Mrs M. V. Hart and Mrs E. Fairclough for skilled technical assistance and to Dr G. N. Rolinson for helpful advice and criticism.

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EXPLANATION OF PLATE

Chromatogram showing the degradation of benzylpenicillin by *Escherichia coli* NCIB 9465

Chromatography system: *n*-butanol + pyridine + water, 1 + 1 + 1 v/v. Descending. Whatman No. 1. Run overnight at 5°. Starch + iodine spray. Samples 1–5 applied at two spots/origin, i.e. 12–16 μl. Reaction mixtures 1–5 consisted of 1 vol. of sodium benzylpenicillin 3000 μg./ml. in 0·2 M-acetate buffer (pH 5·5) plus 5 vol. of one of the following, adjusted to pH 5·5 and incubated for 5 hr at 37°.

1. 40-hr culture of NCIB 9465 grown statically at 37° in nutrient broth containing benzylpenicillin 100 μg./ml.

2. *E. coli* centrifuged from 1 vol. of above culture and resuspended in 1 vol of 0·2 M-acetate buffer (pH 5·5).

3. Filtrate obtained after centrifuging and membrane filtering above culture.

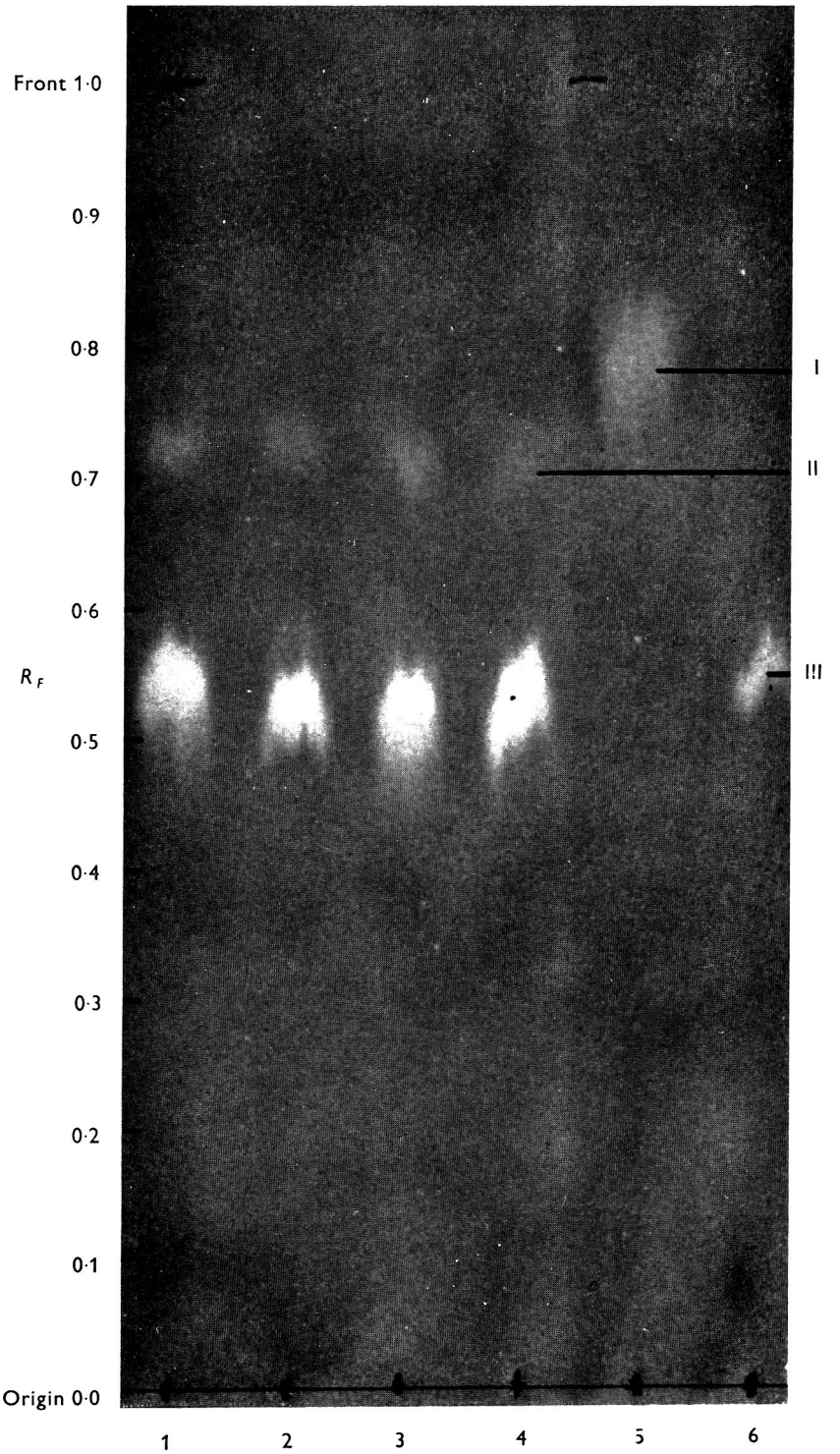
4. Freeze-dried material prepared as in 3 and reconstituted at double original concentration.

5. 0·2 M-acetate buffer (pH 5·5).

6. Benzylpenicilloic acid 500 μg./ml. prepared by treatment of benzylpenicillin with β-lactamase obtained from *B. cereus* (one spot/origin).

I = benzylpenicillin, II, = benzylpenilloic acid, III, = benzylpenicilloic acid.

Butanol + ethanol + water chromotograms of reaction mixtures 1–5 did not reveal any 6-APA after phenylacetylation and contacting with agar seeded with *B. subtilis*. A 6-APA zone was detected for a 5 μg./ml. 6-APA solution.



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Thiosulphate Metabolism and Rhodanese in *Chromatium* sp. strain D

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SUMMARY

Suspensions of Chromatium D oxidized thiosulphate completely to sulphate (in the light) under anaerobic conditions in the presence of carbon dioxide. During the oxidation, intracellular sulphur accumulated transiently and sulphate production followed a biphasic pattern. Using inner-^{35}S-thiosulphate, the initial burst of sulphate production, which accounted for about half of the total yield, occurred principally at the expense of the inner (SO_3-) atom of thiosulphate; there was no intracellular accumulation of labelled sulphur. The radioactivity from outer-^{35}S-thiosulphate accumulated transiently within the organisms and was transferred to sulphate at a rate which was similar to the second phase of sulphate production; most of the outer (S-) atom therefore passed through the stage of endogenous sulphur. Extracts of the organism catalysed the cyanolysis of thiosulphate to give sulphite and thiocyanate. An assay for this enzyme, rhodanese, based on the spontaneous reduction of 2,6-diehlorophenol-indophenol by sulphite was developed. The enzyme was also detected in extracts of Athiorhodaceae. The enzyme was partially purified from extracts of Chromatium D and resolved into two active fractions. It is concluded that endogenous sulphur is an intermediate in the oxidation of thiosulphate by Chromatium D and that the cleavage of the S—S bond in the molecule is a key step in the oxidation process. Rhodanese, which catalyses this type of reaction, may be concerned in this cleavage.

INTRODUCTION

Photosynthetic sulphur bacteria assimilate carbon dioxide into cell material under anaerobic conditions in the light with the concomitant oxidation of a variety of reduced inorganic sulphur compounds to sulphate or to an intermediate oxidation product such as sulphur (van Niel, 1931; Larsen, 1952, 1953). The ability of purple sulphur bacteria (Thiorhodaceae) to accumulate globules of sulphur intracellularly during autotrophic growth on sulphide and thiosulphate is a characteristic feature of these organisms (van Niel, 1931, 1936; Eymers & Wassink, 1937). The role of endogenous sulphur in the oxidation of thiosulphate by Thiorhodaceae is uncertain.

The oxidation of inorganic sulphur compounds has been studied more intensively with thiobacilli which oxidize them under aerobic conditions in the dark to sulphate or intermediate oxidation products such as polythionates and elementary sulphur. The accumulation of extracellular sulphur during such processes has been variously attributed to biological reactions (Starkey, 1935; Peck, 1960) and to spontaneous

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reactions (Tamiya, Haga & Huzisige, 1941; Vishniac, 1952; Rittenberg & London, 1964). The results of experiments both with whole organisms and with extracts has led to two differing schools of thought concerning the mechanism of the initial attack on thiosulphate:

(1) the oxidative coupling of two molecules to give tetrathionate (Trudinger, 1958, 1959; Vishniac & Santer, 1957; Rittenberg & London, 1964);

(2) the reductive cleavage of one molecule to give sulphide and sulphite (Peck, 1960; Peck & Fisher, 1962).

In the present work, the metabolism of thiosulphate and other inorganic sulphur compounds has been studied in the purple sulphur bacterium Chromatium strain D to establish the mechanism of thiosulphate oxidation and the role of intracellular sulphur in this process. In the course of this work thiosulphate sulphur-transferase activity was demonstrated in extracts. This enzyme catalyses the cyanide-dependent cleavage of thiosulphate to form thiocyanate and sulphite (equation 1) and has been extensively studied with purified preparations from mammalian sources such as liver and kidney (Green & Westley, 1961; Westley & Nakamoto, 1962). In the presence of reduced lipoic acid ($Lip(SH)_2$) the enzyme catalyses the reductive cleavage of thiosulphate (equation 2); lipoyl persulphide is an intermediate in this reaction (Villarejo & Westley, 1963*a*, 1963*b*).

$$S_2O_3^{2-} + CN^- \rightarrow SO_3^{2-} + CNS^-, \qquad (1)$$

$$S_2O_3^{2-} + Lip(SH)_2 \rightarrow SO_3^{2-} + Lip\begin{matrix}\diagup SSH \\ \diagdown SH\end{matrix} \rightarrow Lip(S)_2 + S^{2-}. \qquad (2)$$

Thiosulphate sulphur-transferase EC 2.8.1.1. (which will be referred to as rhodanese) has been partially purified from Chromatium D and its possible relevance to thiosulphate metabolism has been examined. This work has been briefly reported elsewhere (Smith, 1964, 1965).

METHODS

Organisms. The culture of *Chromatium* sp. strain D and its maintenance as stab cultures on the thiosulphate + salts medium have been previously described (Hurlbert & Lascelles, 1963). This organism was used throughout these studies except in the investigations with extracts where the Athiorhodaceae, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* were also used. These organisms were grown anaerobically in the light on the malate + glutamate medium described by Lascelles (1959) fortified in the case of *Rhodospirillum rubrum* with yeast extract (0·2%, w/v).

Media. The thiosulphate + bicarbonate + salts medium used for the growth of Chromatium D was that described by Hurlbert & Lascelles (1963). In some experiments organic substrates were used in place of thiosulphate, as described previously.

Growth of cultures. In preparing organisms for experiments with suspensions and cell-free extracts the inoculum was taken from a stab culture or from a liquid culture for volumes in excess of 1 l. (approximately 1 ml. of liquid culture/100 ml. of medium). The cultures were incubated in completely filled glass-stoppered bottles at 34° in a light cabinet fitted with banks of 15 W. tungsten lamps. The organisms were free of endogenous sulphur and fully grown after 2–4 days.

For the quantitative investigation of growth on various inorganic sulphur compounds, sulphide (0·01 %, w/v) and thiosulphate (up to 0·3 %, w/v) were omitted from the complete salts medium and replaced by the appropriate sulphur compound The inoculum, taken from a liquid culture, gave an initial concentration equiv. 30 mg. protein/l.; for growth on endogenous sulphur, the initial concentration equiv. was 70 mg. protein/l. The inoculated medium was incubated in Roux bottles under oxygen-free nitrogen in a glass-sided water bath at 34° illuminated with 150 W. tungsten lamps 15 cm. from the bath; the light intensity was approximately 150 ft.c. at the surface of the vessel. The densities of the cultures were measured at the end of growth as protein determined by the biuret method described by Hurlbert & Lascelles (1963). The protein:dry weight ratios of organisms grown on various inorganic sulphur compounds were in the range 0·5–0·6.

Preparation and incubation of suspensions. Organisms were normally harvested from the thiosulphate + salts medium at the end of growth when largely free of endogenous sulphur by centrifuging at 2500 *g* for 15 min., washed twice in about one-fifth the original culture volume of 0·04 M-potassium phosphate buffer (pH 7·5) and resuspended in 0·1 M-potassium phosphate buffer (pH 6·75) containing sodium bicarbonate (60 mM) and sodium thiosulphate (2–40 mM). Suspensions were incubated at 34° and gassed continuously with N_2 + 5 % CO_2; the final pH was 7·3. For investigations at low intensities of illumination, suspensions containing up to 2·0 mg. protein/ml. were incubated in flat-sided glass bottles in a glass-sided water bath as described above. Incubations at higher intensities of illumination (up to 500 ft.c. at the level of the suspension) were made in a photosynthetic Warburg tank (American Instrument Co., Inc., Washington, D.C.) illuminated from below with a bank of twelve 100 W. tungsten lamps coupled to a rheostat. Suspensions containing 0·2–0·5 mg. of protein/ml. were incubated in this bath in flat-bottomed conical flasks shaken transversely at 100 cyc./min. with an amplitude of 4 cm.

Preparation of extracts. Organisms were normally harvested at the end of growth when largely free of endogenous sulphur by centrifuging at 2500 *g* for 15 min., washed twice in one-tenth the original culture volume of 0·05 M-potassium phosphate buffer (pH 6·1) and resuspended in the same buffer at concentrations to 50 mg. protein/ml. Crude extracts were prepared by the French press or by ultrasonic treatment (Hurlbert & Lascelles, 1963). They were centrifuged at 18,000 *g* for 20 min. to remove whole organisms and debris. Cell breakage and all subsequent fractionations were done at 0–5°; extracts and extract fractions were stored at −20°.

Assay for rhodanese. The reaction mixture contained, in a volume of 3 ml.: tris buffer (pH 8·7), 300 μmole; sodium thiosulphate, 150 μmoles; half neutralized sodium cyanide (NaCN/HCl, 2·5/1., mole/mole), 150–240 μmoles; 2,6-dichlorophenol-indophenol (DCIP), 0·5 μmole; *N*-methylphenazoniummethosulphate (PMS), 0·25 mg.; enzyme preparation equivalent to 0·5 mg. of crude extract protein. The reaction was initiated by the addition of cyanide to the otherwise complete reaction mixture. The reference cuvette contained the complete reaction mixture without thiosulphate. DCIP reduction was followed at room temperature (18–20°) using a recording double-beam grating spectrophotometer (Optica type CF4DR Optica U.K. Ltd., Gateshead-on-Tyne, England). The millimolar extinction coeffi-

cient of DCIP at 600 mμ is 19·1 (Basford & Huennekens, 1955). The unit of enzyme activity was defined as the amount of enzyme which catalysed the reduction of 30 mμmole of DCIP/min.

Purification of rhodanese. Particle-bound pigment was removed from the extract prepared from 12 l. of fully grown culture by centrifuging at 100,000 *g* for 60 min. in a Spinco model L ultracentrifuge. The supernatant fraction was treated with 5% (w/v) streptomycin sulphate (Glaxo Laboratories Ltd., Greenford, England) in 0·04 M-potassium phosphate buffer (pH 6·1) to a final concentration of 0·6 mg. of streptomycin sulphate/mg. extract protein. Precipitated nucleic acid was removed after 15 min. by centrifuging at 18,000 *g* for 20 min. Protein was precipitated from the supernatant fluid with saturated ammonium sulphate (pH 7·0) at a final concentration of 90% saturation (by volume). After 30 min., the precipitated protein was removed by centrifuging at 18,000*g* for 20 min., and was redissolved in a minimum amount of 0·04 M-potassium phosphate buffer (pH 6·1). The concentrated protein fraction was applied to a column (35 × 1·5 cm.) of Sephadex G75 (Pharmacia) and eluted with 0.01 M-potassium phosphate buffer (pH 6·1). The enzyme recovered from the column was transferred to a column (10 × 1·5 cm.) of DEAE-DE50 cellulose (Whatmans.). Protein was eluted with potassium phosphate buffer (pH 6·1) using a concentration gradient (10–500 mM); the flow rate was 2 ml./min. and the eluate was collected in 5 ml. fractions.

Analytical methods. With crude extracts, protein was precipitated and pigments were removed by heating the samples to 65° with 5 ml. of acid ethanol (400 ml. of ethanol, 5 ml. of glacial acetic acid and 100 ml. of water). The extraction procedure was repeated at least twice. The protein suspension was collected by centrifuging and the protein determination carried out as described by Hurlbert & Lascelles (1963). Protein determinations at all stages of extract fractionation after the removal of pigments from the crude extract were made by u.v. absorption (Layne, 1957).

The concentrations of thiosulphate and sulphate in suspensions were determined using samples of the supernatant fluid after the organisms had been removed by centrifuging. Thiosulphate was determined titrimetrically with standardized iodine, and by the methods of Schöön (1959) and Sorbo (1957). Sulphate was determined by the method of Gleen & Quastel (1953).

An approximate estimate of the accumulation of sulphur within the organisms in a suspension oxidizing thiosulphate was made by following the change in turbidity of the suspension with an EEL photoelectric colorimeter with a neutral density filter (Evans Electroselenium Ltd., Halstead, Essex, England.).

Phosphate was determined by the method of Taussky & Shorr (1953).

Measurement of ^{35}S in the organisms and sulphate in suspensions. For determination of radioactivity in the organisms, samples of suspension containing 0·06 mg. protein were drawn through cellulose plastic filters under vacuum (Millipore filters of pore size 0·65 μ held in a Pyrex microanalysis filter-holder; Millipore Filter Corporation, Bedford, Mass., U.S.A.). The organisms on the filter were washed with water. Samples for the determination of ^{35}S in the sulphate were centrifuged to remove the organisms. Supernatant fluid containing up to 6 μmole sulphate was made up to 9 ml. with water and mixed with 1 ml. of reagent (15 g. of barium chloride and 4·8 ml. of concentrated hydrochloric acid in 100 ml. of water); samples of the sus-

pension containing up to 0·7 mg. dry weight of barium sulphate were drawn through cellulose plastic filters under vacuum and washed with water.

The filter pads containing organisms and barium sulphate were dried at room temperature and glued to aluminium foil planchets. Samples were counted with an end-window Geiger–Müller tube (EHM25, General Electric Co., England) in a lead castle coupled to an Ecko Automatic Scaler (type N530D, Ecko Electronics Ltd., England). Self-absorption was negligible and the counts were corrected for background. The efficiency of counting was approximately 3·5%.

Chemicals. Tetrathionate was prepared by the method of Gi man (1946).

The sodium salts of inorganic sulphur compounds were used throughout these studies.

^{35}S-thiosulphates were obtained from the Radiochemical Centre (Amersham, Bucks., England). The original specific activities were in the range 10–20 mc./μmole.

RESULTS

Growth of Chromatium D

Autotrophic growth of the organism was supported by sulphide, sulphur, thiosulphate and sulphite (Table 1); intracellular sulphur accumulated transiently during growth on sulphide and thiosulphate and such endogenous stores of sulphur supported the further growth of the organism on the salts medium in the absence

Table 1. *Growth of Chromatium* D *on inorganic sulphur compounds*

The basal salts medium, containing 0·5% $NaHCO_3$, was supplemented with thiosulphate, sulphide, sulphite or tetrathionate, each at a final concentration of 4 mM. Organisms that were in the stationary phase and largely free of sulphur were used as the inoculum. In the experiment showing growth on endogenous sulphur, organisms were harvested during growth on thiosulphate when packed with sulphur and used as the inoculum for sterile sulphur-free salts medium (see Methods section). Another sample of these organisms was incubated in the suspension medium at a concentration of 1·5 mg. protein/ml. for several hours; the sulphate produced was determined and used as a measure of the intracellular sulphur. Growth was estimated as the increase in protein, when the cultures had reached the stationary phase.

Sulphur compound	Growth yield (mg. protein/mmole)	Reducing capacity (electrons/molecule)
Thiosulphate	27	8
Sulphide	27	8
Sulphite	6	2
Sulphur (endogenous)	19	6
Tetrathionate	No growth	14
Tetrathionate + thiosulphate	No growth	–

of added sulphur compounds (Table 1). Tetrathionate did not support the autotrophic growth of the organism and the polythionate inhibited the growth of the organism on thiosulphate (Table 1). It also inhibited growth on organic compounds such as pyruvate and succinate.

Under autotrophic conditions the extent of growth was dependent on the amount of sulphur compound in the medium and, on a molar basis, was proportional to the number of electrons released during oxidation of the sulphur compound to sulphate (Table 1).

Oxidation of inorganic sulphur compounds by suspensions of Chromatium D

Thiosulphate oxidation. Suspensions of inorganic grown organisms in phosphate buffer and bicarbonate, at pH 7·3, oxidized thiosulphate completely to sulphate under anaerobic conditions in the light (Fig. 1). The production of sulphate from thiosulphate followed a biphasic pattern. The rapid disappearance of thiosulphate was accompanied by the rapid production of up to 60% of the theoretical yield of sulphate and the intracellular accumulation of sulphur. When thiosulphate had disappeared, sulphate was produced at a much slower rate, presumably from the endogenous sulphur which had accumulated during the initial period. Under these conditions, tetrathionate was not detected in suspensions during thiosulphate oxidation. The rates of thiosulphate disappearance and of sulphur accumulation were dependent on the intensity of illumination in the range 0–200 ft.c. Above the light saturation point, the rate of thiosulphate disappearance was strictly dependent on the density of the suspension. The kinetics of thiosulphate utilization and sulphate production by suspensions were, however, similar at both low (150 ft.c.) and high (500 ft.c.) intensities of illumination.

Table 2. *Thiosulphate oxidation by suspensions of organisms under various conditions*

Organisms grown on thiosulphate were harvested and resuspended to a density of 1·5 mg. protein/ml. in the suspension medium containing thiosulphate (2 mM). The suspension was incubated at a light intensity of 150 ft.c., with a gas phase of N_2/5% CO_2 or air/CO_2.

Conditions	Thiosulphate disappearance (μmole/hr/ml.)	Sulphate produced after 20 hr (% theoretical)
Anaerobic dark	0	0
Anaerobic light	2·9	96
Aerobic, dark	2·2	62
Aerobic, light	5·3	72

The oxidation of thiosulphate was not affected by the omission of phosphate, by the addition of organic compounds (pyruvate, 10 mM; succinate, 10 mM), or by the presence of considerable amounts of endogenous sulphur within the organisms of the suspension. Under anaerobic conditions, thiosulphate did not disappear in the absence of the physiological oxidant, bicarbonate, or in the dark. Under aerobic conditions (gas phase, air/CO_2), however, thiosulphate oxidation by suspensions was not light-dependent but was increased by illumination (Table 2).

Oxidation of other inorganic sulphur compounds. Suspensions of thiosulphate-grown organisms harvested during growth, when they contained considerable amounts of intracellular sulphur, oxidized this endogenous sulphur to sulphate under anaerobic conditions in the light. Sulphate production was accompanied by a fall in the turbidity of the suspension (Fig. 2). The fact that organisms oxidized endogenous sulphur to sulphate enabled an estimate to be made of the magnitude of sulphur accumulation during growth on thiosulphate. Determination of the sulphate produced by organisms harvested when they contained much intracellular

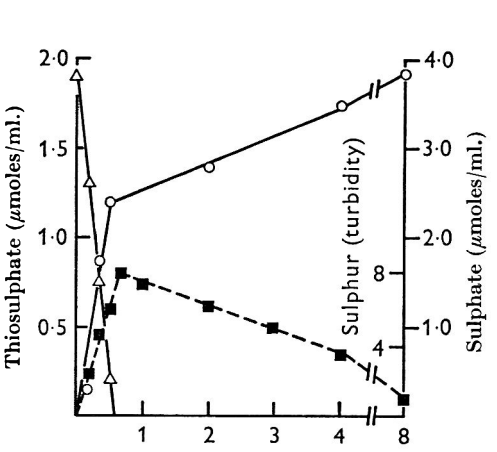

Fig. 1.

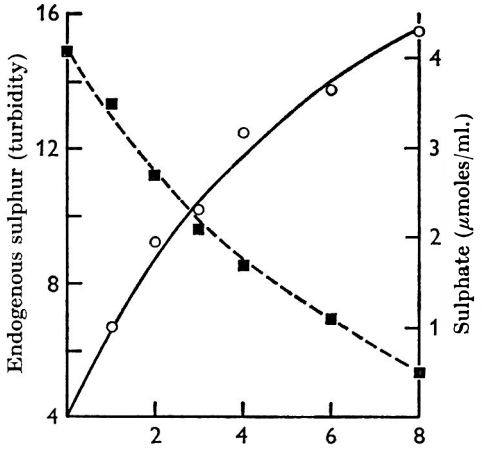

Fig. 2.

Fig. 1. Oxidation of thiosulphate by a suspension of Chromatium D. A suspension of thiosulphate-grown organisms containing 0·25 mg. of protein/ml. was incubated under anaerobic conditions at a light intensity of 500 ft.c. in the presence of thiosulphate (2 mM). Thiosulphate (—△—) disappearance, sulphur (--■--) accumulation and sulphate (—○—) production were determined, as described in the Methods section.

Fig. 2. Oxidation of endogenous sulphur. A suspension of thiosulphate-grown organisms (0·25 mg. of protein/ml.) containing endogenous sulphur was incubated under anaerobic conditions at a light intensity of 500 ft.c. Sulphur (--■--) utilization and sulphate (—○—) production were determined.

Table 3. *Oxidation of inorganic sulphur compounds by suspensions of organisms*

Suspensions of thiosulphate-grown organisms (1·5 mg. of protein/ml.) were incubated under anaerobic conditions at a light intensity of 150 ft.c. with the appropriate sulphur compound—sulphide (10 mM), sulphite (10 mM), thiosulphate (10 mM) or exogenous elementary sulphur. In the experiment showing the oxidation of endogenous sulphur, the organisms contained much intracellular sulphur when harvested. The rate of sulphite oxidation was corrected for the spontaneous reaction and the figures quoted for thiosulphate refer to the first and second phases of sulphate production.

Sulphur compound	Rate of sulphate production (μmole/hr/ml.)
Thiosulphate	6·2
	1·1
Sulphide	1·0
Sulphur (exogenous)	0·4
(endogenous)	1·4
Sulphite	5·4

sulphur showed that endogenous sulphur accounted for up to 27 % of the dry weight of the organisms.

Sulphide, exogenous sulphur and sulphite were oxidized to sulphate by the suspensions (Table 3). Sulphur accumulated intracellularly during the oxidation of sulphide but not of sulphite. The suspensions did not oxidize tetrathionate to sulphate under anaerobic conditions in the light.

Oxidation of ^{35}S-thiosulphates

The kinetics of thiosulphate utilization and of sulphate production suggested that the accumulation of intracellular sulphur represented an intermediate stage in the oxidation of thiosulphate to sulphate by the suspensions. This possibility was examined with ^{35}S-thiosulphate labelled in the inner (SO_3-) and outer (S-) atoms. The magnitude of intracellular sulphur accumulation and the origin of this sulphur was also determined by such isotopic techniques. Radioactivity was measured in the sulphate produced and in the organisms of the suspensions (as described in the Methods section).

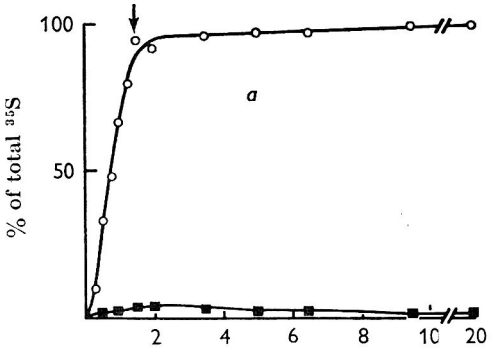

Fig. 3*a*.

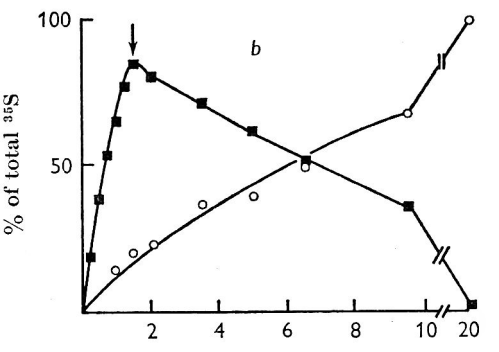

Fig. 3*b*.

Fig. 3. Labelling of organisms and sulphate in suspensions oxidizing ^{35}S-thiosulphates. Similar suspensions of thiosulphate-grown organisms containing 0·25 mg. protein/ml. were incubated under anaerobic conditions at a light intensity of 500 ft.c. in the presence of (*a*) inner (SO_3-) and (*b*) outer (S-) labelled thiosulphate (4 mM; 0·1 μc./μmole). The amounts of radioactivity in the sulphate (—○—) fraction and in the organisms (—■—) were determined. The arrow indicates the completion of thiosulphate utilization.

Oxidation of S-$^{35}SO_3^{2-}$. The radioactivity of the thiosulphate was rapidly transferred to the sulphate fraction, during thiosulphate disappearance; on the completion of thiosulphate utilization, more than 90% of the ^{35}S added originally as thiosulphate was in the sulphate fraction (Fig. 3*a*). There was negligible accumulation of radioactivity in the organisms.

Oxidation of ^{35}S-SO_3^{2-}. During thiosulphate utilization, the organisms were labelled rapidly; on the completion of thiosulphate disappearance this labelling had reached a peak value equivalent to 80% of the radioactivity added initially as thiosulphate (Fig. 3*b*). As the incubation continued, ^{35}S accumulated in the sulphate fraction at the expense of the radioactivity within the organisms.

The labelling of the organisms during the oxidation of outer-labelled thiosulphate coincided with the change in turbidity of the suspension. Turbidity changes have been used in this work as a rough measure of intracellular sulphur accumulation during thiosulphate oxidation by suspensions (Fig. 1) in place of the lengthy analytical methods used by other workers (van Niel, 1936; Trüper & Schlegel, 1964).

The thiosulphate-cleaving enzyme (rhodanese)

Crude extracts of the organism catalysed a cyanide-dependent cleavage of thiosulphate to thiocyanate and sulphite, an activity attributed to the enzyme rhodanese. This reaction did not occur with heated extracts.

Rhodanese assay. An assay for the enzyme based on the spontaneous reduction of DCIP by sulphite in the presence of PMS was developed and used to investigate the properties of the enzyme in crude extracts and to follow activity during enzyme fractionation:

$$S_2O_3^{2-} + CN^- \rightarrow CNS^- + SO_3^{2-},$$

$$SO_3^{2-} + \text{DCIP} \xrightarrow{\text{PMS}} SO_4^{2-} + \text{reduced DCIP}.$$

With partially purified pig kidney rhodanese (Westley & Green, 1959), this assay method was compared with the standard assay for the mammalian enzyme. The reaction mixture used in the conventional enzyme assay (Sorbo, 1963) was supplemented with DCIP (0·5 μmole/3 ml.) and PMS (0·25 mg./3 ml.) and dye reduction was followed spectrophotometrically. Enzyme activity using the dye reduction method was 25% lower than that given by the standard method, but the dye reduction system had the advantage of greater rapidity and sensitivity.

Table 4. *Rhodanese activity in extracts of photosynthetic bacteria*

Extracts were prepared from organisms harvested towards the end of growth. The activity of the enzyme in extracts was determined using samples containing 0·25 mg. protein/ml. and the values were corrected for heat-stable activity.

Organism	Growth substrate	Enzyme activity (units/mg. of protein)
Chromatium D	$S_2O_3^{2-} + CO_2$	9
Chromatium D	Pyruvate	8
Chromatium D	Succinate	7
Rhodospirillum rubrum	Malate + glutamate + 0·2% yeast extract	5
Rhodopseudomonas spheroides	Malate + glutamate	5·5

Properties of the rhodanese activity of crude extracts. The rhodanese activity of crude extracts of the organism was optimal at pH 8·7 and the Michaelis constants of the enzyme for thiosulphate and cyanide were 0·6 and 20 mM respectively. The enzyme was insensitive to EDTA (10 mM), fluoride (10 mM), azide (10 mM), thiocyanate (10 mM), cadmium ions (1 mM), arsenite (1 mM) and iodoacetate (1 mM). Tetrathionate (10–40 mM) added before or after the initiation of the reaction with cyanide inhibited the enzyme-dependent reduction of DCIP.

The enzyme was found in extracts of Chromatium D grown on succinate and pyruvate and of anaerobically grown *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*. The amounts of enzyme in the extracts of the Athiorhodaceae were approximately half that in the extracts of inorganic grown Chromatium D (Table 4). The activity of the bacterial extracts was less than that in the pig kidney extract; this gave a value of 44 units/mg. protein when assayed in the same way.

Partial purification of the enzyme. The enzyme in crude extracts of inorganic grown Chromatium D was partially purified in a six-stage process (Table 5)

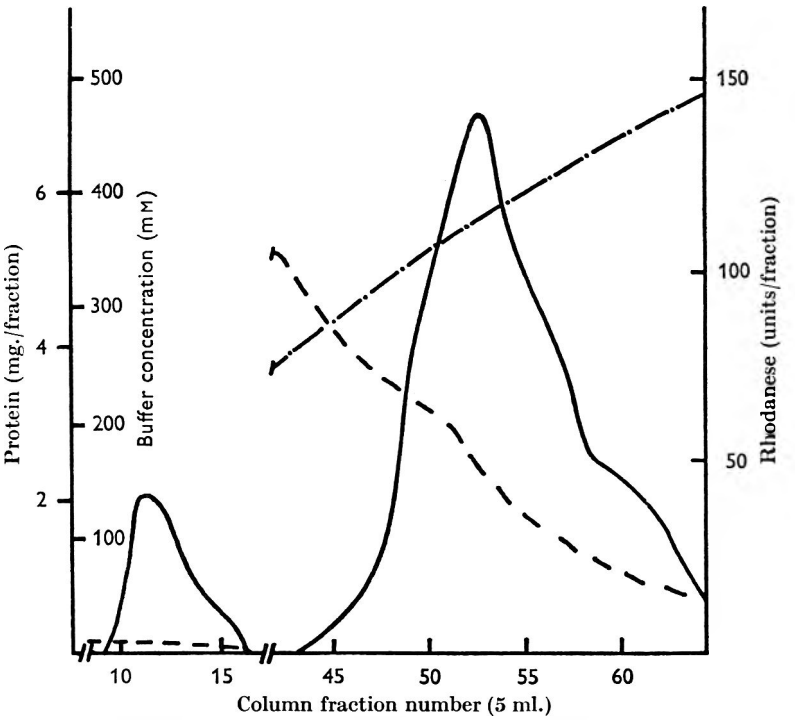

Fig. 4. Chromatography of rhodanese on DEAE cellulose. The protein fraction from stage V was applied to a column of DEAE cellulose. 15 % of the total enzyme recovered passed through the column at low ionic strength (column fractions 10–15 termed fraction A); the remainder of the enzymic activity was eluted from the column with 350–400 mM buffer (column fractions 49–57 termed fraction B). Protein (----), buffer concentration (–·–·–) and enzyme activity (——) were determined.

Table 5. *Partial purification of the rhodanese activity in crude extracts of Chromatium* D

The details of the individual steps are given in Methods. 5 ml. of the concentrated protein fraction from stage IV were applied to the column of Sephadex before chromotagraphy on DEAE cellulose.

Stage	Treatment	Product volume (ml.)	Total protein (mg.)	Total enzyme (units)	Specific activity (units/mg. protein)
I	French press	30	1050	12000	11
II	High-speed centrifugation	25	450	8550	19
III	Streptomycin sulphate	28	336	7050	21
IV	Ammonium sulphate 0–90 % saturation	13	325	6120	19
V	Desalting with Sephadex	12	124	2107	917
VI	Chromatography on DEAE cellulose				
	Fraction A nos. (10–15)	30	0·5	148	296
	Fraction B (nos. 49–57)	45	22	887	40

involving differential centrifuging, the precipitation of nucleic acid and enzyme with streptomycin sulphate and ammonium sulphate respectively, gel filtration on Sephadex and column chromatography on substituted celluloses. The enzyme was recovered from DEAE cellulose as well as from CM cellulose in two distinct fractions (Fig. 4). The smaller fraction (A) was unstable during storage and unsuitable for further study; the enzyme in the other fraction (B) was similar in optimum pH, Michaelis constant for thiosulphate and inhibition by tetrathionate to the activity of crude extracts. The enzyme in fraction B, after dialysis, was eluted from DEAE cellulose as a single peak of activity.

DISCUSSION

The growth of *Chromatium* sp. strain D under autotrophic conditions on sulphide, thiosulphate, exogenous sulphur, and sulphite confirmed the observations of van Niel (1931). The ability to grow on thiosulphate is a feature which Chromatium D has in common with the small Thiorhodaceae (thiopedia and thiocapsa species) and *Chlorobium thiosulphatophilum* and which distinguishes it from the large purple sulphur bacteria (*Chromatium okenii*, *C. warmingii*, *Thiospirillum jenense*) and *Chlorobium limicola* (see Trüper & Schlegel, 1964). Tetrathionate, a suggested intermediate in the microbial oxidation of thiosulphate, did not support the growth of Chromatium D under autotrophic conditions.

Various workers have reported the intracellular accumulation of sulphur during the growth of Thiorhodaceae on sulphide and thiosulphate (van Niel, 1931, 1936; Eymers & Wassink, 1937). Sulphur accumulation was a characteristic feature of the growth of Chromatium D on sulphide and thiosulphate and of the oxidation of these sulphur compounds by suspensions of the organism. This sulphur is an endogenous counterpart of the exogenous sulphur compounds required for autotrophic growth as it supported the further growth of the organism in a sulphur-free medium. Endogenous sulphur accounted for up to 27 % of the dry weight of the organism; similar amounts of sulphur accumulate in other Thiorhodaceae during growth on sulphide (van Niel, 1936; Trüper & Schlegel, 1964). The chemical nature of this endogenous sulphur is not known. Intracellular sulphur bears some analogy to poly-β-hydroxybutyrate, which accumulates in photosynthetic bacteria during growth under certain conditions on organic media (Stanier, Doudoroff, Kunisawa & Contopoulou, 1959; Doudoroff & Stanier, 1959). Endogenous sulphur resembles the polymer in the magnitude of accumulation, in its requirements for utilization and in its ability to support further growth. Both accumulants are a potential source of reducing power within the organism although poly-β-hydroxybutyrate is also a source of fixed carbon.

During thiosulphate utilization by suspensions of Chromatium D, the inner (SO_3-) atom was rapidly oxidized to sulphate whilst the outer (S-) atom largely accumulated as endogenous sulphur. On the completion of thiosulphate disappearance, more than 90 % of the inner atom and 15 % of the outer atom of thiosulphate were in the sulphate fraction; the remainder of the outer atom was within the organisms and was oxidized to sulphate at a relatively slow rate compared with the inner atom. At least 80 % of the outer atom of thiosulphate passed through the stage of endogenous sulphur, implying that it was an intermediate in the oxidation

of thiosulphate to sulphate by Chromatium D. Endogenous sulphur has a similar intermediate role in the oxidation of sulphide by *Chromatium okenii* (Trüper, 1964). In the present work the results indicated that the inner and outer sulphur atoms of thiosulphate were oxidized at different rates and therefore independently to sulphate. This requires the separation of the two sulphur atoms of thiosulphate at an early stage in the oxidation process. The fact that Chromatium D grew on, and oxidized, sulphide, sulphur and sulphite but not tetrathionate was consistent with the key role envisaged for thiosulphate cleavage in the oxidation process (Fig. 5). The role of rhodanese in the oxidation of thiosulphate by Chromatium D remains to be proved, especially since the enzyme was also found in Athiorhodaceae. It could, however, be concerned in the initial cleavage reaction. A double displacement mechanism has recently been proposed for the reaction catalysed by the mammalian enzyme (Green & Westley, 1961; Westley & Nakamoto, 1962; Villarejo & Westley, 1963*a*, *b*) involving the formation of an enzyme–sulphur intermediate.

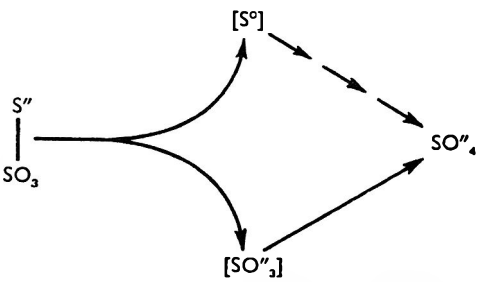

Fig. 5. The complete oxidation of thiosulphate.

The enzyme is regenerated in a reaction with an acceptor molecule such as cyanide or reduced lipoic acid. In the case of the oxidation of thiosulphate by Chromatium D, a reaction mechanism is envisaged in which a physiological acceptor reacts with an enzyme–sulphur intermediate to give sulphur or a closely related compound. Reactions other than the cyanide-dependent cleavage of thiosulphate catalysed by Chromatium rhodanese have yet to be demonstrated.

Although the mechanism of the oxidation of inorganic sulphur compounds by coloured and colourless sulphur bacteria are apparently different in detail, the oxidation process in both groups of organisms is closely associated with the production of reducing power for the fixation of carbon dioxide and subsequent biosynthetic reactions. The growth yields of Chromatium D in media containing a variety of reduced inorganic sulphur compounds were consistent with the electron donor role of the sulphur compound in bacterial photosynthesis (van Niel, 1931). The nature of the electron acceptors directly involved in the oxidation of sulphur compounds by Chromatium D is, however, unknown.

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The Role of Tetrathionate in the Oxidation of Thiosulphate by *Chromatium* sp. strain D

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SUMMARY

Sulphate was progressively replaced by tetrathionate as the end product of thiosulphate oxidation by suspensions of Chromatium D when the pH value was decreased from 7·3 to 6·25; tetrathionate was not itself oxidized to sulphate within this range. The effect of pH value on the oxidation of endogenous sulphur was less marked than on the production of sulphate from thiosulphate. Extracts of Chromatium D catalysed the oxidation of thiosulphate to tetrathionate in the presence of ferricyanide; pH 5·0 was the optimum of the purified enzyme. Tetrathionate inhibited growth and the complete oxidation of thiosulphate by suspensions. At pH 6·75 where sulphur accumulated during thiosulphate utilization and sulphate and tetrathionate were formed, added tetrathionate inhibited the accumulation of endogenous sulphur but not the production of tetrathionate from thiosulphate. Tetrathionate had no effect on the oxidation of endogenous sulphur by suspensions. It is concluded that in Chromatium D thiosulphate is metabolized by two alternative pathways depending on the conditions: (1) the cleavage of one molecule of thiosulphate in a reaction similar to that catalysed by thiosulphate sulphur-transferase E.C.2.8.1.1. (rhodanese); (2) the oxidative coupling of two molecules of thiosulphate to give tetrathionate, catalysed by the thiosulphate-oxidizing enzyme which only operates at low pH values.

INTRODUCTION

Tetrathionate has been suggested as an intermediate in the light-dependent oxidation of thiosulphate to sulphate by purple sulphur bacteria (van Niel, 1936; Eymers & Wassink, 1937; Wassink, 1941). The evidence, largely indirect, was based on the kinetics of carbon dioxide uptake by suspensions of purple sulphur bacteria in the presence of thiosulphate. In similar studies with *Chlorobium thiosulphatophilum* which grows on sulphide and thiosulphate but not on tetrathionate, Larsen (1952, 1953) reported that tetrathionate was oxidized by suspensions with the concomitant fixation of carbon dioxide. He suggested that tetrathionate might be an intermediate in the oxidation of thiosulphate by this organism. Manometric studies, however, gave no indication of an accumulation of tetrathionate during thiosulphate oxidation.

Tetrathionate not only supports the chemo-autotrophic growth of several species of *Thiobacillus*, but also accumulates during their growth on other inorganic sulphur compounds (Parker & Prisk, 1953; Pratt, 1958; Pankhurst, 1964). Several

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workers have reported the accumulation of tetrathionate during the oxidation of sulphur compounds by suspensions of thiobacilli (Vishniac, 1952; Baalsrud & Baalsrud, 1954; Vishniac & Santer, 1957; Trudinger, 1959; Jones & Happold, 1961).

Extracts of *Thiobacillus thioparus* and *T. thio-oxidans* have recently been shown to catalyse the oxidation of sulphide, thiosulphate, tetrathionate, trithionate and sulphite to sulphate (Rittenberg & London, 1964). Tetrathionate was identified as a likely intermediate in the oxidation of sulphide and thiosulphate. Extracts of Thiobacillus X contain an enzyme which catalyses the oxidation of thiosulphate to tetrathionate (Trudinger, 1958); by using soluble and particulate cytochrome preparations isolated from crude extracts, thiosulphate oxidation has been coupled to the uptake of oxygen. This enzyme is thought to catalyse the initial step in the oxidation of thiosulphate by Thiobacillus.

In the present work, the production of tetrathionate from thiosulphate and the effect of tetrathionate on the complete oxidation of thiosulphate and endogenous sulphur by Chromatium strain D were investigated to establish the role, if any, of polythionate in the oxidation of thiosulphate to sulphate. Extracts were used to provide an enzymological basis for the observation made with intact organisms.

METHODS

Organisms, media and growth of cultures. The culture of *Chromatium* sp. strain D was grown as described by Smith & Lascelles, (1966). This organism was used throughout these studies except in some experiments with extracts of the Athiorhodaceae *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*. These organisms were grown as described previously (Smith & Lascelles, 1966).

Preparation and incubation of suspensions. Organisms for suspensions were prepared and incubated as described previously (Smith & Lascelles, 1966). In the investigation of the effect of pH value on the oxidation of sulphur compounds, the concentration of sodium bicarbonate was decreased to 3 mM and the pH value of the potassium phosphate buffer chosen so as to give the required final pH value when gassed with N_2 95% (v/v) + CO_2 5% (v/v).

Assay of the thiosulphate-oxidizing enzyme. Extracts were prepared as described by Smith & Lascelles (1966) except that the washed organisms were finally suspended in 0·04 M-potassium phosphate buffer (pH 7·5). A modification of the Trudinger (1961) assay was used. The reaction mixture contained, in a volume of 3 ml.: acetate buffer (pH 5·5), 350 μmoles; sodium thiosulphate, 25 μmoles; potassium ferricyanide, 7·5 μmoles; enzyme preparation equivalent to 1·5 mg. protein of crude extract. The reaction was started by the adding of enzyme. The reference cuvette contained the complete reaction mixture without thiosulphate. Ferricyanide reduction was followed at room temperature (18 to 20°) by using a recording double-beam grating spectrophotometer (Optica CF4DR, Optica U.K. Ltd., Gateshead-on-Tyne, England). The millimolar extinction coefficient of ferricyanide at 418 mμ is 1·0 (Ibers & Davidson, 1951), and a unit of enzyme activity was defined as the amount which catalysed the reduction of 1 μmole ferricyanide/min.

Purification of the thiosulphate-oxidizing enzyme. Particle-bound pigment was removed from the extract prepared from 12 l. fully grown culture by centrifugation at 100,000 *g* for 60 min. in a Spinco model L ultracentrifuge. The supernatant

fluid fraction of the extract was treated with 5% (w/v) streptomycin sulphate (Glaxo Laboratories Ltd., Greenford, England) in 0·04 M-potassium phosphate buffer (pH 7·5) to give a final concentration of 1 mg. streptomycin sulphate/mg. extract protein. Precipitated nucleic acid was removed after 15 min. by centrifugation at 18,000 *g* for 20 min. The supernatant fluid was adjusted to pH 4·6 with N-hydrochloric acid and precipitated protein removed after 5 min. by centrifugation at 18,000 *g* for 10 min. The supernatant fluid was adjusted to pH 7·0 with N-sodium hydroxide. The enzyme was precipitated by 35–55% of saturation (by volume) with ammonium sulphate. Precipitated protein was removed after 30 min. by centrifugation at 18,000 *g* for 20 min. and dissolved in a minimum amount of 0·04 M-potassium phosphate buffer (pH 7·0). After dialysis for 12 hr against mM-potassium phosphate buffer (pH 7·0), the concentrated enzyme preparation was applied to a column (15 × 1·5 cm.) of DEAE-DE50 cellulose (Whatman, Colnbrook, England). Protein was eluted with potassium phosphate buffer (pH 7·0) by using a concentration gradient (1–200 mM); the flow rate was 1 ml./min. and the eluate was collected in 5 ml. fractions.

Analytical methods. Protein, phosphate, thiosulphate, sulphate and endogenous sulphur were determined as described previously (Smith & Lascelles, 1966). The amount of tetrathionate in suspensions was determined by the method of Sorbo (1957) in samples of the supernatant medium after the organisms had been removed by centrifugation.

Chromatography of inorganic sulphur compounds. Thiosulphate and polythionates were separated by descending chromatography on Whatman no. 1 paper with the solvent systems described by Skarzynski & Szczepkowski (1959) and Trudinger (1959). The chromatograms were dried at room temperature and the sulphur compounds located by the method of Trevelyan (1950).

Inorganic sulphur compounds. Sodium salts were used throughout these studies.

RESULTS

Effect of pH value on growth and the oxidation of inorganic sulphur compounds

Chromatium D grew well on the thiosulphate + salts medium at pH 7·3 whereas the organism did not grow on the same medium at pH 6·5. Growth on pyruvate showed a similar dependence on the pH value of the medium.

Effect of pH value on the oxidation of thiosulphate. At pH 7·3, suspensions of organisms oxidized thiosulphate completely to sulphate under anaerobic conditions in the light in the presence of bicarbonate; there was a transient accumulation of sulphur within the organisms (Fig. 1). At pH 6·25, thiosulphate disappearance was more rapid than at pH 7·3 but sulphate production was less than 25% of the theoretical (Fig. 1) and an accumulation of sulphur within the organisms was not observed. Tetrathionate was found chromatographically in suspensions oxidizing thiosulphate at pH 6·25; there was no trace of polythionate in suspensions oxidizing thiosulphate at pH 7·3. The pattern of tetrathionate production in suspensions oxidizing thiosulphate at various pH values was established by using a quantitative method for determining polythionate (Fig. 1). As the pH value was decreased the amount of tetrathionate produced

from thiosulphate increased until, at pH 6.25, it was the principal product of thiosulphate utilization. At each pH value, the sum of tetrathionate + sulphate produced accounted quantitatively for the thiosulphate used by the suspensions.

Tetrathionate, whether produced from thiosulphate by suspensions under acidic conditions (Fig. 1) or added to suspensions in the absence of thiosulphate, was not oxidized to sulphate in the range pH 7·3–6·25.

There was no disappearance of thiosulphate or production of polythionates in suspensions of heat-killed organisms or in the absence of organisms between pH 7·3 and pH 6·25. The lowering of the pH of suspensions was therefore accompanied by a fundamental change in the pattern of thiosulphate oxidation; sulphur accumulation and sulphate production were progressively replaced by the conversion of thiosulphate to tetrathionate.

Effect of pH on the oxidation of endogenous sulphur. Suspensions of organisms containing considerable amounts of intracellular sulphur oxidized this sulphur to

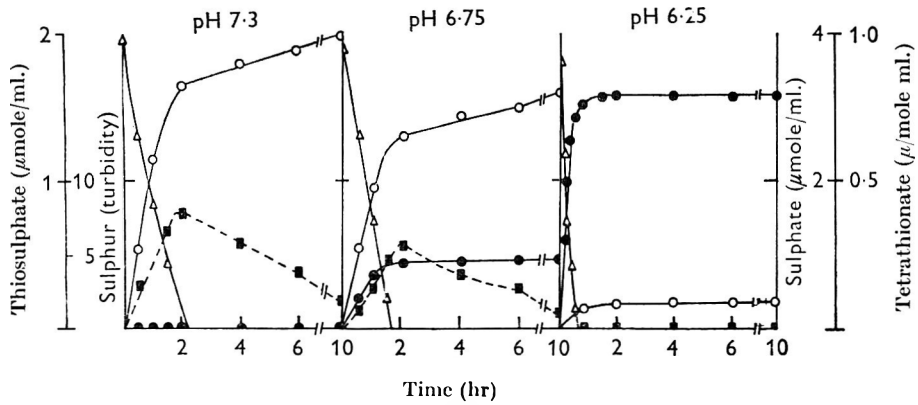

Fig. 1. Effect of pH value on the oxidation of thiosulphate by suspensions. Suspensions of thiosulphate-grown organisms containing 0·25 mg. protein/ml. were incubated at various pH values under anaerobic conditions with a light intensity of 500 ft.c. in the presence of thiosulphate (2 mM). Thiosulphate (—△—) disappearance, sulphur (-- ■ --) and tetrathionate (— ●—) accumulation and sulphate (—○—) production were determined.

Table 1. *Effect of pH value on the oxidation of endogenous sulphur*

Suspensions of thiosulphate-grown Chromatium D containing intracellular sulphur (equiv. 0·25 mg. protein/ml.) were incubated at various pH values under anaerobic conditions at a light intensity of 500 ft.c. The sulphate produced after incubation for 1 hr and 8 hr was determined. The figures in parentheses refer to sulphate production by suspensions of sulphur-free organisms oxidizing thiosulphate (2 mM) under similar conditions.

Time (hr)	pH 7·3	pH 6·75	pH 6·25
	Sulphate production (μmole/hr/ml.)		
1	1·2 (1·5)	1·2 (1·2)	1·2 (0·1)
8	3·1 (4·0)	3·1 (3·2)	2·3 (0·4)

sulphate under anaerobic conditions in the light (Smith & Lascelles, 1966). The effect of pH value on the oxidation of endogenous sulphur was less marked than its effect on the production of sulphate from thiosulphate (Table 1). Sulphate appeared to be the only product of endogenous sulphur oxidation by suspensions in the range pH 7·3–6·25.

Tetrathionate production by extracts

Extracts of Chromatium D catalysed the rapid and complete oxidation of thiosulphate to tetrathionate in the presence of potassium ferricyanide according to the equation

$$2S_2O_3^{2-} + 2Fe(CN)_6^{3-} \rightarrow S_4O_6^{2-} + 2Fe(CN)_6^{4-}$$

The reaction did not occur with heated extracts.

Properties of the thiosulphate-oxidizing enzyme. The optimum pH value of the reaction catalysed by crude extracts was below 5·5. The enzyme in crude extracts was insensitive to EDTA (10 mM), fluoride (10 mM), iodoacetate (10 mM) and arsenite (1 mM); azide (10 mM) decreased the reaction rate by 50 %. The Michaelis constant of the enzyme for thiosulphate was 1·5 mM. Extracts did not catalyse the reduction of ferricyanide when thiosulphate was replaced by tetrathionate.

Extracts of Chromatium D grown on organic carbon had about half the activity of extracts from thiosulphate-grown organisms; the enzyme was not found in extracts of *Rhodospirillum rubrum* or *Rhodopseudomonas spheroides*.

Table 2. *Purification of the thiosulphate-oxidizing enzyme of Chromatium D*

The details of the individual steps are given in Methods. Five ml. of the dialysed protein fraction were applied to the column of DEAE cellulose.

Stage	Treatment	Product volume (ml.)	Total protein (mg.)	Total enzyme (units)	Specific activity (units/mg. protein)
I	French press	27	1350	1000	0·7
II	High-speed centrifugation	22	572	853	1·5
III	Streptomycin sulphate	24	480	804	1·7
IV	Acid precipitation	24	209	670	3·2
V	Ammonium sulphate (35–55 % saturation)	5·2	85	624	7·3
VI	Dialysis	6·6	85	521	6·1
	(Fractions 15–19)				
VII	Chromatography on DEAE cellulose	25	3·2	253	79

Purification of thiosulphate-oxidizing enzyme. The enzyme from extracts of thiosulphate-grown Chromatium D was partially purified in a seven stage process (Table 2) which involved column chromatography on DEAE cellulose. The thiosulphate-cleaving and thiosulphate-oxidizing enzymes were completely separated on the column of substituted cellulose (Fig. 2). The reaction catalysed by the 100-fold purified enzyme was optimumal at pH 5·0 (Fig. 3).

Effect of tetrathionate on the oxidation of inorganic sulphur compounds

Tetrathionate did not support the growth of Chromatium D under autotrophic conditions and it inhibited the growth of the organism on the thiosulphate-salts medium as well as on organic media (Smith & Lascelles, 1966). Thiosulphate utilization and sulphur accumulation by suspensions at pH 7·3 were inhibited by

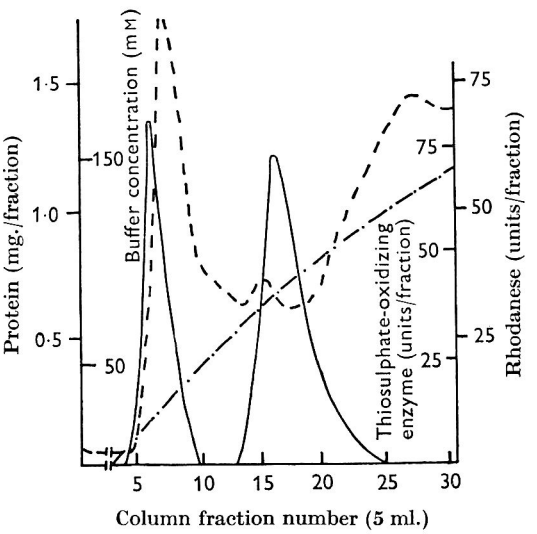

Fig. 2

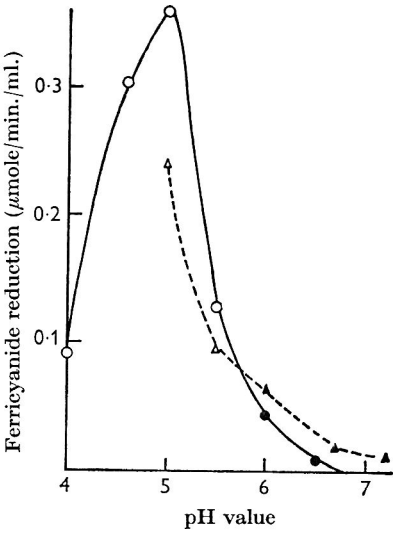

Fig. 3

Fig. 2. Chromatography of the thiosulphate-oxidizing enzyme on DEAE cellulose. The protein fraction from stage VI was applied to a column of DEAE-DE 50 cellulose. The thiosulphate-oxidizing enzyme was eluted with 75–90 mM buffer (column fractions 15–19) whilst rhodanese was eluted from the column with 10–35 mM buffer (column fractions 5–9). Protein (– – –), enzyme activity (——), and buffer concentration (– · – · –) were determined.

Fig. 3. Optimum pH value of the thiosulphate-oxidizing enzyme. Phosphate (full symbols) and acetate (open symbols) buffers of the appropriate pH were used in the determination of the activity of the enzyme in a crude extract (– – – –) and in a purified preparation (——) from the DEAE cellulose column. Protein was precipitated from reaction mixtures containing crude extract below pH 5·5.

Table 3. *Effect of tetrathionate on thiosulphate utilization and sulphur accumulation by Chromatium D at pH 7·3 and 6·25*

Suspensions of thiosulphate-grown Chromatium D (equiv. 0·25 mg. protein/ml.) at pH 7·3 or pH 6·25 were incubated at a light intensity of 500 ft.c. under anaerobic conditions with thiosulphate (5 mM) and various concentrations of tetrathionate.

pH	Tetrathionate concentration (μmoles/ml.)	Thiosulphate utilization (μmoles/hr/ml.)	Sulphur accumulation (maximum turbidity change)
7·3	0	2·7	12·8
	2·5	0·6	2·8
	5	0·3	0·9
6·25	0	4·8	0
	2·5	4·6	0
	5	4·2	0

tetrathionate (Table 3); the inhibition was non-competitive and reversed by washing. The effect of tetrathionate on thiosulphate disappearance was much less at pH 6·25 than at pH 7·3 (Table 3). At the intermediate value pH 6·75, where sulphur accumulated transiently and sulphate and tetrathionate were the products of thiosulphate oxidation, sulphur accumulation was markedly inhibited by added tetrathionate, whereas tetrathionate production from thiosulphate was, if anything, increased (Fig. 4). Added tetrathionate had no effect on the oxidation of endogenous sulphur by suspensions. Tetrathionate reacts spontaneously with sulphide and sulphite and this prevented a study of its effect on the oxidation of these compounds.

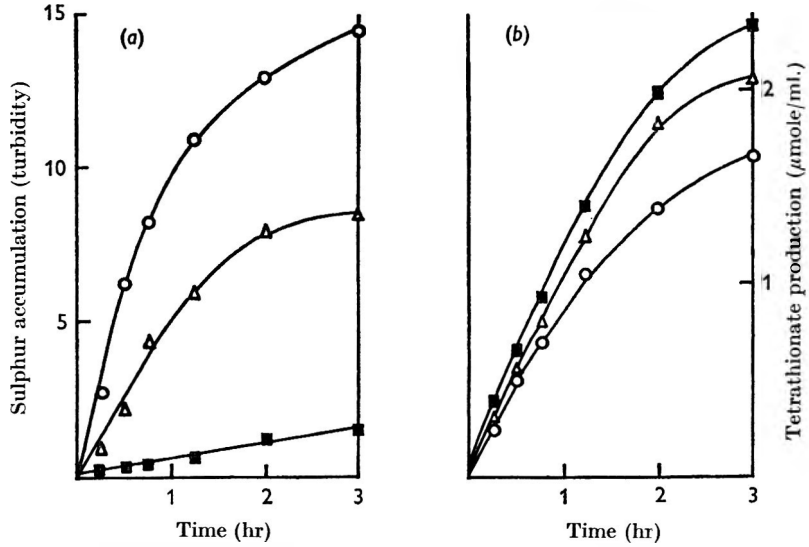

Fig. 4. Differential effect of added tetrathionate on the accumulation of sulphur (*a*) and tetrathionate (*b*) at pH 6·75. Suspensions of thiosulphate-grown organisms containing 0·2 mg. protein/ml. were incubated anaerobically in the light at pH 6·75 in the presence of thiosulphate (6 mM) and various concentrations of tetrathionate: 10 mM (— ■—), 1 mM (—△—) and none (—○—).

DISCUSSION

Chromatium D unlike *Thiobacillus thio-oxidans* did not grow on inorganic media at pH 6·5. Nevertheless, suspensions of Chromatium D utilized thiosulphate at pH values below 6·5. Sulphur accumulation and sulphate production were, however, greatly decreased at pH 6·25; the yield of sulphate under these conditions was less than 25% of the theoretical. Tetrathionate was the principal product of thiosulphate utilization at pH 6·25 whereas at pH 7·3 there was no detectable accumulation of tetrathionate during thiosulphate oxidation. Two possible explanations of the accumulation of tetrathionate at acid pH values were: (1) the accumulation of tetrathionate, a possible intermediate in thiosulphate oxidation, was due to the inactivity at acid pH of the enzymes for its further oxidation; (2) tetrathionate was the product of an enzyme reaction which was only functional at low pH values. The fact that tetrathionate was not oxidized in the range 7·3–6·25 favoured the second explanation.

Extracts of Chromatium D contained a soluble enzyme distinct from rhodanese which catalysed the oxidation of thiosulphate to tetrathionate. Such an enzyme was described by Trudinger (1958) in extracts from Thiobacillus strain X. The enzyme in crude extracts of Chromatium D was only slightly active at pH 7·0; the purified enzyme had an optimum at pH 5·0. In view of its properties it is likely that this enzyme is involved in the production of tetrathionate from thiosulphate by intact organisms at low pH values.

The inhibition by tetrathionate of thiosulphate utilization and of sulphur accumulation at pH 7·3 was an additional complicating factor. At the intermediate value pH 6·75, where both sulphate and tetrathionate were products of thiosulphate oxidation, added tetrathionate inhibited the intracellular accumulation of sulphur but did not affect the production of tetrathionate from thiosulphate.

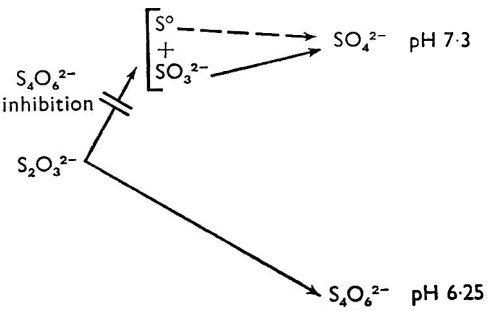

Fig. 5. The oxidation of thiosulphate by Chromatium D.

In contrast, tetrathionate had no effect on the oxidation of endogenous sulphur. The site of the inhibition of the conversion of thiosulphate to sulphate was therefore likely to be at a stage before the oxidation of endogenous sulphur. The results of this and other work (Smith & Lascelles, 1966) suggested that thiosulphate was metabolized by the two mechanisms outlined in the scheme shown in Fig. 5, one catalysed by a rhodanese-like enzyme and the other by the thiosulphate-oxidizing enzyme.

Wassink, Katz & Dorrestein (1941) investigated the fixation of carbon dioxide by suspensions of a species of *Chromatium* and found it to be most rapid at pH 6·3 in the presence of excess thiosulphate. At this pH value carbon dioxide fixation was accompanied by the production of alkali (measured by chemical binding of carbon dioxide) which was attributed to the conversion of thiosulphate to tetrathionate. On this basis, tetrathionate was proposed as an intermediate in thiosulphate oxidation. In the present work direct analysis for thiosulphate and its oxidation products has shown that lowering from pH 7·3 to 6·25 resulted in a fundamental change in the reactions for the oxidation of thiosulphate in which sulphate was replaced by tetrathionate as the end-product of oxidation. Wassink (1941) correctly interpreted the results of his experiments at pH 6·3 in terms of the oxidation of thiosulphate to tetrathionate; his investigation, based only on measurement of carbon dioxide fixation, did not however reveal the change in the pattern of thiosulphate oxidation which accompanied the change in pH. Since

fixation of carbon dioxide can apparently be coupled to the conversion of thiosulphate to tetrathionate at a pH value where growth is not possible, this oxidation process might be of some importance to the organism. The change from sulphate to tetrathionate production at acid pH values would be beneficial to an organism oxidizing thiosulphate in that acidic products would be replaced by the production of alkali: (1) complete oxidation: $Na_2S_2O_3 \rightarrow Na_2SO_4 + H_2SO_4$; (2) incomplete oxidation: $2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaOH$.

The inhibition of the complete oxidation of thiosulphate by tetrathionate would also prevent the further production of acidic oxidation products, whilst permitting the assimilation of carbon dioxide coupled to the oxidation of thiosulphate to tetrathionate.

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Extracellular Polysaccharides and Classification of the Genus *Lipomyces*

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(*Received* 1 *September* 1965)

SUMMARY

Strains classified as *Lipomyces lipoferus* and *L. starkeyi* produce specific extracellular acidic heteropolysaccharides. The polymer from *L. lipoferus* contains mannose and glucuronic acid; the polymer from *L. starkeyi* contains, in addition, galactose and a trisaccharide. This trisaccharide is composed of mannose and glucuronic acid in the molar ratio 1:2. The difference in polysaccharide composition may be the most significant physiological basis yet discovered for separation of species in the genus *Lipomyces*.

INTRODUCTION

During a survey of extracellular yeast polysaccharides, strains designated as *Lipomyces starkeyi* Lodder et Kreger-van Rij and *L. lipoferus* (den Dooren de Jong) Lodder et Kreger-van Rij were found to produce different extracellular polymers. Taxonomic differences based on assimilation tests are not clear-cut in this ascomycete genus. Reinforcement of these data by differences in macromolecular composition and structure, which must in turn reflect genetic distinctions, ought to be of value in species identification. The finding of distinct differences in the extracellular heteropolysaccharides produced by lipomyces strains suggests a basis for separation of the genus into two species.

METHODS

The organisms were grown in yeast extract+malt extract+peptone medium containing 5% glucose hydrate as previously described (Slodki, Wickerham & Bandoni, 1966). Polymers were isolated from cell-free culture liquors by precipitation with 1·2–1·5 volumes of methanol. At these methanol concentrations, precipitation of polymer required addition of a small amount of potassium acetate. The reprecipitated polymers were taken up in water, dialysed and lyophilized.

Methods used for paper chromatographic analysis and determination of component ratios are described elsewhere (Slodki *et al.* 1966).

RESULTS

Table 1 lists the optical rotations and approximate molar ratios of O-acetyl, total hexose and glucuronic acid. Mannose and galactose both occur in the polymers from *Lipomyces starkeyi*. Mannose is the only hexose present in the *L.*

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lipoferus polymers. The difference in hexose composition alone does not account for the optical rotations of the two polymer types. As shown in Fig. 1, hydrolysates of the *L. starkeyi* polysaccharides also contain a characteristic acidic oligosaccharide that gives, on paper chromatograms, a deep red-brown stain with the *o*-aminodiphenyl spray reagent (Timmell, Glaudemans & Currie, 1956). Data in Table 1 and Fig. 1 also serve to characterize culture NRRL Y-6333 as belonging to *L. lipoferus*. Previous taxonomic criteria, based largely on assimilation tests (Wickerham, 1951) failed to place this organism definitively in either lipomyces species.

Table 1. *Properties of lipomyces species and polysaccharides*

						Hexoses present		Molar ratios		
Species	Source	NRRL no.	Lactose assimilation	Polymer yield*	$[\alpha]_D^{25}$†	Mannose	Galactose	Acetyl:	Hexose:	Glucuronate
L. starkeyi	Starkey 74	Y-1388	+	1·01	0°	+	+	0·9	2·2	1
L. starkeyi	Starkey 74*b*	Y-2543	−	0·80	− 4°	+	+	0·6	2·0	1
L. lipoferus	A. C. Thayson	Y-1351	+	0·56	+42°	+	−	1·1	2·5	1
L. lipoferus	Type strain, CBS	Y-2542	+	0·33	+40°	+	−	1·1	3·0	1
L. starkeyi‡	David Jones	Y-6333	−	0·93	+54°	+	−	1·2	2·0	1

* g. 100 ml. culture. † *c*, 0·5, water.
‡ Classification according to Brady & Jones (1964).

A preliminary attempt was made to ascertain the nature of the acidic oligosaccharide in the *Lipomyces starkeyi* Y-2543 polysaccharide. A small portion of the polymer (37 mg.) was hydrolyzed in 2 N-HCl (2 ml., boiling water bath, 1 hr). After neutralization to pH 4·2 with Ag_2CO_3, the mixture was filtered and then decationized by passage through an Amberlite IR-120 (H^+) column. The acidic solution was allowed to percolate into a Dowex 1–4 X, acetate, 200–400 mesh microcolumn (VanEtten & McGrew, 1957). Elution of the column was done with an acetic acid gradient established by passing 1·5 M-acetic acid into 125 ml. water. The fractions (5–7 ml.) were examined for the presence of carbohydrate by the phenol + sulphuric acid reagent (DuBois *et al.* 1956). After pooling, the various peaks were examined by paper chromatography. The free hexoses, mannose and galactose, were not adsorbed and were present in approximately equal amounts. As shown by hydrolysis and paper chromatography, the oligosaccharides in the two major elution peaks contained only mannose and glucuronic acid. This composition was confirmed by the sulphuric acid + carbazole test (Gregory, 1960) for uronic acid and by the sulphuric acid + cysteine test (Dische, Shettles & Osnos, 1949) for hexoses. The latter test confirmed the presence of galactose in the unadsorbed fraction only and indicated that this hexose must not be involved in relatively stable uronide linkages.

The first peak eluted consisted primarily of an aldobiuronic acid contaminated with a much smaller amount of free glucuronic acid. On paper chromatograms, the aldobiuronic acid had the characteristics of 2–0-(β-D-glucopyranosyluronic acid-D-mannose) (Slodki *et al.* 1966; Sloneker & Jeanes, 1962). These characteristics include an orange-brown spot with the *o*-aminodiphenyl spray, a yellow colour with the periodate Schiff spray (Hardy & Buchanan, 1963) and no reaction with alkaline triphenyltetrazolium (Wallenfels, 1950). The occurrence of the aldobiuronic

acid is not surprising. Previous experience at the Northern Laboratory has been that microbial polysaccharides which contain glucuronic acid and mannose generally possess this disaccharide (J. H. Sloneker, personal communication).

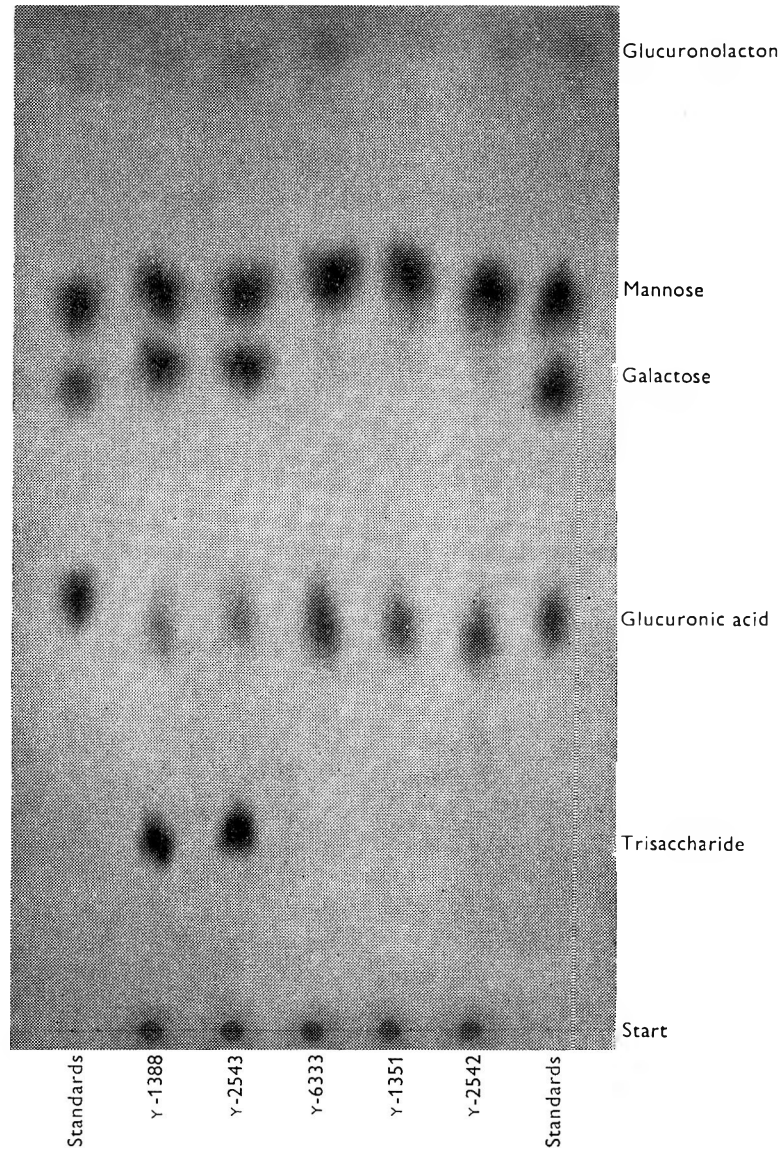

Fig. 1. Chromatogram of lipomyces polysaccharide hydrolyzates. Descending irrigation in ethyl acetate + acetic acid + pyridine + water (5 + 1 + 5 + 3, v/v) for 16 hr; *o*-aminodiphenyl spray reagent (Slodki *et al.* 1966). Strains NRRL Y-1338 and Y 2543 were designated *L. starkeyi*: strains Y-1351 and Y-2542 were classified as *L. lipoferus*.

The second peak contained a single component, the oligosaccharide, giving a red-brown spot on chromatograms sprayed with *o*-aminodiphenyl. The reducing end group was determined by alkaline iodine titration (Slodki, 1963); a mannose:

glucuronic acid:reducing group molar ratio 1·0:2·0:1·1 was obtained. Based on the glucuronic acid analysis, the free acid form gave $[\alpha]_D^{55}+30°$ (*c*, 0·23, water). On paper chromatograms, the substance is tetrazolium negative and gives a yellow spot with the periodate Schiff reagent. Hydrolysis and paper chromatography gave spots visualized by the spray reagents which corresponded to mannose, glucuronic acid and the aldobiuronic acid of the first peak. Thus, the trisaccharide is probably glucuronic acid α-linked to 2-O-(β-D-glucopyranosyluronic acid)-D-mannose.

DISCUSSION

Assignment of lipomyces strains to either of the two species comprising the genus has been difficult on the basis of morphological and taxonomic criteria. The principal criterion for separating lipomyces strains has been the ability of *L. lipoferus* and the inability of *L. starkeyi* to assimilate lactose (Lodder & Kreger-van Rij, 1952). Culture NRRL Y-6333 received from D. Jones under the name *L. starkeyi* did not assimilate lactose. In our experience this strain is unusually weak in its ability to assimilate compounds in still medium. Even shaking does not permit assimilation of more than trace amounts of xylose, erythritol, dulcitol, sorbitol, α-methylglucoside, and salicin, although these compounds are strongly assimilated by strains classified by various investigators as either *L. lipoferus* or *L. starkeyi*. Brady & Jones (1964) have classified Jones strain as *L. starkeyi*. Though it does not assimilate lactose, its polymer is like that of the *L. lipoferus* strains, which are recognized as belonging to a separate species (Lodder & Kreger-van Rij, 1952) because they assimilate lactose. Connell, Skinner & Hurd (1954) suggested that the two species should be considered as one, because they believed that no clear means had been found to differentiate strains at the species level. It is perhaps fortunate that lipomyces strains produce one or the other of two distinctive extracellular polysaccharides differing not only in a particular component sugar (galactose), but also in respect to intrinsic polymer structure. This difference represents strong evidence that at least two distinctive species comprise the genus *Lipomyces*.

Among our data from routine taxonomic studies we have found no easily determined characteristic that correlates with the difference in composition of the polymers. In any event, this work casts additional doubt on the validity of separating the two species of the genus *Lipomyces* on the basis of lactose assimilation.

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The Growth and Respiration of *Nitrosocystis oceanus* at Different Partial Pressures of Oxygen

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SUMMARY

The following effects of different partial pressures of oxygen $p(O_2)$ on the growth and on the oxidation of ammonium to nitrite by *Nitrosocystis oceanus* in liquid and on solid sea-water media were observed. Nearly pure O_2 was toxic to the organism when it was exposed to the oxygen on agar medium. In normal air the growth on agar medium was variable; usually a large proportion of the inoculum organisms never started to divide. At low $p(O_2)$ values the formation of microcolonies took place. In liquid medium the organism was less affected by high $p(O_2)$ values than on agar. The lower limit of oxygen concentration which permitted oxidation of ammonium in liquid medium corresponded to about 0·05 ml. O_2/l. In respiration experiments a high $p(O_2)$ in the gas phase resulted in twice as much oxygen uptake at the beginning as from air. Respiration at low $p(O_2)$ was comparatively high. These results seem to indicate that the inorganic respiration is accelerated by an increased $p(O_2)$ whereas growth is inhibited. Possible explanations are discussed.

INTRODUCTION

In an extensive search for organisms transforming ammonium to nitrite in the open ocean Watson (1961) showed the participation of a hitherto unknown nitrifying bacterium in this process. This organism oxidized ammonium to nitrite in an inorganic sea-water medium and assimilated carbon dioxide as sole carbon source. The organism therefore was classified as an obligately autotrophic nitrifier related to the soil nitrosomonas type of organisms. Since cysts were formed in liquid media the organism was placed with the genus *Nitrosocystis* as a new species, *N. oceanus*; morphological and physiological characteristics were given by Watson (1962*b*) and by Murray & Watson (1963, 1965).

The present paper deals with the effects of different partial pressures of oxygen $p(O_2)$ on *Nitrosocystis oceanus*.

METHODS

The organism. A culture of *Nitrosocystis oceanus* (Watson's strain; Watson, 1962*a*) was used. The culture was purified from a heterotrophic contaminant by picking single isolated colonies which had developed on agar medium after incubation at low $p(O_2)$.

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Media. Stock cultures and liquid experimental cultures were grown in sea-water medium consisting of 2 g. NH_4Cl, 0·1 g. K_2HPO_4, 5·0 g. $CaCO_3$ in a mixture of 750 ml. aged sea water and 250 ml. distilled water; pH 7·6. Solid media were prepared from this liquid medium by adding 10 g. 'Ion-agar' no. 2 (Oxoid)/l. For shaken cultures with dense suspensions of organisms a medium buffered with M/15 phosphate (pH 7·5), and with 3·3 g. $NaHCO_3$/l. rather than with $CaCO_3$, was used.

The purity of the *Nitrosocystis oceanus* cultures was tested occasionally by transfer to a standard medium for marine heterotrophs which consisted of 5·0 g. Bacto peptone, 0·1 g. $FePO_4$, 15·0 g. Bacto agar in a mixture of 750 ml. aged sea water and 250 ml. distilled water; pH 7·5–7·6.

Batch cultures. To obtain large crops of organism, cultures were prepared in 15-l. volumes of the liquid medium in 20-l. Pyrex flasks equipped with aeration and sampling devices. The inoculum consisted of 200 ml. of an actively nitrifying culture. Initially the batch culture was stirred with a Teflon-coated magnetic bar, but when growth was well established air filtered through a glasswool column was blown through the culture. As ammonium was consumed the pH value tended to decrease and more sterile NH_4Cl and $CaCO_3$ were added. Later it was necessary to add $NaHCO_3$ to keep the pH above 7. In the most successful of a series of batch cultures a final crop of 5×10^9 organisms/ml. was obtained in 5 weeks. The organisms were harvested by centrifugation in a Sorvall continuous-flow centrifuge at 10,000 rev./min. after the particulate $CaCO_3$ had been allowed to settle. The organisms were washed free from ammonium and nitrite with sterilized 75% (v/v) sea water in distilled water. A reddish-brown sediment was obtained which was stored in 75% sea water at 4°. The ammonium-oxidizing capability of cold-stored suspensions of organisms decreased slowly, but it was found safe to use the organisms for respiration studies within 4 weeks after harvest.

Incubation. All incubations, when not otherwise stated, were made in darkness at room temperature (25° ± 3°). Anaerobic jars and desiccators were used as incubation chambers when growth in gas mixtures with different $p(O_2)$ values was investigated. The ratio of gas volume to total culture volume in the jars always exceeded 100. Cultures to be shaken during incubation were prepared in 250 ml. Erlenmeyer flasks with bored rubber stoppers equipped with a short piece of glass tubing attached to a vacuum rubber hose closable with a clamp. Sterile glycerol was used to secure tightness between stopper and flask neck. When gas mixtures other than air were used in the flasks a slight under-pressure served to keep the stoppers tight and as a check that tightness had been maintained during the experiment. A rotary shaker with an amplitude of 2 cm. and turning at 300 rev./min. was used.

Counts of organisms were determined either by serial (decimal) dilutions in liquid medium followed by a check on nitrite production after 14 days-incubation, or by direct counting of the organisms in a Petroff–Hauser bacteria counter.

Nitrite determinations were made in a Spectronic 20 spectrophotometer at 540 mμ after colour development with sulphanilamide and *N*-(1-naphthyl)-ethylenediamine solutions. In experiments where nitrite was to be determined in agar, the plates were poured with 20 ml. of agar medium. At the time of analysis the agar was cut into two pieces with a knife and the one half (about 10 ml.) further divided into smaller pieces and quantitatively transferred to 90 ml. nitrite-free water in a covered

beaker. After at least 2 hr of soaking the nitrite was determined as described above. The second half of the agar culture was used for microscopic examination.

Respiration studies. Oxygen uptake measurements were made in a conventional Warburg apparatus with 15 ml. flasks at 28°. When oxygen pressures other than atmospheric were used, the flask + manometer system was evacuated to a calculated pressure and refilled to atmospheric pressure with oxygen or nitrogen as desired while the flasks were shaking in the water bath. The organism suspensions were tipped from the side-arm of the flasks at zero time. Nitrite-N in the reaction mixtures was determined immediately after the conclusion of the last reading of the manometers.

Gas mixtures. When $p(O_2)$ values different from that in air were used, the calculated volume of air was replaced after evacuation by pure nitrogen (N_2 minimum 99·996%, v/v; The Liquid Carbonic Corp., San Diego, Calif., U.S.A.) or by oxygen (grade medically pure; Victor Equipment Co., Los Angeles, Calif., U.S.A.). The oxygen pressures given in the text and the tables below refer to the partial pressures of oxygen in the gas phase in the incubation jars or Warburg flasks. For convenience the relationship between the different partial pressures of oxygen in the gas phase most frequently used and oxygen dissolved at equilibrium in water of a salinity of 25‰ (which is close to the salinity of the media used) at 25° is given in Table 1. An oxygen-free atmosphere was obtained by placing a dish with freshly mixed NaOH + pyrogallol in the incubation jar, evacuating to approximately 25 mm. Hg and refilling with nitrogen. In the Warburg flasks oxygen was similarly removed by adding 0·2 ml. of the NaOH + pyrogallol mixture to the centre well instead of KOH.

Table 1. *Oxygen dissolved in water of 25‰ salinity at 25° when in equilibrium with atmospheres of different partial pressures of oxygen, $p(O_2)$*

Gas phase		Dissolved oxygen		
$p(O_2)$ (mm. Hg)	O_2 (% v/v)	ml./l.	mg./l.	mM
684	90	22·4	32·0	1·0
160 (air)	21	5·2	7·5	0·23
16	2·1	0·52	0·75	0·023
5·3	0·7	0·17	0·25	0·007
1·6	0·21	0·05	0·08	0·002

RESULTS

Growth and nitrite production in liquid medium

Early experiments showed that the oxidation of ammonium by *Nitrosocystis oceanus* in liquid shaken cultures was slower than in stationary cultures at the same temperature. There was also better growth and more dividing organisms in the static cultures than in the shaken ones. This observation was followed up by a series of experiments designed to examine what effect the oxygenation of the sea-water medium might have on the growth of *N. oceanus* and on the oxidation of ammonium to nitrite. Since the mechanical shaking of the cultures was thought to introduce uncontrollable effects on these processes, it was decided to make the experiments with stationary cultures under atmospheres of different $p(O_2)$ values.

Figure 1 shows that the oxidation of ammonium took place over a wide range of oxygen concentrations. Maximum oxidation took place with normal air as gas phase. Small but significant amounts of nitrite (about a doubling of the amount carried over from the inoculum) were produced at the two lowest $p(O_2)$ values; however, it cannot be excluded that this nitrite was preformed by the organisms and had merely leaked from them into the culture fluid during incubation.

Table 2. *Growth of Nitrosocystis oceanus and production of nitrite in liquid static cultures incubated at different $p(O_2)$ values*

First 2 weeks			Next 2 weeks		
$p(O_2)$ (mm. Hg)	NO_2-N (μg./ml.)	Growth index*	$p(O_2)$ (mm. Hg)	NO_2-N (μg./ml.)	Growth index*
684	0·5	1	684	0·7	1
			160	3·0	3
			5·3	1·0	2
160 (air)	15·2	3	684	60·0	5
			160	51·0	5
			5·3	30·0	4
5·3	3·1	2	684	4·7	3
			160	10·0	4
			5·3	4·5	3

* The growth index is based on the appearance of nitrite in decimal dilutions in liquid medium from the original cultures. 1 means last positive tube dilution 10^{-1}; 2, dilution 10^{-2}, etc. The test was made after 14-day incubation of the dilution tubes.

Table 2 shows the results of an experiment where three sets of identical cultures had first been kept for 2 weeks at three different oxygen pressures and then changed to higher and to lower $p(O_2)$ values for the following 2 weeks. An initial incubation at $p(O_2)$ 684 mm. Hg harmed the organisms severely, resulting in less growth and less nitrite production during the subsequent incubation at lower $p(O_2)$, and complete stagnation of the cultures kept continuously at this pressure. One further feature of this experiment was that the higher $p(O_2)$ seemed to stimulate nitrite production and growth in those cultures which had initially been incubated in air. Short-term experiments with shaken cultures also showed clearly that oxygen stimulated the oxidation of ammonium to nitrite but at the same time inhibited the growth of the organism.

Growth and nitrite production on agar medium

The sensitivity of *Nitrosocystis oceanus* to oxygen was most clearly demonstrated when the organism on agar medium was exposed to the gas. The growth was completely inhibited in an atmosphere of $p(O_2)$ 684 mm. Hg. The inhibition was irreversible since subsequent incubation in an atmosphere of lower $p(O_2)$ which should otherwise have permitted growth did not result in resumed growth.

When plates on which growth had already been established during a period of incubation in air or at lower $p(O_2)$ were subjected to the higher $p(O_2)$, growth was also inhibited, but the oxidation of ammonium to nitrite continued, and at an increased rate (Table 3).

In air growth was very irregular; usually it was considerably delayed and took place only after a lag of 3 weeks or more (Fig. 2). On the other hand, the growth of

Nitrosocystis oceanus at low $p(O_2)$ was always rapid, with a lag period of less than 1 week (Fig. 2; Table 4). A $p(O_2)$ corresponding to about one-tenth of the $p(O_2)$ of air was optimal for growth on agar medium, although considerable growth was sometimes obtained as low as $p(O_2)$ 5·3 mm. Hg.

Growth and nitrite production in agar cultures incubated at the same $p(O_2)$ throughout a 4-week period as compared with growth and nitrite production in cultures incubated at higher or lower $p(O_2)$ from the third week are shown in Table 3.

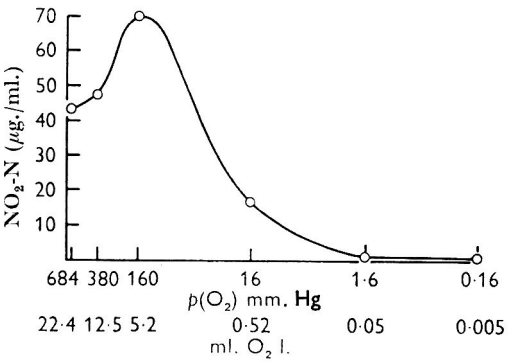

Fig. 1

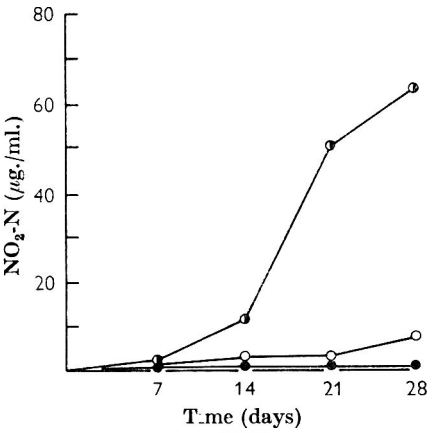

Fig. 2

Fig. 1. Nitrite production by *Nitrosocystis oceanus* in liquid sea-water medium incubated for 2 weeks under atmospheres of different values of $p(O_2)$. The dissolved oxygen values (ml. O_2/l.) corresponding to the $p(O_2)$ of the gas phase at equilibrium are added in the abscissa.

Fig. 2. Nitrite production by *Nitrosocystis oceanus* grown on agar medium in air (○), at $p(O_2)$ 16 mm. Hg (◑) and at $p(O_2)$ 684 mm. Hg (●).

Table 3. *Growth of Nitrosocystis oceanus and nitrite production on agar medium after incubation at different $p(O_2)$ values*

First 2 weeks				Next 2 weeks			
		Growth				Growth	
$p(O_2)$ (mm. Hg)	NO_2-N (μg./ml.)	Colonies per field	Colony diameter (μ)	$p(O_2)$ (mm. Hg)	NO_2-N (μg./ml.)	Colonies per field	Colony diameter (μ)
684	0·2*	Scattered single cells		684	0·2	0	—
				160	0·2	0	—
				5·3	0·2	0	—
160 (air)	2·4	1·0	13·8	684	35·0	0·8	14·0
				160	27·0	1·0	46·2
				5·3	18·1	0·9	40·8
5·3	1·9	0·9	11·9	684	21·2	0·5	11·6
				160	21·2	1·0	41·5
				5·3	14·0	0·8	37·4

* From the inoculum.

Table 4 shows clearly what took place on the agar media during incubation in air and at low $p(O_2)$. On the low-oxygen plates the organism had already passed through three generations after 1 week whereas only half of the organisms grown in air had started to divide (most probably the apparent onset of growth in the latter case was false: the organisms may well have been in the dividing stage in the inoculum,

Table 4. *Comparison of growth of Nitrosocystis oceanus on agar plates incubated in air (A) and at $p(O_2)$ 5·3 mm. Hg (B)*

	7 days		28 days	
	A	B	A	B
Colonies/field:	2·4*	2·5	0·12	2·9
Organisms/colony:	1·5	8·1	—	—
Colony diameter (μ):	—	—	86·5	34·7

* Mean values from 25 microscopical fields/plate: two plates.

Table 5. *Oxygen consumption and nitrite production by washed suspensions of Nitrosocystis oceanus at different values of $p(O_2)$*

To each Warburg flask was added 1 ml. organism suspension equiv. 0·2 mg. dry wt.; *plus*: to flask no. 1, 2 ml. buffer; to flask no. 2, 1 ml. buffer + 1 ml. NH_4Cl, 150 mM; to flask no. 3, 1 ml. NH_4Cl, 150 mM, + 1 ml. $NaHCO_3$, 15 mM. Total volume 3·0 ml. + 0·2 ml. 20 % KOH in the centre well, + pyrogallol also for the anaerobic flasks. The organism suspensions as well as the ammonium and bicarbonate solutions were made up in M/15 phosphate buffer + 3 % (w/v) NaCl, pH 7·0. Bath temperature 28°. Shaking rate 65 strokes/min. Duration of experiment 6 hr.

$p(O_2)$ (mm. Hg)	Flask no.	Oxygen consumed		Nitrite produced		$\frac{O_2^*}{NO_2^-}$
		μl.	μM	μg. N	μM	
684	1 (endogenous)	9	0·39	0·7	0·05	—
	2	164	7·32	67·8	4·85	1·53
	3	171	7·64	74·0	5·29	1·39
160 (air)	1 (endogenous)	4	0·18	0·3	0·02	—
	2	141	6·30	62·5	4·46	1·38
	3	153	6·82	69·5	4·96	1·35
16	1 (endogenous)	4	0·18	0·3	0·02	—
	2	126	5·62	56·1	4·01	1·37
	3	137	6·10	58·3	4·16	1·43
0	1 (endogenous)	0	—	0·02	—	—
	2	0	—	0·02	—	—
	3	0	—	0·02	—	—

* Corrected for endogenous values.

which had been taken from an actively nitrifying culture). The number of micro-colonies/field agreed well after the first week. After 4 weeks, however, only the low-oxygen plates had the original number of colonies, whereas most of the air-grown 'colonies' had disappeared. On the other hand, the very few colonies which had developed were considerably larger than the colonies on the plates incubated at low $p(O_2)$.

Although *Nitrosocystis oceanus* was able to proliferate at quite low $p(O_2)$, strict anaerobiosis was as harmful to the organism as a high $p(O_2)$; growth did not occur in air at low $p(O_2)$ when plates inoculated with *N. oceanus* had first been incubated for 2 weeks in the complete absence of oxygen.

Respiration experiments

The consumption of oxygen and the production of nitrite in systems of four different $p(O_2)$ values are shown in Table 5. As expected, no nitrite was formed in the complete absence of oxygen. With the highest $p(O_2)$ oxygen was taken up twice as fast as from normal air during the first 30 min. of the experiment, but this high rate of respiration did not persist (Fig. 3). With a $p(O_2)$ corresponding to one-tenth of the $p(O_2)$ of air the respiration was lower but still surprisingly high in all of a series of experiments. More than one-third of the 300 μl. oxygen available in these flasks was consumed during the experimental period. The respiration generally was a little higher in flasks to which carbonate had been added than in flasks without any carbonate.

There was very little difference in the ratios O_2 consumed:NO_2^- produced, at the different $p(O_2)$ values. The observed values decreased slightly below the theoretical for the oxidation of ammonium to nitrite:

$$2NH_4Cl + 3O_2 \rightarrow 2HNO_2 + 2HCl + 2H_2O,$$

which gives the O_2:NO_2^- ratio 1·50.

Addition of reducing substances

The results of the experiments on the agar medium seemed to show that the organism was most sensitive to oxygen in the early stages of growth but that when growth was first established the organism became less sensitive. Since this might have been the result of the production by the organism itself of stabilizing substances, an experiment was run on agar medium to which sterile-filtered 14-day-batch culture fluid of *Nitrosocystis oceanus* (pH 5·8; NO_2-N, 324 μg./ml.) was added. The culture fluid was adjusted to pH 7·8 before sterilization and diluted in sterile medium to give final concentrations 25, 2·5, 0·25 and 0·025% (v/v). The plates were incubated in air and the growth followed microscopically for 2 weeks. The culture filtrate enhanced the growth of the organism to some degree; after 2 weeks tetrads and microcolonies consisting of 8–32 organisms were observed with the highest concentration, whereas the controls and the plates containing more dilute culture filtrate hardly showed any growth.

In the next series of experiments three different reducing substances (glutathione, Na thioglycollate, L-cysteine) were added to the agar medium in the following final concentrations: 0·2, 0·02, 0·002 and 0·0002% (w/v). The plates were incubated in air. Cysteine and thioglycollate had no growth-promoting effect whereas 0·02% glutathione accelerated division. After 2 weeks the glutathione plates contained a large number of colonies, most consisting of 30 or more organisms, as compared to the single organisms, pairs and a few tetrads observed on the control plates. Also the production of nitrite was high in the 0·02% glutathione plates; the higher concentration was inhibitory, the lower without effect (Fig. 4).

Yeast extract (0·1 %, w/v) also exerted a stimulatory effect on the growth of *Nitrosocystis oceanus* although different from the effect found with glutathione and low $p(O_2)$ values. On yeast extract plates the colonies were few in number but considerably larger than in any previous experiment.

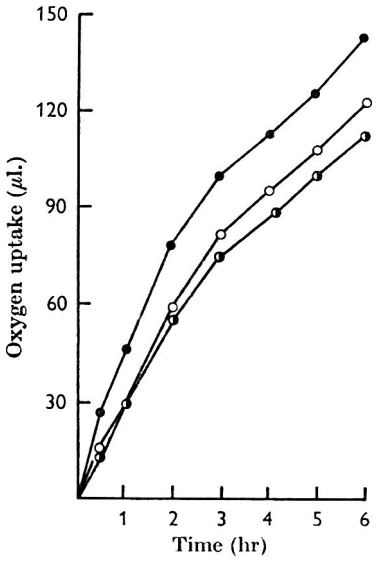

Fig. 3

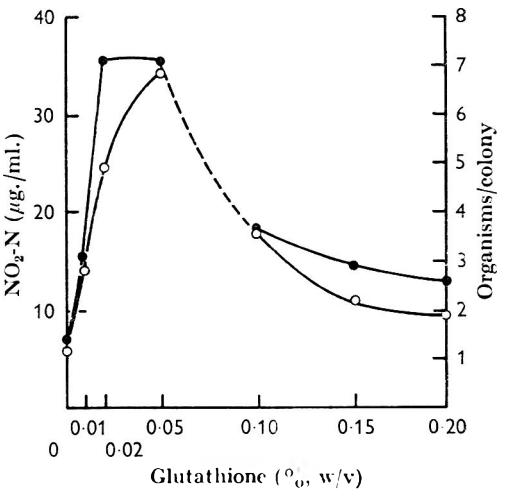

Fig. 4

Fig. 3. Oxygen uptake by suspensions of *Nitrosocystis oceanus* exposed to different values of $p(O_2)$ in a Warburg apparatus. Symbols as Fig. 2.

Fig. 4. Growth and nitrite production by *Nitrosocystis oceanus* on agar medium to which different amounts of glutathione had been added. Incubation for 7 days in air. Organisms/colony ●; NO_2-N, O.

DISCUSSION

That oxygen may be toxic to *Nitrosocystis oceanus* seems to have been well established by the present observations. Several other nitrifying bacteria and *Thiobacillus thiooxidans* have also been found to be irreversibly harmed or strongly inhibited by pure oxygen (Meyerhof, 1917; Schön, 1965; Gundersen, Carlucci & Boström, 1966). It is a question whether the frequent failure to grow nitrifiers on agar media in air is due to oxygen inhibition. Division will take place in *N. oceanus* when the $p(O_2)$ value is somewhat less than in normal air. However, when growth has once been established *N. oceanus* is no longer so sensitive to oxygen, except to very high concentrations. Thus there seems to exist a critical moment in the life of the organism during which growth will not begin unless the $p(O_2)$ is decreased *or* the conditions in the medium made more reducing, e.g. by the addition of certain concentrations of glutathione.

It has been suggested (Gundersen, Boström & Carlucci, 1966) that this sensitivity to oxygen has to do with the partition of reducing equivalents (electrons and reduced NAD or similar hydrogen carrier which have been produced by dehydrogenations of the inorganic substrate) between oxygen (energy metabolism) and the assimilatory system. This explanation seems to derive support from a finding by Schön (1965)

that in *Nitrobacter winogradskyi* the assimilation of CO_2 was completely inhibited by 95% (v/v) oxygen but not nitrite oxidation. It has been shown in respiration experiments with resting suspensions (e.g. Meyerhof, 1917; Hofman & Lees, 1952) that the ammonium oxidizers consume 1·5 moles oxygen/mole ammonium oxidized. This implies that electrons and reducing equivalents from the substrate react readily with oxygen to form water (and a maximum of energy). However, when the oxidation of the substrate takes place according to the equation shown above, no reducing equivalents are available for the reduction of CO_2 in assimilation and no growth can occur.

The possible significance of the redox potential on the activities of the nitrifying bacteria has occasionally been emphasized. ZoBell (1935) observed that growth and substrate oxidation of marine nitrifying bacteria had optima at different redox potentials, ammonium oxidation being optimal at E_h 0·30 to 0·55 V and growth at a somewhat lower potential. The growth-promoting effect of Nährstoff-Heyden (a preparation of egg albumin) on nitrosomonas was ascribed by Kingma Boltjes (1935) as a redox-stabilizing effect of the material. These findings, it is felt, are in favour of the explanation of the oxygen inhibition outlined above, but more comprehensive measurements of the redox fluctuations in cultures of nitrifying bacteria are desirable.

The age and the condition of the inoculum material apparently influences the sensitivity of *Nitrosocystis oceanus* to oxygen. When exposed to air on the surface of an agar plate, the majority of the organisms are incapable of growth, and only a small fraction may overcome the adverse condition and start dividing. Apparently the organism may itself, at a certain active stage of its life, adjust the environment in a direction favourable for its growth (provided the $p(O_2)$ is not too high), possibly by the production of reducing substances.

The observation that oxygen was considerably less toxic in static liquid cultures than on agar medium seems to be explicable by the much shorter diffusion path of oxygen in the latter case, resulting in a constant high oxygen concentration at the cell surface. Nitrifying bacteria, in spite of their motility and basically aerobic nature, never accumulate at the surface of liquid media (as do many other motile aerobic bacteria) but are almost exclusively found near the bottom of tubes and flasks, eventually adhering to calcium carbonate or other particles. From an ecological point of view the finding that *Nitrosocystis oceanus* is able to oxidize ammonium to nitrite in sea-water medium containing very little dissolved oxygen is interesting. It has generally been considered by chemical oceanographers that nitrification in the ocean was only possible in well-oxygenated waters. The coincidence of nitrite and low oxygen contents usually has been taken as evidence of nitrate reduction rather than nitrification (Brandorst, 1959; Braarud & Klem, 1931). However, if *N. oceanus* is widely distributed in the oceans of the world (as may reasonably be expected) then the possibility of nitrification in waters (and sediments) low in oxygen seems as likely as nitrate reduction except, of course, in completely anaerobic environments where nitrate reduction would be the only alternative. Virtually nothing is known about the organisms which carry out the final step of nitrification in the sea, namely the oxidation of nitrite to nitrate. However, if these organisms resemble their soil counterparts (e.g. nitrobacter species) in their relation to oxygen (Gundersen, Carlucci & Boström, 1966) then the oxidation of

nitrite in the sea would be much slower than the oxidation of ammonium at low oxygen concentrations, with the result that nitrite could accumulate until sufficient oxygen were again available for the complete nitrification.

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Haemagglutination with Plant and Insect Viruses

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SUMMARY

The passive haemagglutination test and the sensitivity of the haemagglutination inhibition test were investigated by using two plant viruses (tobacco mosaic virus and latent carnation virus) and an insect virus, tipula iridescent virus. The minimum quantities detectable by these methods were of the order of 0·26 to 0·015 μg. There was no direct agglutination by tobacco mosaic virus or tipula iridescent virus of erythrocytes from a variety of animals.

INTRODUCTION

The passive haemagglutination test (HA) with tanned erythrocytes and the related haemagglutination-inhibition test (HI) can be used to detect very small quantities of antibody and antigen. These tests are based on the technique of Boyden (1951) who found that the treatment of sheep erythrocytes with suitable concentrations of tannic acid rendered them capable of adsorbing a variety of proteins. The advantages, limitations, sensitivity and specificity of these methods have been discussed and illustrated experimentally by Stavitsky (1954). The passive haemagglutination technique has been widely used in medical virology (Friedman & Bennett, 1957; Scott, Felton & Barney, 1957; Benedict & O'Brien, 1958; McKenna, Zuschek & Frankel, 1958; Garabedian, 1965). Matthews (1957) suggested that this technique might have applications in plant virology. Passive haemagglutination with six plant viruses was shown by Saito & Iwata (1964). Their results showed that the haemagglutination titre was 100 to 40,000 times higher than the complement-fixation titre. They described three methods for detecting virus by the haemagglutination-inhibition test including a technique in which the γ_2 globulin fraction of the antiserum was purified and used to sensitize the tanned erythrocytes which were then agglutinated in the presence of homologous virus. The smallest quantity of virus detected was reported to be 10^{-8} g. Haemagglutination inhibition (HI) has been used with insect viruses to detect free virus particles in the haemocoele (MacGregor, 1956). Although the HA test is of potential value in plant and insect virology, the HI test is likely to prove a more useful technique and its sensitivity was thought worthy of investigation.

Direct haemagglutination of erythrocytes from a variety of species has been reported for several animal viruses and it provides a simple and rapid means of virus assay. There are no reports of direct haemagglutination with plant or insect

viruses, so erythrocytes of some common laboratory animals were tested with plant and insect viruses. The viruses used were tobacco mosaic virus (TMV), latent carnation virus (LCV) and tipula iridescent virus (TIV).

METHODS

Purification of virus. Highly purified preparations of virus are required in haemagglutination techniques to decrease non-specific reactions. TMV was purified by two ammonium sulphate precipitations, followed by two cycles of high- and low-speed centrifugation.

Latent carnation virus and its homologous antiserum were kindly supplied by Mr M. Hollings, Glasshouse Crops Research Institute, Rustington, Sussex.

TIV was purified by cycles of high- and low-speed centrifugation and finally in a sucrose gradient.

Preparation of antisera. Rabbits were immunized with TMV and TIV by a total of six intravenous injections, each containing 3 mg. virus and given at 3-day intervals. The rabbits were bled 10 days after the last injection and the sera preserved by the addition of equal volumes of glycerol and stored at 4°.

Passive haemagglutination. Rabbits were bled from the ear and the blood collected in Alsever's solution. The red cells were washed thrice in normal saline and after the third washing 1 ml. packed red cells was diluted in 40 ml. phosphate-buffered saline (pH 7·2). Three volumes of diluted red cells were incubated with three volumes of 1/25,000 tannic acid (Analar) at 37° for 10 min. The tanned red cells were then washed with 3 vol. of phosphate buffer (pH 7·2) and finally resuspended in 2 vol. of normal saline (0·85 %, w/v, NaCl).

In sensitizing the tanned red cells, two factors had to be considered: (1) the optimal pH value for the reaction; (2) the optimal concentration of antigen/ml. tanned red cells. A series of tests was arranged for each antigen, phosphate + citrate buffered saline at pH 6·4, 5·8 and 5·0 being used with various dilutions of antigen. pH 5·0 was found to be the optimal value for all the viruses. Spontaneous agglutination occurred when too much virus was added to the tanned red cells.

To sensitize 1 ml. tanned red cells, 4·0 ml. buffered saline (pH 5·0), a selected concentration of antigen in 0·5 ml. saline and 1 ml. tanned red cells in saline were added to a tube in that order and incubated for 20 min. at room temperature. The red cells were sedimented by low-speed centrifugation (100*g*) and washed in 1·5 ml. of 1/100 normal rabbit serum diluted in 0·8 % saline. The sensitized red cells were finally resuspended in 1·5 ml. 1/100 normal rabbit serum.

Control red cells were prepared by adding 0·5 ml. saline to 4·0 ml. buffer (pH 5·0) followed by 1 ml. tanned red cells and then treated like the sensitized cells.

Titration of antiserum. Serial 2-fold dilutions of antiserum were made in 9 × 75 mm. tubes using 0·2 ml. antiserum and 0·2 ml. 1/100 normal rabbit serum; 0·2 ml. normal rabbit serum was added to each tube, bringing the total volume to 0·4 ml. Samples (0·05 ml.) of sensitized red cells were added to each tube in the titration, 0·05 ml. of control cells were added to a tube containing the highest concentration of antiserum, and the tubes were kept at room temperature. Readings were taken after 3 hr and again after 12 hr; the results were interpreted by using the standards suggested by Stavitsky (1954).

Haemagglutination inhibition test. Serial 2-fold dilutions of antigen (0·2 ml.) of known concentration in 1/100 normal rabbit serum were made in 0·2 ml. of 1/100 normal rabbit serum. The highest dilution of antiserum which gave marked haemagglutination was chosen for HI tests and 0·2 ml. of this diluted antiserum was added to each tube. Controls consisting of antiserum + 1/100 normal rabbit serum, virus + 1/100 normal rabbit serum and 1/100 normal rabbit serum alone were arranged and all the tubes incubated at 37° for 3 hr; 0·05 ml. of sensitized red cells were then added to each tube and readings taken after 3 and 12 hr. The last tube which showed no haemagglutination or only doubtful haemagglutination was taken as the end-point of the titration.

Direct haemagglutination. Perspex plates containing 80 small wells were washed before use in the sequence of 2% caustic soda, tap water, 5% HCl, tap water and thrice in distilled water. Red cells were collected in Alsever solution and washed thrice with normal saline. A range of pH values from 4·2 to 8·0, in steps of 0·2 pH unit, was used. Phosphate + citrate buffers were used from pH 4·2 to 6·0 and phosphate buffers from pH 6·2 to 8·0. 0·5 ml. of each buffer was added to two rows of wells in each plate. 0·1 mg. virus in saline was added to the first row and saline alone to the second as a control. On the third washing the red cells of the species being tested were centrifuged at 500*g* for 5 min. One ml. of packed red cells was added to 100 ml. of saline containing 0·2% bovine γ-globulin to prevent non-specific haemagglutination; 0·5 ml. of diluted red cells were added to each well. The results were clearly visible in 1 hr.

Table 1. *Haemagglutination by antisera to tipula iridescent virus (TIV), tobacco mosaic virus (TMV) and latent carnation virus (LCV)*

	Reciprocal of dilution of antiserum					
Virus	1,000	2,000	4,000	8,000	16,000	32,000
TIV	++++	++++	++++	++++	++++	± +
TMV	++++	++++	++++	++++	++++	− + + +
LCV	++++	+++	++++	++++	++++	− + + +
	64,000	128,000	256,000	512,000	1,024,000	2,048,000
TIV	±	±	−	−	−	−
TMV	++	+	±	−	−	−
LCV	++++	+++	+++	++	±	−

RESULTS

Passive haemagglutination

All the antisera were found to have very high HA titres and the dilutions were started at 1/1000. The antisera were diluted with 1/100 normal rabbit serum. In all tests controls of red cells sensitized with saline instead of antigen, and antiserum replaced by 1/100 normal rabbit serum, were used and no haemagglutination was observed.

The optimal quantities of virus necessary to sensitize 1 ml. red cells were found to be: TIV 0·3 mg. virus; TMV 0·23 mg.; LCV 0·05 mg. These quantities did not necessarily give the highest titre when reacted with their homologous antisera but they did give marked haemagglutination to a dilution very near the end-point.

The results of the HA titrations are given in Table 1. The TIV antiserum gave a titre of 1/32,000, the TMV 1/128,000 and the LCV 1/512,000.

Haemagglutination inhibition

The sensitivity of the HI test was considerably increased by the selection of the highest dilution of antiserum which caused agglutination. The antisera dilutions selected were those which gave the last strong HA reaction (+ + + + or + ± +) in the HA titration and were: TIV 1/16,000; TMV 1/30,000; LCV 1/250,000.

The quantity of virus in the first tube was arbitrarily selected and serial 2-fold dilutions made. The results of the test are shown in Table 2. The minimum quantity of virus detected by HI was found to be: 0·26 μg. for TIV; 0·015 μg. for TMV; 0·024 μg. for LCV.

Direct haemagglutination

TMV did not agglutinate fowl, rabbit, rat, mouse, hamster, human or sheep erythrocytes in a range pH 4·2 to pH 8·0 at room temperature. Considerable lysis was noted below pH 5·0. TMV and TIV did not agglutinate goose erythrocytes between pH 4·2 and 8·0 at 37° or 4°.

Table 2. *Dilution end points of tobacco mosaic virus (TMV), tipula iridescent virus (TIV) and latent carnation virus (LCV) determined by the haemagglutination-inhibition test*

Virus	Mg. virus in first tube	Serial double dilutions of virus									
		1	2	3	4	5	6	7	8	9	10
TIV	0·00012	−	−	−	±	+ + +	+ + +	+ + +	+ + +	+ + +	+ + −
TMV	0·00175	−	−	−	−	−	−	−	−	+ +	+ + + +
LCV	0·00039	−	−	−	−	±	+ + +	+ + +	+ + + +	+ + + +	+ + + +

DISCUSSION

The haemagglutination inhibition (HI) test is more sensitive for the detection of virus in plant and insect pathology than any existing method. The selection of an antiserum dilution very close to the end-point of the haemagglutination (HA) titration greatly enhanced the sensitivity of the reaction. The results of HA tests were subject to variation but this was decreased by using fresh blood in each experiment and an antiserum dilution near the end-point can be used with confidence.

The minimum quantities of virus detected were of the same order as those reported by Saito & Iwata (1964) who used sensitization of tanned erythrocytes with γ_2 globulin. The two rod-shaped plant viruses were detectable in slightly lower concentration than was the spherical insect virus.

Attempts to obtain direct haemagglutination of tobacco mosaic virus failed, though it is possible that erythrocytes from other animals than those tested and at wider pH ranges might yield positive results.

Since the HI test is capable of detecting very small quantities of virus it might be useful in a study of the early stages of virus synthesis. It would be particularly valuable with insect viruses as the only method of assay is by LD50 tests which are both time-consuming and equivocal.

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Loss of Antibiotic Resistance in *Staphylococcus aureus* Resulting from Growth at High Temperature

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SUMMARY

Fifty strains of *Staphylococcus aureus* resistant to penicillin or tetracycline or both were examined for loss of these two resistances as the result of growth at 43–44°. Twelve strains showed a loss of penicillin resistance under the experimental conditions, and of these twelve, three showed a loss of tetracycline resistance. The two resistances were lost independently; in strains in which both resistances were lost most of the sensitive variants had lost one or other resistance but not both. However, penicillin resistance was lost only in strains that were also tetracycline-resistant and *vice versa*. All strains in which a loss of resistance occurred had similar phage-typing patterns and all belonged to the '52, 52A, 80, 81 complex' of strains. Not all strains in the complex, however, showed a loss of resistance. Both penicillin and tetracycline resistance were transduced into suitable sensitive recipients. The results showed that the heat-sensitivity of the transduced resistance was the same as in the donor in which the transducing phage was propagated. The results are consistent with the hypothesis that these two resistances are carried by two separate plasmids, at least in certain strains of *S. aureus*.

Nine multiply-resistant strains of *Staphylococcus aureus* were examined for loss of resistance to antibiotics other than penicillin and tetracycline as the result of growth at elevated temperatures. These other antibiotics included streptomycin, erythromycin, novobiocin, oleandomycin, neomycin and bacitracin. In no case was any loss of resistance observed.

INTRODUCTION

Penicillin-resistance in most naturally occurring strains of *Staphylococcus aureus* is due to the production of the enzyme penicillinase (Kirby, 1944, 1945). This character was believed to be a stable one until Barber (1949) showed that it was lost at a rather high rate in old cultures maintained in the laboratory under a variety of conditions. Bondi, Kornblum & de Saint Phalle (1953) confirmed this finding and showed that growth of the organism at 45° increased the rate of loss of penicillinase production. Fairbrother, Parker & Eaton (1954) also found that growth at elevated temperatures (44°) increased the rate of loss of penicillin resistance in some but not all strains of staphylococci. May, Houghton & Perret (1964) showed loss of both penicillin and tetracycline resistance during growth of a strain of *S. aureus* at 43–44°. These two resistances were lost independently; the authors concluded that they were probably carried on different plasmids, and that, during growth at elevated temperatures, the plasmids failed to replicate at the same rate as the bacteria and were therefore 'diluted out'.

The experiments reported here were done to see whether this phenomenon occurred in other strains of staphylococci and whether loss of resistance to antibiotics other than penicillin and tetracycline was accelerated by growth at elevated temperatures.

METHODS

Strains of Staphylococcus aureus. Fifty-two strains of *S. aureus* chosen from material sent to this laboratory for routine phage typing and one propagating strain (PS 80, the propagating strain for typing phage 80) were examined. They represented several different phage-typing patterns from fourteen different hospitals, and all except one were resistant to penicillin. The majority were also resistant to tetracycline and nine of the strains were resistant to other antibiotics. Fifty of the strains were examined for loss of resistance to penicillin, or tetracycline, or both during growth at 37° and 43–44°. These fifty strains are listed in Table 1. Six of these 50 strains and 3 additional multiply-resistant strains were examined for loss of resistance to antibiotics other than penicillin and tetracycline. They are listed in Table 4.

A single colony was picked from a blood agar culture of each strain. After growth in broth it was phage-typed and tested for resistance to antibiotics.

Growth at 43–44°. Overnight broth cultures of the strains were diluted 1/4 in fresh nutrient broth and incubated at 37° for 1½ hr. with aeration. Samples (0·04 ml.) of this were then added to each of two tubes containing 5·0 ml. nutrient broth pre-warmed to 37° and to 43–44°, respectively. The tubes were then incubated at the appropriate temperature for 5½ hr without aeration. The cultures were then suitably diluted so that 0·1 ml. contained 100–200 viable units and this amount was spread on nutrient agar plates or on starch agar plates. Plates were incubated overnight at 37° and the resulting colonies counted and then tested for loss of antibiotic resistance.

Testing for antibiotic resistance. Two methods were used to screen colonies for penicillin resistance: (*a*) Suitably diluted cultures were plated on starch agar plates and screened for penicillinase production by a modification of Perret's iodometric method (Perret, 1954). (*b*) Cultures were plated on nutrient agar plates and the colonies replicated on to agar plates containing benzylpenicillin 0·2 μg./ml.

Resistance to other antibiotics was tested by replicating colonies grown on nutrient agar plates to agar plates containing the following concentrations of antibiotics: streptomycin calcium chloride, 100 μg./ml. (73·5 units/ml.); novobiocin (sodium salt), 1·0 μg./ml. (0·895 unit/ml.); tetracycline HCl, 5·0 μg./ml. (5 units/ml.); erythromycin base, 2·0 μg./ml. (1·8 unit/ml.); oleandomycin phosphate, 2·0 μg./ml.; neomycin sulphate, 5·0 μg./ml. (3·4 units/ml.); bacitracin, 5·0 units/ml.

Colonies which failed to appear on the appropriate antibiotic plate or appeared to be penicillinase-negative on starch plates were subcultured to nutrient agar plates. They were phage-typed to ensure that they corresponded to the parent culture and the loss of antibiotic resistance was confirmed by restreaking on the appropriate antibiotic-containing agar plate.

Transduction of antibiotic resistance. Penicillinase production and tetracycline resistance were transduced to sensitive recipients by using typing phage 80 as transducing phage. The phage was grown on an appropriate resistant donor strain by

Table 1. *Effect of growth at 43–44° on resistance to penicillin and tetracycline in strains of Staphylococcus aureus*

Strain no.	Phage type	Resistance to P and T	% losing resistance during growth: At 37° — No. colonies screened	At 37° — P^s	At 37° — T^s	At 43–44° — No. colonies screened	At 43–44° — P^s	At 43–44° — T^s
1	80	P^rT^r	199	–	–	56	23·2	–
2	52/52A/80		219	–	–	118	4·2	9·3
3	52/52A/80		200	–	–	52	15·3	7·7
4	80/81		201	–	–	148	8·1	–
5	*52/52A/80/81*		361	–	–	144	4·1	–
6	52/52A/80/81		241	–	–	72	4·1	–
7	52/52A/80/81		321	–	–	345	1·1	–
8	52/52A/80		364	–	–	167	1·7	–
9	52/52A/80/81		777	–	–	1078	3·5	–
10	52/52A/80		490	–	–	380	4·0	–
11	80/81		554	–	–	468	7·5	–
12	52/52A/80		455	–	–	260	2·3	0·4
13	29/+		230	–	–	128	–	–
14	52A/79		407	–	1·0	224	–	1·2
15	*29*		512	0·4	–	900	0·2	–
16	52		340	–	–	696	–	–
17	52A/79	P^rT^s	309	–	·	314	–	·
18	29		343	–	·	191	–	·
19	52/52A/80/81		275	–	·	684	–	·
20	80/81		366	–	·	248	–	·
21	29/52/52A/80		350	–	·	79	–	·
22	81		240	–	·	192	–	·
23	52/80		326	–	·	248	–	·
24	81		470	–	·	462	–	·
25	29/52/52A/80/77	P^rT^s	139	–	·	205	–	·
26	80/81	P^sT^s	525	·	–	521	·	–
27	55/71	P^rT^r	215	–	0·5	720	–	0·7
28	3C	P^rT^s	350	–	·	180	–	·
29	3C/55	P^rT^s	616	–	·	1680	–	·
30	83A	P^rT^r	211	–	–	237	–	–
31	53/75/77		183	–	–	143	–	–
32	7/47/54/75/77/83A		169	–	–	122	–	–
33	83A		290	–	–	360	–	–
34	7/47/54		207	–	–	197	–	–
35	83A		400	–	–	174	–	–
36	77		544	–	–	984	–	–
37	83A		360	–	–	292	–	–
38	75/77	P^rT^s	130	–	·	188	–	·
39	77		176	–	·	98	–	·
40	47/81		306	–	·	243	–	·
41	6/7/47		292	–	·	200	–	·
42	77		280	–	·	465	–	·
43	7/42E/54/81/+		300	–	·	450	–	·
44	77		763	–	·	1600	–	·
45	6/7/47		592	–	·	3000	–	·
46	6/7/47/53/54/83A/81		520	–	·	507	–	·
47	42E/42D	P^rT^r	210	–	–	160	–	–
48	N.T.		486	–	–	400	–	–
49	N.T.		405	–	–	2905	–	–
50	N.T.		96	–	–	188	–	–

–, no loss observed; · = not tested; phage patterns shown in italics were obtained at 1000 × routine test dilution (RTD); N.T. = not typable. The three strains reported as N.T. (nos. 48, 49, 50) are the same as those described by Jevons & Parker (1964) and are common epidemic strains. P = penicillin; T = tetracycline; P^s = penicillin-sensitive; P^r = penicillin-resistant; T^s = tetracycline-sensitive; T^r = tetracycline-resistant.

the soft agar layer method of Swanstrom & Adams (1951). After filtration through sintered glass filters (A.P.D. 1·5 μ), the filtrates were tested for bacterial sterility. Sufficient phage was added to log-phase broth cultures of the recipient strain to give a multiplicity of infection of from 0·4 to 0·7. After allowing 30 min. for phage adsorption the mixtures were centrifuged and the bacteria resuspended in broth containing 0·02 M-sodium citrate to prevent further phage adsorption. Incubation was continued for a further 2–3 hr before screening for transductants by plating the organisms on plates containing either tetracycline 5·0 μg./ml. or penicillin 0·1 μg./ml.

RESULTS

Table 1 lists the 50 strains of *Staphylococcus aureus* which were tested for loss of resistance to penicillin or tetracycline or both during growth at 43–44°. The phage type of the strains and their resistance to the two antibiotics are also shown. Thirteen of the 50 strains tested showed a loss of penicillin resistance when grown at 43–44°. With twelve of these strains no loss of penicillin resistance was detected when the strains were grown at 37°. The loss observed during growth at 43–44° can therefore be assumed to have resulted from growth at this higher temperature.

With one strain (no. 15), however, the loss of penicillin resistance that occurred during growth at 43–44° was no greater than that observed when the strain was grown at 37°. Loss of tetracycline resistance occurred with 5 out of 28 strains tested; but with two of these (nos. 14, 27) it was no greater than the rate of spontaneous loss at 37°. If these three strains be excluded, certain conclusions can be drawn: (1) Loss of penicillin resistance as a result of growth at 43–44° occurred with greater frequency than loss of tetracycline resistance. (2) Strains which lost tetracycline resistance always lost penicillin resistance as well, but these two resistances were lost independently, i.e. it was rare to find a clone which had lost both resistances, although this did occur occasionally. For example, with strain no. 2, loss of resistance to penicillin occurred in 2·5 % of clones, loss of resistance to tetracycline in 8 % of clones, loss of resistance to both antibiotics in 1·7 % of clones.

An examination of the phage-typing pattern of those strains in which loss of resistance was accelerated by heating revealed that they all belonged to the '52, 52A, 80, 81 complex'. This complex, which comprises strains lysed by various mixtures of the four typing-phages (52, 52A, 80, 81) is believed to contain organisms which arose as a result of lysogenizations of a single 'basic' strain by a number of different temperate phages. However, not all members of this complex showed a loss in antibiotic resistance when grown at 43–44°. Eighteen such strains were tested and six of these (nos. 19, 20, 22, 23, 24, 26) did not show any loss in penicillin resistance. None of these six was resistant to tetracycline. On the other hand, all of the twelve strains in the complex in which loss of penicillin resistance or tetracycline resistance, or both, occurred, were naturally resistant to both antibiotics. It seemed possible, therefore, that it was necessary for a strain to be doubly resistant and possibly, therefore, to carry two plasmids, for loss of one or both to occur during growth at 43–44°.

To test this possibility strain no. 2 (phage pattern 52/52A/80), which was resistant to both penicillin and tetracycline (P^rT^r) and consistently showed a loss of both resistances during growth at 43-44°, was examined further. From this strain two

P^sT^r (penicillin-sensitive, tetracycline-resistant) clones and two P^rT^s (penicillin-resistant, tetracycline sensitive) clones were isolated after growth at 43–44°. These 4 clones, together with the original P^rT^r parent, were tested for loss of resistance as result of growth at 43–44°. The results are summarized in Table 2. The P^rT^s isolates lost their penicillin resistance, and the P^sT^r isolates their tetracycline resistance, at approximately the same rate as the P^rT^r parent lost each resistance separately. It seems therefore that the presence of more than one plasmid is not necessary for this loss to occur.

Table 2. *Loss in antibiotic resistance during growth at 43–44° in a strain of Staphylococcus aureus resistant to penicillin and to tetracycline (P^rT^r) and isolates from this strain resistant to penicillin only (P^rT^s) and to tetracycline only (P^sT^r)*

Strain	Colonies screened (no.)	% losing resistance during growth at 43–44°	
		Penicillin	Tetracycline
Strain 2 P^rT^r	934	1·1	2·2
Strain 2 P^rT^s—1	794	1·6	.
Strain 2 P^rT^s—2	1207	1·0	.
Strain 2 P^sT^r—1	601	.	2·1
Strain 2 P^sT^r—2	1360	.	2·4

. = not done.

Table 3. *Loss in antibiotic resistance during growth at 43–44° in strains of Staphylococcus aureus transduced to resistance with phage 80/2 and phage 80/80*

Recipient strain	Transducing phage	Resistance transduced	Clone no.	Colonies screened (no.)	% losing transduced resistance during growth at 43–44°	
					Penicillin	Tetracycline
Strain 2 P^sT^s	80/2	Penicillin	1	348	6·0	.
	80/2	Penicillin	2	316	2·5	.
Strain 2 P^sT^s	80/80	Penicillin	1	1410	< 0·07	.
	80/80	Penicillin	2	335	< 0·3	.
Strain 2 P^sT^s	80/2	Tetracycline	1	541	.	3·3
	80/2	Tetracycline	2	323	.	4·3
PS 80 P^rT^s	80/2	Tetracycline	1	1260	.	1·1
	80/2	Tetracycline	2	987	.	2·0

. = not done.

Transduction experiments

Transduction of antibiotic resistance was done with typing phage 80 grown on two different donor strains: (1) strain 2, which is P^rT^r, and in which both resistances are susceptible to loss during growth at high temperatures; (2) PS 80, the propagating strain for phage 80 (strain 20 in Table 1) which is penicillin-resistant and tetracycline sensitive, and in which the penicillin resistance was not lost during growth at 43–44°. These two phage preparations will be referred to as phage 80/2 and phage 80/80.

The recipient strains used were (1) a P^sT^s clone isolated from strain 2 after heat treatment. This strain was transduced to penicillin resistance with both phage

80/2 and phage 80/80, and also to tetracycline resistance with phage 80/2. (2) PS 80, the propagating strain for phage 80 (strain 20 in Table 1). This strain was transduced to tetracycline resistance with phage 80/2.

Two of each class of transductants were tested for loss of antibiotic resistance during growth of 43–44°. The results of these experiments (Table 3) show that the heat sensitivity of a particular antibiotic resistance was transduced with the resistance itself. Thus, strain 2 P^sT^s transduced to penicillin resistance by phage 80 grown in strain 2 P^rT^r, where the penicillin resistance is sensitive to heat, showed a similar loss in penicillin resistance when grown at 43–44°. The same strain, when transduced to penicillin resistance by phage 80 grown in strain PS 80, where the penicillin resistance was not lost during growth at 43–44°, showed no loss in resistance when grown at this elevated temperature. Similarly, both strain $2P^sT^s$ and PS 80 P^rT^s, when transduced to tetracycline resistance by phage 80/2, showed a loss in tetracycline resistance when grown at 43–44°. The heat stability of the plasmid, therefore, is characteristic of the plasmid itself and seems to be independent of the cell which carries it.

Table 4. *Effect of growth at 43–44° on loss of resistance to streptomycin, erythromycin, novobiocin, oleandomycin, neomycin and bacitracin by strains of Staphylococcus aureus*

Strain	Phage pattern	Resistance pattern	Growth temperature	No. colonies screened	% losing resistance to S*	E	Nv	Ol	Neo	Bac
1	80	$P^rT^rE^r$	37°	495	.	–	.	.	.	.
			43–44°	156	.	–	.	.	.	.
15	*29*	$P^rT^rS^rE^rOl^r$	37°	360	–	–	.	–	.	.
			43–44°	420	–	–	.	–	.	.
16	52	$P^rT^rS^rE^rNv^r$	37°	340	–	–	–	.	.	.
			43–44°	696	–	–	–	.	.	.
30	83A	$P^rT^rE^rNv^r$	37°	723	.	–	–	.	.	.
			43–44°	290	.	–	–	.	.	.
33	83A	$P^rT^rS^rE^r$	37°	197	.	–	.	.	.	.
			43–44°	498	.	–	.	.	.	.
50	n.t.	$P^rT^rS^rE^r$	37°	390	–	–	.	.	.	.
			43–44°	410	–	–	.	.	.	.
51	83A	$P^rT^rS^rE^rNv^r$	37°	505	.	–	–	.	.	.
			43–44°	660	.	–	–	.	.	.
52	n.t.	$P^rT^rS^rE^rNeo^rBac^r$	37°	383	–	.	.	.	–	–
			43–44°	715	–	.	.	.	–	–
53	n.t.	$P^rT^rS^rE^rNeo^r$	37°	623	–	–	.	.	–	.
			43–44°	831	–	–	.	.	–	.

* S, streptomycin; E, erythromycin; Nv, novobiocin; Ol, oleandomycin; Neo, neomycin; Bac, bacitracin; . = not done; – = no loss observed; n.t. = not typable.

Effect of growth at 43–44° on loss of resistance to antibiotics other than penicillin and tetracycline

Nine strains of *Staphylococcus aureus* which were resistant to other antibiotics in addition to penicillin and tetracycline were examined to see whether growth at elevated temperatures accelerated the loss of resistance to these other antibiotics. The results are summarized in Table 4. Five strains were tested for loss of strepto-

mycin resistance; eight strains for loss of erythromycin resistance; three strains for loss of novobiocin resistance; one strain for loss of oleandomycin resistance; two strains for loss of neomycin resistance and one strain for loss of bacitracin resistance. In no case was there a detectable loss of resistance to these antibiotics, either during growth at 37° or at 43–44°.

DISCUSSION

The results reported here confirm those of other workers, particularly those of May *et al.* (1964) and support the hypothesis that resistance to penicillin and tetracycline is carried cytoplasmically, at least in some strains of *Staphylococcus aureus*. However, of the 50 strains examined only 12 strains showed a loss of penicillin resistance as the result of growth at 43–44°, and of these 12 only three showed a loss of tetracycline resistance. The two resistances were lost independently but penicillin resistance was lost only in strains that were also tetracycline resistant and *vice versa*.

All strains in which a loss of resistance occurred shared very similar phage-typing patterns (80, 52/52A/80, 80/81, 52/52A/80/81) characteristic of strains in the '52, 52A, 80, 81 complex'. All the evidence suggests that these strains are closely related and that the difference in typing patterns is the result of lysogenization of a single 'basic' strain by a series of temperate phages (Asheshov & Winkler, 1966). Staphylococcus strain E 169 of May *et al.* (1964) had the phage pattern 52/52A/80/81 and is, therefore, also a member of this complex of strains. In view of this it seemed possible that the loss of two plasmids in these closely related strains was a characteristic of the bacteria carrying them and independent of the plasmids themselves. The results of the transduction experiment would seem to rule this out, however, since they showed that the heat sensitivity of the plasmids was transferred together with the antibiotic resistance, which suggests that the heat sensitivity is a characteristic of the plasmid itself.

Failure to show a loss of resistance to penicillin or tetracycline in the other 38 strains may mean that the method used was not sufficiently sensitive (e.g. not enough colonies were screened) to detect a lower rate of loss. It seems possible that the rate of loss observed as the result of growth at 43–44° simply reflects an increase in the normal rate of spontaneous loss which occurs on storage. Two strains in which a high rate of loss of penicillin resistance occurred as the result of growth at elevated temperature (strains 1 and 2 of Table 1) also showed a very high rate of loss of penicillin resistance on storage. In the case of strain 1, 8% of colonies were penicillin sensitive after 4–5 months storage at room temperature: during the same period 40% of the bacteria in strain 2 showed a similar loss.

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Liberation of Protoplasts from the Mycelium of Phytophthora

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SUMMARY

Spherical protoplast-like bodies were released from the mycelia of *Phytophthora cinnamomi* and *P. parasitica* on digestion of their hyphal walls with an extracellular enzyme preparation from *Streptomyces* sp. in media of appropriate osmotic pressure. Protoplast liberation was observed when using as osmotic stabilizer either sucrose or mannitol (0·2 M–0·8 M); maximum response at about 0·4 M–0·6 M. Although hyphal wall digestion was always extensive, the amount of protoplast release varied widely under seemingly similar conditions. Protoplasts were liberated from the phytophthora mycelium by two mechanisms: bud-like emergence in which cytoplasm was squeezed through a small pore in the wall usually located at or near a hyphal apex; or intercalary hyphal swelling, which consisted of the swelling and rounding off of short hyphal segments whose walls became progressively thinner until they were no longer visible. Both types of protoplast were osmotically sensitive and disintegrated upon dilution of the suspending media.

INTRODUCTION

A crude extracellular enzyme preparation from *Streptomyces* sp. strain QMB 814 (Reese, Smakula & Perlin, 1959) can almost completely dissolve the hyphal walls of *Phytophthora cinnamomi* (Bartnicki-Garcia & Lippman, to be published). The preparation effectively digested isolated purified hyphal walls as well as the walls of living mycelium. The lytic action is believed to be chiefly due to β-glucanases which cleave the major components of the wall, namely cellulose and other glucan(s), to soluble carbohydrate molecules of a wide range of sizes. The present work was prompted by the availability of this enzyme preparation which is capable of rapidly lysing the hyphal walls of phytophthora and a desire to prepare cellular forms of phytophthora devoid of cell walls which could be used for studies of cell-wall biosynthesis. The release of seemingly wall-free protoplast-like bodies from the mycelia of *P. cinnamomi* and *P. parasitica*, described here, was attained by digesting hyphal walls of living mycelia in an osmotically favourable environment.

METHODS

The strains of *Phytophthora cinnamomi* and *P. parasitica*, and the culture media used were as described by Bartnicki-Garcia (1966). Protoplast release was followed under a bright-field Reichert Zetopan microscope equipped with a Polaroid camera. Sterile micro-chambers were improvised with ordinary microscope slides and cover-slips. A square frame of silicon lubricant (Dow Corning, high vaccum grease), the

size of a coverslip, was applied to the centre of a microscope slide. After the slide was sterilized in the autoclave, a small piece of mycelium was placed on its centre and excess liquid removed with a capillary pipette. The mycelium was rinsed once with a drop of enzyme solution. A fresh drop was then added, and a sterile coverslip pressed against the silicon grease to form a liquid-tight chamber. Incubations were at room temperature (21–26°).

Mycelium for incubation in the micro-chambers was prepared by the following technique. A small portion of a mycelial mat from a 7–10-day stationary culture in liquid V-8 juice medium (Campbell Soup Co.) was homogenized at slow speed in 15 ml. of fresh V-8 solution in a Sorvall Omni-mixer for 3 min. The resulting suspension of mycelial fragments was poured into a Petri dish and incubated for 1 or sometimes 2 days, at 27°. Thus were obtained small actively growing pieces of mycelium which could be transferred intact to the micro-chambers with a Pasteur pipette.

The streptomyces enzyme preparation was kindly given by Dr E. T. Reese (U.S. Army, Quartermaster Research and Engineering Centre, Natick, Massachusetts). Enzyme solutions containing 120 Cx cellulase units/ml. were made in 0·05 M citrate buffer (sodium citrate + citric acid; pH 5·8) or in 0·1 M-phosphate buffer ($K_2HPO_4 + KH_2PO_4$; pH 6·8). Buffers were adjusted to various concentractions of sucrose (A.R.), mannitol (commercial grade) or KCl (A.R.) to increase the osmotic pressure. Enzyme solutions were sterilized through Millipore filters.

RESULTS

By providing adequate osmotic protection during the enzymic hydrolysis of living mycelial walls of *Phytophthora cinnamomi* or *P. parasitica*, the formation of large spherical protoplast-like bodies was commonly observed (Pls. 1, 2). Protoplasts were liberated in digestion media which contained mannitol, sucrose or KCl as osmotic regulators in citrate or phosphate buffers. Protoplasts appeared in sucrose or mannitol concentrations ranging from 0·2 M to 0·8 M with better responses at about 0·4 M to 0·6 M. Potassium chloride was similarly effective, except at 0·8 M at which concentration no protoplasts were released. Without osmotic stabilizers no protoplasts were released; instead, amorphous masses of protoplasm were extruded, mainly through the burst tips of some hyphae.

Autolysis of phytophthora mycelium was not detected even after prolonged incubation in the micro-chambers. Thus, control specimens containing no Streptomyces enzyme, showed no sign of hyphal wall degradation, nor any evidence of protoplasm release by either amorphous discharge or protoplast liberation.

Liberated mycelium-protoplasts were usually spherical, but occasionally irregularly bulged bodies were also observed (Pl. 2, fig. 6). Protoplast size ranged from 10 to 40 μ and was considerably greater than the diameter of the parental hyphae. The internal morphology of the protoplasts varied considerably, reflecting in this respect the variability of cytoplasmic structure of the hyphae from which they derived.

From both phytophthora species, protoplasts appeared by two distinct processes, namely a bud-like emergence or intercalary hyphal swelling. Early protoplasts, which under optimum conditions could begin to emerge after incubation for 1 hr, were released even before the walls showed any visible sign of digestion

and always emerged in a bud-like manner, i.e. a variable amount of protoplasm was squeezed through a small not readily visible pore in the wall, forming on the outside a growing spherical body. The final size and time of detachment of protoplasts varied greatly. Sometimes more than one protoplast was formed on a single hypha from what appeared to be a continuous mass of discharging protoplasm (Pl. 2, fig. 7). The liberation of large protoplasts required the mobilization of protoplasm from relatively distant hyphal regions. Thus it was calculated (neglecting volume changes due to osmotic pressure differences) that a protoplast 40 μ in diameter contained protoplasm equivalent to a 1185 μ length of hypha of average diameter 6 μ.

In a more advanced digestion stage, i.e. after incubation for 12 hr, when the hyphal walls were perceptibily thinner, the appearance by intercalary swelling was more common. This consisted of the swelling and rounding off of short hyphal segments whose walls became progressively thinner; protoplasm from adjacent hyphal regions emptied into the swellings which assumed a spherical form. Several such swellings and their resulting protoplast-like bodies could form from a single hypha (Pl. 2, fig. 8). Careful observation revealed that intercalary protoplasts, derived from one hypha, sometimes remained connected in a chain by a barely visible thread, presumably the collapsed remnant of the hyphal wall. No cell wall was readily evident on the protoplasts themselves.

Protoplasts were stable and retained their external morphology for several days when kept in the sealed micro-chambers where they had been liberated. There were, however, marked internal changes, such as the cytoplasm becoming coarsely granulated.

The osmotic stability of the protoplasts was tested by running water through the micro-chamber (silicon grease omitted). Addition of distilled water caused the protoplasts to swell slightly and to burst with disintegration of their membrane, which then ceased to be visible. Concomitant with bursting, a spherical vesicle, about the original size of the protoplast, was frequently formed. Conceivably these vesicles represent swollen cytoplasmic vacuoles and, characteristically, protoplast debris remained attached to them from without (Pl. 2, figs. 9, 10).

The extent of protoplast liberation, from one experiment to the next under seemingly similar conditions, was widely variable. However, duplicates within one experiment were almost always in agreement. In some rare instances, there was observed a massive liberation of protoplasts as illustrated in Pl. 2, fig. 5; on other occasions, however, complete failure to release protoplasts was encountered. Intriguingly, hyphal walls seemed to have been extensively digested in both cases. The usual degree of hyphal wall digestion of phytophthora can be appreciated by comparing figs. 1 and 2 of Pl. 1.

Protoplasm was not always discharged as protoplasts even under favourable osmotic conditions. Thus in a given preparation, obviously suitable for protoplast formation since many such bodies were actually formed, certain hyphae would discharge their cytoplasm into amorphous masses devoid of a limiting membrane, while simultaneously neighbouring hyphae gave normal protoplasts. On one occasion cytoplasm was seen to discharge amorphously through the same pore from which protoplast had emerged immediately before. On comparing mycelial pieces grown for 1 or 2 days no consistent response was obtained indicative of an optimum age for protoplast release.

Mycelial pieces under digestion were still capable of further growth despite concomitant extensive wall lysis. This renewed growth was especially noticed after incubation for about 24 hr with enzyme solutions containing 0·2 M- to 0·6 M-sucrose; it consisted of long branched hyphae with walls of normal appearance radially protruding around the periphery of the extensively digested initial mycelial piece. That the enzyme preparation was active during the growth of these new hyphae may be deduced from the fact that, on continued incubation for another day their walls also were lysed, yielding a second crop of intercalary protoplasts. This alternating sequence of digestion and growth suggests that the hyphal walls of Phytophthora vary in their susceptibility to enzymic attack.

No significant differences in protoplast liberation or behaviour were detected between *Phytophthora cinnamomi* and *P. parasitica* except that the latter seemed to liberate protoplasts somewhat more readily.

DISCUSSION

Protoplast liberation in filamentous fungi was first observed in *Neurospora crassa* (Emerson & Emerson, 1958; Bachmann & Bonner, 1959) by the action of a hemicellulase preparation of snail digestive juice on its mycelial or conidial walls. Subsequently, Villanueva and co-workers released protoplasts and like structures from other chitinous filamentous fungi by using snail juice (Rodriguez-Aguirre & Villanueva, 1962; Garcia Acha & Villanueva, 1963), or a lytic preparation from a streptomyces (Garcia Acha & Villanueva, 1963; Rodriguez-Aguirre, Garcia Acha & Villanueva, 1964). The present communication, we believe, is the first to describe the artificial release of protoplasts from a fungus with cellulosic walls.

A set of requirements specified by Brenner *et al.* (1958) to define a bacterial protoplast could also be extended to fungal protoplasts. The main criterion is the complete absence of cell wall from osmotically sensitive spherical protoplasts. The spherical bodies released from phytophthora mycelia were osmotically sensitive and appeared, under the microscope, to lack most, if not all, of the original cell wall; however, definite evidence for absence of cell wall is not available. Whether or not the 'protoplasts' from phytophthora mycelia are entirely devoid of the original cell wall may be a question related to their genesis. Thus protoplasts which arise by emergence through a pore are probably entirely deprived of cell wall; on the other hand, it is possible that some undigested wall remained attached to the membrane of protoplasts formed by swelling of hyphal sections. In support of this view, studies on the digestion of isolated hyphal walls of phytophthora (Bartnicki-Garcia & Lippman; to be published) have indicated that a small portion of the wall was not dissolved by the streptomyces enzyme preparation even after digestion for 24 hr at 50°.

The phytophthora mycelium is coenocytic; therefore protoplast extrusion may involve the artificial separation of a continuous protoplasm to form spherical units at the time of liberation; see, for example, the simultaneous emergence of two protoplasts through separate pores, but both arising from the same protoplasmic mass (Pl. 2, fig. 7). Bachmann & Bonner (1959) considered the protoplasts from neurospora as artifacts since even the protoplasm confined within two septa could give rise to more than one protoplast.

Protoplast release from phytophthora mycelia was widely variable under seemingly equal conditions even though hyphal walls were always extensively digested. Factors other than wall digestion and adequate osmotic pressure must also intervene in the successful liberation of a protoplast. The extrusion of protoplasm without a limiting membrane and occurring under conditions favourable for protoplast formation might be an indication of variations in the fragility of the cytoplasmic membrane and of its failure to maintain continuity under the pressure of expulsion.

Phytophthora, like other aquatic Phycomycetes, release during their life cycles flagellated zoospores which may be regarded in some respects as natural protoplasts. Although zoospores are not quite spherical, and will withstand very low osmotic pressure without bursting, they seemingly lack a cell wall. Although the internal fine structure of phytophthora zoospores has not yet been reported, electron microscopy of thin-sectioned zoospores of other fungi (e.g. *Blastocladiella emersonii*; Cantino, Lovett, Leak & Lythgoe, 1963) indicate the absence of a cell wall. Questions then arise as to whether the membrane of an artificial protoplast is of the same nature as that of a zoospore and whether phytophthora protoplasts are able to regenerate a cell wall *de novo*.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Mycelium of *Phytophthora cinnamomi* incubated with streptomyces enzyme in phosphate 0·8 M mannitol buffer. Zero time. (× 170.)

Fig. 2. Same as fig. 1 after incubation for 68 hr. (× 170.)

Fig. 3. Protoplast of *P. cinnamomi* released in phosphate 0·6 M-mannitol buffer. (× 825.)

Fig. 4. Protoplasts of *P. parasitica* released in phosphate 0·6 M-mannitol buffer. (× 825.)

PLATE 2

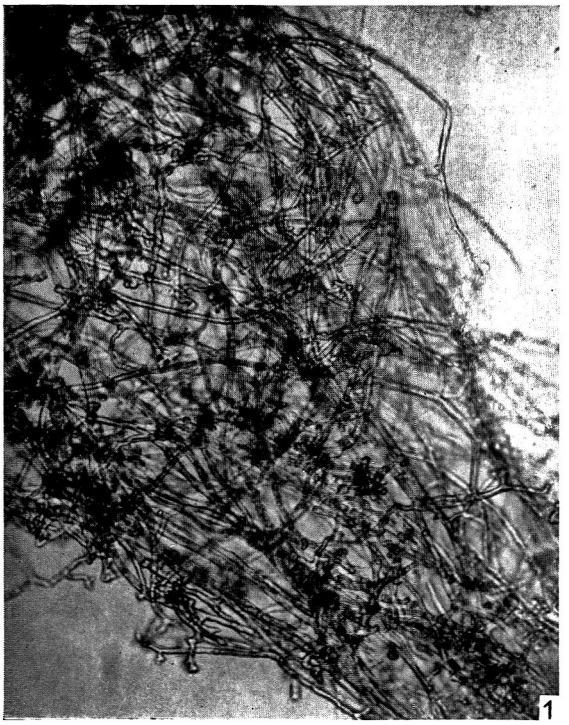
Fig. 5. Abundant release of protoplasts from mycelium of *P. cinnamomi* after digestion for 1 day with Streptomyces enzyme in phosphate 0·6 M-mannitol buffer. (× 115.)

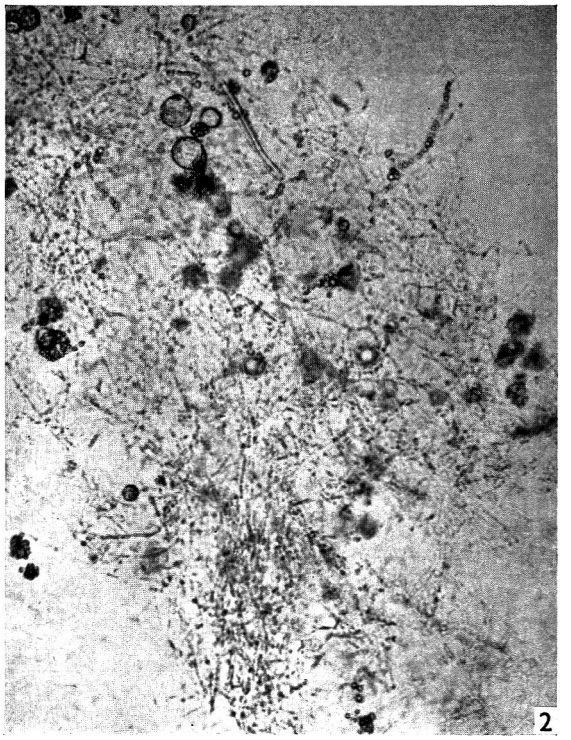
Fig. 6. Irregularly shaped protoplast-like bodies from *P. cinnamomi* produced in same slide as that of Pl. 1, fig. 3. (× 710.)

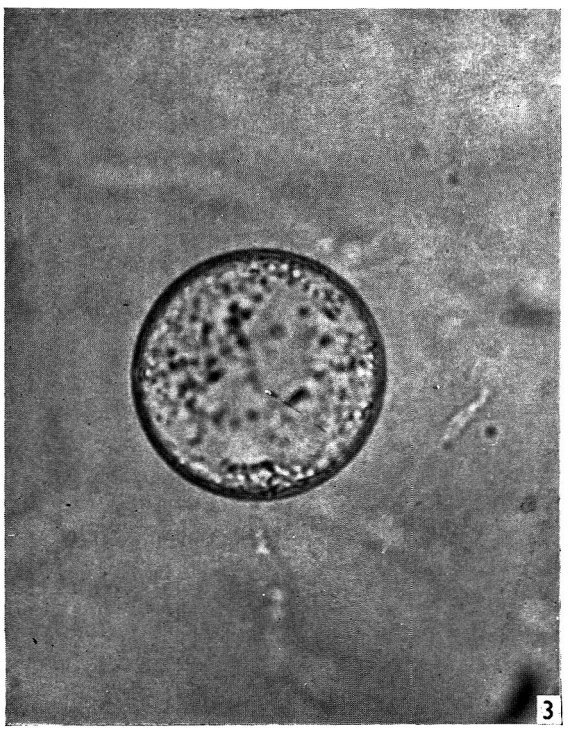
Fig. 7. Simultaneous release of two protoplasts from a hyphal tip of *P. parasitica* after digestion for 2 hr with Streptomyces enzyme in phosphate 0·6 M-mannitol buffer. (× 1140.)

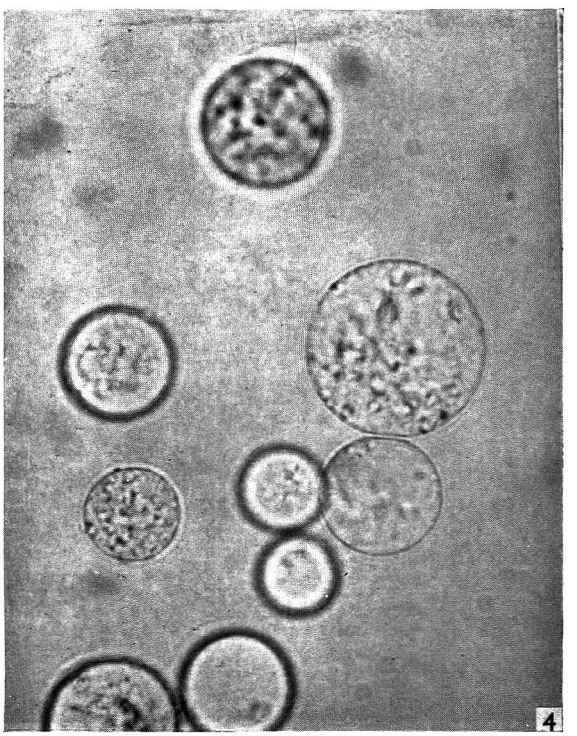
Fig. 8. Chain of protoplasts (right) formed by intercalary swelling of a hypha from *P. cinnamomi* digested with Streptomyces enzyme in phosphate 0·6 M-mannitol buffer for 1 day. (× 115.)

Figs. 9, 10. Protoplasts of *P. cinnamomi* osmotically lysed with distilled water. (× 670.)









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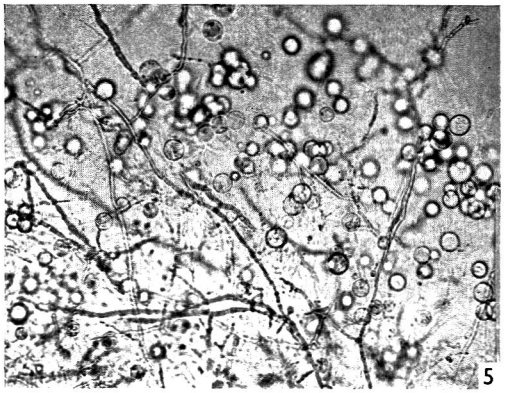
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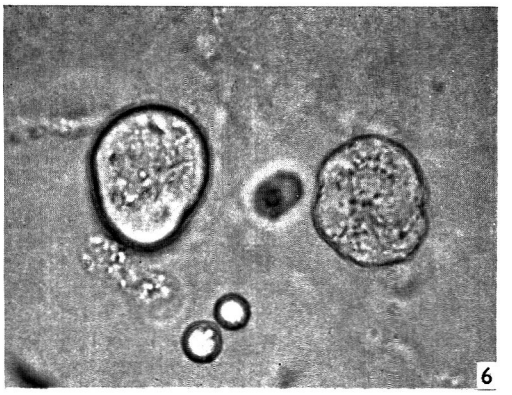
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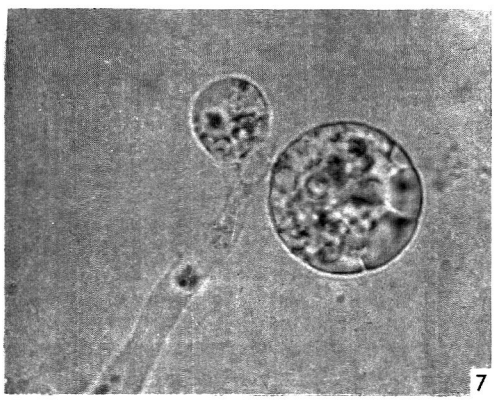
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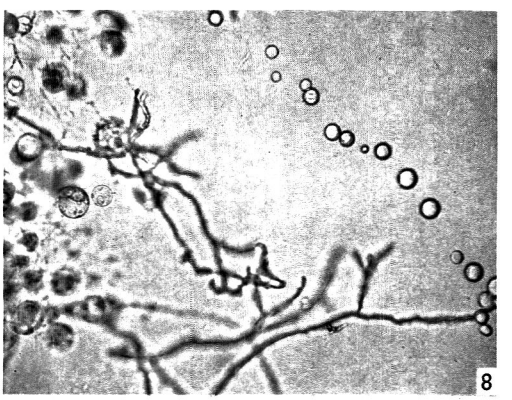
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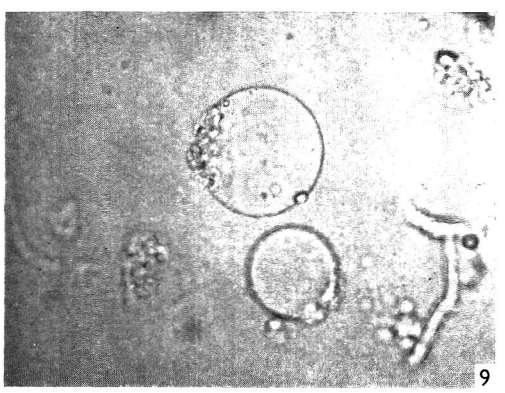
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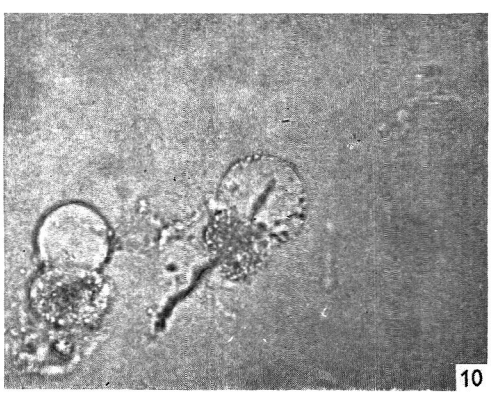
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Observations on the Effects of a Chromosome Duplication in *Aspergillus nidulans*

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SUMMARY

During a study of genetic factors affecting the morphology of *Aspergillus nidulans* a novel and characteristic variant was observed among the progeny of crosses involving certain morphologically normal parents. This type, which had a diminished linear growth rate, segregated in constant proportion; it was designated 'crinkled'. It carried a duplication for a chromosome segment as a result of an unequal translocation in one parent. Crinkled colonies showed vegetative instability and sectored types which, in varying degree, approached normal morphology and growth rate. These revertants probably arose by loss of a variable part of the chromosome segment carried in duplicate. The loss occurred from either the translocated or untranslocated segment.

INTRODUCTION

During a study of genetic factors affecting morphology in *Aspergillus nidulans* a novel and characteristic variant, designated 'crinkled' (*cr*); was observed; *cr* types posed two main problems. They arose regularly and in constant proportion from crosses of particular parents which had normal morphology. Furthermore, *cr* colonies showed vegetative instability in giving sectors which, in varying degree, approached normal morphology and growth rate. The present paper describes an investigation into the genetic determination of *cr* and the nature of its instability.

METHODS

The general techniques used in this work were those of Pontecorvo *et al.* (1953). Incubation was at 37°.

Media. Minimal medium (MM), Czapek-Dox with 1% glucose. Complete medium (CM), a complex medium containing yeast extract, hydrolysed casein, hydrolysed nucleic acid, vitamins, etc. Solid media contained 2% agar.

Organisms. Aspergillus nidulans strains were maintained on CM. Details of mutant alleles, and their locations, are given by Pontecorvo *et al.* (1953), Roper & Käfer (1957), Käfer (1958) and Warr & Roper (1965). Käfer (1961, 1962*a*, *b*, 1965) described the chromosome translocations in many of the currently used strains.

The mutants of main importance in this work were: *y*, yellow conidia; *cha*, chartreuse conidia; *w*2 and *w*3, white conidia; *ad*3 and *ad*20, *arg*1, *bi*1, *cys*2, *lys*5,

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*nic*2 and *nic*8, *paba*6, *phen*2, *pro*1, *pyro*4 and *pyro*12, *ribo*1 and *ribo*2, *s*1, *s*3 and *s*12, growth requirement, respectively, for adenine, arginine, biotin, cystine, lysine, nicotinic acid, *p*-aminobenzoic acid, phenylalanine, proline, pyridoxin, riboflavine and sulphite; *su*1-*ad*20, suppressor of *ad*20; *Acr*1, resistance to acriflavine. Translocations are designated by the symbol (T) after the genotype.

Genetic analysis. The general techniques of analysis used were those of Pontecorvo *et al.* (1953) with modified techniques for ascus dissection (Bainbridge, 1964) and for certain crosses (Bainbridge, 1965).

RESULTS

The segregation of crinkled

Table 1 shows the segregations from a number of crosses between particular parents with normal morphology. The *cr* segregants had a characteristic phenotype (Pl.1, fig. 1) and a diminished linear growth rate; *cr* segregants from the five crosses were phenotypically indistinguishable, suggesting a common mode of origin. However, in case they differed in origin, *cr* segregants were numbered consecutively at isolation.

Table 1. *Aspergillus nidulans: segregation of crinkled from crosses between morphologically normal parents*

Parents	Segregants		P*
	Normal	Crinkled	
*bi*1; *w*3; *cys*2 × *y*; *ad*3 s1	398	193	0·7
*bi*1; *w*3; *cys*2 × *ribo*1 *y*; *nic*8	192	98	0·8
*bi*1; *w*3 × *su*1-*ad*20 *y ad*20; *Acr*1; *phen*2; *pyro*4; *lys*5; *s*3; *nic*8; *ribo*2	250	108	0·2
*bi*1; *w*3; *pyro*12 × *s*12; *pyro*4; *nic*2	294	148	> 0·9
*bi*1; *w*3; *pyro*4 × *ribo*1 *y*; *nic*8	884	424	0·4

* χ^2 test for fit to 2:1 ratio of normal:crinkled. Results from the last two crosses were kindly supplied by Dr J. R. Warr.

The segregation data were statistically inconsistent with 1:1 or 3:1 ratios but showed good agreement with a ratio of 2 normal:1 *cr*. This suggested the possibility of two freely-recombining interacting genes in which one recombinant class was nonviable and the other *cr*. Ascus analysis was undertaken on only a small scale. However, the results indicated inviability of a type which could not be ascribed readily either to technique or to chance inviability of a proportion of ascospores. From the cross, *bi*1 × *y*; *ad*3; *s*1 *cys*2, four classes of asci were found in the 10 asci analysed. Five asci were of type I with 4 spore pairs giving morphologically normal colonies; 1 type II ascus with 2 *cr*, 2 nonviable spore pairs; 2 type III asci with 2 normal, 1 *cr*, 1 nonviable spore pairs and 2 type IV asci with 4 nonviable spore pairs. These segregation patterns corresponded to asci showing the following spore sizes: I, 8 normal; II, 4 normal, 4 small; III, 6 normal, 2 small, and IV, 8 small. Overall ascospore viability, both from ascus analysis and random spore platings, was about 60%.

Two freely-recombining genes with the interactions outlined above would yield asci of types, I, II and III but type IV would not be expected and it was necessary

to seek an explanation of *cr* in other terms. The data of Käfer showed that in all the crosses of Table 1 one or other parent carried a chromosome translocation involving linkage groups III and VIII and it seemed reasonable to explore the possible association of this translocation and *cr*. Pritchard (1960; and personal communication) had already reported a strain of *Aspergillus nidulans* carrying a chromosome duplication and with a morphology somewhat similar to *cr*. Furthermore, from a cross in *Neurospora crassa* involving an unequal translocation in one parent, McClintock (1945) reported ascus analysis data similar in patterns of defective spores to those given above.

Many crosses were examined for the segregation of *cr* but consideration will be given here only to those crosses involving parents of known status in respect of the III–VIII translocation. There were 28 crosses involving 27 different, morphologically normal parent strains. Those crosses in which both parents or neither parent carried the translocation did not yield *cr* segregants; all crosses in which only one parent carried the translocation did yield *cr* among the progeny.

The segregation data could be accommodated if the translocation were unequal. A cross heterozygous for this translocation would yield 25% of the progeny with a deficiency and 25% with a duplication. The former would certainly be nonviable and the latter, presumably, *cr*. It remained to test this idea and to determine the direction of the III–VIII translocation. Mitotic haploidization is used to detect heterozygous translocations (Käfer, 1962*b*). During haploidization of diploid nuclei of *Aspergillus nidulans* there is no crossing-over and markers on the two linkage groups involved in a translocation show complete linkage, in contrast to the free recombination expected in translocation-free diploids. However, this first analysis did not reveal either the direction or extent of the translocation.

Table 2. *Aspergillus nidulans: segregations from crosses involving a crinkled parent*

Cross	Segregants		P*
	Normal	Crinkled	
*cr*14 *y*; *ad*3 × *bi*1; *w*3; *cys*2 (T)	73	74	> 0·9
*cr*15 *pro*1 *y* × *bi*1; *w*3; *pyro*12 (T)	48	54	0·5
*cr*16 *y* × *bi*1; *w*3; *cys*2 (T)	102	97	0·7
*cr*4 *bi*1 × *y*; *w*2; *arg*1	134	143	0·5

(T) indicates a strain carrying the III–VIII translocation.
* χ^2 test for agreement with 1:1 ratio.

As a first step towards testing the idea of an association of *cr* and a chromosome duplication, *cr* segregants from various crosses were backcrossed to strains of normal morphology with or without the III–VIII translocation. In both types of cross the only chromosomal difference between the parental strains of each cross was the suspected presence of a duplicated chromosome segment. A 1:1 ratio of *cr* type to normal was therefore expected in the progeny. Table 2 shows a selection of the results obtained and these are all consistent with the expected ratio. Certain anomalous backcross results, obtained in other crosses, are discussed later.

Anomalous segregations of nutritional markers among cr progeny

Normal and *cr* progeny from various crosses of normal parents were classified for the segregation of a number of nutritional markers. Allele ratios for both morphological classes were 1:1 except in the case of *cys*2 which is located on the right arm of linkage group III. From a cross of an untranslocated strain to a strain carrying *cys*2 and the III–VIII translocation, normal and *cr* segregants were tested for cystine requirement. This marker gave a 1:1 segregation among morphologically normal progeny but of 70 *cr* segregants only 6 required cystine. This suggested that

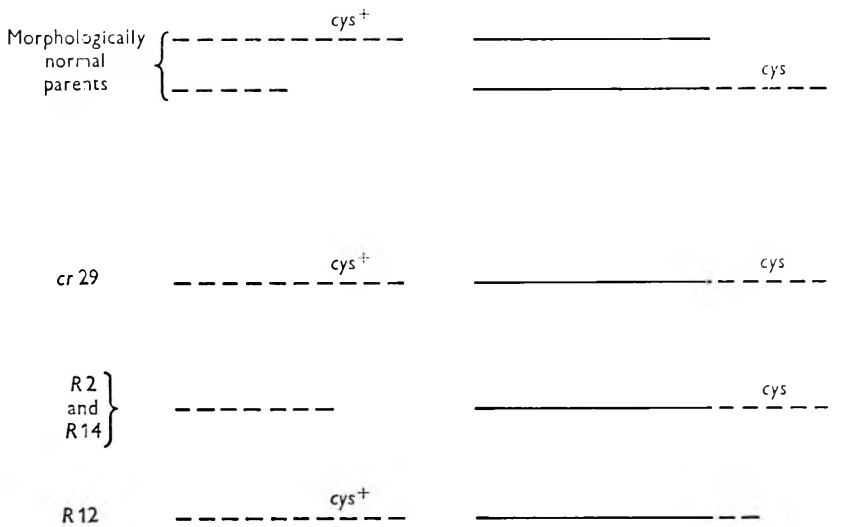

Fig. 1. *Aspergillus nidulans*. A diagrammatic representation of the origin of *cr* and revertants of *cr*. Broken and unbroken lines represent chromosomes III and VIII respectively.

the second parent in the above cross had a translocation to chromosome VIII of the segment of chromosome III which carries *cys*2. The *cr* segregants would then carry this segment of chromosome III in duplicate (Fig. 1). Most *cr* segregants would be *cys*2/*cys*2$^+$ and only those which resulted from crossing-over between *cys*2 and the point of translocation, followed by appropriate segregation, would be *cys*2/*cys*2. The alternative, a translocation from chromosome VIII to chromosome III, would have given *cr* types with duplication of a segment of chromosome VIII. In this case at least 50 % of *cr* segregants would have required cystine. The frequency of *cr*, cystine-requiring segregants gave a measure of the extent of the translocation and indicated that *cys*2 shows about 17 % recombination with the point of break.

A number of cystine-independent *cr* segregants were selected from crosses involving the marker *cys*2. Each was crossed to a morphologically normal, cystine-independent strain with the III–VIII translocation. Several crosses gave no segregants requiring cystine and the *cr* parents in these were presumably *cys*2$^+$/*cys*2$^+$. Two crosses yielded cystine requirers. Of 25 normal segregants from the cross, *cr*15 *pro*1 *y* × *bi*1; *w*3; *pyro*12, 12 required and 13 were independent of cystine. From the cross, *cr*29 *bi*1; *w*2; *arg*1 × *ad*3, both crinkled and normal segregants were

classified. The normal segregants were 23 independent and 14 requirers while of 29 crinkled segregants tested only 1 required cystine. These crosses showed unequivocally that certain *cr* strains were heterozygous *cys*2/*cys*2$^+$ and carried, therefore, a duplication for a segment of chromosome III.

Instability of cr strains

Table 3 lists further segregations from backcrosses of *cr* strains and the results are in sharp contrast to those of Table 2. These results indicated possible instability of *cr* strains; they were later explained in terms of vegetative instability.

Table 3. *Aspergillus nidulans: anomalous results from crosses involving a crinkled parent*

Cross	Segregants		P*
	Normal	Crinkled	
*cr*6 *bi*1; *ad*3 × *bi*1; *w*3 (T)	284	137	≪ 0·001
*cr*4 *bi*1 × *bi*1; *w*3; *cys*2 (T)	515	236	≪ 0·001
*cr*4 *bi*1 × *pro*1 *paba*6 *y*†	118	10	≪ 0·001
*cr*4 *bi*1 × *pro*1 *paba*6 *y*†	308	12	≪ 0·001
*cr*10 *bi*1; *w*3 *ad*3 × *pro*1 *paba*6 *y*	149	71	≪ 0·001

(T) indicates a strain carrying the III–VIII translocation.
* χ^2 test for agreement with 1:1 ratio.
† Results from different individual perithecia.

Three *cr* strains were selected from the cross *bi*1; *w*3; *cys*2 (T) × *y*, *ad*3; *s*1. Each was inoculated at a single point in the centre of several plates of CM. After 10-days incubation more than half of the colonies showed 'revertant' sectors (Pl. 1, fig. 2). The *cr* parents had a linear growth rate about 53% that of wild type and the revertant sectors ranged between 58 and 97% of wild type. All the revertant sectors could still be distinguished from the morphologically normal parents in showing some degree of the general phenotypic features of *cr*. It seemed likely that reversion could be explained in one of two ways. Revertants might arise from mutations which suppressed, in varying degree, the morphological effects of the duplication. Alternatively, there might have been loss at mitosis of a part, presumably a variable part, of one of the chromosome segments carried in duplicate.

A number of *cr* segregants were selected from the cross *bi*1; *w*3; *cys*2 (T) × *y*; *w*2; *arg*1. These segregants, and revertants derived from them, were classified for cystine requirement with the results shown in Table 4. *cr* strains 34, 35 and 36 are presumably *cys*2/*cys*2 in genotype; the genotypes of strains 25, 26, 28, 30 and 33 cannot be determined from the data available; 23, 29 and 31 showed vegetative segregation indicating a genotype *cys*2/*cys*2$^+$. These results confirmed the duplication, in *cr* strains, of a segment of chromosome III and they also strongly indicated the process of reversion. It seemed highly probable that the revertants arose by loss, at mitosis, of part or all of the duplicated chromosome segment. Furthermore, *cr*31 and *cr*29 segregated both cystine-requiring and cystine-independent revertants indicating that the proposed chromosome loss could take place from either the translocated or untranslocated segment.

In an attempt to confirm this last point, three of the revertants from *cr*29 were

crossed to morphologically normal strains with or without the III–VIII translocation. The results are shown in Table 5; no distinction was made in classification between segregants with revertant and those with wild type morphology. Revertants 2 and 14 gave segregations consistent with a 2:1 ratio when crossed with an untranslocated strain but not when crossed with a strain carrying the translocation. The reverse situation obtained for revertant 12. These results could be explained in the terms shown diagrammatically in Fig. 1. *cr*29 is shown with a duplication

Table 4. *Aspergillus nidulans: characterization of cr segregants and their revertants for cystine requirement*

Parent *cr* strain	Requirement for (−)/independence of (+) cystine		
	Parent	Revertants	
		+	−
34	−	0	1
35	−	0	2
36	−	0	2
23	+	0	1
25	+	1	0
26	+	3	0
28	+	1	0
29	+	11	5
30	+	2	0
31	+	1	1
33	+	2	0

The *cr* parents were segregants from the cross *bi*1; *w*3; *cys*2 (T) × *y*; *w*2; *arg*1.

Table 5. *Aspergillus nidulans: segregations from crosses of cr revertants*

Revertant*	Cross to *y*; *ad*3 (T)			Cross to *ribo*1 *y*; *nic*8		
	Normal†	Crinkled	P‡	Normal†	Crinkled	P‡
2	165	10	⩽ 0·001	226	114	> 0·9
12	104	51	0·9	199	3	⩽ 0·001
14	221	7	⩽ 0·001	202	125	0·05

* Spontaneous revertants from *cr*29 *bi*1; *w*2; *cys*2/*cys*2+; *arg*1.
† Revertants and normal were similar in morphology and were not distinguished in classification.
‡ χ^2 test for agreement with 2:1 ratio of normal:crinkled.

of a segment of chromosome III. It was certainly heterozygous *cys*2/*cys*2+ and it was highly likely, from consideration of its pedigree, that *cys*− was carried on the untranslocated segment. In crosses with translocation-free strains, *R*2 and *R*14 behaved like morphologically normal strains carrying the III–VIII translocation; they yielded a 2:1 ratio of normal:*cr*. They had presumably lost, from the previously intact chromosome III, most of that segment of III which was also represented in the translocation to VIII. However, they could not have lost the whole of the segment. From crosses with normal strains carrying the translocation they yielded a low frequency of *cr* segregants and this would not have been the case for two morphologically normal translocated parents. The low proportion of *cr* could

arise by crossing-over in, and appropriate segregation from, the quadrivalent formed at meiosis. A similar argument could be applied to *R*12 except that in this case the behaviour of the revertant approached that of an untranslocated normal strain; it yielded very low frequencies of *cr* segregants in a cross with a translocation-free strain and gave a 2:1 ratio normal:*cr* in a cross with a normal strain carrying the translocation.

It was suggested earlier that mutations at suppressor loci might be invoked to explain reversion. It could be supposed that such mutations would completely or partially suppress the phenotypic effects of the duplications. The results of Tables 4 and 5 excluded the possibility of suppressors which recombined freely with the point of translocation. To maintain this possibility, in view of the vegetative segregation shown in Table 4, it would have been necessary to suggest that each suppressor was specific for one or other of the duplicated segments; this seemed very unlikely. Moreover, certain crosses of revertants (Table 5) yielded only a low frequency of *cr* segregants; a freely-recombining suppressor would have yielded a far higher frequency. However, there remained the possibility, admittedly remote, that reversion might be explained not by chromosome loss but by mutations at suppressor loci linked to one or other duplicated chromosome III segment. Such mutations would have to suppress both the linked *cys* allele and the phenotypic effects of the segment of chromosome distal to the point of mutation. *R*14 required cystine; on the basis of this argument it would have carried a suppressed *cys*2$^+$ allele which it should have been possible to reveal in appropriate crosses. *R*14 was crossed with *bi*1; *cys*2; *lys*5; *cha* and a total of 20,000 ascospores, of hybrid origin, plated on MM supplemented with all requirements except cystine. From platings at various concentrations no cystine-independent segregants were recovered.

DISCUSSION

The observed morphological effects of a chromosome duplication were not surprising since such effects are well known in a wide variety of organisms. However, the vegetative instability was unexpected. So far we have observed this instability through the sectors formed from *cr* colonies. Our data do not permit a calculation of the reversion rate. Such a calculation could be based on the frequency of revertants obtained by plating conidia from *cr* strains. Both from such platings, and from the frequency of sectors yielded by *cr* colonies, it may also be possible to investigate genetic and environmental factors which influence the reversion rate. For the present we can only say that the frequency of spontaneous reversion of *cr* was far greater than that of gene-determined slow-growing variants.

Reversion could best be explained by spontaneous chromosome or chromatid breakage, followed by loss of the fragment at mitosis. It could also be explained by an error in replication which resulted in a break in the continuity of the chromosomal material. We were fortunate in having a system which selects for revertant events but our results do not yet permit distinction between these two possible modes of origin.

When this work was done there were few known markers on the relevant segment of chromosome III. A search is now being made for further markers in this segment and for a similar situation involving other well-marked chromosome segments. It

should then be possible to define the positions of chromosome loss more precisely. Such a system might lend itself to a study of chromosome structure, especially if it were possible to find agents or conditions which selectively produced losses in particular regions of the duplicated segment. The positions of loss would correspond to points sensitive either to breakage or to the interruption of replication.

The authors are grateful to Dr A. J. Clutterbuck and Dr J. R. Warr for valuable discussions. They are particularly indebted to Dr E. Käfer-Boothroyd who made strains and unpublished data freely available; without this help the work could not have been completed. An A.R.C. Postgraduate Studentship to one of us (B. W. B) is gratefully acknowledged.

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EXPLANATION OF PLATE

Fig. 1. A crinkled colony and, for comparison, the edge of a normal colony. × 3.

Fig. 2. A crinkled colony with revertant sectors. × 1.

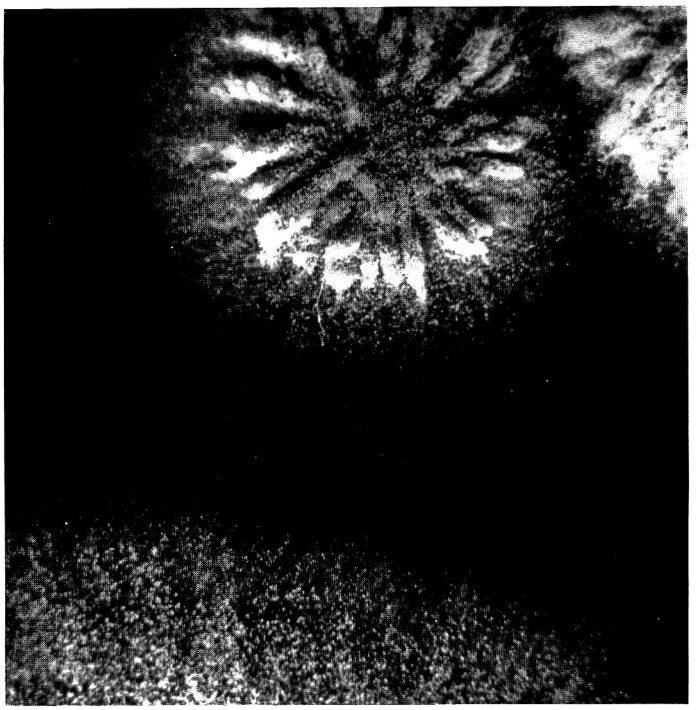

Fig. 1

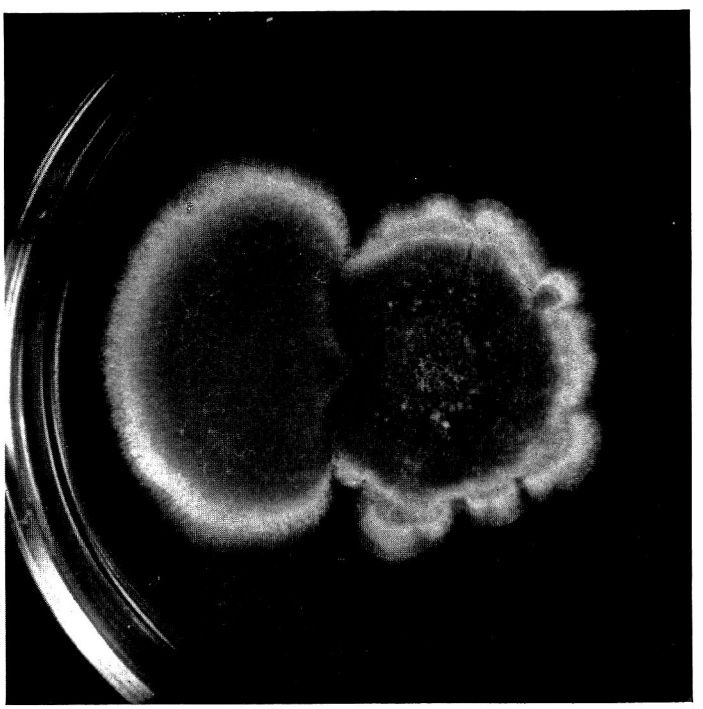

Fig. 2

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The Sterol Requirement of *Phytophthora cactorum*

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SUMMARY

Several sterols and related substances were tested for ability to promote sexual reproduction in *Phytophthora cactorum*. These compounds were classified as *active* (oospores formed), *partially active* (oogonia and antheridia formed, but no oospores), or *inactive* (no oogonia). Quantitative differences between active substances were noted in respect of the numbers of oospores produced and the time of their appearance. The most active substances tested were 29-isofucosterol and β-sitosterol. Loss of activity was associated with shortening of the side chain of the sterol molecule or change in its configuration, absence of a double bond in the B ring or of a hydroxyl group at position 3 on the A ring.

INTRODUCTION

In media which lack sterols the growth of species of the fungal genera *Pythium* and *Phytophthora* tends to be purely vegetative. However, in media to which certain sterols are added, vegetative growth is increased and, more significantly, sexual organs (oogonia and antheridia) develop and oospores are formed (Haskins, Tulloch & Micetich, 1964; Hendrix, 1964, 1965; Elliott, Hendrie, Knights & Parker, 1964; Leal, Friend & Holliday, 1964; Harnish, Berg & Lilly, 1964). In this paper we consider what structural features of the sterol molecule are necessary to confer this physiological activity on it in the case of *Phytophthora cactorum* (Leb. & Cohn) Schroet.

METHODS

Fungus. The strain of *Phytophthora cactorum* used was IMI 21168, obtained from the Commonwealth Mycological Institute, Kew.

Media. The basal medium had the following composition: sucrose, 10·0 g.; L-asparagine, 1·0 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·25 g.; trace element solution, 1·0 ml.; thiamine hydrochloride, 1·0 mg.; Difco Bacto-Agar, 10·0 g.; water, 1·0 l. The trace element solution contained: $Na_2B_4O_7.10H_2O$, 88 mg.; $CuSO_4.5H_2O$, 393 mg.; $Fe_2(SO_4)_3.6H_2O$, 910 mg.; $MnCl_2.4H_2O$, 72 mg.; $Na_2MoO_4.2H_2O$, 50 mg.; $ZnSO_4.7H_2O$, 4403 mg.; EDTA, 5 g.; water 1 l.

Sterols and other substances to be tested were dissolved in diethyl ether and added to the medium after autoclaving and while it was still warm. The concentration of sterol was such that as a rule 1 ml. of ether solution was added to each 20 or 25 ml. of medium to give the desired final concentration. Pure ether was added to the basal medium as a control.

Except where otherwise stated the substance under test was added to the medium at the rate of 10 mg./l.

Culture methods. Small Petri dishes (5 cm. diameter), each containing 5 ml. medium, were used in general; measurements of linear growth rate were made in 9 cm. Petri dishes containing 16 ml. medium. The inoculum was a plug of agar 4 mm. diameter cut from a colony growing on the basal medium. For dry weight measurements the fungus was grown in 25 ml. liquid medium in 100 ml. conical flasks. Incubation was at 25° in the dark.

Linear growth rate was determined by measuring the diameter of the growing colonies at intervals and calculating regression coefficients by the usual least squares method.

Oospore counts. The number of oospores was counted in a radial transect, the width of a low-power microscope field, from the central inoculum plug to the edge of the dish. Two or three transects were counted in each of several dishes. For the analysis of variance the count per transect, x, was transformed to $(x+\frac{1}{2})^{\frac{1}{2}}$.

To provide a basis for comparison between experiments, tests on both basal medium and cholesterol were always included.

Chemicals. In the following list, * indicates that the sample was pure according to gas–liquid chromatography (GLC). β-Sitosterol was extracted from oat seed, and was 90% pure by GLC, the 10% impurities being mainly campesterol and cholesterol (Knights, 1965). A mixture of the acetates of isofucosterol and $\Delta^{7,24(28)}$-stigmastadienol was also obtained from an extract of oat seed. From this, isofucosterol, sample 1, was prepared, and this contained 10% stigmasterol (Knights, 1965) according to GLC. Isofucosterol, sample 2, was synthetic material obtained from Dr J. Dusza.

Ergosterol, sample 1, was obtained from Mr D. J. Austin (University of Glasgow), and was about 80% pure by GLC; sample 2 was obtained from British Drug Houses Limited, and purified by recrystallization from acetone. Fucosterol* was supplied by Dr G. Wood (Organon Limited, Newhouse, Lanarkshire), stigmasteryl acetate* by Professor D. H. R. Barton (Imperial College, London), ergosterol peroxide by Mr I. M. Campbell (University of Glasgow) and 24-dihydrolanosterol by Mr D. J. Austin. Dr C. J. W. Brooks (University of Glasgow) supplied Δ^7-cholestenol*, Δ^5-cholesten-3-one*, $\Delta^{4,6}$-cholestadien-3-one*, Δ^4-cholesten-3-one*, desmosterol*, and a mixture of β-sitosterol and campesterol (3:1); 7-dehydrocholesterol was obtained from Sigma Chemical Co., and recrystallized from acetone*. Δ^5-cholenol* was synthesized from commercial cholenic acid.

RESULTS

The substances tested are listed in Table 1, in which they are classified as active, partially active and inactive. The addition of some substances to the medium resulted in the production of oogonia and antheridia followed two days or so later by oospores; these are called active. With other substances oogonia and antheridia were produced but no oospores were formed, and generally the contents of the oogonia degenerated after about 4 days; these substances are called partially active. With inactive substances no oogonia or antheridia were produced, or exceptionally a few appeared at a late stage of growth and then at the same time in both supplemented and basal medium.

Active substances

In addition to the substances shown in the first part of Table 1 mixtures of β-sitosterol and campesterol (3:1) and of the acetates of 29-isofucosterol ($\Delta^{5,24(28)}$-stigmastadienol) and $\Delta^{7,24(28)}$-stigmastadienol (2:1) were active.

The effects of varying concentrations of β-sitosterol and cholesterol on vegetative growth and on oospore production were examined and are shown for β-sitosterol in Figs. 1, 2 and Table 4, Expt. 4. At high concentrations the hyphal growth rate was decreased but the dry weight of organism was relatively little affected; the colony was correspondingly denser. Hendrix (1965) also found increased vegetative growth in cholesterol medium.

Table 1. *List of substances tested and summary of results*

Active (oogonia, antheridia and later oospores)	*Partially active* (oogonia and antheridia only)
Cholesterol	Cholestanol
Cholesteryl acetate	Epicholestanol
β-Sitosterol	Δ^5-Cholenol
β-Sitosteryl acetate	Δ^5-Cholesten-3-one
29-Isofucosterol	Δ^4-Cholesten-3-one
Stigmasteryl acetate	$\Delta^{4,6}$-Cholestadien-3-one
Ergosterol	
Fucosterol	
Δ^7-Cholestenol	
Inactive	
7-Dehydrocholesterol	Coprostanol
Desmosterol	Cholestane
Lanosterol	Squalene
24-Dihydrolanosterol	Calciferol
Cycloartenol	DL-α-Tocopherol
Ergosterol peroxide	Diosgenin
Δ^5-Cholenic acid	Tomatidine
Bisnorcholenic acid	Solanidine
Δ^5-Pregnenol	Coumarin
Δ^5-Pregnen-3β-ol-20-one	Polyporenic acid A
Progesterone	Lecithin
Testosterone	Oleic acid
Dehydroepiandrosterone	Sodium acetate
Oestrone	

The various active compounds were compared in a series of tests in which the sterols were added at 10 mg./l. The relative activities were assessed by three criteria: (1) by the time of appearance of oogonia and oospores; (2) by the relative numbers of oospores; (3) by the effect on linear growth rate. The data for the first of these criteria are the most complete and Figs. 3–6 show the proportion of plates in which oogonia and oospores were seen on successive days. On this basis the greatest activity was shown by isofucosterol and β-sitosterol. Cholesterol promoted slightly less rapid development, while ergosterol and stigmasteryl acetate were somewhat slower. Development with Δ^7-cholestenol was very slow.

The time of appearance of sexual organs in the culture was related to the linear growth of the hyphae. Generally reproductive bodies did not appear until after the

hyphae had reached the edge of the Petri dish. The most rapid growth was promoted by β-sitosterol and cholesterol among substances tested in this way (Table 2); oogonia and oospores appeared sooner with these substances than with ergosterol, with which hyphal growth was slower.

As regards the number of oospores produced in a culture (Tables 3–4), isofucosterol and β-sitosterol were easily the most active, but considerable numbers were also produced with cholesterol. Stigmasteryl acetate and ergosterol were less active and few oospores were found with fucosterol and Δ^7-cholestenol.

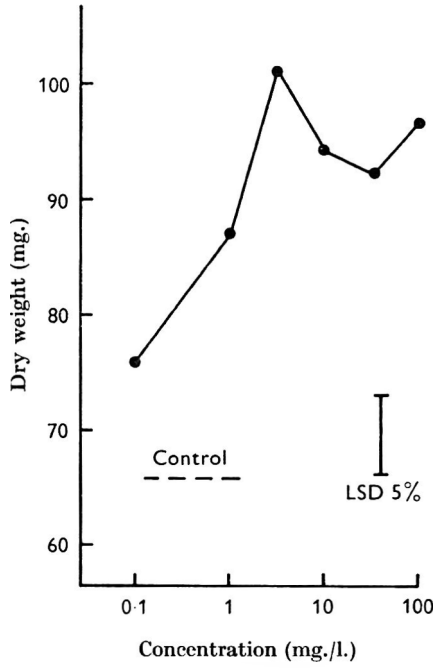

Fig. 1

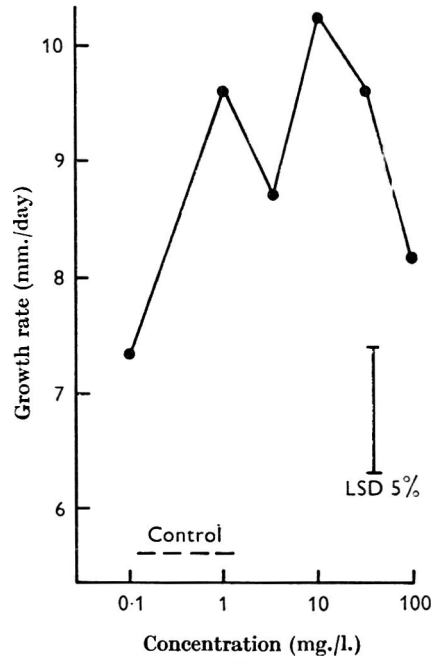

Fig. 2

Fig. 1. Effect of concentration of β-sitosterol on growth (dry weight) of *Phytophora cactorum*. Mycelium harvested after 18 days incubation at 25°.

Fig. 2. Effect of concentration of β-sitosterol on linear growth rate of hyphae of *Phytophthora cactorum*. Scale refers to mm. increase per day in colony diameter.

With fucosterol at 10 mg./l. oogonia appear early (Fig. 4) and in great numbers, but oospores were slow to appear, not being seen until 4 days later, and then only a very small proportion (about 1%) of the oogonia formed them. However, the oospore count was considerably increased at fucosterol 100 mg./l. (Table 4, Expt. 5).

A few oogonia but no oospores were formed with β-sitosterol at 0·1 mg./l. and with fucosterol at 1 mg./l.

There was great variation from one experiment to another. This was especially true with the growth rate on basal medium, less so on cholesterol medium (Table 2). These differences might have been due in part to differences in the way the medium was made, but were largely due to variation in the fungus. In some experiments a fast-growing strain was used, in others a slow one (Table 2, Expt. 3); these arose

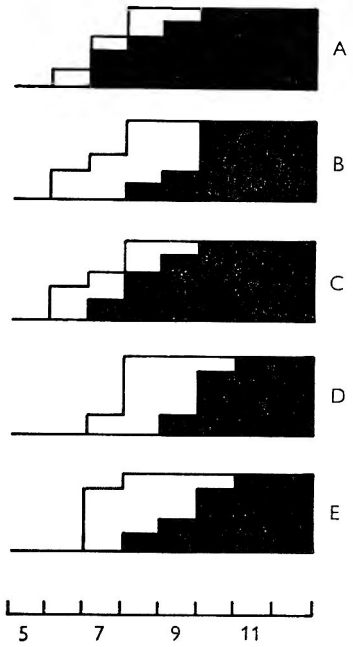

Fig. 3

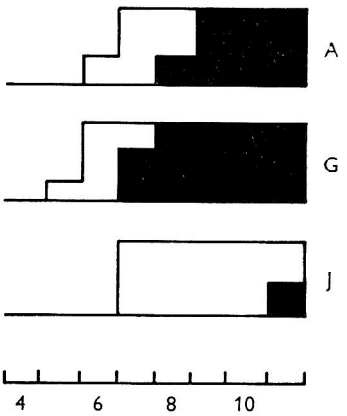

Fig. 4

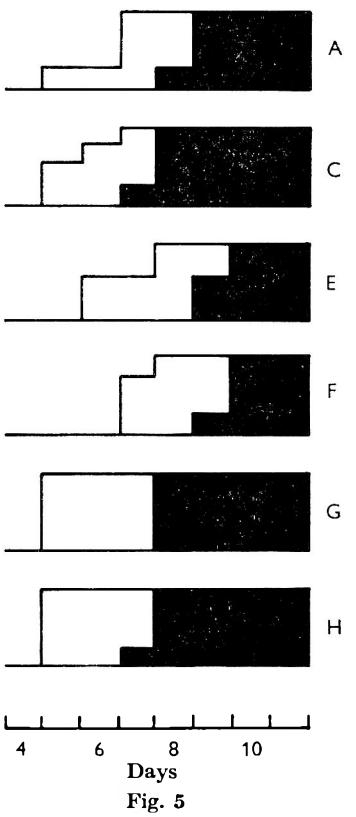

Fig. 5

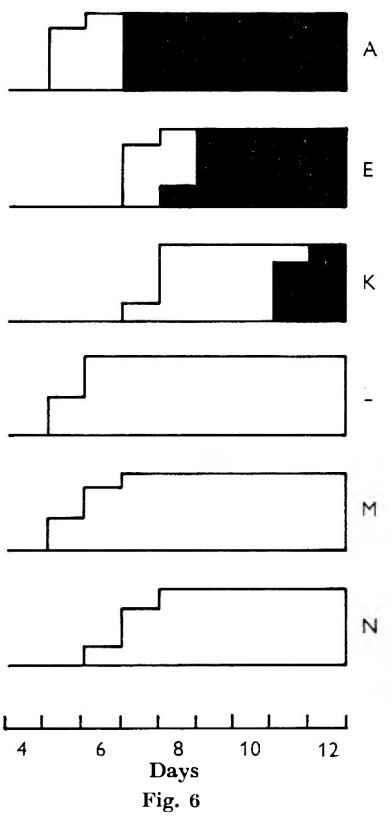

Fig. 6

Figs. 3–6. *Phytophthora cactorum*: course of development of sexual organs in variously supplemented media in four experiments. The vertical axis represents the proportion of cultures having oogonia (white) and oospores (black) on successive days.

A, Cholesterol; B, cholesteryl acetate; C, β-sitosterol; D, stigmasteryl acetate; E, ergosterol, sample 1; F, ergosterol, sample 2; G, isofucosterol, sample 1; H, isofucosterol, sample 2; J, fucosterol; K, Δ^7-cholestenol; M, Δ^6-cholenol; N, Δ^5-cholesten-3-one.

Table 2. *Phytophthora cactorum: growth rates (mm. increase in diameter of colony/day) in 9 cm. Petri dishes in basal medium with sterols added at 10 mg./l.*

Sterol added	Experiment 1 Mean	1 Rel. value	2 Mean	2 Rel. value	3 Mean	3 Rel. value	4 Mean	4 Rel. value	5 Mean	5 Rel. value
	Growth rate									
Cholesterol	7·52	1·57	6·10	2·34	7·26 f 5·86 s	2·47 2·79	10·08	1·29	8·53	1·13
β-Sitosterol	7·17	1·50	6·28	2·41	—	—	10·10	1·30	—	—
Stigmasteryl acetate	—	—	—	—	—	—	7·60	0·97	—	—
Ergosterol (sample 1)	5·48	1·15	3·64	1·40	—	—	8·23	1·06	—	—
Cholestanol	7·34	1·54	5·04	1·94	—	—	9·43	1·21	—	—
Δ^5-Cholenol	5·15	1·08	6·44	2·48	—	—	8·60	1·10	—	—
Δ^5-Cholesten-3-one	4·18	0·87	2·98	1·15	—	—	7·28	0·93	—	—
Δ^4-Cholesten-3-one	—	—	—	—	—	—	7·83	1·00	—	—
Basal medium	4·78	1·00	2·60	1·00	3·09 f 2·14 s	1·00 1·00	7·80	1·00	7·56	1·00
Least significant difference between means (5 % level)	1·21	—	0·91	—	0·61	—	1·14	—	1·48	—

In Expt. 3, f and s were fast-growing and slow-growing segregants from one colony.

Table 3. *Phytophthora cactorum: oospore counts in relation to activity of sterols*

Counts were made in three radial transects in each of 5 dishes (day 1) and again in the same dishes one week later (day 2). Sterol concentration = 10 mg./l. in all cases.

Sterol	Day	Dish 1	2	3	4	5	Mean count/ transect
		Counts/dish (3 transects)					
Cholesterol	1	111	41	33	24	20	
	2	110	32	15	28	39	15·1
Cholesteryl acetate	1	5	0	10	18	1	
	2	4	7	2	28	0	2·5
β-Sitosterol	1	148	193	41	87	101	
	2	222	145	36	223	74	42·3
Stigmasteryl acetate	1	4	1	7	169	16	
	2	0	1	4	93	42	11·2
Ergosterol (sample 1)	1	30	32	18	5	29	
	2	73	32	13	6	11	8·3

Analysis of variance

	D.F.	Mean square	*F*
Compounds	4	95·43	6·82*
Dishes within compounds	20	14·00	10·45*
Days	1	0·01	
Days × compounds	4	0·73	
Days × dishes of one compound	20	1·77	
Within dishes on same day	100	1·34	

* Significant at 0·1 % level.

by segregation from the original strain. Again, in different experiments with cholesterol medium, oogonia appeared in 4 to 8 days. There were also considerable differences in the number of oospores produced in different experiments (Table 4). Nevertheless, a similar pattern was seen in each experiment when we compared the activities of different sterols relative to the standards, basal medium and cholesterol.

β-Sitosterol and isofucosterol are the principal sterols present in oats (Knights, 1965). Since these are the most active substances tested it is evident why oatmeal agar has always been found to be a most satisfactory natural medium for sexual stages of the phytophthora species.

Table 4. *Phytophthora cactorum: oospore counts as affected by sterols in five experiments*

Sterol	Concentration (mg./l.)	Experiment 1*	2	3	4	5
		Mean count/transect				
Cholesterol	10	15·1	5·4	2·0	—	—
Cholesteryl acetate	10	2·5	—	—	—	—
β-Sitosterol	100	—	—	—	120·2	—
	10	42·3	—	22·0	126·0	68·0
	1	—	—	—	17·8	1·7
	0·1	—	—	—	0	0
Isofucosterol, sample 1	10	—	52·6	8·1	—	—
Fucosterol	100	—	—	—	—	5·6
	10	—	—	—	—	1·0
	1	—	—	—	—	0
Ergosterol, sample 1	10	8·3	8·2	—	—	—
sample 2	10	—	—	0·2	—	—
Stigmasteryl acetate	10	11·2	—	—	—	—

* See Table 3.

Partially active substances

The substances found to be partially active are listed in the second part of Table 1.

When cholestanol was added to the medium at 2·5, 10, 40 and 160 mg./l., oogonia developed at all concentrations, but no oospores were seen. With Δ^5-cholesten-3-one and $\Delta^{4,6}$-cholestadien-3-one, oogonia but no oospores were produced when the substances were added at 1, 10, and 100 mg./l. In these cases, increasing the concentration of the substance did not lead to oospore production. The distinction between active and partially active substances would thus appear to be a real qualitative one.

The addition of cholestanol and Δ^5-cholenol to the medium resulted in the production of oogonia early, generally at the same time as with cholesterol (Fig. 6). These substances significantly increased the hyphal growth rate (Table 2). With the three cholestenones, the oogonia appeared later, about 2 days after the cholesterol controls. These compounds did not increase hyphal growth as much as did cholestanol and Δ^5-cholenol.

Inactive substances

Substances found to be inactive are shown in the third part of Table 1. Inactive substances were not always without morphogenetic effects. Irregular swelling of the hyphae was observed especially with 7-dehydrocholesterol and ergosterol peroxide, and with coprostanol many curiously coiled and twisted hyphae appeared where one would expect oogonia and antheridia. Lecithin and oleic acid were both inactive, although oleic acid has been found to stimulate vegetative growth (Hendrix, 1965). However, the addition of lecithin or oleic acid to medium with cholesterol at 10 mg./l. increased the numbers of oospores (see Table 5) although the scale of the experiments was inadequate to give a significant result in the case of oleic acid. Oleic acid and lecithin enhance the activity of cholesterol in the sterol-requiring protozoan *Tetrahymena corlissi* (Holz *et al.* 1961).

Table 5. *Phytophthora cactorum: effect of lecithin and oleic acid on oospore production in medium containing cholesterol 10 mg./l.*

Concentration of lecithin or oleic acid (mg./l.)		Dish 1	2	3	4	Mean count/ transect
		Counts/dish (3 transects)				
Lecithin	0	57	23	21	—	11·2
	10	78	60	111	—	27·7
	100	99	111	41	—	27·9
Oleic acid	0	0	7	2	6	1·3
	10	18	9	1	—	3·1
	100	11	11	19	9	4·2

Analysis of variance

	Lecithin		Oleic acid	
	D.F.	Mean square	D.F.	Mean square
Between concentrations				
Presence *vs.* absence	1	22·338†	1	3·228*
100 *vs.* 10 mg./l.	1	0·018	1	1·033
Between dishes at one concentration	6	3·434	8	0·838
Between transects in one dish	18	1·114	22	0·470

* Significant at 5% level compared with counts in one dish, not significantly greater than difference between dishes.

† Significant at 0·1% level compared with counts in one dish, and at 5% level compared with differences between dishes.

An effect of agar

We previously reported the occurrence of oospores in small numbers in cultures grown on basal medium, and with some substances now classified as inactive and partially active (Elliott *et al.* 1964). This was due to the use at that time of Oxoid agar No. 3. Subsequently we used only Difco Bacto-Agar and, with this, no oospores were formed on the basal medium, although in some of the tests a few oogonia appeared at a late stage of growth. With partially active substances, the use of Oxoid agar sometimes resulted in the early appearance of oospores, whereas with the Difco agar the oogonia appeared early but were not followed by oospores. It would appear that some factor in Difco agar is limiting and this emphasizes the differences in activity between various steroids in promoting sexual reproduction.

DISCUSSION

A comparison of the molecular structures of the active compound, cholesterol Fig. 7, II) with the partially active ones, Δ^5-cholesten-3-one (VI), cholestanol (V), and Δ^5-cholenol (III), each of which differs from cholesterol in one point only, shows

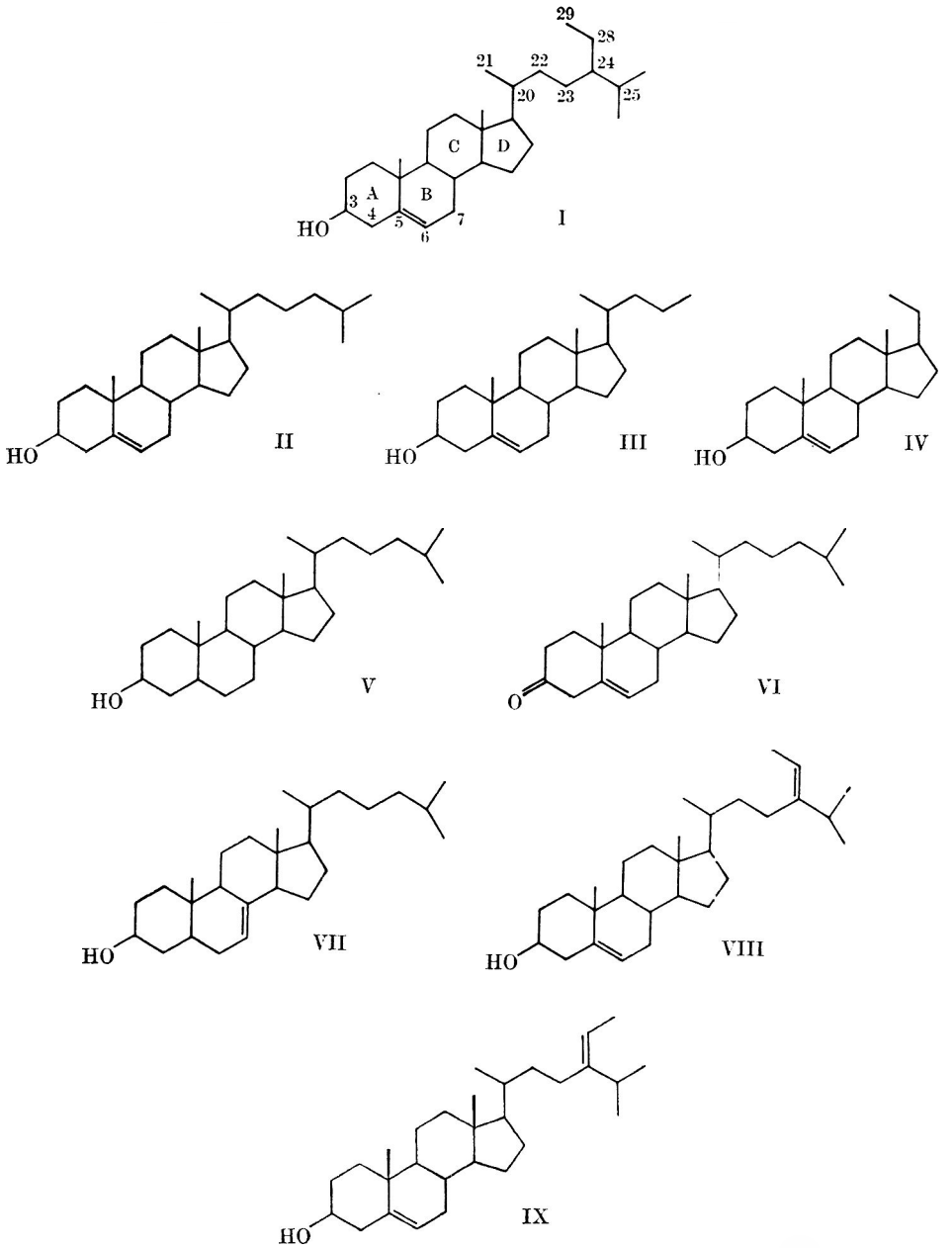

Fig. 7. Structures of sterols tested: I, β-sitosterol; II, cholesterol; III, Δ^5-cholenol; IV, Δ^5-pregnenol; V, cholestanol; VI, Δ^5-cholesten-3-one; VII, Δ^7-cholestenol; VIII, 29-isofucosterol; IX, fucosterol.

that to induce sexual activity and allow its completion in *Phytophthora cactorum* the sterol nucleus is required with a hydroxyl group at carbon 3, a double bond in the B ring, and a side chain of more than five carbon atoms.

Only compounds with a hydrocarbon side chain are active. For example, while cholenol has some partial activity, cholenic acid (Fig. 8, XV) has none, and such

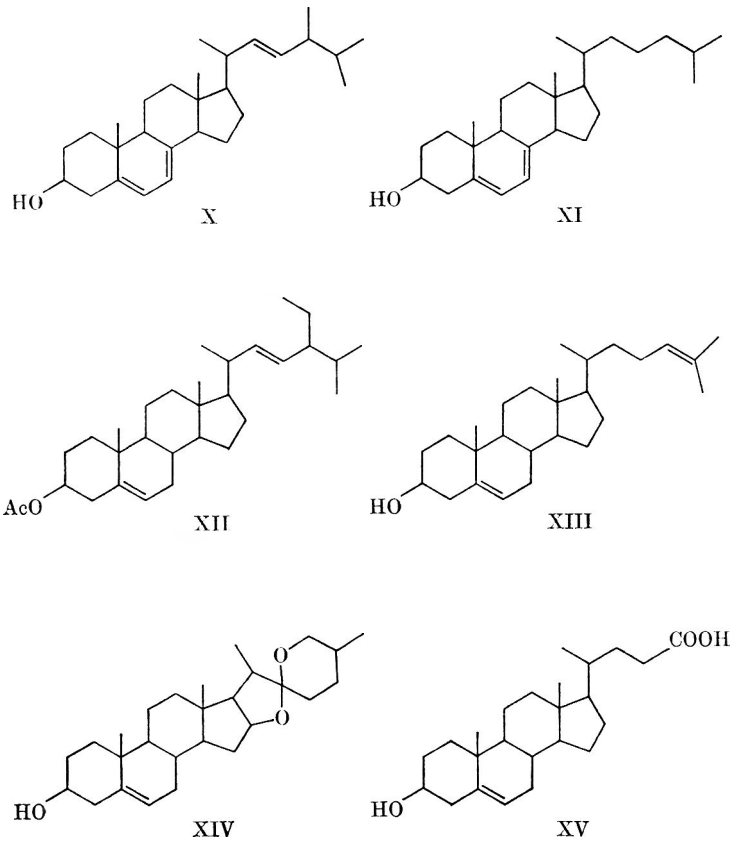

Fig. 8. Structures of sterols tested: X, ergosterol; XI, 7-dehydrocholesterol; XII, stigmasteryl acetate (Ac = $CH_3.CO$—); XIII, desmosterol; XIV, diosgenin; XV, cholenic acid.

substances as diosgenin (XIV) and solanidine with their much modified side chains are also inactive. The mammalian sex hormones with their short and modified side chains are inactive. Increasing the length of the side chain results in increasing activity, as shown by the series pregnenol (IV), cholenol (III), cholesterol (II), β-sitosterol (I).

One of the most striking results obtained relates to the position of the 29th carbon atom. The double bond linking carbons 24 and 28 holds no. 29 in one position in 29-iso-fucosterol (VIII) and in the alternative position in fucosterol (IX). The first of these is perhaps the most active substance found; with the second few oospores were formed although oogonia were very numerous. In β-sitosterol, the 29th carbon is free to rotate; it could presumably readily adopt the isofucosterol configuration in fitting into some cellular structure.

A double bond in the side chain at carbon 22 results in slower development, stigmasteryl acetate (Fig. 8, XII) being less active than β-sitosteryl acetate. A double bond at carbon 24 as in desmosterol (XIII), however, surprisingly results in no activity. Ergosterol (X) has a double bond at carbon 22, but it also has an extra methyl group at carbon 24 and this would increase the resemblance of the side chain to that of β-sitosterol.

7-Dehydrocholesterol (XI) was inactive. It has two double bonds in the B ring. Ergosterol has the same B ring structure, and its activity (in contrast to the inactivity of 7-dehydrocholesterol) is presumably due to a compensatory effect of its side chain configuration.

7-Dehydrocholesterol, Δ^7-cholestenol and ergosterol all support the growth of *Tetrahymena corlissi* (Holz *et al.* 1961) and overcome the inhibition of growth of *T. pyriformis* induced by triparanol (Holz, Erwin, Rosenbaum & Aaronson, 1962). However, only the first two support the growth of the beetle *Dermestes vulpinus* (Clayton & Bloch, 1963). On the other hand (and as we find in *Phytophthora cactorum*) ergosterol is active but 7-dehydrocholesterol inactive in promoting growth of a parasitic mycoplasma (Smith & Lynn, 1958). Ergosterol is slightly active in inducing oospore formation in a pythium s.o. (Haskins *et al.* 1964).

The partially active compounds, which allowed the appearance of oogonia and antheridia but not the consummation of the sexual process, are interesting. A knowledge of why the course of development breaks down with them would go far towards explaining the regulation of sexual processes by sterols in these fungi.

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Bacterial Genetics. By W. Braun. Published by W. B. Saunders Co. Ltd., 12 Dyott Street, London, W.C. 1. 380 pp. Price £3. 10*s.*

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Le Epatiti Sperimentali da Virus MHV-1 e MHV-3. By M. Piazza. Published by Minerva Medica, Turin, Italy. 240 pp. Price L. 6500.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-fifth General Meeting at Imperial College, London, S.W. 7, on Thursday and Friday, 6 and 7 January 1966. *On Thursday the Society joined with the British Mycological Society for a Symposium. Abstracts of the Contributions to the Symposium on 'Chemical, serological and other techniques for the identification of fungi', and of Original Papers are below.*

The Virus Group met at the Wellcome Buildings, Euston Road, London, N.W. 1, *on Tuesday and Wednesday,* 4 *and* 5 *January. On the morning of Tuesday there was a Symposium on 'Tumour Viruses' and Abstracts of the Contributions to this Symposium are given below.*

SYMPOSIUM: CHEMICAL, SEROLOGICAL AND OTHER TECHNIQUES FOR THE IDENTIFICATION OF FUNGI

Biochemical Differences Between Yeasts. By J. A. BARNETT (*Low Temperature Research Station, Cambridge*)

The nutritional tests used in classifying yeasts mainly concern minor differences of carbohydrate catabolism, rather than differences of major metabolic pathways. Two alternative reasons for a yeast's inability to grow aerobically upon a particular carbohydrate (or derivative) are: (i) the substrate does not penetrate the cells; or (ii) despite penetration, it is not metabolized because the organism lacks one or more enzymes to convert the substrate into an intermediate metabolite of a major pathway. *Saccharomyces cerevisiae* cannot use citrate for growth, not from lack of appropriate catabolic enzymes, but probably because citrate does not get into the cells (Barnett, J. A. & Kornberg, H. L. (1960), *J. gen. Microbiol.* **23**, 55). On the other hand, a strain of *Candida utilis* which cannot use D-ribose appears to be deficient in both ribokinase and ribulokinase; one of these enzymes is essential for each of the two most likely routes of D-ribose catabolism (Horecker, B. L. (1962), in *Pentose Metabolism in Bacteria*, New York: Wiley).

For various reasons, the ability to use one substrate may be related to the ability to use others. For example: (i) an initial catabolic enzyme may be shared: a single β-glucosidase may hydrolyse arbutin, salicin and aesculin (Herman, A. & Halvorson, H. (1963), *J. Bact.* **85**, 895); or (ii) substrates may follow a common route to a major pathway: if yeasts convert both D-xylose and L-arabinose via xylitol to D-*threo*-pentulose (D-xylulose), then the xylitol $\rightleftharpoons$ D-xylulose enzyme could be crucial for the utilization of both L-arabinose and D-xylose:

$$\text{L-arabinose} \xrightarrow{*} \text{L-arabinitol} \rightarrow (?)\,\text{L-}\textit{threo}\text{-pentulose} \rightarrow \text{xylitol}, \quad (1)$$

$$\text{D-xylose} \xrightarrow{*} \text{xylitol}, \quad (2)$$

$$\text{xylitol} \xrightarrow{**} \text{D-}\textit{threo}\text{-pentulose} \rightarrow \text{pentose cycle}. \quad (3)$$

$$\text{D-ribose} \rightarrow \text{ribitol (adonitol)} \xrightarrow{**} \text{D-}\textit{erythro}\text{-pentulose} \rightarrow \text{pentose cycle}. \quad (4)$$

* Enzyme A. ** Enzyme B

How far does the hypothetical scheme above (based on Horecker, *loc. cit.*) for the catabolic routes of four test substrates (in bold type) apply to yeasts in general? If (*a*) the scheme shown in the equations were true and complete, and (*b*) the substrates were effectively unimpeded by cell membranes, then no yeast could be (I) [D-ribose +, ribitol −] or (II) [L-arabinose +, D-xylose −]. A recent taxonomic survey of the genera *Pichia*, *Debaryomyces* and *Endomycopsis* (Kregervan Rij, N. J. W. (1964), University of Leiden, Thesis) gives the scheme some support.

No exception was found either to statement (I) or (II) in this survey of 51 species, though a significant proportion of [L-arabinose −] yeasts utilized D-xylose, and a number of [D-ribose −] yeasts utilized ribitol. The results of the survey suggest that [D-xylose −] yeasts lack enzyme A rather than B, and should therefore be [ribitol −] too; *Endomycopsis capsularis* [L-arabinose −, D-ribose +] was the only exception: perhaps this yeast catabolizes

ribitol → ribose → ribose phosphate → pentose cycle.

There are at least two major hazards in such attempts to interpret the results of taxonomic tests: (i) It may be impracticable to decide from growth tests alone whether different responses to a substrate concern a permeability barrier or catabolic enzymes. (ii) There is the widespread practice of testing with inadequate aeration and long incubation (e.g. Wickerham, L. J. (1951), *Tech. Bull. U.S. Dep. Agric.* no. 1029); after many days' incubation, a positive response to a test may depend not on the characteristics of the original intact yeast cells, but on selection of a mutant or initial break-down of the substrate by enzymes released from autolysed cells (cf. De Ley, J. (1961), *J. gen. Microbiol.* 24, 31). Overcoming such difficulties should make it easier to understand the biochemistry of the overt differences between yeasts.

Some Aspects of the Biochemical Differentiation of Pathogenic Fungi. By I. G. MURRAY (*Mycological Reference Laboratory. London School of Hygiene and Tropical Medicine, London, W.C.* 1)

In a sense, biochemical differentiation of pathogenic fungi has been with us since the beginning of medical mycology. Sabouraud in *Les Teignes* (Masson et Cie, Paris, 1910) noted well the effects of medium variation on the growth and appearance of dermatophytes, and indeed it is very difficult in the absence of the ingredients he used to reproduce his work accurately now. That the behaviour of fungi varies on different substrates is well known, and the importance of peptone in this respect was forcibly brought home to us by the import restrictions of 1946 (Carlier, G. I. M. (1948), *Brit. J. Derm. Syph.* **60**, 61). However, by and large, fungi of medical importance continued to be identified by morphological means rather than by a study of their nutritional requirements. This is still largely true today but, what the eye can see is being increasingly supplemented by other tests, biochemical and immunological, which often allow an earlier or a more precise identification to be made.

Biochemical tests currently in use to assist in the differentiation of pathogenic fungi can be divided into two categories:

(1) Tests to assess what particular compounds fungi assimilate or degrade in their growth processes. Alternatively, the identification of metabolites produced under controlled conditions may serve as a test.

(2) Tests to assess the constituents of fungi.

Assimilation tests are extremely important in the taxonomy of yeasts but are of much less value in the study of pathogenic filamentous fungi. The latter are extraordinarily catholic feeders and it is difficult to find compounds of sufficient selectivity. Nevertheless, occasionally such tests can be of great value in differentiating species which are very much alike.

In the field of dermatophytes there are several outstanding examples of biochemical tests: the use of histidine to distinguish *Trichophyton megninii* from *T. rubrum* and *T. gallinae* or of nicotinic acid to separate *T. equinum* from *T. mentagrophytes* (Georg, L. K.

& Camp, L. B. (1957), *J. Bact.* **74**, 113); and *Madurella grisea* and *M. mycetomi* differ in their capacity to utilize sucrose and lactose. It is when one comes to deal with organisms virtually identical morphologically that nutritional tests are of the greatest value. In the identification of species of *Nocardia*, for example, the assessment of capacity to attack a variety of compounds such as casein, tyrosine, xanthine, gelatine and various sugars is of paramount importance (Georg, L. K. *et al.* (1961), *Amer. Rev. resp. Dis.* **84**, 337 and (1964), *J. Bact.* **88**, 477; Mariat, F. & Lavalle, P. (1955), *C.R. Acad. Sci.* **240**, 255; Gordon, R. E. & Mihm, J. (1959), *J. gen. Microbiol.* **20**, 129, and *ibid.* (1962), **27**, 1, and *J. Bact.* (1957), **73**, 15).

The chemical composition of the cell-walls of Actinomycetes is of great assistance in their identification. Most workers have concentrated on the demonstration of the presence of the pentose sugar arabinose in organisms like *Nocardia* and *Mycobacteria* and its absence in certain morphologically similar organisms (Bishop, C. T. & Blank, F. (1958), *J. gen. Microbiol.* **4**, 35; Cummins, C. S. (1962), *ibid.* **28**, 35; Cummins *et al.* (1958), *ibid.* **18**, 173). Diaminopimelic acid has also shown itself to be a useful marker in the differentiation of this group of organisms (Becker, B. *et al.* (1964), *Appl. Microbiol.* **12**, 421). Lastly one may note that the principal sterol of *Trichophyton mentagrophytes* is ergosterol (Audette, R. C. S. *et al.* (1961), *Can. J. Microbiol.* **7**, 282; Prince, H. N. (1960), *J. Bact.* **79**, 154), whereas that of *T. rubrum* appears to be brassicasterol (Wirth, J. C. *et al.* (1961), *J. invest. Derm.* **37**, 153).

Chemical Tests in Agaricology. By R. WATLING (*Royal Botanic Garden, Edinburgh*)

Only comparatively recently have chemical tests played any major role in the identification of fresh or herbarium collections of agarics. In fact, it is now as familiar to see chemical characteristics of the carpophore included in a species diagnosis, as the inclusion of chemical reagents in the agaricologist's field-kit. However, it is seventy years since it was first shown that certain agaric pilei gave a deep blue coloration when treated with alkali and nearly forty since Melzer and Zvara utilized reagents in their monograph on *Russula* (Melzer, V. (1927), *Arch. Prirodo v Vyzkum Cech.* **17**, 1). Since this Czech publication appeared, many authorities have utilized inorganic and/or organic compounds in the study of agarics (Moeller, F. H. (1950), *Friesia*, **4**, 1).

Forty-two different chemical compounds or mixtures are available for tests by the agaricologist and the time is ripe to undertake a critical revaluation of the use of chemical reagents in agaricology. Although colour changes obtained on application of chemicals to agaric basidiospores and hyphae are often striking, the chemical pathways and nature of the substrates are unknown for the vast majority of reactions; the whole field is instilled with a considerable degree of alchemy despite the fact that some reactions are constant and correlatable with other morphological characters.

Careful consideration is given to the reactions involving the use of Melzer's solution and of aqueous solutions of alkali and simple iron salts. These reagents have been chosen because they may be used macro- and microscopically, with fresh or herbarium material, and because they illustrate some of the problems experienced with chemical tests. Emphasis throughout the paper will be placed on the Bolbitiaceae and Boletaceae. General reference should be made to the review by Singer ((1962), in *Agaricales in Modern Taxonomy*, 2nd edition). Tastes and odours are constant in cultural experiments; even though subject to the same general considerations applied to colour reactions, they are not dealt with in this paper.

Isolation and analysis of the crystals in *Conocybe* spp. similar to those observed by Kühner (Kühner, R. (1935), 'Le Genre Galera', *Encyclopédie Mycologique*, VII, P. Le Chevalier, Paris) shows them to consist of calcium oxalate. Crystal formation has been used in the separation of infrageneric groups. Cultural experiments and field observations on *Conocybe subovalis* indicate that crystal formation is exceedingly variable between members of a single population and members of different populations.

The significance of colour changes of the flesh of the Russulaceae, Hygrophoraceae, etc. and the darkening of certain agaric basidiospores after the application of alkali solutions is discussed.

Two distinct series of colour changes, one red and one green, are observed when agaric tissue is treated with aqueous solutions of simple iron salts. The validity of selecting a single point on such a series for utilization in taxonomy is criticized.

Differences in the colours produced by a single chemical when applied to carpophores of a single species at different stages of maturity are described in relation to the increase in toxicity with age of certain members of the Helvellaceae. Spontaneous colour changes of the flesh of certain agarics are briefly mentioned.

Iodine in a mixture of aqueous solutions of potassium iodide and chloral hydrate is one of the most important and widely used reagents in agaricology today. It is argued from careful laboratory observations that the reactions brought about by this reagent are far from as simple as is indicated by the three terms, dextrinoid, non-amyloid and amyloid: other colour changes which are recordable are considered just as important. Artifacts resulting from pigment masking are described as well as results indicating a chemically distinct layering within the pilei and stipes of boleti.

Lichen Substances and Lichen Identification. By P. James (*British Museum (Natural History), London, S.W.* 7)

A number of unique compounds are produced by the mycobiont of many lichens. Several of these substances, often loosely termed 'lichen acids', may occur in a single taxon and are there present as extracellular, non-wettable incrustations of the hyphae. They may comprise as much as 38 % of the dry weight of the lichen. Lichen substances may be uniformly distributed throughout the thallus or confined to specific parts such as the cortex, pycnidium, epithecium, excipulum, etc. Though many are closely related to those occurring in non-lichenized fungi, of the *c.* 95 compounds in which the chemical structure has been ascertained, only very few, such as polyporic acid, thelephoric acid and endocrocin, are common to both groups. Though mainly considered as complex waste products derived from the metabolism of the mycobiont, several subsidiary roles, such as chelating agents, or the maintenance of air spaces within thalli has been ascribed to them. They exhibit marked antibiotic properties which may be an important factor in the relative longevity and known disease resistance of lichens. The pigments, such as parietin and colycin, which are chiefly located in the upper cortex, serve to restrict the amount of light reaching the phycobiont layer in heteromerous lichens.

The extraction and chemistry of lichen substances, unlike their biochemical properties, has been studied in considerable detail (Asahina, Y. & Shibata, S. (1954), *The Chemistry of Lichen Substances*, pp. 1–240). According to their structure they may be grouped as follows:

(*a*) Alicyclic and aliphatic compounds, including fatty, straight chain and lactonic acids, triterpenoids, polyalcohols and oligosaccharides.

(*b*) Aromatic compounds which may either be: (i) coloured and include quinones, derivatives of xanthene, dibenzofuran, diketopiperazine and pulvic acid; or, (ii) the most widespread and important colourless compounds which include depsides, depsidones, and depsones (all derived from orcinol and β-orcinol).

The taxonomic value of the various distinctive colorations obtained with potassium hydroxide, calcium hypochlorite, *p*-phenylenediamine, nitric acid and iodine (for isolichenin and lichen) has assisted in the separation of closely related or morphologically identical entities. The problems in lichen taxonomy resulting from the elevation of 'chemical species' dates from the discovery of the K and C tests by Nylander in 1866. More refined methods such as micro-chromatography, spectrophotometry, fluorescence analysis and crystal extraction are now widely used to supplement the findings of the crude chemical tests outlined above. Crystal extraction involves the extraction of the lichen substance with acetone or benzene and the resulting evaporate recrystallized in one

of several glycerol-based solvents containing acetic acid, pyridine, *o*-toluidine, aniline, ethanol, or quinoline. The shape of the crystals in the various solvents is diagnostic for a particular lichen substance.

Whereas fungi and bacteria are commonly given species status on their chemical characteristics there is a considerable divergence of opinion in adopting a similar view for the lichens. The problem is aggravated by the paucity of knowledge of their synthesis *in vivo* and the total ignorance of the lichen genetics. Such assumptions on taxonomic importance of lichen substances can only be related to similarities in other plant groups. The linkage between structurally related substances could be the result of the presence or absence of a single enzyme in the synthetic chain as has been demonstrated in flower pigments and metabolic pathways in fungi such as *Neurospora*. Such pathways in lichens as suggested by Asahina and Shibata could be the result of an allelomorphic series in which the consideration of chemical variation at species level would be unjustified. The meagre information on sexuality in lichens suggests a parallel with that of apomicts in higher plants. In the absence of relevant biochemical data specific or subspecific rank should only be considered where other factors such as differences in habitat or geographical distribution are known.

Application of the Fluorescent Antibody Technique to the Identification of Plant Pathogenic Fungi. By T. F. PREECE (*Department of Agriculture, University of Leeds*)

Although little is known about the antigenic composition of fungi, the fluorescent antibody technique (F.A.T.) has been reported to be of value in their identification in several applied fields: detecting certain medically important fungi (*Candida*, *Histoplasma*, *Cryptococcus*, *Blastomyces*, *Sporotrichum*) (McEntegart, M. G. (1964), *Fluorescent Protein Tracing*, p. 173, ed. R. C. Nairn, Edinburgh: Livingstone); distinguishing wild yeast contaminating bakers' yeast (Kunz, C. & Klaushofer, H. (1961), *Appl. Microbiol.* **9**, 469); identification of *Streptomyces* species (Arai, T. *et al.* (1962), *J. Bact.* **83**, 20); detection of *Aspergillus flavus* on buried slides (Schmidt, E. L. (1962), *Science*, **136**, 776); and of some *Cladosporium* species, principally of medical importance (Al-Doory, Y. & Morris, A. G. (1963), *J. Bact.* **86**, 332; and (1965), *ibid.* **87**, 551).

I am interested in using the method for mapping and identifying as far as possible the fungi found on entire plant organs especially leaf surfaces during the early stages of attack by pathogenic fungi (Preece, T. F. (1963), *Trans. Brit. mycol. Soc.* **46**, 523). A similar problem is the direct identification of spores on spore trap slides (Hirst, J. M. (1953), *Trans. Brit. mycol. Soc.* **36**, 375). In addition some pathogens apparently exist in the mycelial state, such as the Grey Sterile Fungus causing corky roots on tomato (Preece, T. F. (1964), *Trans. Brit. mycol. Soc.* **47**, 375) and a further test to identify such non-sporing fungi would be valuable.

Three problems in the application of the F.A.T. in plant diseases caused by fungi are: damaged tissue fluoresces; some fungi are autofluorescent, and cross-reactions occur due to common antigenic components. The fact that damaged or diseased tissue fluoresces may be due to production of scopoletin (Udenfriend, S. (1964), in *Fluorescence Assay in Biology and Medicine*, p. 383, New York: Academic Press) but the impermeable nature of the undamaged entire leaf to stains and reagents (Preece, T. F. (1959), *Plant Pathology*, **8**, 127) promoted the use of the F.A.T. technique, in spite of the presence of scopoletin.

A simple F.A.T. using fluorescein isothiocyanate conjugated α-globulins and examinations by u.v. light and darkground microscopy is likely to be valuable in ecological studies at least as far as identifying the *genus*. The fungi being studied are: *Mycosphaerella citrullina* and *M. pinodes*; *Phytophthora parasitica* and *P. cactorum*; *Fusarium culmorum* and *F. avenaceum*; *Aspergillus flavus* and *A. niger*; *Pyrenochaeta terrestris* and a Sterile Grey Fungus causing corky rot of tomato.

Taxonomic Applications of Gel Electrophoresis. By J. R. NORRIS (*Milstead Laboratory of Chemical Enzymology, 'Shell' Research Ltd., Sittingbourne*)

Much of microbial taxonomy is concerned with the study of enzyme reactions of cultures or with the nature of cell components. The discovery that two micro-organisms perform a particular enzyme reaction tells us only that they use a similar method to deal with a particular aspect of their environment. The enzyme molecules involved may be widely different in terms of size, shape, molecular weight and primary structure of their protein components and we cannot imply any relationship at the genetic level from such observations. Serological studies of cell components provide the taxonomist with more detailed information about molecular structure but again this often concerns only certain sites on the molecule and results may be difficult to interpret.

Electrophoretic separation of cell components in starch or polyacrylamide gels enables us to characterize large molecules not only in terms of net surface charge and, in the case of many enzymes, in terms of biological activity, but also, because of the molecular sieving properties of the gels, in terms of their size and shape. The application of these techniques in fields outside microbiology has given information of considerable taxonomic significance and the last two years have seen the extension of these methods into microbial taxonomy.

Starch gel analysis of the esterase systems of strains of *Bacillus thuringiensis* enabled this group of bacteria to be subdivided into ten esterase types. There was a close correlation between esterase types and groups defined by the possession of H antigens (Norris, J. R. (1964), *J. appl. Bact.* **27**, 439). A similar approach to the taxonomy of the non-pathogenic mycobacteria gave results which correlated closely with other schemes of classification (Cann, D. C. & Willox, M. E. (1965), *J. appl. Bact.* **28**, 165).

Lund has used polyacrylamide gels to separate the cell components of Group D streptococci and found that she could distinguish between *Streptococcus faecalis* on the one hand and *S. faecium* and *S. durans* on the other, both by esterase patterns and by the patterns of proteins obtained when cell disintegrates were analysed (Lund, B. M. (1965), *J. gen. Microbiol.* **40**, 413).

Catalases, esterases and peroxidases have been studied by Robinson (pers. comm.) using starch gel electrophoresis in an investigation of corynebacteria. It was possible to define several groups of which one contained the parasitic diphtheriae–ulcerans–xerosis–hofmanni bacteria, another *Corynebacterium pyogenes* strains, and another certain plant pathogens.

The use of gel electrophoresis in microbial taxonomy is at present in an exploratory phase and more information is needed before the potential of the method, or the significance of the groupings indicated may be ascertained. The indications at present are that these techniques have a valuable part to play in the future development of the subject.

Application de l'Immunoélectrophorèse et de l'électrophorèse en Gel Polyacrylamide à la Taxonomie Fongique. By J. BIGUET, S. ANDRIEU et F. ROSE (*Faculté de Médecine et de Pharmacie, Lille*)

The Application of Numerical Taxonomy to the Genus *Helminthosporium*. By F. M. IBRAHIM* and R. J. THRELFALL (*Department of Botany, Imperial College, London, S.W. 7*)

Numerical taxonomy may be defined as the numerical evaluation of the affinity or similarity between taxonomic units and the ordering of these units into taxa on the basis of their affinities (Sneath, P. H. A. & Sokal, R. R. (1962), *Nature, Lond.* **193**, 855). Its aim is to provide a stable unbiased classification by the analysis of large numbers of characters each of equal weight.

The heterogeneity of the genus *Helminthosporium* has been partially clarified by the division of the graminicolous species into the form genera *Drechslera* (Ito, S. (1930), *Proc.*

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Imp. Acad., Tokyo, VI, 8, 352) with cylindrical or semi-cylindrical spores germinating by any cell and having *Pyrenophora* (or *Pleospora*) as perfect state and *Bipolaris* (Shoemaker, R. A. (1959), *Can. J. Bot.* 37, 879) with fusoid conidia germinating by polar cells and with *Cochliobolus* as perfect state. However, within these groups considerable confusion still exists. It was hoped that the application of numerical taxonomy to this group would provide a more stable classification.

Twenty-nine operational taxonomic units (OTU's) have been examined, perfect states where known being treated in the same OTU as the conidial state. The OTU'S are: (1) all known *Drechslera* spp., 11 collected in the field, 8 obtained from other laboratories and 5 for which descriptions from the literature were used; (2) descriptions from the literature of *Pleospora herbarum* and its conidial state *Stemphylium botryosum*; (3) a strain of *Bipolaris sorokiniana* (*H. sativum*); and (4) strains of *H. triseptatum* and *H. biforme* isolated by us and of *H. dematoideum* from the Commonwealth Mycological Institute.

Each OTU was examined for a number of pathological, cultural, physiological and morphological characters of perfect and imperfect states. These characters were of three kinds: (*a*) with mutually exclusive alternatives, (*b*) with multiple alternatives, and (*c*) quantitative characters. Characters of types (*b*) and (*c*) were scored non-additively (system I), additively (system II) or some scored one way and some the other (system III).

For any pair of OTU's, e.g. *X* and *Y* scored for all unit characters then:

			Strain X No. of unit characters	
			Positive	Negative
Strain Y	No. of unit characters	Positive	+ + (*a*)	+ − (*b*)
		Negative	− + (*c*)	− − (*d*)

In the calculation of similarity (*S*) Sneath ((1957), *J. gen. Microbiol.* 17, 201) used the formula $S\ \% = a/a+b+c$ (Formula 1) ignoring negative matches because they might be confused with inapplicable ones while Sokal & Michener ((1958), *Kans. Univ. Sc. Bull.* 38, 1409) included them in their formula, $S\ \% = a+d/a+b+c+d$ (Formula 2). Formula 1 was applied to all three scoring systems while System II was also analysed by Formula 2. In System III those negative matches thought to be significant were included in the computation of *S* by splitting the character into two. Sets of data for the three scoring systems were analysed by the Mercury Computer of the University of London to give a sorted similarity matrix and to arrange the OTUs in groups of 5 % levels of similarity.

No important differences were found between additive (II) and non-additive (I) scoring. Additive scoring might be expected to accentuate similarities and differences but the range of characters studied has minimized this effect. Formula 2 applied to System II resulted in quite different organisms appearing similar but this is because many of the characters which are counted as similar are really inapplicable. The results of this work are: (1) a new grouping at the generic level of *Helminthosporium biforme*, *H. triseptatum* and *H. dematoideum*; (2) *Drechslera*, *Bipolaris* and *Stemphylium* are confirmed as separate genera; (3) *H. avenae* and *H. siccans* shared 95 % similarity and seem to be con-specific, the disease caused by them on oat and rye-grass being identical; (4) *H. avenaceum* is shown to be the same as *D. avenacea*; (5) *D. dictyoides* f. *dictyoides* and *D. dictyoides* f. *perenne* are confirmed but species formed from these by Schariff ((1959) *Ph.D. Thesis, University of London*) are rejected; (6) *H. dematoideum* and *H. biforme* are possibly isolates of the same species; and (7) a new species, *D. intermedia* is made from a new form isolated from *Dactylis glomerata*.

Physical and Biochemical Techniques in the Identification of Fusaria. By C. Booth (*Commonwealth Mycological Institute, Kew*)

The overriding view that fungal species should be based on morphological rather than biochemical characteristics is a sound concept that will undoubtedly always dominate mycological taxonomy in its wider sense. The great majority of fungal species are suffi-

ciently stable in the morphological characteristics of their fructifications that a knowledge of these is all that is required for their accurate identification.

However, stability and uniformity is not a feature of all fungal species. Many important fungal pathogens are extremely variable in their morphological characteristics and in an effort to identify these, other characters, particularly those produced by pure culture techniques, have long been used as a basis for speciation. *Fusarium* species are both outstanding examples of fungi which show extreme variability and are economically important. If we are going to move away from the personal judgement of the taxonomist to a more experimental analysis of the species then we are almost obliged to adopt biochemical techniques. In fact, in the most elementary sense we have long been using a biochemical basis for our species. That is since we began to require cultures for the purposes of identification. Fusaria are greatly influenced by the environment. The influence of light, carbon dioxide concentration of the atmosphere and the hydrogen-ion concentration, together with the carbon/nitrogen/phosphate ratio of the media has long been known to have a marked effect on both the morphological and cultural characteristics of the species. Such influences have been overcome by careful standardization of cultural conditions and it is on these that our present concept of the species is based. If this concept is correct then we should find that the more critical biochemical tests now available will support it. Serological investigations of *Fusarium oxysporum*, *F. moniliforme* and *F. solani* has already shown that they can be clearly differentiated and also that *F. oxysporum* is more closely related to *F. moniliforme* than to *F. solani*, a relationship already believed to exist from morphological and cultural evidence.

Orthodox techniques for critical morphological and cultural studies coupled with mating experiments have already been developed to their maximum in establishing our present system of *Fusarium* classification. But this system does not answer the pathologist's most obvious question—which is pathogenic, which is saprophytic? It has become apparent from our morphological and cultural studies that the genes governing these characters are not linked to those governing pathogenicity. If we as taxonomists are ever going to be able to give the pathologist this information or even indicate from a range of isolates which may be pathogenic, we shall have to use biochemical techniques. Physiologic specialization has in the past generally been determined by the inoculation of an indicator host. This method although slow and laborious is reasonably satisfactory for rapidly growing plants raised from seed, but it is not so satisfactory in vegetatively propagated plants or for trees. Much work has recently been done that shows the production of pectolytic and cellulolytic enzymes on suitable media is much higher in pathogenic than saprophytic strains. Tests *in vitro* for these can be easily made in the laboratory and they may come to form a reliable indication of the nature of the isolate tested.

Serological methods have also been used successfully to effectively separate pathogenic strains of *Fusarium oxysporum* and specific antisera for a particular strain of *Fusarium* pathogen may soon become a feature of the mycological laboratory.

A further method is the extraction and separation of intracellular proteins by acrylamide-gel electrophoresis. This simple precise method shows the protein bands to vary with the species and it is believed that further experimentation will show that some are specific for the particular strain under examination.

ORIGINAL PAPERS

The Role of Copper and Caramelized Sugar in the Effect of Inoculum-Size on Growth of *Penicillium chrysogenum*. By M. N. Ojha, J. Meyrath and E. O. Morris (*Department of Applied Microbiology and Biology, University of Strathclyde, Glasgow*)

It has been shown previously (Meyrath (1963), *Antonie von Leeuwenhoek*, **29**, 57; Meyrath & McIntosh (1963), *J. gen. Microbiol.* **33**, 47) that under a variety of conditions

Aspergillus oryzae and other moulds show strong effects of inoculum size on growth. Under most circumstances, growth rate and maximum yield of mycelium have been shown to be larger with large inocula than with small ('usual effects of inoculum size'), but 'reversals' of these effects have also been shown to occur, for example in the presence of small amounts of Cu^{2+} in media poor in trace elements (McIntosh & Meyrath (1964), *Arch. Microbiol.* **48**, 363).

A similar 'reversal' effect has now been shown to occur also with *Penicillium chrysogenum* and if aluminium sulphate is added with the copper sulphate the effect is even more pronounced.

Caramelization of the (purified) sugar results in almost complete inhibition of large-inoculum cultures without the addition of Cu^{2+}, whereas the growth of small-inoculum cultures is hardly affected. Since in these caramelized substrates small amounts of Cu^{2+} stimulate large-inoculum cultures very markedly, it appears that these cultures require some Cu^{2+} and that the assimilation of this element has been prevented by the caramelized sugar. This hypothesis is strongly supported by the fact that caramelized sugar has been shown to act as a chelating agent for heavy metals with an outstandingly high affinity for Cu^{2+}.

Influence of Vermiculite and Metal Salts on Amylase Production by *Aspergillus oryzae*. By J. MEYRATH and M. H. IKRAM (*Department of Applied Microbiology and Biology, University of Strathclyde, Glasgow*)

Further to earlier observations (Meyrath (1965), *J. Sci. Fd Agric.* **16**, 14) on amylase production by *Aspergillus oryzae* on vermiculite, it has now been shown that the rate of amylase production in 'solid' cultures is strongly stimulated under a great variety of conditions. It appears that this stimulation is due to the chemical rather than the physical or structural nature of the vermiculite. The influence is probably due to vermiculite functioning as cation exchanger; it is known that vermiculite has a particularly high affinity for heavy metals. An excess of the trace elements Fe^{3+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, added singly or in combination has less influence on amylase production when vermiculite is present, since these elements are then less available to the mould. Excess of trace elements has little influence on amylase production in presence of vermiculite, but deficiency of trace elements inhibits formation of the enzyme considerably. Exhaustive treatment of vermiculite with salts of Ca^{2+} or Mg^{2+} results in a decrease in both the rate of production and the maximum yield of amylase. A similar but less pronounced result is obtained by charging vermiculite with K^+ but not with Na^+. The inhibition is probably due to removal of some naturally occurring trace elements since Mg^{2+} and Ca^{2+} could replace most of the exchangeable cations; K^+ would do this to a smaller extent but Na^+ only to a very small extent.

If vermiculite is charged with H^+ or Fe^{3+}, only very little growth and amylase production take place. It is not easy to give an explanation for this effect since the pH in the substrate is not low, nor is the amount of Fe^{3+} present excessively large. The effect may be due to an absolute removal of naturally occurring trace elements from vermiculite.

Influence of Trace Elements and Chelating Agents on Growth and Amylase Production by *Aspergillus oryzae*. By J. MEYRATH (*Department of Microbiology, Swiss Federal Institute of Technology, and Department of Applied Microbiology and Biology, University of Strathclyde, Glasgow*)

Earlier (Meyrath (1965), *Zentbl. Bakt.* II, **119**, 53; Steiner (1961), *Zentbl. Bakt.* II, **114**, 47), it has been shown that substrates with a trace element composition suitable for growth do not give high specific amylase production (i.e. amylase production as compared with growth). The present results show that the stage of culture at which the trace elements are added is important, e.g. Zn^{2+} added at advanced stages does not reduce the maximum yield of amylase as does Zn^{2+} added at inoculation or during early growth. This underlines

earlier conclusions that the stage at which self-stimulating and self-inhibiting substances act is important for the final development of the culture.

The variation of specific amylase production (occurring generally at more advanced stages of the culture development) which occurs with various brands of maltose, dextrin and starch is probably partly due to their varying content of trace elements. However, besides trace element contamination one has also to consider possible contamination with chelating agents. It is significant that a yellow dextrin is under most circumstances a better source of carbon for specific amylase production than several other brands of maltose, dextrin or starch. Furthermore, caramelization of maltose stimulates amylase production but not growth. These stimulations are probably due to chelation of Cu^{2+} (Ojha, Meyrath and Morris, accompanying abstract) since it has been shown that minute amounts of Cu^{2+} reduce the yield of amylase under the present circumstances (Steiner, *loc. cit.*). Thus, the effects are comparable to those of vermiculite (Meyrath and Ikram, accompanying abstract). Similar effects can also be produced by adding well-known chelating agents. These investigations stress also the importance and possible errors in removing trace elements from some substrates by certain methods.

Relation between F and other Fertility Factors. By ELINOR MEYNELL and NAOMI DATTA (*Medical Research Council Microbial Genetics Research Unit, and Postgraduate Medical School, Hammersmith Hospital, Ducane Road, London, W.* 12)

Drug resistance (R) factors resemble the sex factor, F, in enabling their host bacteria to conjugate with recipient strains to whom the factor is then transferred, occasionally with part of the host's chromosome. However, R^+ cells mate less frequently than F^+, and R^+ cultures show no lysis by specific F phage. We found that this results from repression, for R^+ cells carry the gene determining synthesis of the specific F phage receptor.

R factors are known to be of two kinds, fi^+ which suppress F function in F^+ cells, and fi^- which do not. Only fi^+ R factors show functional homology with F. If the R factor carries its own repression system, and fi^+R factors are related to F, the extension of their repression to F is not surprising. When F^- strains carrying *col* factors were tested, F phage sensitive cells were present with *col* V or *col* B, but not with *col* I. It thus appears that factors mediating bacterial conjugation are of at least two kinds, only one of which is similar to F.

F *itself* might be exceptional among sex factors, while repressed behaviour was the norm. If so, F might revert to the repressed state, so that its host would now appear F^- by the usual criteria. F^- derivatives of K12 F^+ did in fact prove to be of two sorts, those in which F was totally lost and those where it was evidently only repressed. Moreover, 1/17 strains of *Escherichia coli* without drug resistance or colicinogeny showed some F phage sensitive cells. Thus, while unrepressed sex factors, recognizable solely by fertility, are rare, repressed factors may be common, but are identified only if they carry genes altering some property of the cell. This would explain why the F phage, with so few known hosts, occurs abundantly in nature. R factors may originate rarely from these repressed F, or other, sex factors by acquiring drug-resistance genes, and become prevalent through the intense selection for drug-resistant bacteria.

Colicin Resistance Associated with Drug-Resistance Factors. By A. G. SICCARDI and ELINOR MEYNELL (*Medical Research Council, Microbial Genetics Research Unit, Hammersmith Hospital, London, W.* 12)

A Resistance Factor (R Factor) consists of genes conferring drug resistance linked to a Resistance Transfer Factor responsible for conjugation. The observed patterns of transmissible drug resistance correspond to the frequency with which each drug is used, presumably because of selective pressure. It follows that the natural occurrence of an antibacterial agent should result in the emergence of similar factors giving resistance to its action, and we have therefore looked for transmissible resistance to colicins, the antibiotics produced

by many enterobacteria. We tested for colicin resistance a large number of strains of *Escherichia coli* K12 carrying R factors transferred by conjugation from 150 strains of *Salmonella*.

Transmissible resistance to one or more colicins, usually colicin I, was associated with 41 of the R factors. The same R factor behaved similarly in different lines of strain K12. The strains carrying these R factors were further tested for the ability to produce colicins, to exclude the possibility that their resistance was simply due to simultaneous transfer of a *col* factor; 65 % were not colicinogenic, while the remainder were associated with colicinogeny in all the bacteria.

One *Salmonella* which was itself colicinogenic transmitted colicin resistance without colicinogeny to the K12 strains.

Interactions Between Single-step Mutations for Chloramphenicol Resistance and R-factor Genes in *Escherichia coli*. By E. C. R. Reeve (*Institute of Animal Genetics, Edinburgh* 9)

Single-step mutations to chloramphenicol resistance (*Cm-r*) in *Escherichia coli* have been found to confer low levels of resistance to tetracyclines (Tc) and puromycin (Pm) as well as to Cm, the level of resistance to each antibiotic varying independently from one mutant to another. This suggests that the *Cm-r* mutations act on a rather general permeability mechanism of the cell (Reeve, E. C. R. & Bishop, J. O. (1965), *Genet. Res.* **6**, 310). In contrast, R factor mediated resistance to either Cm or Tc can be separated from other R-carried resistance genes, and each has been claimed to act on some permeability mechanism specific for that antibiotic (Watanabe, T. (1963), *Bact. Rev.* **27**, 87; Datta, N. (1965), *Brit. med. Bull.* **21**, 254).

Two infective R factors, R-Cm and R-Tc (my notation), conferring high-level resistance to Cm and to Tc, respectively, were each introduced by conjugation into a Cm-sensitive strain of *Escherichia coli* and 4 single-step *Cm-r* mutants of this strain. Sensitivity to Cm, Tc and Pm was estimated by streaking on antibiotic plates. R-Cm gave 10 × and R-Tc 40 × the resistance conferred by our strongest single-step mutants.

Neither R factor modified the resistance to the antibiotics to which it did not itself confer high-level resistance. However, the various mutants still expressed their increased resistance to Tc in the presence of R-Tc and to Cm in the presence of R-Cm, to an extent roughly proportional to the resistance conferred in the absence of an R-factor. Clearly the low-level resistance of mutational origin was not masked by high-level R-determined resistance. This suggests an interaction between the two mechanisms of resistance (mutational and R-mediated), whose nature is still quite obscure.

The Identity of Immunogenic Material Purified from Culture Filtrates with that from Cell Walls of *Brucella abortus*. By D. C. Ellwood, J. Keppie and H. Smith* (*Microbiological Research Establishment, Porton, near Salisbury, Wilts*)

Immunogenic material of *Brucella abortus* has been found in the cell-wall and also in filtrates of cultures grown in a synthetic medium; the question arose whether the materials from the two sources were the same or different (references see Macrae & Smith (1964), *Brit. J. exp. Path.* **45**, 595). This paper describes the purification of culture filtrate material and immunogenic material extracted from the cell-walls of intact *B. abortus* with sodium dodecyl sulphate. The ability of these immunogenic materials to inhibit the bactericidal action of bovine serum on *B. abortus* was used as a rapid biological test for fractionation.

Culture filtrate and *Brucella abortus* organisms were obtained as described by Macrae & Smith (1964). Small molecular material was removed from the freeze dried culture filtrate with Sephadex G.25. The high molecular material was heterogeneous when examined by cellulose acetate electrophoresis and by gel diffusion against appropriate antiserum. Fractionation on three small columns (active material in effluent) and on one large column

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(active material eluted with 0·5 M-TRIS) of diethyl amino-ethyl cellulose followed by a column of Sephadex G.200 yielded material showing only one band on cellulose acetate electrophoresis and producing no precipitin lines in gel diffusion (although precipitation did occur in free solution). The immunogenic material was finally sedimented from inactive material by ultracentrifugation (173,000 *g*, 10 hr). About 40 mg. of final material was obtained from 50 l. culture filtrate.

Brucella abortus ($3{\cdot}0 \times 10^{11}$ per ml.) were extracted with sodium dodecyl sulphate (0·5 %, w/v, 4°, 17 hr) with no significant reduction in the total number of organisms. The extracted immunogenic material was fractionated by a similar procedure to that described above except Sephadex G.200 was used in the first stage in which sodium dodecyl sulphate was removed. Chemical analysis and biological tests indicated that the two immunogenic materials (phosphorus containing complexes of protein, carbohydrate and lipid) were similar if not identical.

Bacterial Population of a Rumen Ciliate *Entodinium caudatum* cultured *in vitro*. By R. W. White (*Institute of Animal Physiology, Babraham, Cambridge*)

Gutierrez & Davis ((1959), *J. Protozool.* **6**, 222) found that their rumen oligotrich protozoa contained large numbers of bacteria as demonstrated by staining methods, but they could only culture 3–10 bacteria/protozoon. The purpose of the work described here was to isolate and try to identify bacteria present in *Entodinium caudatum* which has been cultured *in vitro* for six years (Coleman, G. S. (1960), *J. gen. Microbiol.* **22**, 565). The medium of Bryant & Robinson ((1961), *J. Dairy Sci.* **44**, 1446), designed for the growth of rumen bacteria, was used; for ease of counting cultures were made in modified Carrel flasks. The protozoa were washed free of external bacteria, and the intracellular organisms cultured after disintegration of the protozoa by sonication. For this, stoppered tubes of protozoa suspension were immersed in a ultrasonic cleaning bath (80 W., 80 kcyc.) for 1–160 sec. Maximum total viable counts of 15–30 bacteria/protozoon were obtained after 5 sec. sonication, when 90 % or more of the protozoa had been broken. After 60 sec. sonication, bacterial counts dropped by 50 %. For isolating individual bacterial species, colonies from roll bottles, and portions of medium from deep tube cultures were subcultured in Oxiod U.S.P. thioglycollate medium. Those organisms most readily isolated were two aerobic gram negative bacilli which overgrew other isolates. One of these bacilli was a non-motile coliform organism, the other non-lactose fermenting and actively motile. Other bacteria cultured and recognized morphologically were all anaerobic: they were (1) streptococci, mainly paired, with some short chains, (2) Gram-positive non-sporing bacilli exhibiting diphtheroid and fusiform characteristics, and (3) small Gram-negative curved and filamentous bacilli similar to butyrivibrio.

Metabolic-Inhibition Colour Tests for Mycoplasma Species. By D. Taylor-Robinson, R. H. Purcell and R. M. Chanock (*M.R.C. Common Cold Research Unit, Salisbury, and Laboratory of Infectious Diseases, N.I.H., Bethesda, U.S.A.*)

Certain mycoplasmas (formerly termed pleuropneumonia-like organisms; PPLO) ferment glucose. When this sugar and phenol red indicator were added to a broth medium containing a glucose-fermenting strain, a shift in pH occurred from alkaline (pH 7·8) to acid (pH 6·5 or less) and this was associated with a change in colour of the medium from pink to yellow. Titration of antibody was possible, since specific antiserum inhibited the growth of mycoplasmas and the concomitant production of acid, thus preventing a change in colour of the indicator. Those mycoplasma strains which did not ferment glucose metabolized arginine; this was associated with an alkaline shift in the pH of the medium and this could be inhibited by specific antiserum. An accessory factor present in guinea-pig serum was necessary to demonstrate inhibition of growth of some strains by specific antiserum. Tests were reproducible and were performed in microtitre plastic plates in which the serum dilutions were made with Takátsy spiral wire loops. The technique was specific and sensitive enough to measure antibody in human serum.

The Nature of the Lactone in Aromatic Ring Fission by Bacteria. By R. B. CAIN (*Microbiology Group, Department of Botany, The University, Newcastle upon Tyne* 1)

Ornston, L. N. & Stanier, R. Y. ((1964), *Nature*, **204**, 1279) have established that *p*-hydroxybenzoate-grown *Pseudomonas putida* A3.12 converts the initial ring-fission product, β-carboxymueonate, to β-oxoadipate through an unstable γ-carboxymuconolactone; this lactone is metabolically quite distinct from the corresponding β-isomer formed by *Neurospora crassa*.

During a survey of the enzymic patterns of aromatic ring breakdown by over 150 different bacteria, it has been found that the γ-isomer was, in every instance, the intermediate in protocatechuate metabolism by those species cleaving the ring by a protocatechuate-3,4-oxygenase. Extracts of these bacteria purified by a combination of salt fractionation, gel and heat treatment, and chromatography on Sephadex columns accumulated a lactone identical in properties with those reported for γ-carboxymuconolactone. Furthermore, the lactone which purified extracts accumulate is degraded by crude extracts of these bacteria at an identical rate with the authentic γ-isomer made with purified lactonizing enzyme from *P. putida* A3.12. The following organisms, when suitably induced by growth on *p*-hydroxybenzoate, have been found to metabolize γ-carboxymuconolactone: *Flavobacterium* sp. (2 isolates), an Achromobacter, *Pseudomonas fluorescens* (71 isolates), *P. ovalis* (3 strains), *P. percolans*, *P. cuneatus*, '*Vibrio*' *cyclosites*, *Moraxella* (Vibrio 01), *Mycobacterium* sp. (3 isolates), *M. rhodochrous*, *Nocardia erythropolis* (3 strains), *N. opaca* (5 strains), *N. globerula*, *Jensenia canicruria*.

From the comparative standpoint, it was interesting that three *Streptomyces* isolates metabolized the γ-isomer but were unable to degrade the β-isomer. In contrast, several yeasts (Bilton, R. F. & Cain, R. B. (1965), *J. gen. Microbiol.* in press) and three moulds so far examined, as well as *Neurospora crassa* itself, utilized β- but not γ-carboxymuconolactone.

VIRUS GROUP SYMPOSIUM: TUMOUR VIRUSES

The Nucleic Acid of Tumour Viruses. By L. V. CRAWFORD (*Institute of Virology, Glasgow, Scotland*)

The number of known tumour viruses has recently increased to such an extent that a survey of these viruses is little short of a survey of animal viruses in general. Unfortunately little is known of the nucleic acid of many tumour viruses and for the time being we must assume that the nucleic acid of such viruses is similar to that of better known viruses of similar morphology. In some cases the viruses considered below have not been proved to cause the tumours with which they are associated and these should therefore be considered as tumour-associated viruses rather than tumorigenic viruses.

Many of the most important tumour viruses are thought to contain RNA, including the avian leukosis group and the murine leukaemia group. Morphologically the virus particles are rather uniform in appearance with an outer lipoprotein membrane enclosing a ribonucleoprotein nucleoid. The RNA of Rous sarcoma virus is the best characterized in this group, having a molecular weight (MW) of about 10 million with non-complementary base ratios and probably therefore a single stranded structure.

DNA tumour viruses are much more varied in their size and structure than RNA tumour viruses. The largest viruses are members of the pox group including YABA monkey virus and Tana virus. By analogy with other pox viruses these may be expected to contain double stranded DNA between 80 and 160 million MW with a base composition in the region of 36 % guanine plus cytosine (GC).

Viruses which appear to be related to the herpes group include Lucke frog tumour virus, a virus from Burkitt lymphoma cells and Allerton lumpy skin virus. Probably these have

double stranded DNA about 90 million MW and base composition in the region of 64 % GC although only the Allerton virus has been analysed.

Many of the mammalian adenoviruses appear to be capable of causing tumours to some extent. The tumorigenic adenoviruses contain double stranded DNA close to 20 million MW and 48 % GC. This is slightly lower GC than the other adenoviruses which have been analysed.

Papilloma viruses of many mammalian species have been described. The DNA of these viruses has a molecular weight of just over 5 million and base composition varying between 40 and 48 % GC. These DNA are double stranded, circular and supercoiled.

The polyoma group of viruses also have circular supercoiled DNAs but the significance of this peculiarity is not clear. The best characterized members of this group, polyoma and SV40, have DNA of 3 million MW and 48 and 41 % GC respectively.

The smallest viruses to be discussed are tumour associated rather than tumorigenic. The minute viruses include Kilham rat virus, H1, H3 and probably the particles associated with human and murine adenoviruses. The particles of these viruses are 20 mμ in diameter and contain DNA. The size of the particle makes it likely that these viruses contain DNA of lower molecular weight than any other animal virus.

Several members of the different groups of DNA animal viruses show interactions with each other or are found in association with each other. The extent to which such interactions may involve the nucleic acids of the viruses will be discussed.

Studies on a Murine Sarcoma Virus (MSV). By M. H. SALAMAN (*London Hospital Medical College*)

An oncogenic virus, isolated by Dr J. J. Harvey, was described last year (Harvey, J. J. (1964), *Nature, Lond.* **204**, 1104). It has been further studied by my colleagues and me at the London Hospital, and by Drs F. C. Chesterman and R. R. Dourmashkin at the Imperial Cancer Research Laboratory (Chesterman *et al.* (1966), in press).

The source was plasma of a rat with Moloney virus (MLV)-induced leukaemia, which when injected into mice, rats, or hamsters, produced effects quite different from those of MLV. Tumours developed rapidly in most mice, rats, and hamsters, inoculated when newborn, and sometimes in mice inoculated as adults. Splenomegaly developed in almost all inoculated mice, and in rats but not hamsters inoculated as adults. The lesions were of two kinds, solid tumours, and cysts filled with clear fluid or blood. Sites of solid tumours depended on route of injection and lymphatic drainage. Cysts were often related to lymph nodes. Death was often from rupture of a blood-filled cyst, or an enlarged spleen.

Incidence and latent period

Incidence approached 100 % in mice, rats, and hamsters, inoculated when newborn. In older mice incidence of splenomegaly remained high, but that of tumours declined. Latent periods after injection into newborn animals were 10–30 days, depending on dose, in mice, 3–7 weeks in rats, and a little longer in hamsters. In older mice and rats latent periods were longer.

Histology

Enlarged spleens seen in mice and rats (but not hamsters) resemble those of Friend and of Rauscher diseases showing proliferation of reticulum cells and erythroblasts. These cells also appear in the blood. Solid tumours were pleomorphic sarcomas with features suggesting a vascular origin. They formed little collagen. Some contained cleft-like spaces lined by endothelial cells. They infiltrated and destroyed adjacent tissues. Sarcomas of hamsters often contained multinucleate giant cells, with 50 or more nuclei. In cystic lesions both inflammation and neoplastic changes were seen. There was destruction of lymphoid tissue and loss of pattern and ectasia in adjacent lymph nodes. The cysts were lined with endothelium, which formed ingrowths into the lumen. Several mouse sarcomas were transplanted, one 18 times. Hamster sarcomas were also transplantable.

Recovery of virus

In mice inoculated newborn or adult, and in rats inoculated newborn, there is persistent viraemia; the tumours contain large amounts of virus. In rats inoculated when adult there is transient viraemia only (4th to 8th day). No virus was found in plasma or tumours of hamsters inoculated newborn.

Physical and chemical properties

The virus was inactivated at 56° for 30 min. It survived at −60° for ⩾ 18 weeks. It was partially inactivated by ether at 4° overnight, and completely by chloroform at 22° for 10 min. Over 99 % was sedimented by centrifugation at 200,000 *g* for 1 hr. Particle diameter, estimated by Gradocol filtration, was similar to that of the Friend virus—about 100 mμ. Apparent diameter in electronmicrographs was consistent with this estimate. However, in morphology the particle resembled MLV more closely than FLV.

Serology

MSV was neutralized by antisera against Friend, Rauscher and Moloney viruses.

Relation to other viruses

MLV has been recovered free of MSV from several preparations. The converse has not been achieved. No procedure so far tried (e.g. serial dilution, heat, ether, neutralizing sera, or passage through tissue culture) has separated the spleen-enlarging from the tumour-producing action of MSV. Several properties definitely distinguish MSV from polyoma. These will be described. Dr Maloney recently isolated an agent with some similarities to MSV. The relation between these agents will be discussed.

Avian Leukosis. By P. M. Biggs (*Houghton Poultry Research Station, Houghton, Huntingdon*)

Avian leukosis includes a number of pathologically defined conditions, which can be divided into two groups on the basis of the characteristics of their aetiological agents.

Leukosis/sarcoma group

This group is comprised of viruses responsible for neoplasias of precursors of blood cells, and rapid-acting tumour viruses affecting tissues of mesenchymal origin. Myeloid leukosis, erythroid leukosis and sarcomas are relatively rare in the domestic fowl compared with lymphoid leukosis. Lymphoid leukosis has been estimated to be responsible for between 10 and 30 % of all mortality in commercial poultry flocks.

Viruses belonging to the group resemble in some ways myxoviruses. They are RNA viruses with a lipid containing outer protein coat, and are produced by budding at the cell surface. Morphologically they have a central core bounded by a double membrane and measure between 80 and 120 mμ in diameter. They induce in infected cells a common complement-fixing antigen, a property which forms the basis of the COFAL test (Complement Fixation Avian Leukosis). On the basis of susceptibilities to infection of cells of known genotype the leukosis and sarcoma viruses can be divided into two subgroups. Recent results, using neutralization tests, indicate that there are a number of serologically distinct types of leukosis and sarcoma viruses, but those belonging to each subgroup bear some serological relationship to one another.

The rapid-acting sarcoma viruses produce neoplastic transformation of chick embryo fibroblasts in tissue culture. The leukosis viruses readily replicate but produce no morphological alteration in such cells. Prior infection of cells with leukosis virus interferes with subsequent infection with Rous sarcoma virus (RSV) of the same subgroup (this property forms the basis of the RIF test). The Bryan strain of RSV has been shown to be a defective virus requiring an associated leukosis virus for replication of infective particles. Evidence

has been presented to suggest that the associated leukosis virus provides the RSV with its outer protein coat, and therefore the properties which determine its serological type and subgroup. This has not been shown for other strains of RSV, but some results have illustrated that co-infection of cells with the Schmidt-Ruppin strain of RSV and a leukosis virus results in phenotypic mixing.

A wide range of types of tumour may occur in transmission experiments with virus isolated from field cases of lymphoid leukosis. It is unclear at present whether such isolates consist of a number of unipotential viruses or whether leukosis viruses are oncogenically multipotential.

Leukosis viruses are widespread in poultry populations and in most cases appear to have a commensal relationship with the host. Only a proportion of infected birds ultimately succumb to pathological disease.

Marek's disease (fowl paralysis, neural lymphomatosis)

Marek's disease is a common lympho-proliferative disease of the domestic fowl. Peripheral nerves are primarily involved, but in a proportion of affected birds visceral lymphoid tumours are also present. A more acute form of this disease has appeared recently in Great Britain, in which the majority of affected birds have visceral lymphoid tumours. This form has been termed 'acute leukosis' in the U.S.A.

The nature of the causative agent of Marek's disease is not clear. It is present in whole blood, washed blood cells, plasma and most tissues of affected birds. Highest titres are found in cellular preparations; procedures which disrupt cells such as homogenization with high-speed blenders, sonication and freezing and thawing destroy infectivity. There is evidence to suggest that this is due to the release of inactivating substances from disrupted cells. The agent does not induce in cells the COFAL antigen and does not interfere with subsequent infection by RSV.

Studies on the Possible Aetiology of Burkitt's Lymphoma. By B. G. ACHONG and Y. M. BARR (*The Bland-Sutton Institute of Pathology, The Middlesex Hospital Medical School, London, W.* 1)

Burkitt's lymphoma is an unusual fatal tumour of children which is commoner in certain parts of Africa than all other childhood malignancies put together. Its distribution in regions of high and low incidence will be considered together with the indirect evidence suggesting that the condition might be dependent on an infectious aetiological agent.

The laboratory investigations which have been undertaken into the possibility that Burkitt's tumour is virus-induced will be described. Strains of cells have been established in continuous culture from a number of cases of Burkitt's lymphoma. These cells grow in suspension without attachment to glass. The mode of growth and characteristics of the cells will be described and the attributes will be given on which their identification as unusual lymphoblasts is based.

The fine structure of the cells has been determined and has confirmed their lymphoblastic nature; the subcellular organization of the cultured cells will be compared to that of the malignant cells in Burkitt's lymphoma.

Each of the established tissue culture strains of Burkitt tumour cells has been found to carry a similar virus. The growth and maturation of this virus will be illustrated together with the cytopathological changes it induces. The nature of the virus will be discussed and reasons will be given for believing it to be a new hitherto unidentified agent; the possibility that the virus might play a role in the aetiology of this human malignancy will be considered.

Reversion in Hamster Cells Transformed by Rous Sarcoma Virus—its Significance in Relation to Viral Integration with the Cell. By IAN MACPHERSON (*Institute of Virology, Glasgow*)

In cells rendered neoplastic by DNA viruses the common finding is that infectious virus is no longer detectable. There is, however, good evidence for the persistence of at least part of the viral genome in these cells and this may be necessary for the continued expression of neoplastic properties. Since small oncogenic DNA viruses replicate in the nucleus during their vegetative cycle and probably rely on the host cell ribosomes to carry their messenger RNA the notion that a closely integrated association may occasionally occur and result in a neoplastic transformation has found general acceptance as a working hypothesis.

In RNA virus-induced tumours and transformed cells the situation is very different. Here the common picture in both mouse and avian leukosis is one of continued virus production following transformation of cells. However, studies with the Bryan strain of Rous sarcoma virus (RSV) have shown that, like DNA viruses, this virus may also persist in cells in a defective form. Also some strains of RSV may induce transformations of mammalian cells that are not associated with the production of infectious virus. Such transformed cells may be cultured through many generations without ever releasing virus yet may still be capable of inducing virus-releasing tumours when implanted in chickens. This apparently occurs as a result of their viral genome being transferred to host cells where maturation of infectious virus can take place. The mechanisms controlling the persistence of RNA virus genome in transformed cells are unknown and few facts are available on which to base a hypothesis. However, some light has been shed on this problem as a result of comparative studies made with polyoma and RSV transformed hamster cells of the BHK21 cell line. These indicate that the integration of RSV genome in transformed cells may be unstable in certain circumstances.

When BHK21 cells were infected with the Schmidt–Ruppin strain of RSV and cultured in agar suspension, a small proportion of the cells formed colonies. These were found to consist of cells that grew in disarray on glass like polyoma-transformed BHK21 cells. When plated to produce discrete colonies some of the colonies were found to consist of cells growing with parallel orientation like the original untransformed cells. It was established that these cells were revertants when two cycles of single cell cloning of transformed cells resulted in a population that gave rise to some progeny cells which formed colonies with parallel orientation.

A comparison of RSV transformed clones with clones of revertant cells derived from them indicated that reversion was correlated with loss or reduction of the cells' content of RSV genome. The presence of the RSV genome in the transformed cells was demonstrated by injecting the cells into chickens and showing that the sarcomas produced were composed of chicken cells and that the chickens bearing these tumours possessed neutralizing antibody to the Schmidt-Ruppin strain of RSV. Homogenates of the transformed cells did not produce tumours in chickens suggesting that complete virus was not present in these cells. The revertant cells did not produce tumours in chickens. Significant amounts of the specific complement-fixing antigen that is possessed in common by viruses of the avian leukosis complex (COFAL antigen) were found in the transformed cells but not in the revertant cells.

These results suggest that the integration of RSV genome with the hamster cell it transforms is unstable and is required for the expression of its transformed character. The stability of polyoma-transformed hamster cells is shown by the fact that in observations on many thousands of transformed colonies on glass no colonies with reverted morphology have been found.

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