J. gen. Microbiol. (1962), 28, 571–578
Printed in Great Britain

A Study by Fluorescence Microscopy of the Replication of Inclusion Blennorrhoea Virus in HeLa Cell Monolayers

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(*Received* 3 *October* 1961)

SUMMARY

The infectious agent of inclusion blennorrhoea was seen by fluorescence microscopy as a particle of deoxyribonucleic acid (DNA) 0·2 μ diam. These infectious particles and particles inactivated at 4° and 56° were adsorbed by HeLa cell monolayers. The inactivated particles were visible for at least 6 hr., distributed at random on the cells, while the infectious particles rapidly disappeared. Multiple infections of the cells readily occurred and in cells so infected, a cluster of ribonucleic acid (RNA) particles was first observed at 3 hr. adjacent to the cell nucleus. Since the number of RNA particles, each of which contained a DNA core, was equal to the number of infectious particles adsorbed and since singly infected cells contained only one RNA particle, the infectious unit is almost certainly a single DNA particle. The RNA particle increased in size without dividing and the surrounding cell cytoplasm gradually dissolved to form the inclusion body. DNA particles which were protected from deoxyribonuclease by a layer containing RNA were first observed in the inclusions at 21 hr. and an average of one infective progeny was detected in each infected cell at 22–23 hr. indicating that infectivity was associated with the DNA particles. RNA, DNA and infectious DNA particles reached their maxima between 27–30 hr., 39–42 hr. and 34–38 hr. respectively and decreased thereafter. Infectious DNA particles were released from the inclusions. The total number of DNA particles/inclusion always exceeded the number of infectious DNA particles/inclusion. Glycogen was not detected in the inclusions suggesting that it may not be essential for the formation of virus.

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INTRODUCTION

The total number of particles/inclusion seen in Giemsa stained preparations of HeLa cells infected with inclusion blennorrhoea virus exceeds the number of infectious units/inclusion, suggesting that only a proportion of these particles are infectious (Furness & Fraser, 1962). Both ribonucleic acid (RNA and deoxyribonucleic acid (DNA) particles are seen in the inclusion found in human amnion and synovial cells infected with trachoma virus (Pollard, Starr, Tanami & Moore, 1960). In the present study, the formation of RNA and DNA particles in HeLa cells infected with inclusion blennorrhoea virus was observed by fluorescence microscopy and their relationship to infectivity determined.

METHODS

Infection of HeLa cell monolayers. The methods used to assay the LB1 strain of inclusion blennorrhoea virus in HeLa cell monolayers and to obtain one-step growth curves have been described (Furness, Graham & Reeve, 1960; Furness & Fraser, 1962). Monolayers required for staining were infected by the same method. The monolayers on coverslips in Leighton tubes were covered with 0·25 ml. tissue culture medium and inoculated with 0·1 ml. of a suitable virus suspension which was allowed to adsorb for 2 hr. at 30°. They were then freed from unadsorbed virus by washing with phosphate buffered saline (Dulbecco & Vogt, 1954), covered with 2 ml. medium and incubated at 37°. Sample monolayers were removed at intervals for staining and for the assay of intracellular infectious units. Some microscopic appearances were more easily studied in heavily infected monolayers inoculated with a virus suspension containing 2–4 infectious units/cell. When it was desired to infect cells with a single infectious unit, a diluted suspension which infected less than 5 % of the cells was used.

Acridine orange staining. The cell monolayers were washed in phosphate buffer (pH 6·8; G. T. Gurr Ltd., London) fixed in Carnoy's fluid for 10 min., rinsed in ethanol and washed in acetate buffer pH 3·0 (N-sodium acetate, 100 ml; N-HCl 99·5 ml.; water, 300·5 ml.). They were stained in 0·5 % (w/v) acridine orange (Imperial Chemical Industries stain R. 150) in acetate buffer (pH 3·0) for 30 min., washed for 30 min. in three changes of acetate buffer (pH 3·0) and sealed in acetate buffer (pH 3·0) with a mixture of beeswax and colophonium.

Fluorescence microscopy. A monocular microscope with apochromatic lenses was mounted horizontally and illuminated by a 250 watt high-pressure mercury vapour lamp of the Metro Vickers glass envelope type, the light being concentrated by a collecting lens and an Abbé condenser. The Wratten exciting filter No. 50 (Kodak Ltd.) was protected by a heat absorbing filter. Observations were made with a Watson oil immersion lens N.A. 1.37 and ×10 ocular containing a minus blue barrier filter (Ilford Ltd.). With these filters, ribonucleic acid (RNA) appeared orange and deoxyribonucleic acid (DNA) green. The magnification was ×950. Measurements were made with ×10 and ×17 micrometer oculars.

Specificity of acridine orange stain. To confirm the specificity of the acridine orange stain for both cell and viral RNA and DNA, uninfected and infected monolayers were stained and compared with replicate monolayers in which the RNA and DNA had been digested with ribonuclease (RNase) and deoxyribonuclease (DNase)

before staining. Aqueous stock solutions, 0·1 % (w/v), of crystalline RNase grade A (California Corporation for Biochemical Research, Los Angeles) were kept at −30° and diluted 1/10 in McIlvaine's buffer (pH 5·6) before use. DNase grade B (California Corporation for Biochemical Research, Los Angeles) 0·01 % (w/v) was prepared in veronal buffer as required. The monolayers were washed in phosphate buffer (pH 6·8), fixed in ethanol for 5 min., washed in veronal buffer, incubated in diluted enzyme for 1 hr. at 37°, washed in acetate buffer (pH 3·0), fixed in Carnoy's fluid for 10 min. and stained.

Iodine staining for glycogen. Monolayers were stained with 1 % (w/v) iodine in 2 % (w/v) aqueous solution of potassium iodide for 15 min., blotted dry, covered with immersion oil and examined at a magnification of ×240 (Collier, 1961).

RESULTS

Identification of cell and viral RNA and DNA by fluorescence microscopy

Treatment with RNase and DNase abolishes the specific colour reactions of cell RNA and DNA with acridine orange (Armstrong, 1956; Armstrong & Niven, 1957). The specificity of our staining and the activity of our enzyme preparations were confirmed by this method. RNase 0·01 % (w/v) digested the orange-staining material within the inclusions in 40 min. and within the cells in 60 min.

Cell DNA was digested by 0·01 % (w/v) DNase in 20 min. at 37°, but the viral DNA was not affected by incubation with 0·1 % (w/v) DNase for 2 hr. at 37°. However, when the preparations were treated with 0·01 % (w/v) RNase for 60 min. at 37°, subsequent treatment with 0·01 % (w/v) DNase for 60 min. at 37° abolished the staining reaction of the viral DNA in the inclusion, indicating that the viral DNA was protected from the enzyme activity of DNase by a layer containing RNA. The specific colour reactions of both cell and viral DNA and RNA were unaffected in controls incubated in buffer only.

Staining of virus suspension. Virus was released from the inclusions, 72 hr. after infection of the cells, by ultrasonic vibrations (Furness & Fraser, 1962). Smears were prepared on coverslips, air dried, fixed in Carnoy's fluid for 10 min. and stained with acridine orange. Large numbers of DNA particles were seen similar in size to those in inclusions. No RNA particles were identifiable.

Adsorption of inactivated and infective virus by HeLa cells. Since virus suspensions contained both infectious and non-infectious particles, the adsorption of both infective and inactivated virus to HeLa cell monolayers was investigated. Inclusion blennorrhoea virus was completely inactivated in 10–15 min. at 56°; at 4°, 99 % became non-infective in 14 days (Fig. 1). To ascertain whether the temperature used for inactivation affected adsorption of the particles, monolayers were inoculated with 0·1 ml. of a virus suspension reduced in infectivity by holding for 14 days at 4° and with the same suspension heated for 10–15 min. at 56°.

After adsorption of the inactivated virus for 2 hr. at 30°, the cells were well washed, covered with 2 ml. medium and incubated at 37°. At intervals monolayers were stained and examined. There was no significant difference in the results after inactivation at the two temperatures (Table 1); the virus was adsorbed, remained visible for over 6 hr. and then disappeared. To compare the rate of adsorption and disappearance of infective and inactivated virus a suspension was prepared from

cells 40 hr. after infection, when the inclusions contained the greatest number of infectious units and release of progeny had not begun (Furness & Fraser, 1962); part was inactivated by heating for 15 min. at 56°. Immediaely after adsorption (Table 1) and 6 hr. later, there were fewer infectious particles than inactivated DNA particles. It was impossible to decide whether the particles were within the cells.

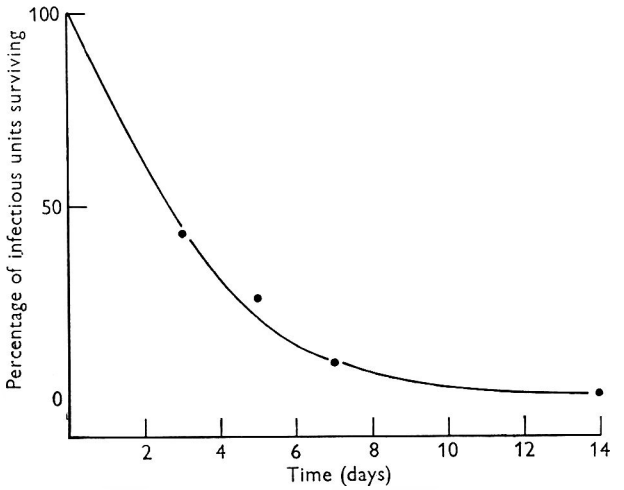

Fig. 1. The loss of infectivity for HeLa cells of inclusion blennorrhoea virus suspended in cell culture medium at 4°.

Table 1. *DNA particles adsorbed to HeLa cell monolayers inoculated with infective and inactivated inclusion blennorrhoea virus*

	After adsorption, incubated at 37° for (hr.)			
	0	3	6	9
Inoculum	Relative no. partieles/field			
Inactivated at 4°	+ + + +	+ + + +	+ + + +	+
Inactivated at 4° followed by 56° for 15 min.	+ + + +	+ + + +	+ + + +	+ +
Inactivated at 56° for 15 min.	+ + + +	+ + + +	+ + + +	+ +
Infective virus	+ +	+	—	—

+ + + + Max. no. particles/field.
+ + Significant reduction in no. particles/field.
+ An occasional particle/field.

Formation of RNA, DNA and infectious DNA particles in infected HeLa cells. The viral RNA stained a different shade of orange from that in the cell cytoplasm so that even small viral RNA particles could be distinguished from the cell cytoplasm In heavily infected monolayers inoculatedwith 2–4 infectious units/cell, a single cluster of RNA particles about 0·5μ diam. was seen adjacent to the cell nucleus at 3 hr. Monolayers were inoculated with dilutions of virus suspensions and the RNA particles in the inclusions counted after 9 hr. incubation at 37°, when the particles were readily identified. The number of RNA particles was proportional to the

dilution of the inoculum (Table 2). In cells estimated to have adsorbed 1·8 infectious particles from the inoculum there was an average of 1·4 RNA particles/inclusion suggesting that each infectious particle was the precursor of one RNA

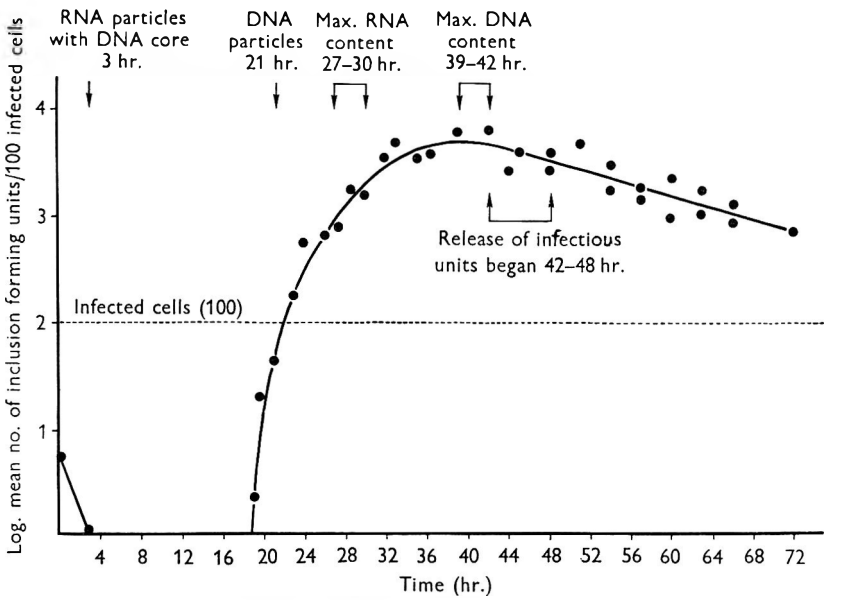

Fig. 2. The formation of RNA, DNA and infectious units in HeLa cell monolayers infected with inclusion blennorrhoea virus. Ordinate: The mean number of intracellular infectious units per 100 infected cells plotted on a logarithmic scale.

Table 2. *The relationship of the infectious units in the inoculum to the number of RNA particles/inclusion*

(*a*) Dilution of inoculum	(*b*) No. of inclusions examined	(*c*) RNA particles in inclusions	(*d*) Average no. of RNA particles/ inclusion (*c*/*b*)
	Expt. no. T. 92		
0	50	71	1·4*
1/10	50	51	1·0
	Expt. no. T. 96		
0	100	1365	13·65
1/2	100	558	5·58
1/4	100	281	2·81
1/8	100	192	1·92

* From titration of original suspension, estimated number of infectious units adsorbed from undiluted inoculum 1·8/cell, so that at 1/10 dilution about 20 % cells were infected with one infectious particle.

particle which was not infectious (Fig. 2). This was confirmed by examining every 2 hr. monolayers in which less than 5 % of the cells were infected with a single infectious particle. With a single exception, the inclusions contained only one RNA

particle which increased in size without any evidence of binary fission, reaching a maximum between 27–30 hr. Thereafter the amount of RNA decreased. DNA particles, about 0·2 μ diam. were first observed in the inclusions at 21 hr. but treatment of the earliest RNA particles with RNase revealed a DNA core which increased in proportion to the RNA during the development of the inclusion. The relation between RNA and DNA particles and infectious units found is summarized in Fig. 2. The virus was non-infective 4 hr. after adsorption to the cell. An average of one infective virus progeny/cell was detected at 22–23 hr. suggesting that infectivity was associated with DNA. The number of DNA particles reached a maximum between 39–42 hr. and infectious particles between 34–38 hr. An accurate total count of the DNA particles/inclusion was impossible, but their number exceeded the number of infectious units/inclusion. After reaching a maximum both infectivity and DNA particles decreased, suggesting release from the inclusion. In monolayers in which about 5 % of the cells were infected, secondary RNA inclusions were seen in previously uninfected cells 69–72 hr. after the initial infection confirming that the infective virus was released and adsorbed to uninfected cells (Furness & Fraser, 1962).

The formation of inclusion bodies. When first detected the RNA particles were surrounded by cell cytoplasm. By 10–15 hr. the cytoplasm immediately around the particles had dissolved leaving the particles often arranged round the periphery of the clear area which enlarged to form the typical cytoplasmic inclusion body adjacent to the cell nucleus. At all stages inclusion bodies unaccountably varied greatly in size.

Glycogen. Although glycogen has been detected in inclusion bodies in HeLa cells infected with this strain of inclusion blennorrhoea virus (Furness, Graham, Reeve & Collier, 1960) it was not found in the present work within 72 hr. of infection.

DISCUSSION

The stages in the replication of inclusion blennorrhoea virus in HeLa cells infected with several infectious particles are given schematically in Fig. 3*a–c*. (*a*) After the adsorption of both infective and inactivated virus, DNA particles, 0·2 μ diam. are distributed at random on the HeLa cells. It is probable that the particles lie within the cells which are known to be phagocytic but this was not demonstrable. Most of the infectious particles disappear immediately after adsorption, i.e. during the adsorption period and at this stage there is no evidence of any gross cellular abnormality. (*b*) At 3 hr. a single cluster of RNA particles 0·5 μ diam. appears in the cytoplasm near the nucleus of each cell. (*c*) These RNA particles do not divide but enlarge, arrange themselves in a ring similar to that seen in Giemsa stained preparations of chick embryo cell cultures infected with trachoma virus (Gordon, Quan & Trimmer, 1960) and then seem to coalesce. At the same time, the cell cytoplasm gradually dissolves leaving the particles within a vacuole which increases in size to form the distinctive cytoplasmic inclusion body. (*d*) By 21 hr. DNA particles are also seen within the inclusion body and are presumably released from within the RNA which reaches its maximum between 27–30 hr. The appearance of DNA particles roughly coincides with the detection of infective virus (*e*) Only an occasional RNA particle 0·5 μ diam. and many DNA particles are found in the inclusion bodies at 66–72 hr. From these results we tentatively conclude that the

infectious unit is a single DNA particle which is the precursor of the one RNA particle seen in the cytoplasm of cells infected with a single infectious unit.

Treatment of the particles with RNase and DNase shows that the RNA particle contains a DNA core, and the DNA particle is covered with a substance which contains RNA but not enough to be visible by its fluorescence. Although the possibility that the enzyme preparations contained effective enzymes other than

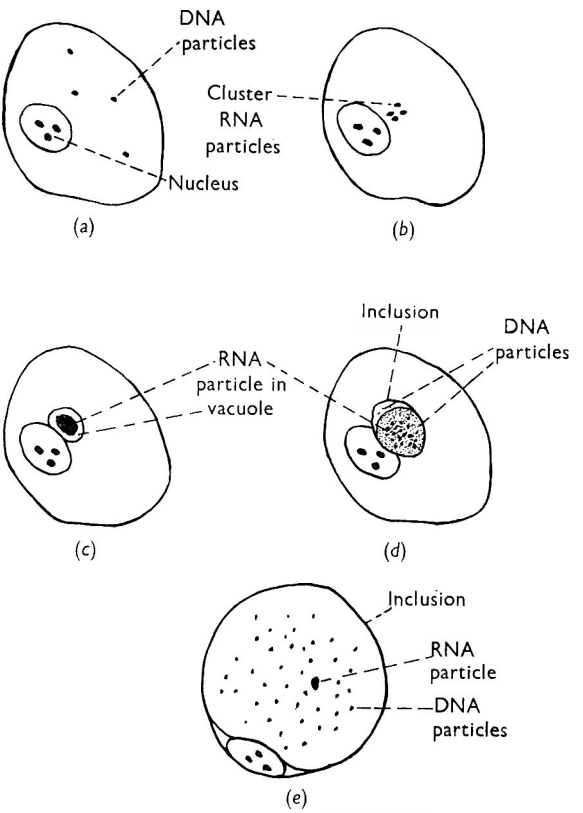

Fig. 3. Schematic representation of the stages in the replication of inclusion blennorrhoea virus in HeLa cells infected with several infectious DNA particles. (*a*) After adsorption a few DNA particles 0·2μ diam. may be seen on the HeLa cell. (*b*) 3 hr. infectious DNA particles have disappeared and a cluster of RNA particles 0·5μ diam. appears near the cell nucleus. (*c*) 18 hr. RNA particles increase in size and finally coalesce. A vacuole is formed around particles 10–15 hr. (*d*) 27 hr. RNA particle reaches maximum. Many DNA particles which first appear at 21 hr. seen within the inclusion. (*e*) 66–72 hr. Large inclusion body contains many DNA particles and an occasional RNA particle about 0·5μ diam.

the two nucleases cannot be excluded, one possible interpretation of the results is that the RNA particle is an enlarged DNA particle, in which the RNA has increased sufficiently to be detectable.

Similar stages in the growth of the related organisms of the psittacosis-lymphogranuloma group were first described for psittacosis virus by Bedson, and his coworkers (Bedson, 1933; Bedson & Bland, 1932, 1934; Bland & Canti, 1935) and their reports have been amply confirmed by other authors. It was generally believed that

the virus remained at least partially infective throughout the growth cycle; though Girardi, Allen & Sigel (1952) concluded that psittacosis virus underwent a non-infective phase. Our observations are consistent with this conclusion.

Other animal viruses so far investigated in this way have proved to lose their infectivity and their original structure during the eclipse phase, their nucleic acids being dispersed in the host cell. Inclusion blennorrhoea virus qualifies for classification as a typical animal virus in that it has a non-infective phase, during the first 3 hr. of which it also became undetectable by the available techniques. Nevertheless, the effective part of the virus material from each particle appears to migrate to a single site near the nucleus, suggesting that during the eclipse, the infective material continues to exist as a discrete particle. By itself, this difference does not, in our view, at present constitute a very cogent reason for excluding the agent of inclusion blennorrhoea from the class of animal viruses, particularly since, for many of the animal viruses, the dispersal of viral nucleic acids during the eclipse phase of replication has been assumed by analogy with those for which dispersal has been proved.

We wish to thank Miss Margaret Ham for her excellent technical assistance. The work of one of us (G.W.C.) was supported by a U.S. Public Health Service Grant administered by the Medical Research Council Working Party on Non-Specific Urethritis.

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J. gen. Microbiol. (1962), 28, 579–584
Printed in Great Britain

Decline in the Cellular Content of RNA, Protein and Dry Weight during the Logarithmic Growth of *Euglena gracilis*

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(*Received* 3 *October* 1961)

SUMMARY

Determinations of the cellular content of RNA, total protein and dry weight of *Euglena gracilis* at different times during logarithmic growth indicated that these parameters did not reflect the constant reproductive rate of these organisms during this period of growth. During exponential growth, the RNA content was reduced by 37 %, the total protein by 45 % and the dry weight by about 76 %. In addition, the RNA and protein content of Euglena showed a similar pattern of decline as growth progressed; the DNA content, however, remained constant throughout the logarithmic growth phase.

INTRODUCTION

Various growth phases are observed when micro-organisms are grown in culture flasks. One of these growth phases, the logarithmic phase, is characterized by a constant reproductive rate, and it is often assumed that this constancy is reflected in other cellular properties. Consequently, this period of growth has been used when cells in the same physiological state are required for biochemical and biophysical experimentation. The question arises whether or not this assumption of constancy in cellular properties other than the reproductive rate is valid. The problem can be investigated by determining the content/cell of various constituents at different times during logarithmic growth. The constituents to be studied would logically include deoxyribosenucleic acid (DNA), ribosenucleic acid (RNA) and proteins, especially since these are involved in the synthesis of other cellular materials (Brachet, 1957, 1960; Chargraff & Davidson, 1955). The present paper reports such an analysis of the DNA, RNA, total protein content and dry weight of the flagellate protozoan *Euglena gracilis* at different times in its logarithmic phase of growth.

METHODS

Growth. Experimental and stock cultures of a streptomycin-bleached strain, SM-Ll, of *Euglena gracilis* var. *bacillaris* were grown in the dark on a defined medium (Cramer & Myers, 1952) with Na acetate (0·061 M) as sole carbon source. All growth studies were carried out at 25° and pH 6·8. The generation time of Euglena on this medium was about 22 hr. (Buetow & Levedahl, 1960).

Dry-weight determination. Triplicate samples of 1 to 2×10^6 organisms were dried

to constant weight at 105° on previously weighed planchets. The weight of the dried organisms was determined on a Sartorius-Werke microbalance.

Total protein determination. Total protein was first determined by the biuret reagent described by Knights, MacDonald & Ploompuu (1957). Each sample contained approximately 10^6 organisms. In later studies, total protein was determined in quadruplicate samples by the Folin phenol reagent method of Lowry, Rosebrough, Farr & Randall (1951). The Lowry method allows the use of fewer organisms (i.e. 10^5 organisms) per sample. The two methods yielded comparable results.

Nucleic acid determination. The method of Schmidt & Thannhauser (1945) was considerably modified to determine the RNA and DNA content of the Euglena. The modified method was as follows: duplicate samples $2\text{–}3 \times 10^6$ organisms were concentrated by centrifugation and successively extracted with 1 ml. absolute ethanol, twice with 1 ml. of a 50-50 mixture absolute ethanol-ether (v/v), and then with 1 ml. absolute ethanol. The organisms were concentrated by centrifugation between each extraction and only the resulting pellets saved. Acid-soluble materials were extracted twice in ice for 15 min. with 1 ml. cold 0·2 N-$HClO_4$ and the samples were centrifuged for 1 min. at 2°–4°. The resulting pellet was incubated with 0·5 ml. N-NaOH at 30° for 16 hr., neutralized with 0·5 ml. N-HCl and chilled in ice for 10 min. The samples were next extracted twice with a total of 3 ml. cold N-$HClO_4$ and centrifuged at 2°–4° for 5 min. The resulting supernatant fluids were read at 260 and 315 mμ in a Beckman DU spectrophotometer against a N-$HClO_4$ blank. The difference in readings (i.e. $OD_{260} - OD_{315}$) gives the RNA fraction.

The pellet was twice incubated with a total of 1·3 ml. N-$HClO_4$ at 90° for 10 min., cooled in ice and centrifuged for 4 min. The resulting supernatant fluids were read in a Beckman spectrophotometer at 267 and 315 mμ. The difference in readings (i.e. $OD_{267} - OD_{315}$) gives the DNA fraction (DeDeken-Grenson & DeDeken, 1959). It was found that a single extraction with $HClO_4$ for the DNA fraction gave variable results with Euglena, whereas double extractions with $HClO_4$ gave consistent results. The DNA values reported in the present paper agree with those reported by Neff (1960) for colourless Euglena. The amounts of RNA and DNA present in a sample were determined by using the optical density conversion factors derived by DeDeken-Grenson & DeDeken (1959); these values were converted to μg. RNA or μg. DNA/10^6 organisms.

RESULTS

During logarithmic growth, both the dry weight and total protein content of the Euglena decreased as growth progressed (Table 1). There was an early threefold decrease, a levelling-off, and then further decline in the dry-weight measurements. The total protein content per million organisms decreased from about 500 to 285 μg. in Expt. 1 and from 536 to 295 μg. over a comparable growth range (Expt. 2). This latter measurement represents a 45% decrease in the total protein content per average organism during the logarithmic phase of the growth cycle.

The RNA and DNA contents of the Euglena were also determined at various points in their logarithmic growth phase. Results of these experiments are presented in Table 2. Expt. 1 indicated a decrease in the RNA content as growth progressed. Expt. 2 was so planned that measurements were taken at Euglena concentrations

intermediate between those reported in Expt. 1. In Expt. 2, the RNA content/million Euglena declined from 34·7 to 21·9 μg., a decrease of 37 % during logarithmic growth. The DNA content of the Euglena remained constant during the exponential growth phase in all experiments. However, at the beginning of the stationary phase of the growth cycle (i.e. 335,000–340,000 Euglenas/ml.), both the RNA and

Table 1. *Total protein and dry weight of Euglena during logarithmic growth*

Expt.	Concentration (organisms/ml. medium)	Total protein* (μg/10^6 organisms)	Dry weight (mg./10^6 organisms)
1	20,200†	498 ± 34·8	3·57 ± 0·04
	36,600	—	1·19 ± 0·01
	123,000	—	1·06 ± 0·02
	158,000	348 + 26·0	0·84 ± 0·02
	170,000	329 ± 29·9	—
	237,000	285 ± 24·1	—
2	13,000	551 ± 9·5	—
	23,400	536 ± 26·9	—
	38,100	375 ± 17·6	—
	64,500	378 ± 11·5	—
	120,000	386 ± 13·3	—
	259,000	295 ± 11·2	—
	335,000‡	302 ± 21·0	—

* Measured by the method of Knights *et al.* (1957) in Expt. 1 and by the method of Lowry *et al.* (1951) in Expt. 2. Values given are the mean ± standard deviation.
† End of the lag phase, beginning of the logarithmic growth phase.
‡ Beginning of stationary phase of growth cycle.

Table 2. *RNA and DNA content of Euglena during logarithmic growth*

Expt.	Concentration; organisms/ml. medium	RNA* (μg./10^6 organisms)	DNA* (μg./10^6 organisms)
1	42,800	30·2	4·34
	87,700	30·2	4·34
	175,000	27·8	4·08
	290,000	26·9	4·14
	340,000†	32·3	4·72
2	23,400	34·7	4·17
	38,100	30·2	4·63
	64,500	29·2	4·60
	120,000	27·7	4·39
	259,000	21·9	—
	335,000†	31·0	5·12

* Mean of duplicate samples; in these experiments, the average deviation between duplicate samples was 1·1 μg./10^6 organisms for RNA and 0·4 μg./10^6 organisms for DNA.
† Beginning of the stationary phase of the growth cycle.

the DNA content increased (Table 2). Such a finding probably results from the fact that a culture in the stationary phase of the growth cycle is essentially a non-dividing one. Under such conditions, the cell constituents would be expected to 'pile up', at least initially. Of further interest is the similar decrease of RNA and total protein of Euglena during exponential growth. Figure 1 presents the

results of an experiment in which both RNA and protein were measured in the same culture during its exponential growth phase. Although the protein initially decreased faster than the RNA, its overall pattern of decline was similar to that of the RNA. After an initial sharp decrease, there is a levelling-off of in the decline of RNA and protein during the middle of logarithmic growth, followed by another sharp decline late in the exponential growth period. This observation appears to be in line with the often demonstrated influence of RNA and protein syntheses on each other (Brachet, 1957).

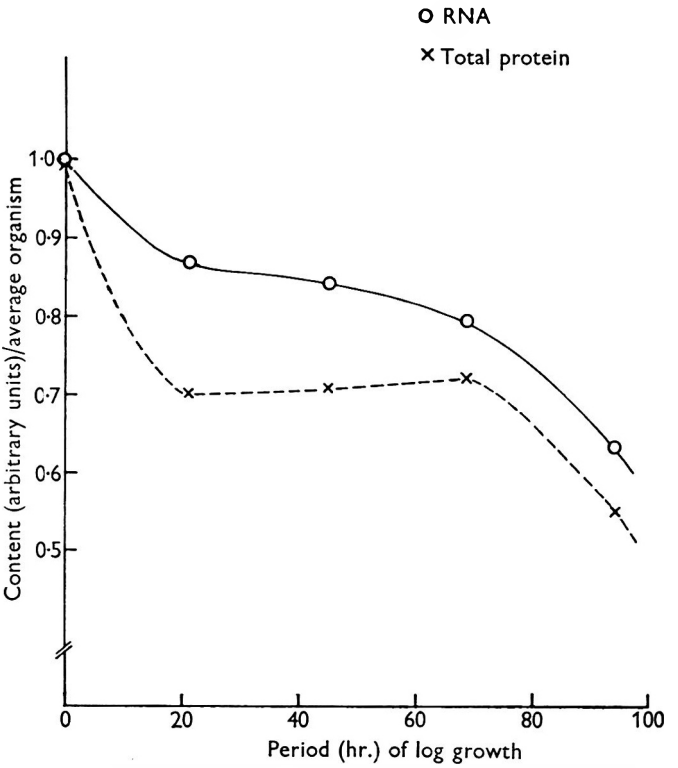

Fig. 1. Decline in mean RNA and total protein content per average Euglena cell during logarithmic growth. Zero time represents the beginning of logarithmic growth. The generation time of Euglena is about 22 hr. In order to show the similarity in decline, both the RNA and total protein content per average cell are given in arbitrary units. (See Tables 1 and 2 for actual values.)

DISCUSSION

The constant reproductive rate of *Euglena gracilis* during logarithmic growth is not reflected in the RNA or total protein contents or dry weight of these organisms. The authors believe the present observations are the first to demonstrate a variability in such cellular parameters during the logarithmic growth of protozoa. These results are reminiscent of those obtained with bacteria and mammalian cell lines maintained in culture. The HeLa cell (Salzman, 1959) as well as other mammalian cells of carcinogenic origin (Swaffield & Foley, 1960) show systematic fluctuations in cellular protein, RNA and DNA content and protein composition (Kruse, Schooler &

White, 1960) during logarithmic growth. Morse & Carter (1949) showed a rapid decline in the RNA content of *Escherichia coli* during the early part of its logarithmic growth phase. The data of Dean & Hinshelwood (1959) on *Aerobacter aerogenes* and of Belozersky (1960) on *Azotobacter agile* indicate a continuous decline in the RNA content of these organisms during exponential growth. The cell size of *A. aerogenes* also decreased during this period of growth (Dean & Hinshelwood, 1959). The decrease in dry weight as well as in the cellular content of RNA and protein during the exponential growth of *Euglena gracilis* examined here suggests that these substituents are synthesized and accumulated during the lag phase of growth. Such a synthesis and accumulation of nucleic acid and protein during the lag phase was shown for bacteria such as *A. agile* (Belozersky, 1960), *A. aerogenes* (Dean & Hinshelwood, 1959; Neidhardt & Magasanik, 1960) and *E. coli* (Morse & Carter, 1949) and various mammalian cells (Salzman, 1959; Swaffield & Foley, 1959). These observations indicate that the lag phase is a period of intense metabolic activity related primarily to protoplasmic growth rather than cell division. The decline in RNA, protein and, especially, dry weight during the exponential growth of *Euglena gracilis* suggests that other cell parameters, i.e. lipids, carbohydrates, etc., may also change during this period of growth. Therefore, before any such parameter is used as an indication of the amount of cell population, its measurement should be completed throughout the growth cycle.

The authors wish to thank Dr J. J. Blum of the Gerontology Branch, National Heart Institute, for aid with the RNA–DNA method, and Mrs M. Robinson for technical assistance. Some of these data are taken from a dissertation presented (by D.E.B.) in partial fulfilment of the requirements for the degree of Doctor of Philosophy, University of California, Los Angeles, 1959. This work was aided by a contract between the office of Naval Research, Department of the Navy, and the University of Califormia, Los Angeles, NR120-336.

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J. gen. Microbiol. (1962), **28**, 585–598
With 1 *plate*
Printed in Great Britain

The Survival of *Escherichia coli* on Drying and Rehydration

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(*Received* 20 *October* 1961)

SUMMARY

By direct observation of individual organisms in a micro-culture chamber and by viable plate counts, a study has been made of the function of sugar/colloid mixtures in promoting the survival of Gram-negative bacteria on drying. The high death rate after drying in sugar alone was due mainly to cell-wall damage caused during rehydration by the temporary osmotic pressure set up by the sugar within the cell, leading to the formation of spheroplasts not capable of division. Spheroplast formation was largely prevented and survival greatly enhanced by controlled rehydration, showing that the sugar component was the primary protective agent. The complementary role of the protein or 'protective colloid' appears to lie in its ability to compress the cell wall against the contracted plasma membrane in plasmolysed cells, thus decreasing the volume of the interspace between the two membranes and so limiting the sugar trapped therein to a safe amount. These observations were corroborated by cell volume measurements in the ultracentrifuge.

INTRODUCTION

Although the prime importance of the suspending medium in promoting a high survival of bacteria on drying has long been recognized (see review by Fry, 1954), the mode of action of an effective medium has not been satisfactorily explained. Broth and serum had been used with moderate success in most of the earlier work, but it was not until Fry & Greaves (1951) introduced their *mist. desiccans*, a serum+broth mixture containing 7·5% glucose, that sugar-containing media came into general use for the preservation of bacteria in the dried state. Fry & Greaves suggested that the function of the sugar lay in its ability to trap the small amount of moisture which they believed necessary for survival, although Record & Taylor (1953) concluded (in view of the high survival maintained after prolonged rigorous drying) that the amount of moisture, if any, must be small indeed. Scott (1959) cast doubt on the moisture theory and proposed a chemical hypothesis based on carbonyl-amino group interactions. Neither hypothesis however explains the rather poor survival obtained in sugar alone nor the enhanced survival, first noted by Heller (1941) which results from the addition of colloidal material. The present paper reports certain physicochemical phenomena concerning both these factors.

METHODS

Organism. The Gram-negative organism *Escherichia coli* strain Jepp was chosen for most of the present work as an average representative of the coli group, being neither as robust as the 'Communis' strain nor as fragile as the American type 'B'

(see Record & Taylor, 1953). It was grown at 37° in tryptic meat broth adjusted to pH 7·4.

Viable counts (Miles & Misra, 1938) were made on tryptic meat agar (35 ml.) in Petri dishes (8·7 cm.). The nutrient agar was dried, after setting, for 1 hr. in an inverted position at 60° to render it absorbent; 0·5 ml. of suspension containing 100–200 viable organisms was spread on the agar surface by rotation in an inclined position. The plates were incubated at 37° overnight. Survivals are expressed as a percentage of the initial viable count.

Drying procedure. Samples of cultures grown in tryptic meat broth as above were diluted with buffer to about 10^8 organisms/ml. and then further diluted to about 10^6 organisms/ml. in the drying medium. Single drops (about 0·02 ml.) of this suspension were delivered to the bottom of small glass ampoules which were then attached by rubber connexions to a manifold system containing P_2O_5, evacuated to 0·1 mm. Hg or less (unless otherwise stated) and dried for 18–24 hr. The drops were weighed or delivered from a calibrated dropping pipette (Snyder, 1947). The dried drops were reconstituted with 5 ml. tryptic meat broth and diluted further with broth as required for viable counts.

Materials. Buffer contained (g./l.): KH_2PO_4 4·5; $(NH_4)_2SO_4$, 0·5; NH_4Cl 0·5; adjusted to pH 7·6 by addition of NaOH. Peptone water was a solution of Evans 'bacteriological' peptone at 10 g./l. D-Glucose was British Drug Houses Ltd. analytical grade. Bovine serum albumin (dried fraction V) was obtained from Armour Laboratories. Polyethylene glycol (PEG), average molecular weight 10,000, obtained from L. Light and Co., was dialysed in cellophane bags against distilled water for 3 days and freeze dried from a 100 g./l. solution.

All reagents were adjusted to pH $7·3 \pm 0·3$.

Microscopy. The study of the behaviour of individual organisms when subjected to various treatments was greatly facilitated by the use of a micro-culture chamber (Powell, 1956). The apparatus was set up in a temperature-controlled room at 30°. Observations were made by the use of a phase contrast optical system. For studies on freely growing organisms, peptone water was circulated beneath the cellophan membrane until two or three generations had appeared, after which the peptone water was replaced by the test solution. When the test solution contained a high molecular weight component which would not diffuse readily through the membrane, a centrifuged deposit of freely-growing organisms was taken in the test solution, the same solution being circulated through the chamber.

Pellet volume measurements. The analytical ultracentrifuge offers a convenient means of measuring pellet volumes at any desired centrifugal force over a wide range. A Spinco 'Model E' was used. Photographs were taken with the diagonal *Schlieren* bar angle set at 90° to give, in the absence of any steep concentration gradient above the surface of the pellet, the position of the pellet boundary. A control run with water in the optical cell fixed the position of the cell bottom. Frequent observations were also made with a bar angle setting of 45°, to reveal the presence of any appreciable concentration gradients such as might result, for example, from the disintegration of some of the bacteria. On account of their fragility, bacteria in the logarithmic phase were generally unsatisfactory and pellet volume measurements were confined to bacteria obtained from cultures in tryptic meat broth incubated for 18 hr. at 37°. The culture was centrifuged,

the deposit either washed with a large volume of water and taken up in water or washed and resuspended in 0·15 M-NaCl. The composition of the suspending medium was then adjusted by the addition of the required amount of NaCl or other material in the solid (usually the finely divided freeze-dried) state with gentle stirring. The resulting dilution of the bacterial suspension was calculated from the partial specific volume of the added substance. The suspensions used contained the equivalent of 25–50 mg. dry wt. bacteria/ml. (determined by heating a known volume of the suspension in an air oven at 100°) giving, in the case of aqueous suspensions at high speed, a pellet layer 1·0–2·0 mm. thick. The avoidance of excessive concentration gradients restricts the choice of colloid component and a polyethylene glycol of average molecular weight 10,000 was selected for these studies.

Osmotic and diffusion pressure measurements. Bags, sealed at one end and provided with a simple capillary manometer filled with mercury were made from Viscose dialysis tubing (18 mm. diam.), filled with the solution under test (20 ml.) supported with an outer perforated metal tube and placed in a 500 ml. cylinder of water at 20°.

RESULTS

The effect of some factors on survival on drying and rehydration

The growth phase. The enhanced sensitivity of organisms in the logarithmic phase of growth to a variety of physical and chemical agents has been noted by numerous workers (e.g. Hegarty & Weeks, 1940; Fry & Greaves 1951; Annear, 1956). As seen in Fig. 1, *Escherichia coli* (Jepp) when subjected to drying and rehydration exhibited a similar behaviour, i.e. a decrease in survival as the organisms entered the logarithmic phase and a rapid increase on entry into the stationary phase. Tryptic meat broth was inoculated with an 18-hr. culture of the organism to give an initial concentration of about 10^5 orgs./ml. and incubated at 37°. During the experiment samples were withdrawn and diluted with the glucose peptone medium before drying in order to maintain the concentration at about 10^6 orgs./ml. The results emphasize the importance, in studies on survival, of stating the phase of growth of the organisms and avoiding, during an experiment, any transition from one phase to another. All the drying experiments described in this paper were carried out on organisms in the stationary phase by the use of 18 hr. 37° cultures.

The temperature and conditions of drying. Although the glucose-containing media commonly used for preservation usually exhibit the normal appearance of a frozen mass during the initial evaporative freeze stage, they frequently show signs of bubbling and shrinkage during drying, leading finally to a glassy and scaly residue; it seems doubtful whether true freeze-drying from the solid phase is realized from such media. The survivals in these circumstances were compared with those obtained by drying in conditions when freezing could not take place, namely, by introducing a controlled leak into the vacuum system to maintain the pressure at 5 mm Hg. during the first hour (the primary drying) thereafter decreasing the pressure to 0·1 mm. Hg. for the remainder of the 24 hr. drying period. The results (Fig. 2) show that for *Escherichia coli* in the stationary phase, survival on drying in the glucose peptone medium was actually enhanced by avoiding the sharp decrease in temperature which accompanies evaporative freezing in high vacuum. Annear (1958) reached similar conclusions with organisms of the Salmonella group.

Measured samples of the same suspensions in glucose peptone were also dropped on to the freeze-dried peptone starch plugs described by Annear (1956), thereby exposing the organisms to very high concentrations of peptone from the onset of drying. The survivals (Fig. 2) thus obtained showed no improvement over ordinary vacuum drying procedure.

The composition of the suspending fluid. Table 1 illustrates the complementary effect of sugar and protein in promoting survival on drying. The total concentration of glucose and bovine serum albumin was maintained constant at 200 g./l. In either sugar or protein alone survival was low, but good survival was obtained over a fairly wide range of mixtures of the two components. There appeared, however,

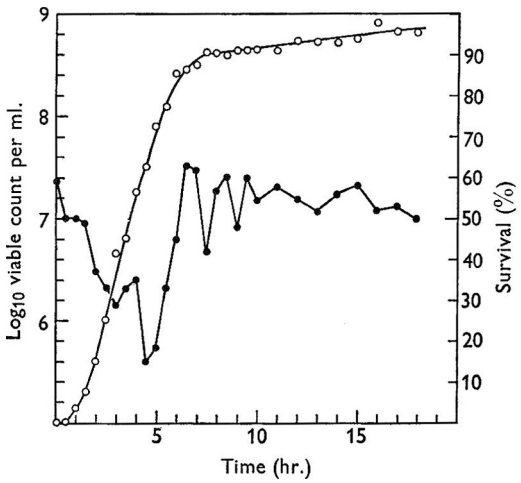

Fig. 1

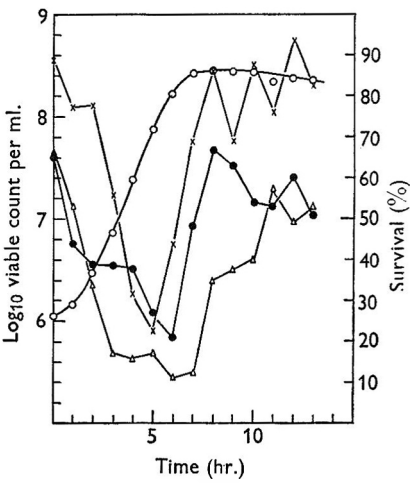

Fig. 2

Fig. 1. Growth of *Escherichia coli* and survival on drying. ○, Static growth in tryptic meat broth at 37°. ●, % survival after vacuum drying in 10 % glucose + 10 % peptone.

Fig. 2. Growth of *Escherichia coli* and survival on drying. ○, static growth curve in tryptic meat broth at 37°. ●, % survival after vacuum drying in 10 % glucose + 10 % peptone. △, % survival after vacuum drying on peptone + starch plugs. ×, % survival after primary drying at +1° (5 mm. Hg pressure).

Table 1. *Survival of* Escherichia coli (*grown for* 18 *hr. at* 37°, *in tryptic meat broth*) *after drying in glucose + bovine serum albumin mixtures*

Glucose (%)	20	18	15	10	5	2	0
Bovine serum albumin (%)	0	2	5	10	15	18	20
Survival (%)	1·5	26	33	35	14	4	0·1

to be no unique requirements for either sugar or protein for good survival. For example, similar results were obtained when the protein was replaced by transfusion dextran with a molecular weight 135,000. Good survival was still obtained when the glucose was replaced by various polyhydric alcohols and some preparations of tryptic meat broth offered excellent protection without any other additive, suggesting that the function of an effective preservative may be physical rather than chemical in character.

The concentration of the components of the suspending fluid. The results of a more detailed study of the effect of varying the concentration of each component in a two component system is shown graphically in Fig. 3 for glucose and polyethylene glycol (PEG). The survival in either glucose or PEG alone was poor at any concentration; optimum survival was obtained in mixtures containing 5–10 % glucose and 2–10 % PEG. Although a 5 % glucose + 5 % PEG mixture gave a good survival, the same mixture diluted fivefold gave only about one-fifth the survival.

The rehydration procedure. Although drying in 10 % glucose followed by direct rehydration with water or buffer gave only a few % survival this could be improved

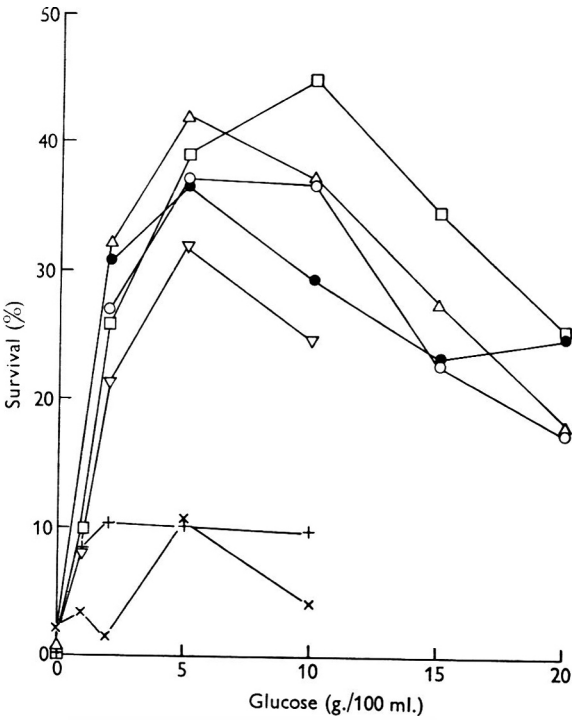

Fig. 3. Survival of *Escherichia coli* (stationary phase) on drying in glucose + polyethylene glycol mixtures. Polyethylene glycol concentration (w/v): ×, 0 %; +, 1 %; ▼, 2 %; □, 5 %; △, 10 %; ●, 15 %; ○, 20 %.

upon considerably by careful control. By reconstituting the dried sample in 50 g. glucose/100 ml. solution, transferring to a cellophan dialysis bag immersed in 50 g. glucose/100 ml. solution at 2° and slowly diluting the outer solution with buffer, with continuous stirring, during 6 hr. a recovery of 44 % viable organisms was obtained.

Observations on individual bacteria

Information about the nature of the damage caused by rapid rehydration of *Escherichia coli* dried from 10 % glucose was revealed by observations on individual bacteria in the micro-culture chamber.

Plasmolysis. Escherichia coli when placed in hypertonic solutions of various electrolytes or sugars exhibited the characteristic appearance of plasmolysis result-

ing from the contraction of the plasma membrane away from the cell wall (see Pl. 1, *b*). Normally growing *E. coli* is shown in Pl. 1, *a*. The plasmolysis was permanent when the plasma membrane was impermeable to the solute. The motility was unimpaired at moderate solute concentrations.

Spheroplasts. Stationary phase *Escherichia coli* after vacuum drying in 10% glucose followed by reconstitution with peptone water presented on examination a high proportion of spherical forms (Pl. 1, *c*) closely resembling protoplasts. Although many were still motile, none has so far been observed to divide or revert to its original shape and it seems unlikely that they are capable of producing colonies on nutrient agar. The proportion of spheroplasts formed could be greatly decreased by controlled rehydration similar to that already found to be effective on a macro scale. For example, a suspension of stationary phase *E. coli* dried in 10% glucose was reconstituted in 50% glucose solution, a drop placed on the micro-culture chamber membrane and 50% glucose solution circulated beneath. The circulating glucose solution was slowly diluted by a drip feed of peptone water over a period of 4 hr. after which 68% of the bacteria in the fields examined were seen to be dividing.

It was possible to produce spheroplasts without recourse to the preliminary drying procedure by using bacteria harvested in the fragile logarithmic phase of growth. After standing for 1 hr. in 50% sucrose on transfer to peptone water over 90% of the organisms assumed the spherical shape. Here again a gradual transfer to peptone water over a period of a few hours practically eliminated sphere formation and the majority of organisms resumed their normal role of growth and division.

The effect of sugar concentration on spheroplast formation. To produce the maximum number of spheroplasts it was found necessary to store the organisms in the sugar solution for a certain minimum time before transfer to peptone water. One hour was sufficient with sucrose over the concentration range examined (up to 2·5 M); glucose required a shorter time. Figure 4 shows the percentage of logarithmic phase organisms which formed spheroplasts after exposure for 1 hr. to glucose and sucrose at various concentrations. Spheroplast formation did not occur spontaneously on transfer to peptone water and counts were made 1 hr. after the transfer to allow completion of the process. Spheroplast formation commenced at a certain threshold sugar concentration and increased with rising concentration, rapidly in the case of sucrose, more gradually with glucose. With *Escherichia coli* in the stationary phase, spheroplasts were formed only after a longer period in the sugar solution, presumably owing to the enhanced thickness and strength of the cell wall in this phase.

Suppression of spheroplast formation by polyethylene glycol. High molecular weight substances which are unable to permeate the cell wall will exert their full osmotic pressure against it and compress it against the plasma membrane. Thus, the plasmolysis seen in 10% sucrose disappeared on the addition of 10% polyethylene glycol and the bacteria assumed a marked flattened appearance (Pl. 1, *d*). This could best be seen when the organisms were freely moving in the surrounding fluid so that they presented a continually changing orientation. A supersaturated solution of raffinose (50%) produced flattening in an enhanced degree and this persisted for some hours, suggesting that the trisaccharide was permeating the cell wall only very slowly. A similar appearance resulted on suspending the organisms in 50% sucrose, but in this case the flattening was only transient, giving way after about

15 min. to a pronounced plasmolysis, presumably after equilibrium had been reached across the cell wall. However, the flattened shape could be restored at this stage by the addition of 10% (i.e. 0·01 M) polyethylene glycol and appeared to be permanent. These observations suggest that the cell wall lacked any appreciable rigidity since mild external forces caused its collapse on to the contracted protoplast.

The effect of the colloid concentration. A suspension of *Escherichia coli* (logarithmic phase) in 2·5 M-glucose containing various concentrations of polyethylene glycol was placed on the cellophan membrane of the micro-culture chamber. After 1 hr. the glucose was removed by circulating peptone water beneath the membrane. After a further hour the percentage of organisms converted to spheroplasts was assessed by counting several fields comprising about 1000 organisms. As seen in Fig. 5, spheroplast formation decreased progressively with increasing concentrations of polyethylene glycol, from 80% in the sugar alone down to 5% in the presence of 10% polyethylene glycol.

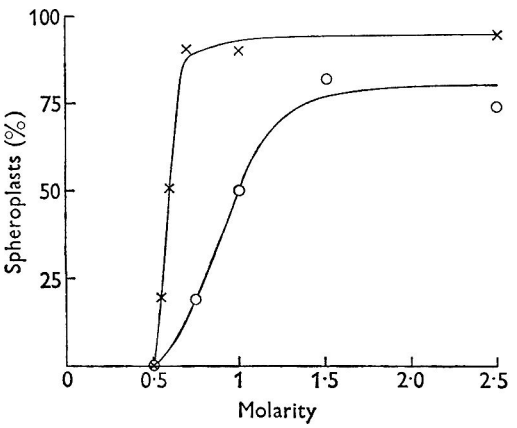

Fig. 4

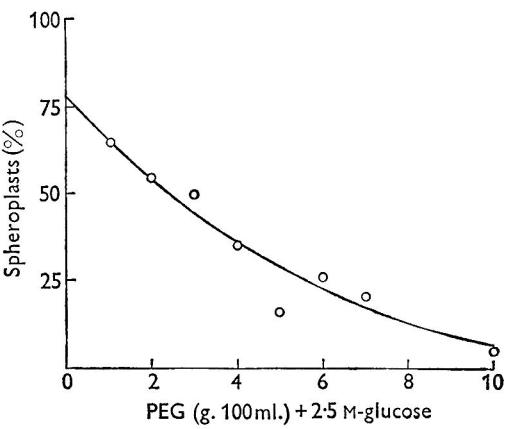

Fig. 5

Fig. 4. Spheroplast formation in *Escherichia coli* (logarithmic phase) due to glucose (○), and sucrose (×).

Fig. 5. Suppression of spheroplast formation in *Escherichia coli* (logarithmic phase) by PEG.

Pellet volume measurements

Independent evidence of the contraction in the bacterial cell volume, caused in plasmolysed bacteria by high molecular weight substances, was obtained from measurements of pellet volume in the analytical ultracentrifuge.

The effect of centrifugal force on the pellet volume of Escherichia coli *in various media.* Figure 6 shows the pellet volumes expressed as ml./g. dry wt. organism over a wide range of centrifugal forces. The pellet volumes refer to the volume as measured after running for 1 hr. at each speed by which time an equilibrium value was closely approached. Measurements made on deceleration of the rotor after reaching full speed, again running for 1 hr. at each speed, gave pellet volumes in close agreement with those measured during acceleration in the case of suspensions in water and 0·15 M-NaCl, but showed a marked hysteresis in 0·5 M-NaCl especially in the presence of polyethylene glycol. The actual curve obtained was to some extent dependent on

the pretreatment of the bacteria; for example, water-washed organisms transferred to 0·15M-NaCl showed substantially smaller pellet volumes than when washed directly in 0·15M-NaCl; they also showed plasmolysis. Attempts were made to measure pellet volumes at zero centrifugal force after removing the ultracentrifuge cell from the rotor at the end of an experiment, but these showed a progressive increase with time. This was especially marked in the case of the polyethylene glycol runs when the pellet at the end of the run still had the same volume as when at full speed and showed a slow expansion over many hours, presumably depending on the rate of diffusion of polyethylene glycol away from the pellet. As seen from Fig. 6 the pellet volume decreased with increasing centrifugal force, no doubt due partly to the expulsion of interstitial fluid from between the bacteria as they suffered deformation under the applied force, and partly to loss of fluid from within. The rate of decrease in pellet volume with increasing centrifugal force was greatest at low centrifugal forces, as would be expected from osmotic considerations.

The force acting on the pellet. At a depth d cm. below the surface of the pellet, the compressive force P acting on the organisms, mean density ρ_1, in a medium of density ρ, will amount approximately (when d is small) to

$$P = \frac{w^2 r}{g}(\rho_1 - \rho)\ dg./\text{cm.}^2$$

$$= \frac{w^2 r (\rho_1 - \rho)\, d}{g \times 76 \times 13{\cdot}6} \text{ atmospheres,}$$

where w = angular velocity of rotor (radians/sec.) and r = distance of pellet layer from centre of rotation (see Sharp, Beard & Beard, 1950). Thus for the case of washed organisms in a field of 100,000 g, where according to the graph (Fig. 6) the volume of organisms per g. dry weight = 4·3 ml., and the mean organism density (assuming a value of 0·71 for the partial specific volume of the cell material), is then about 1·07, the compression at the bottom of a 1 mm. thick layer amounts to about 0·70 atmosphere. The force of compression decreases practically uniformly to zero at the surface of the pellet, the average force at the centre being 0·35 atmosphere.

The hydrodynamic volume of the organisms. As a result of the unknown amounts of interstitial and intracellular fluid associated with the organisms in the pellet and their dependence on the centrifugal force, pellet volume measurements are not adaptable to the determination of the true hydration of bacteria. We have been unable to substantiate the claim made by Bendet, Smith & Lauffer (1960) that these difficulties are overcome by sedimenting the organisms through an immiscible solvent and measuring the upward shift of the solvent boundary. Our measurements with a bromobenzene+kerosine lower layer of density 1·05 showed the same variation in pellet volume with increasing centrifugal force as pellet volumes measured directly, although of course the reversible effect on deceleration was no longer seen. Thus, although the mean hydration value for *Escherichia coli* given by Bendet *et al.* is of the same order as that calculated from the graph (Fig. 6) at the speed of 12,590 rev./min. used by these authors, it cannot be regarded as having any real significance. Nevertheless, pellet volume measurements made under comparable conditions at the same speeds provide an indication of changes in the volume and compressibility of organisms resulting from changes in the suspending media and are therefore of importance in the present study.

The effect of NaCl *concentration.* The pellet volume of *Escherichia coli* in 0·15 M-NaCl showed a marked diminution, at comparable centrifugal forces, of the volume in water (Fig. 6), no doubt as a result of a decrease in tension in the bacterial cell wall. Plasmolysis induced by increasing the NaCl concentration to 0·5 M resulted in still further decrease in volume of pellet.

The effect of glucose and sucrose. The effect of glucose and sucrose at similar molarities was compared from measurements of the limiting pellet volumes attained at a given speed. The results (Fig. 7) are expressed as a percentage of the volume of the original (stationary phase) suspension of water washed *Escherichia coli* run under

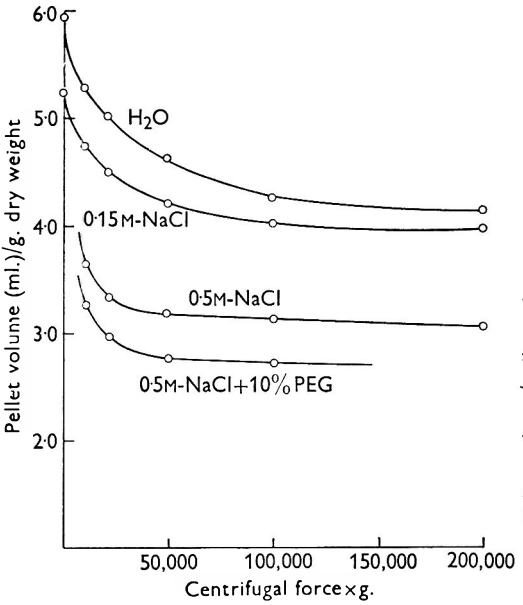

Fig. 6

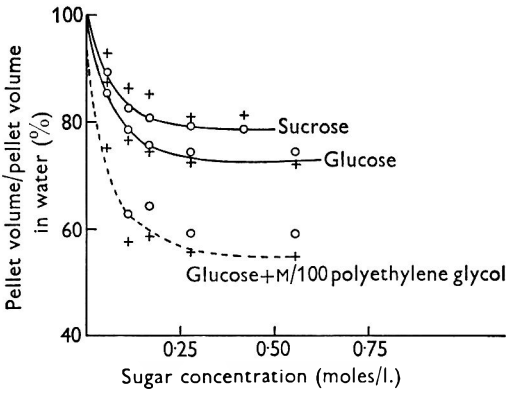

Fig. 7

Fig. 6. Variation in pellet volume of *Escherichia coli* (stationary phase) with centrifugal force.

Fig. 7. Contraction in pellet volume in glucose, sucrose and glucose + M/100 PEG. ○, at 22,000*g*.; +, at 100,000*g*.

identical conditions at each of two speeds (12,590 and 37,000 rev./min.), until no further contraction in the pellet volume occurred (1–2 hr.). On a molarity basis, the two sugars appeared to have a similar effect on the reduction in the pellet volume produced. (The slightly higher curve in the case of sucrose solutions may be the result of some reduction in compressive force due to their higher density.) Above 0·3 M, at which concentration plasmolysis was clearly evident, further increases in concentration had little effect in further reducing pellet volume.

The effect of polyethylene glycol. The addition to the bacterial suspension of a high molecular weight solute such as polyethylene glycol which does not penetrate the bacterial cell wall was found to cause a decrease in pellet volume at a given speed. The effect was most marked when the bacteria were already plasmolysed, e.g. in 0·5 M-NaCl (Fig. 6) or in the higher concentrations of glucose (Fig. 7). Although the

concentration of the 10% polyethylene glycol solutions tested was low in terms of molarity (0·01 M), the osmotic pressure of such a solution approached 1 atmosphere (see next section), a value in excess of that imposed by the centrifugal force at the maximum safe rotor speed (60,000 rev./min.).

Experiments with cellulose membranes

To assess the magnitude of the external pressure exerted by high molecular weight materials against the bacterial cell wall, osmotic pressure (Π) measurements were made in simple rise-tube osmometers with cellophan dialysis tubing (see methods). In addition, the temporary pressures produced by various low molecular weight substances, which freely permeated the cellophan membrane, were studied in relation to concentration (c) and molecular weight.

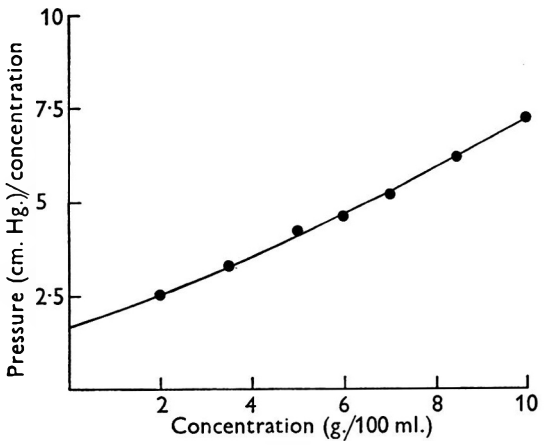

Fig. 8

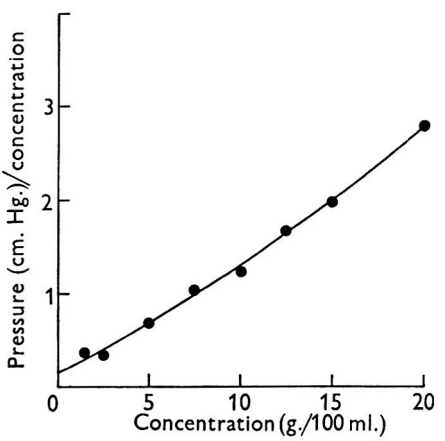

Fig. 9

Fig. 8. Osmotic pressure of PEG.

Fig. 9. Osmotic pressure of dextran.

High molecular weight substances. Osmotic pressure measurements made on polyethylene glycol (undialysed) at varous concentrations showed very little falling off with time in spite of the poly-dispersity of the material. The plots of Π/c against c (Fig. 8) showed a marked concentration dependence; the pressure of nearly 1 atmosphere for a 10% solution was over four times that calculated from the limiting value of Π/c at zero concentration. Dextrans have proved effective and have been widely used as the colloid component in drying media. Figure 9 shows the Π/c against c relationship for a transfusion dextran of molecular weight 135,000 (Glaxo Laboratories Ltd.). Here also, considerable deviations from ideality occurred; the osmotic pressure of 12 cm. Hg recorded for a 10% solution rose to 56 cm. for a 20% solution.

Low molecular weight substances. When the solute is able to permeate the membrane, the pressures developed are transient and less than the true osmotic pressures. To obtain comparative results, the cellophan bags were tested with 0·3 M-sucrose and selected to give peak pressures under the experimental conditions within ±5% of 25 cm. Hg. The rate at which the pressure in the bag increased is shown for glucose at various concentrations in Fig. 10. A peak pressure was reached in

each case in 1·5 –2 hr. and thereafter fell away. Sucrose and raffinose attained peak pressures after a similar period of time. The peak pressures attained with NaCl and three different sugars (a mono-, di, and tri-saccharide) over a range of concentrations are shown in Fig. 11. The ratio of peak pressure to concentration increased in each case with rising concentration and at similar molarities the peak pressures increased with the molecular weight. Peak pressures of this order may possibly be sufficient to cause rupture of the bacterial cell wall, though in view of its relatively low permeability the pressures developed by glucose and sucrose within the cell are probably markedly greater. However, in the absence of any close structural analogy between cellophan and a bacterial cell wall, such deductions must be treated with some reserve.

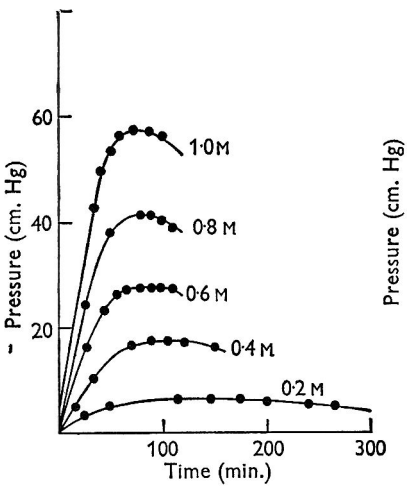

Fig. 10

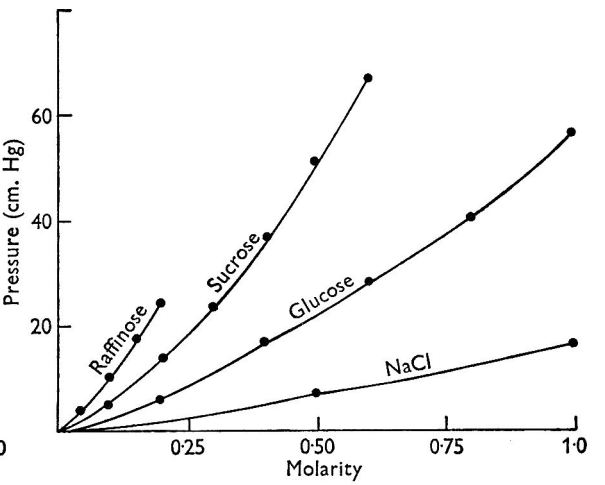

Fig. 11

Fig. 10. Diffusion pressures of glucose.

Fig. 11. Maximum pressures due to NaCl and sugars.

DISCUSSION

The concept of a bacterium as confined by a thin, rather impermeable plasma membrane supported by an outer, stronger and more permeable cell wall is now generally accepted. The phenomenon of plasmolysis, as displayed by various Gram-negative organisms in hypertonic solutions of electrolytes or sugars, such as glucose or sucrose, which readily permeate the cell wall but not the plasma membrane, is regarded as the result of the retraction of the plasma membrane away from the cell wall, due to osmotic forces. A hypothesis to explain the observations made in this paper is suggested on the basis of this concept. The plasmolysis induced by the sugar solution will result in an increased volume between the cell wall and plasma membrane and this space will, at equilibrium, be occupied by sugar solution of the same concentration as that outside the cell. On transfer to peptone water, the sugar solution, temporarily trapped between the bacterial cell wall and plasma membrane, will set up diffusion pressures which may be of sufficient magnitude to rupture the cell wall and release the spheroplast which, being no longer constrained by the

tough cell wall envelope, assumes a spherical shape. The operation of such a mechanism will depend on the permeability properties to the sugar concerned, of both the cell wall and the plasma membrane. The cell wall, although permeable to the sugar, must present a sufficient barrier to its return passage to create temporary osmotic forces large enough to rupture the wall. On the other hand, unless the plasma membrane is impermeable to the sugar, the plasmolysis will be only temporary (e.g. as in the case of erythritol) and the cell as a whole may then suffer rupture on transfer to peptone water. The observation that the bacteria must remain for some time in the sugar solution in order to produce the maximum number of spheroplasts is in accord with the hypothesis, this time being that required for the sugar solution to reach equilibrium across the cell wall. The further observation that a period of time, up to 1 hr., was necessary after transfer to peptone water to produce the maximum number of spheroplasts suggests, however, that completion of the process may be dependent on some enzymic breakdown. How far, if at all, the spherical forms thus produced differ from the protoplasts prepared by the enzymic digestion of certain bacterial cell walls (see e.g. Weibull, 1953) is not at present known, but the technique may be of general interest in providing a new method of forming spherical bodies from Gram-negative bacteria without the agency of a foreign enzyme.

Although the survival of *Escherichia coli* when dried in a glucose solution and reconstituted in the ordinary way is poor, the large improvement in survival resulting from carefully controlled rehydration suggests that glucose alone has excellent preservative properties and that the low molecular weight component of effective preservation media is the primary protective agent. One of the consequences of the flattening in the shape of plasmolysed bacteria observed after the addition of a colloidal solution is a decrease in the amount of sugar held between cell wall and plasma membrane, thus mitigating the tendency to cell wall rupture on transfer to peptone water or, in the case of dried organisms, on reconstitution. It appears to be in this manner that the protective colloid plays its complementary role. The relatively small osmotic forces due to high molecular weight material acting against the cell wall appear to be sufficient to cause it to collapse upon the plasmolysed interior, although as a consequence of the marked deviations from ideality revealed by direct measurements of osmotic pressure, substantial pressures can result with such substances in the high concentrations which would occur during drying. No evidence was found to suggest a threshold concentration where the cell wall, if a rigid structure, might be expected to collapse suddenly under a certain critical pressure.

Most of our observations and experiments were made in liquid suspending media. It is necessary to be cautious in applying these results to the complex process which occurs during drying, especially when approaching dryness. It is clear, however, that if the low molecular weight component permeates the plasma membrane the high molecular weight material cannot fulfil its function of controlling the amount within the cell. On the other hand, effective agents such as glucose and sucrose which induce a more or less permanent plasmolysis of the cells and do not pass the plasma membrane barrier, presumably cannot afford any protection to the components of the cytoplasm and the nucleus which might have been expected to be most susceptible to damage on drying. The need for protection on drying therefore appears to lie mainly in protection of the cell wall and/or the outer surface

of the protoplast where a number of enzymes are believed to be located. Thus, although the precise role played by the low molecular weight component in protecting the bacteria on drying is still largely a matter of conjecture, it appears that one of its most important functions is to preserve the cell wall intact. This it may do by replacing, rather than retaining, some of the water in the pores of the cell wall and so preventing its total collapse on drying.

The most effective preservative substances are generally very water-soluble and difficult to freeze-dry in the true sense, because of their tendency to supersaturate and to contract on drying to a glass-like residue which loses moisture extremely slowly in the final stages of drying. If drying proceeds in fact from the liquid phase, most of the cellular water will be removed by osmotic exchange rather than by direct evaporation or sublimation, and it is possible that less damage is thereby caused to the membrane structure.

In view of the marked flattening of bacteria observed after transfer to a 50% sucrose solution, giving way after 15 min. to the characteristic plasmolysed appearance, it is conceivable that with the rise in concentration during drying from a liquid or semi-solid phase, certain substances having the appropriate physical properties could exert a self-limiting control on the amount entering the cell and thus give a satisfactory survival even in the absence of any high molecular weight component. The maintenance of survival on storage would then be very dependent on the completeness with which drying had been carried out since trace amounts of water, especially with a rise in storage temperature, would facilitate diffusion of more material into the cell, leading to an increased risk of cell wall rupture on reconstitution. The presence of a protective colloid would prevent such an occurrence in the manner already described, although the possibility that it may promote survival by the alternative process of modifying the physical properties, viscosity, etc., of the low molecular weight component cannot be dismissed.

These conclusions have been arrived at by study of *Escherichia coli* Jepp, but other Gram-negative bacteria namely *Chromobacterium violaceum*, *Serratia marcescens* and another strain of *E. coli* (Porton, 164) all showed a similar behaviour in exhibiting a well-defined plasmolysis (presumably as a result of the ease with which the plasma membrane contracts away from the cell wall in hypertonic media) and all formed spheroplasts after transfer from concentrated glucose to peptone water. On the other hand, the Gram positive bacteria, *Bacillus cereus* in the vegetative form and *Corynebacterium diphtheriae* (gravis) and *C. xerosis* did not show plasmolysis, spheroplast formation or rupture after similar treatment. This accords with the tougher character of the Gram-positive group.

We wish to record our debt to Dr D. W. Henderson, F.R.S. for his special interest and encouragement in this work.

Our thanks are due to Mr K. H. Grinstead for valuable assistance in carrying out the ultracentrifuge measurements.

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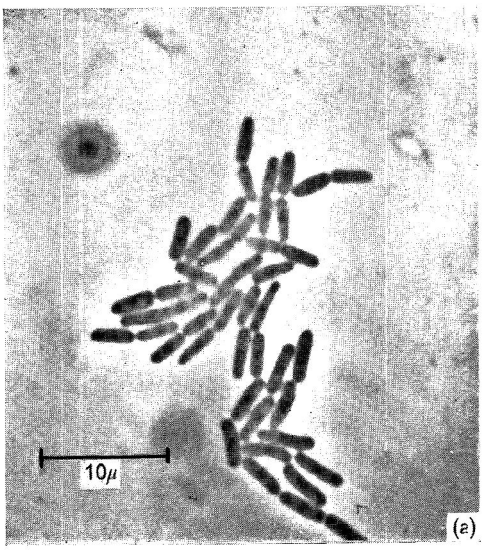
EXPLANATION OF PLATE

Plate I

Escherichia coli (logarithmic phase) viewed by phase contrast illumination: (*a*) Growing freely in peptone water. (*b*) Plasmolysed in M-glucose. (*c*) Spheroplasts formed after transfer from 1·5 M-sucrose to peptone water. (*d*) Flattened bacteria in M glucose with 10 % polyethylene glycol.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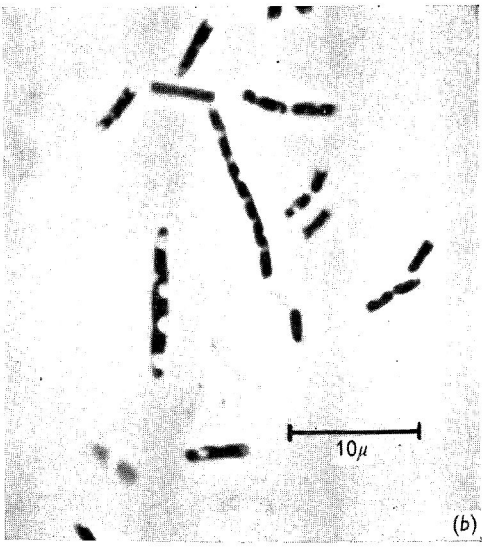
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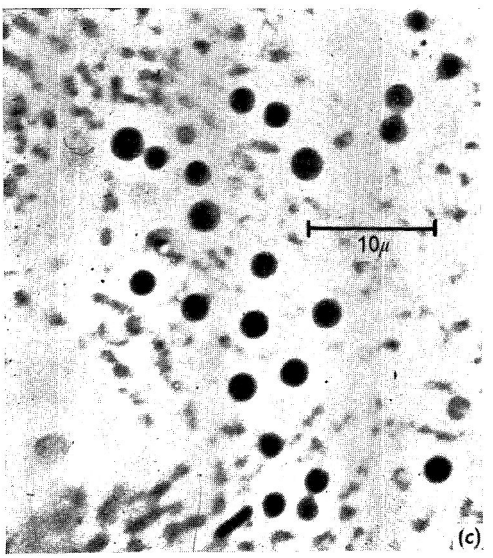
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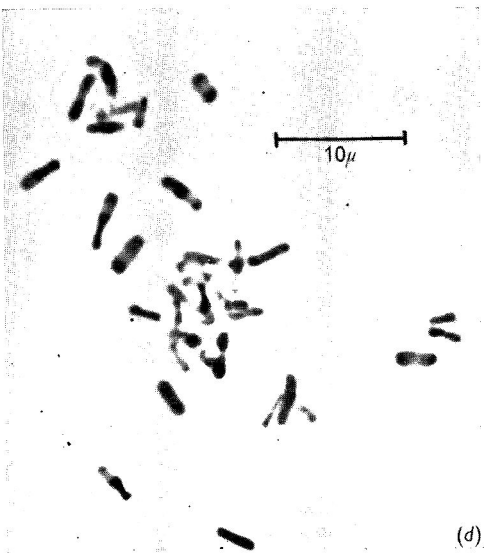
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J. gen. Microbiol. (1962), **28**, 599–605
Printed in Great Britain

Observations on the Relationship between the Formation of Photopigments and the Synthesis of Protein in *Rhodopseudomonas spheroides*

By W. R. SISTROM

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(*Received* 30 *October* 1961)

SUMMARY

Cells of *Rhodopseudomonas spheroides* cannot increase their specific photopigment content in the absence of protein synthesis. The significance of this finding for an understanding of the structure of the photosynthetic apparatus in bacteria is discussed.

INTRODUCTION

Several years ago it was shown that in the non-sulphur purple bacteria the pigmentation of the cells is markedly influenced by oxygen and by the light intensity. When grown under highly aerobic conditions these bacteria contain only traces of bacteriochlorophyll and carotenoid pigments. When the bacteria are grown anaerobically in the light, the concentrations of the pigments in the cells depend upon the light intensity: the lower the intensity the greater the pigment concentrations. If the light intensity is suddenly reduced during growth, the differential rate of pigment synthesis (relative to the increase in turbidity of the culture) immediately increases and the pigment concentrations approach the values characteristic of the lower light intensity. On the other hand, if the light intensity is increased, pigment synthesis stops, and, since the culture continues to grow, the concentrations of the pigments fall to the values characteristic of this light intensity—pigment synthesis then recommences (Cohen-Bazire, Sistrom & Stanier, 1957).

More recently it has become clear that more profound changes in cellular structure and metabolism accompany these variations in pigment content. Electron micrographs of light grown *Rhodopseudomonas spheroides* or *Rhodospirillum rubrum* show the cytoplasm to contain round, electron transparent structures which increase in number as the pigment content of the cell increases. These structures are not seen in micrographs of aerobically grown cells (Hickman & Frenkel, 1959; Flexer, Sistrom & Chapman, 1960). Newton (1960) has shown that pigmented cells of *R. rubrum* contain antigens which are not present in aerobically grown cells. Similar observations have been made in this laboratory on *R. spheroides*. Lascelles (1959, 1960) has shown that marked changes in the activities of enzymes concerned in porphyrin biosynthesis are initiated when aerobically grown cells are transferred to photosynthetic conditions.

It seems reasonable then to think that changes in the amounts of photopigments involve much more than merely variations in the rates of synthesis of the pigments

themselves. If this is true, one would expect to find some relationship between the synthesis of the pigments and of other cell components. In this paper one such relationship is described; it is shown that pigment synthesis is obligatorily coupled to protein synthesis.

METHODS

Rhodopseudomonas spheroides, strain Ga, has been used throughout. This strain is a mutant which differs from the wild-type in that it contains only neurosporene and hydroxyneurosporene in place of the array of carotenoid pigments of the wild-type (Griffiths & Stanier, 1956). In some experiments a proline requiring mutant of strain Ga (Ga M7) was used. Both strains were obtained from Dr R. Y. Stanier.

Medium A of Sistrom (1960) was used. Succinic acid is the carbon source, and the medium is supplemented with glutamic acid and aspartic acid to give more rapid growth. This medium is termed AG Su. For experiments on the uptake of radioactive sulphate, the medium was made with ammonium chloride in place of ammonium sulphate and the sulphur supplied as sodium sulphate.

For photosynthetic growth the special culture vessels previously described were used (Cohen-Bazire *et al.* 1957). Recently, vessels constructed of lucite have also been used. These were 10 cm. wide, 20 cm. high and 2·5 cm. thick. The cultures were incubated in aquaria at 34°. Light was provided by arrays of 300, 150 or 75 W. reflector flood lamps. The light intensity was measured at the entrance window of the aquarium with a Weston illumination meter calibrated in foot-candles. The cultures were aerated continuously with 5% carbon dioxide (v/v) in either nitrogen or helium.

In some experiments it was necessary to have cultures with extremely low specific pigment concentrations, lower than result from growth in any reasonable light intensity. Such cultures were obtained in the following way, making use of the fact that aerobiosis stops pigment synthesis but does not destroy the pigments already present. A culture was first grown photosynthetically and its bacteriochlorophyll concentration measured; it was then diluted to give the bacteriochlorophyll concentration per unit volume called for. The diluted culture was placed under highly aerobic conditions and allowed to grow until the pigment concentration per cell reached the desired level. Such cultures were always pre-incubated anaerobically in the light before the start of the experiment.

Experience in this laboratory has shown that the relation between the optical density of a culture and its protein content varies with the conditions of growth. For this reason cell protein was determined directly.

Protein was estimated by the Folin–Lowry method (Lowry, Rosebrough, Farn & Randall, 1951). Whole cells were digested in 1 M-NaOH for 1–2 hr. at 40° before analysis. Neither bacteriochlorophyll nor carotenoid pigments interfere with this determination.

Bacteriochlorophyll and carotenoid pigments were estimated from the optical densities at 775 and 468 mμ of methanolic extracts (Cohen–Bazire, Sistrom & Stanier, 1957).

Radioactivity measurements

Uniformly labelled L-^{14}C-phenylalanine was obtained from Nuclear-Chicago; ^{35}S labelled sulphuric acid was obtained from Oak Ridge National Laboratory.

The method used to determine the radioactivity of cell protein depended upon the specific activity expected. When the activity was low, a sample of the culture was added to one fifth its volume of ice-cold 50% trichloroacetic acid (TCA); after 15–20 min. in the cold the suspension was centrifuged, washed once with cold 5% TCA, and resuspended in a small volume of water containing Dupanol C (200 μg./ml.) (sodium lauryl sulphate obtained from E. I. DuPont, Inc., Wilmington, Delaware). A portion of this suspension was dried on a previously weighed planchet and the radioactivity determined. Corrections for self absorption were applied if necessary.

When the specific activity was high a more convenient procedure could be used. An equal volume of ice-cold 15% TCA was added to the sample. After 15–20 min. in the cold a portion of this suspension, containing not more than 500 μg of protein, was filtered through a 'Millipore' filter (22 mm. diam., 0·65 μ pore size; Millipore Filter Corp., Watertown, Massachusetts). The filter was washed with cold 5% TCA and then with a little water, cemented to a planchet and the radioactivity determined.

The samples were counted with a thin-window Geiger tube; in every case a total of at least 1000 counts was recorded.

When cells were labelled with ^{14}C-phenylalanine, treatment with boiling TCA removed only about 10% of the radioactivity in the cold TCA insoluble fraction. The more extensive washing procedure of Mandelstam (1958) failed to remove any further radioactivity and the cold TCA extraction described was therefore used routinely.

RESULTS

The experiments described below all demonstrate an obligatory coupling of pigment synthesis and protein synthesis. In each of these experiments cells initially deficient in photopigments are allowed to begin synthesis of these pigments and then protein synthesis is interrupted. They differ in the means used to obtain depigmented cells and to stop protein synthesis.

Experiment 1. In this experiment the cells were grown in bright light (5000 ft.c.) overnight, the culture was centrifuged and resuspended in the same medium containing phenylalanine (1 μg./ml.; 1485 c.p.m./μg.) The culture was exposed to dim light (50 ft.c.) under anaerobic conditions. After 245 min. chloromycetin was added to a final concentration of 50 μg./ml. The results of this experiment are shown in Fig. 1.

It is clear that the synthesis of both bacteriochlorophyll and carotenoid pigments and the uptake of phenylalanine stop at the same time. The specific content of bacteriochlorophyll at the time of addition of chloromycetin was 2·2 μg./100 μg. of protein, which is a little less than one-third the value of 6·5–7·0 eventually reached under these conditions.

Experiment 2. In this experiment the proline auxotroph (Ga M7) was used. A culture was grown in the usual medium supplemented with L-proline (20 μg./ml.) under highly aerobic conditions. This culture was centrifuged, washed once and resuspended in proline-free medium and divided in two parts. At the start of the experiment, L-proline was added to give a concentration of 3 μg./ml. in one culture and of 10 μg./ml. in the other, and each received radioactive phenylalanine (4 μg./

ml.; 108 c.p.m./μg.). The cultures were aerated with a gas mixture consisting of 5% CO_2, 5% O_2 and 90% N_2. In these semi-aerobic conditions pigment synthesis begins immediately. The results are depicted in Fig. 2; it can be seen that these results are in accord with those of the first experiment. In the culture with 3 μg.

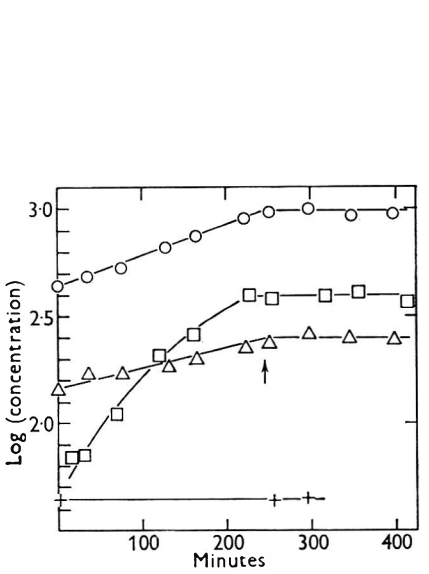

Fig. 1

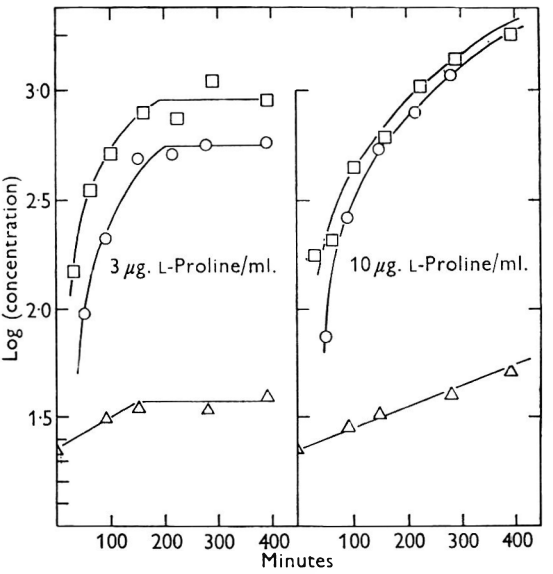

Fig. 2

Fig. 1. Inhibition of bacteriochlorophyll synthesis by chloromycetin. A culture of *Rhodopseudomonas spheroides* was grown photosynthetically in a light intensity of 5000 ft.c.; at the start of the experiment the intensity was reduced to 50 ft.c. At the time shown by the arrow chloromycetin was added to give a concentration of 50 μg./ml. Protein, μg./ml., +; bacteriochlorophyll, mμg./ml., ○; carotene, mμg./ml., Δ; phenylalanine, mμg./ml., □.

Fig. 2. Requirement for proline for bacteriochlorophyll synthesis in proline auxotroph of *Rhodopseudomonas spheroides*. A culture of strain Ga M7 was grown aerobically in medium containing proline. At the start of the experiment it was divided into two parts and L-proline added to the concentrations shown. The cultures were incubated semi-aerobically in the dark. Protein, μg./ml., Δ; bacteriochlorophyll, mμg./ml., ○; phenylalanine, mμg./ml., □.

Table 1. *Specific bacteriochlorophyll content and chromatophore protein of extracts of* Rhodopseudomonas spheroides *grown under different conditions*

Growth conditions	Specific bacteriochlorophyll content (μmole/100 mg. of protein) Fraction: Crude extract	Fraction: Chromatophore	% total protein in chromatophore fraction*
Photosynthetic:			
30 ft.c.	7·9	11·2	60
12,000 ft.c.	0·81	1·6	45
Aerobic	0·004	—	50

* This is the material sedimented after 1 hour in a centrifugal field of 100,000g.

of L-proline per ml., uptake of phenylalanine and the synthesis of bacteriochlorophyll stop at about 200 min. Synthesis of the carotenoid pigments also stops at this time, but this result is not included in the figure for the sake of simplicity.

Experiment 3. In this experiment protein synthesis was limited by allowing growth to exhaust the medium of sulphur. The culture used in this experiment was obtained by alternate photosynthetic and aerobic growth as described under Methods. The culture was washed once with sulphate-free medium and resuspended in the same medium. At zero time radioactive sulphate was added to give a final

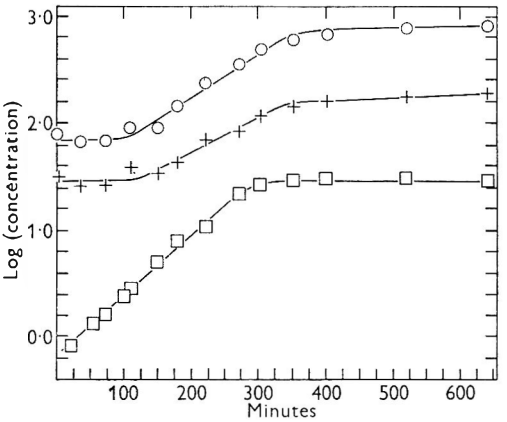

Fig. 3

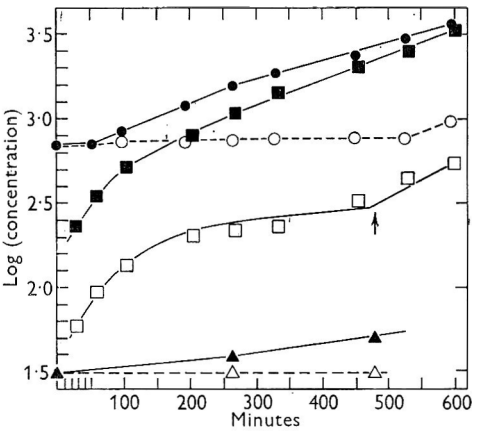

Fig. 4

Fig. 3. Inhibition of the synthesis of photopigments by sulphur starvation. A photosynthetically grown culture on *Rhodopseudomonas spheroides* was depigmented by a period of aerobic growth. During the experiment the culture was incubated anaerobically in light of 600 ft.c. The medium initially contained 0·04 μg. of ^{35}S per ml. (as Na_2SO_4). Sulphur, mμg./ml., □; bacteriochlorophyll, mμg./ml., ○; carotene, mμg./ml., +.

Fig. 4. Synthesis of bacteriochlorophyll and protein in proline auxotroph in presence and absence of proline. A culture of *Rhodopseudomonas spheroides* (Ga M7) was grown photosynthetically in a light intensity of 6000 ft.c. in medium with proline. After depletion of the endogenous proline the culture was divided into two parts. One part was incubated in the absence of proline (open points) and the other in the presence of proline (50 μg./ml., filled points). Both cultures were incubated anaerobically in a light intensity of 50 ft.c. At the time shown by the arrow L-proline was added to the first culture. Protein, μg./ml., Δ; bacteriochlorophyll, mμg./ml., ○; phenylalanine, mμg./ml., □.

concentration of 0·04 μg./ml. (100,000 c.p.m./μg.) and the culture exposed to 600 ft.c. of light under anaerobic conditions. The results are shown in Fig. 3. The initial specific content of bacteriochlorophyll was 0·2 μg./100 μg. of protein and the final value was 2·0.

The specific content eventually attained under these conditions is around 3·0 μg./100 μg. of protein.

Experiment 4. In this experiment the proline-requiring mutant was used. The culture was grown anaerobically in bright light (6000 ft.c.), washed once in proline-free medium and resuspended in the same medium. This suspension was incubated anaerobically in bright light for 2 hr. to deplete the proline pool, centrifuged and resuspended in the same medium, and distributed in two growth flasks. To one, L-proline was added to a final concentration of 50 μg/ml. Both suspensions were

incubated anaerobically in dim light (50 ft.c.). At zero time radioactive phenylalanine was added (3·3 μg./ml.; 200 c.p.m./μg.). The results are depicted in Fig. 4. There is no pigment synthesis in the flask without added proline.

Experiment 5. Lascelles has shown that when a culture of *Rhodopseudomonas spheroides* is suspended in an iron-free medium, synthesis of bacteriochlorophyll ceases and porphyrins are excreted (Lascelles, 1956). It was of interest to see if porphyrins or other intermediates of bacteriochlorophyll synthesis could be detected after the addition of chloromycetin. This was attempted in an experiment similar to Expt. 1. At intervals after the addition of chloromycetin, 25 ml. samples were taken, acidified with HCl, centrifuged and the supernatant adjusted to pH 3·5 with sodium acetate. This was extracted with two 15 ml. portions of ether and the ether extracted with 4·0 ml. of 1·5M-HCl. No porphyrin could be detected spectrophotometrically in either the original acidified supernatant or in the final hydrochloric acid extract, even 4 hr. after the addition of chloromycetin. Also, no porphobilinogen could be detected by the method of Mauzerall & Granick (1956). Since the rate of bacteriochlorophyll synthesis was approximately 0·5 μg./ml./hr. at the time chloromycetin was added, had either porphyrin or porphobilinogen formation continued at this rate, an easily detectable amount would have been present after 4 hr. It is concluded that the biosynthesis of bacteriochlorophyll is inhibited at an early stage.

DISCUSSION

The experiments reported here demonstrate that in the absence of protein synthesis, neither bacteriochlorophyll nor carotenoid pigments are synthesized by cells of *Rhodopseudomonas spheroides*. Lascelles (1959) reported that chloromycetin as well as *p*-fluorophenylalanine and 8-azaguanine inhibit the formation of bacteriochlorophyll. The experiments described here have extended this observation. Neither Lascelles's experiments nor those reported here can be interpreted by assuming that the inhibition is due to an inability to form enzymes concerned in bacteriochlorophyll synthesis, since the cultures were synthesizing bacteriochlorophyll before protein synthesis was inhibited.

Two other explanations are possible. In the first, it is assumed that the immediate effect of inhibition of protein synthesis is an increase in the concentration of some metabolite which in turn stops the synthesis of photopigments. It is, however, difficult to see how the formation of both bacteriochlorophyll and carotenoid pigments could be inhibited to the same extent by one compound.

In the second explanation, it is assumed that the synthesis of photopigments cannot proceed independently of the synthesis of the rest of the photosynthetic apparatus. It is interesting to examine some consequences of this hypothesis. The photopigments and some of the enzymes concerned in photosynthesis are associated with the particulate fraction of cell-free extracts of photosynthetic bacteria: the chromatophore fraction. Several analyses of the pigment and protein composition of chromatophores have been published (Cohen-Bazire & Kunisawa, 1960; Newton & Newton, 1957; Bergeron, 1959). Table 1 shows some typical results for *Rhodopseudomonas spheroides* grown under different conditions. The molecular ratio of bacteriochlorophyll to chromatophore protein varies from 1·6 to 11·2 if an average molecular weight of 100,000 is assumed for the protein. Cohen–Bazire has found a

similar variation in the specific bacteriochlorophyll contents of chromatophore preparations from *R. rubrum* grown in different light intensities. On the other hand, the specific photophosphorylating activity calculated on a protein basis is constant regardless of the light intensity in which the cells were grown (Cohen-Bazire & Kunisawa, 1960). In other words, these results suggest that cells in different light intensities differ only in their pigment contents.

In order to reconcile this conclusion with the fact that pigment synthesis cannot occur in the absence of protein synthesis the following hypothesis is suggested. It is assumed that the photosynthetic apparatus once formed cannot be modified, and that the amount of pigment associated with a unit of this apparatus depends upon the light intensity at the time the unit is formed. When a culture is subjected to a sudden change in light intensity, the photosynthetic apparatus already present does not change in composition. The chromatophore material subsequently formed will, however, have a different composition determined by the new light intensity; the average chromatophore material will therefore gradually change. Experiments to test this hypothesis further are in progress.

This work was supported in part by a Grant (G-14983) from the National Science Foundation, and in part by a Grant (G-232) from the National Council to Combat Blindness.

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The Kinetics of the Synthesis of Photopigments in *Rhodopseudomonas spheroides*

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(*Received* 30 *October* 1961)

SUMMARY

The magnitude of self-shading in light-grown cultures of *Rhodopseudomonas spheroides* has been estimated. When a culture of *R. spheroides* is transferred from bright light to dim light there is preferential synthesis of bacteriochlorophyll and carotenoid pigments relative to net protein synthesis. Protein turnover does not account for this.

The kinetics of the synthesis of bacteriochlorophyll relative to the incorporation of amino acids into protein have been estimated under a variety of conditions. On the basis of these findings a mechanism for the cellular control of pigment synthesis is presented.

INTRODUCTION

Experiments of Cohen-Bazire, Sistrom & Stanier (1957) demonstrated that there is an inverse relation between the concentration of bacteriochlorophyll and carotenoid pigments in cells of *Rhodopseudomonas spheroides* and the light intensity in which the cells have been grown. When a culture is transferred from dim to bright light, synthesis of the photopigments stops, while cell growth continues; thus the specific pigment contents (μg. pigment/100 μg. cell protein) decrease to the levels characteristic of the higher light intensity. On the other hand, when a culture is transferred from bright to dim light, the growth rate, as measured by increase in turbidity, is markedly reduced, while the photopigments continue to be synthesized. Thus, the specific pigment contents increase to the levels characteristic of the lower intensity. As these levels are approached, cell growth begins again and eventually both the turbidity of the culture and the photopigments are increasing at the same rate (Cohen-Bazire *et al.* 1957).

This behaviour is an example of regulation of cellular metabolism by 'feed-back' control. In order to gain a better understanding of the mechanism of this regulation, it was necessary to undertake a more detailed analysis of the kinetics of photopigment synthesis than had been attempted by Cohen-Bazire and co-workers. This paper reports the results of experiments on the differential rate of photopigment synthesis relative to the rate of synthesis of cell protein. These results provide a basis for a more complete description of the control of pigment synthesis in *Rhodopseudomonas spheroides* than was hitherto possible.

METHODS

Rhodopseudomonas spheroides strain Ga was used throughout. Medium A of Sistrom (1960) was used, with succinic acid as carbon source, and supplemented with L-glutamic acid (100 μg./ml.) and L-aspartic acid (40 μg./ml.); this is medium

AG Su. The conditions of growth and the general methodology have been described in the preceding paper (Sistrom, 1962). The details of the procedures used in the several experiments are described separately.

RESULTS

The effect of self-shading

Before attempting measurements of the differential rates of photopigment synthesis, it was necessary to assess the effect of self-shading in cultures of *Rhodopseudomonas spheroides*, since it is clear that pigment synthesis is influenced not by the amount of light incident on a culture but by the light absorbed by it.

Preliminary experiments showed that under the conditions employed, self-shading is not appreciable unless the bacteriochlorophyll concentration is greater than 5–6 μg./ml. At pigment concentrations greater than this the amount of light absorbed is no longer proportional to the pigment concentration. Consequently, in a growing culture, the specific pigment content will be constant only if the bacteriochlorophyll concentration is less than about 5 μg./ml. In these experiments the effect of self-shading has been circumvented by keeping the bacteriochlorophyll concentration as low as possible.

Cohen-Bazire *et al.* (1957) reported the growth rates and specific pigment contents of cultures grown in different light intensities. In these experiments no account was taken of self-shading; it was of interest to repeat these measurements under more carefully controlled conditions. To obviate the effect of self-shading, the experiments were conducted so that at each light intensity employed the bacteriochlorophyll concentration increased from about 0·5–1·5 μg./ml. during the time analyses were made. The specific bacteriochlorophyll content and the growth at a bacteriochlorophyll concentration of 1·0 μg./ml. were calculated by interpolation. The results are incorporated in Fig. 1. Two points are of interest here. In the first place, the maximum specific growth rate is about 0·75 doublings per 100 min. which is equivalent to a generation time of a little over 2 hr. This growth rate is considerably greater than the maximum reported by Cohen–Bazire and co-workers. The second point is that any decrease in the light intensity causes not only an increase in pigment content but also a decrease in growth rate. In other words, the increased pigment content is never equivalent to the decrease in light intensity. This point will be considered further in the discussion.

Chlorophyll synthesis and protein turnover

The experiments of Cohen-Bazire *et al.* showed that photo-pigments are apparently synthesized preferentially when a culture of *Rhodopseudomonas spheroides*, grown in bright light, is transferred to dim light. This recalls the synthesis of β-galactosidase during adaptation of *Escherichia coli* to lactose (Rickenberg & Lester, 1955). Mandelstam (1960) has shown that in this case preferential synthesis is not the result of an increased differential rate of enzyme synthesis during adaptation, but that the extent of protein synthesis is masked by a simultaneous breakdown of pre-existing protein. Protein turnover appears to be of general occurrence in micro-organisms whenever the cells are starved for nitrogen or carbon. It was of interest to see if protein turnover occurs during the period of rapid bacteriochlorophyll synthesis after a culture of *R. spheroides* is transferred to dim light, and

while the cells are presumably starved for energy. The extent of turnover was estimated by comparing the amount of phenylalanine incorporated into protein with the amount released from protein under identical conditions (Mandelstam, 1958).

Three hundred ml. of medium ASu (without glutamic or aspartic acid) containing uniformly labelled ^{14}C-L-phenylalanine (25 μg./ml.; 42 c.p.m./μg.) was inoculated with *Rhodopseudomonas spheroides*. The culture was grown in a light intensity of 12,000 ft.c. for at least five generations to assure complete labelling of the protein. The culture was centrifuged and resuspended in the same volume of

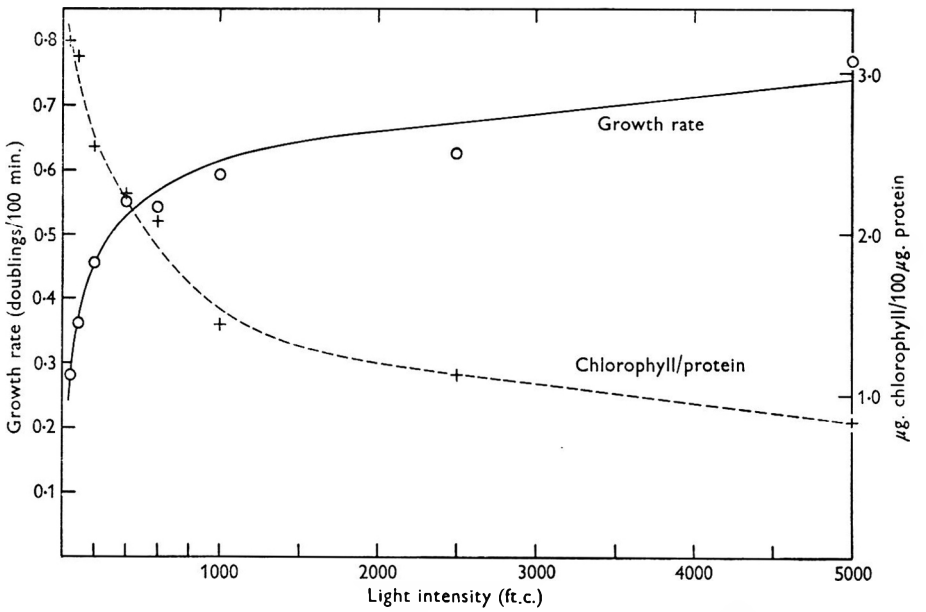

Fig. 1. The specific growth rate constant and the specific bacteriochlorophyll content of *Rhodopseudomonas spheroides* at various light intensities. The values in each case are for a culture with 1 μg. of bacteriochlorophyll/ml. Medium AG Su; aeration mixture 5% (v/v) in N_2.

medium containing non-radioactive L-phenylalanine (25 μg./ml.), and allowed to grow under the original conditions for 1 hr. This served to reduce radioactivity of the phenylalanine pool. The culture was centrifuged, washed once and resuspended in 300 ml. of medium ASu with 100 μg. L-phenylalanine/ml., incubated anaerobically (5% (v/v) CO_2 in N_2) in a light intensity of 80 ft.c. Protein and bacteriochlorophyll were determined periodically. At intervals, 16 ml. samples were taken to measure the radioactivity in protein and in the medium. 1·6 ml. of cold 50% trichloroacetic acid was added to each sample, after 20 min. in the cold the suspension was centrifuged. The pellet was washed once with cold 5% TCA containing L-phenylalanine (1 mg./ml.) and resuspended in 4 ml. of water containing 200 μg. Duponal C per ml. (obtained from E. I. Dupont Company, Wilmington, Delaware). The radioactivity of duplicate 1 ml. samples was measured. The cold TCA supernatant was put on a Dowex 50-W column (1·5 × 4·0 cm.); the resin was washed with 50 ml. of water and eluted with 1·5 M-NH_4OH. The first 10 ml. of eluate was discarded, the next 50 ml. was collected in a beaker and evaporated to dryness. The residue was dissolved

in a little water and quantitatively transferred to planchets and the radioactivity determined. In preliminary experiments this method gave about 90% recovery of radioactive phenylalanine.

A non-radioactive culture was prepared in a similar fashion. It was suspended in 300 ml. of medium ASu containing radioactive phenylalanine (10 μg./ml.; 157 e.p.m./μg.), and incubated anaerobically in 80 ft.c. of light. Protein and bacteriochlorophyll were determined periodically. At intervals, samples were taken and treated with 5% TCA in the cold for 20 min. The suspension was centrifuged and the supernatant discarded. The pellet was resuspended in boiling 5% TCA containing 1 mg. L-phenylalanine per ml. and centrifuged. The pellet was dissolved in 2 ml. of 2 M-NaOH and reprecipitated by the addition of 2 ml. of 20% TCA. This precipitate was washed twice with 5 ml. portions of acetone and finally dissolved in 1·4 ml. of M-NH_4OH. Duplicate 1 ml. samples were counted. Preliminary experiments showed that this treatment removed only a negligible fraction of the radioactivity of the cold TCA precipitate.

Figure 2 shows the fraction of the initial phenylalanine released or incorporated during the time bacteriochlorophyll synthesis is proceeding rapidly. The amount of phenylalanine released amounted to less than 10% of the amount incorporated; it is concluded that there is virtually no protein turnover during this time. This finding is substantiated by the experiment described in the previous paper which showed that there is no detectable synthesis of bacteriochlorophyll in cells deprived of an essential amino acid.

The differential rate of pigment synthesis

Cohen-Bazire and co-workers presented an hypothesis to explain the control of photopigment synthesis they had observed in the non-sulphur purple bacteria. According to this hypothesis, the differential rate of pigment synthesis at a given light intensity should be inversely proportional to the specific pigment content of the cells, if the pigment content is less than the steady-state value. The experiments described below were designed to test this prediction. They provide a detailed description of the kinetics of photopigment synthesis, especially of the differential rate of pigment synthesis relative to protein synthesis.

The differential rate in depigmented cells. In these experiments the effects on the differential rate of the light intensity and of the initial specific bacteriochlorophyll content were studied. The general procedure was to expose a culture of *Rhodopseudomonas spheroides* to two light intensities, 80 and 500 ft.c.; bacteriochlorophyll, carotenoid pigments and protein and the uptake of ^{14}C-phenylalanine into protein were determined at intervals. In order to reduce the effect of self-shading, these experiments were usually terminated before the cultures had become fully pigmented.

The details and results of a typical experiment are given in Fig. 3. In this experiment the culture was first grown in a light intensity of 12,000 ft.c. and then exposed to 80 ft.c. during the experiment. The amount of protein synthesized did not greatly exceed the limits of error of the Folin–Lowry method (Lowry, Rosebrough, Farn & Randall, 1951). The turbidity of the culture actually decreased during the first 200 min. or so. Clearly, the differential rate relative to either turbidity or protein

cannot be determined accurately. It is for this reason that the uptake of phenylalanine has been used as a measure of cell growth.

In Fig. 4 the increase in bacteriochlorophyll has been plotted relative to the increase in phenylalanine; the slope of this curve gives the differential rate directly. It can be seen that except for a small lag the differential rate is constant throughout the experiment; this constancy is maintained in the face of a nearly threefold increase in specific bacteriochlorophyll content. The final bacteriochlorophyll concentration was 5 μg./ml.; self-shading can accordingly be neglected.

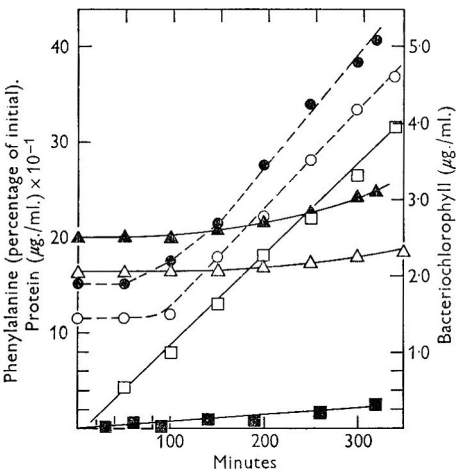

Fig. 2

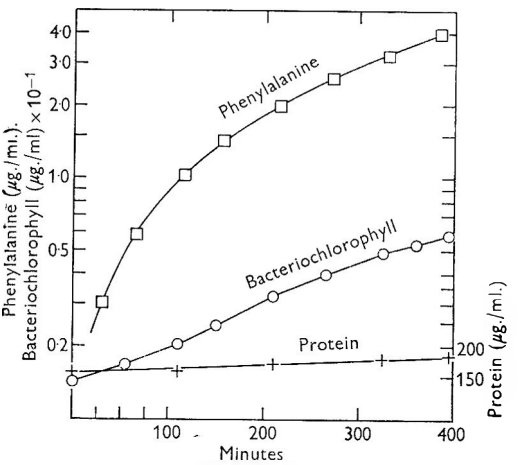

Fig. 3

Fig. 2. Uptake and release of ^{14}C-phenylalanine during preferential synthesis of bacteriochlorophyll. See text for experimental details. Results with prelabelled culture shown by filled points, those with initially unlabelled culture by open points. □, Phenylalanine (% initial); ○, bacteriochlorophyll (μg./ml.); △, protein (μg./ml.).

Fig. 3. Bacteriochlorophyll synthesis and uptake of ^{14}C-phenylalanine by a depigmented culture of *Rhodopseudomonas spheroides*. The culture was grown in a light intensity of *c.* 12,000 ft.c. in medium AG Su with 25 μg. L-phenylalanine/ml. It was suspended in AG Su and aerated with 5 % (v/v) CO_2 in N_2 for 10 min. in the dark. At zero time ^{14}C-L-phenylalanine (60 c.p.m./μg.) was added to a final concentration of 20 μg./ml. and the culture illuminated with 80 ft.c. The radioactivity of the material precipitated by 5 % TCA was determined. □, L-phenylalanine (μg./ml.); ○, bacteriochlorophyll; +, protein.

This result was unexpected and not in accord with the hypothesis of Cohen-Bazire *et al.* which predicts that the differential rate should decrease as the specific pigment content increases. This result became even more difficult to understand when the experiment was repeated with cultures of different initial specific bacteriochlorophyll contents. The results of several such experiments are collected in Table 1. The culture grown in 500 ft.c. was fully pigmented at the start of the experiment; and that grown in 1000 ft.c. became so during the experiment. The differential rate in these two cultures is the same as that for fully pigmented cells under these conditions. Inspection of this table shows that the differential rate increases with decreasing initial specific bacteriochlorophyll content and has a maximum of about 1·4 μg. bacteriochlorophyll/μg. phenylalanine, which is about 3 times the rate in fully pigmented cells.

In Table 2 the differential rates in cultures exposed to 500 ft.c. are shown. These results are subject to greater error than those of Table 1, since the cultures become fully pigmented quickly and since the bacteriochlorophyll concentration soon becomes high enough to cause self-shading. Nevertheless, it seems clear that here too the differential rate is higher in cultures with low specific pigment contents and furthermore that the maximum value of 0·8 is less than the maximum differential rate found in cultures exposed to 80 ft.c.

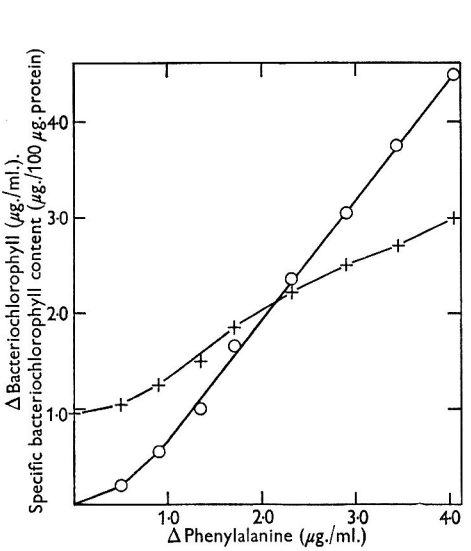

Fig. 4

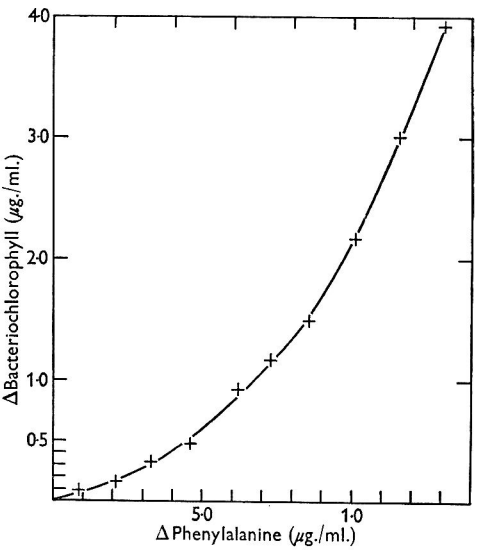

Fig. 5

Fig. 4. The differential rate of bacteriochlorophyll synthesis. The results shown in Fig. 3 have been replotted. ○, Increase in bacteriochlorophyll (μg./ml.); +, specific bacteriochlorophyll content (μg./100 μg. of protein). Each is plotted relative to the increase in TCA-insoluble phenylalanine (μg./ml.). The differential rate is 1·2 μg. bacteriochlorophyll/μg. phenylalanine.

Fig. 5. The differential rate of bacteriochlorophyll synthesis following repression by high light intensity. A culture of *Rhodopseudomonas spheroides* was grown in a light intensity of 80 ft.c.; at the start of the experiment the light intensity was increased to 500 ft.c. and ^{14}C-L-phenylalanine (175 c.p.m./μg.) added to a concentration of 25 μg./ml. Bacteriochlorophyll synthesis did not begin until 250 min. In the figure the increase in bacteriochlorophyll has been plotted against the increase in TCA-insoluble phenylalanine.

These results are paradoxical. On the one hand, the differential rate in any given culture is independent of specific pigment content; but, on the other hand, the differential rate depends on both the initial pigment content of the culture and on the light intensity to which it is exposed. A possible resolution of this paradox is offered in the discussion.

It is clear that the differential rate in an initially depigmented culture must eventually decrease and become equal to that in a fully pigmented culture. The experiments just described suggest that the decrease does not begin until the culture has become very nearly fully pigmented. This was confirmed by the following experiment. A culture, grown in 10,000 ft.c. of light, was exposed to 100 ft.c. At intervals the culture was diluted with fresh medium so that the bacteriochlorophyll

concentration was maintained between 1·0 and 1·5 μg./ml. The effect of self-shading was thereby kept to a minimum. The differential rate remained constant at a value of about 1·0 μg. bacteriochlorophyll/μg. amino acid incorporated until the specific pigment content was within 10–15% of the value finally attained. The

Table 1. *Differential rate of bacteriochlorophyll synthesis in* Rhodopseudomonas spheroides *exposed to light intensity of* 80 *ft.c.*

Light intensity during growth	Bacteriochlorophyll μg./ml. Initial	Bacteriochlorophyll μg./ml. Final	Bacteriochlorophyll μg./100 μg. protein Initial	Bacteriochlorophyll μg./100 μg. protein Final	Differential rate*
12,000	1·4	5·8	0·9	3·2	1·25
12,000	1·4	6·0	0·85	3·1	1·35
10,000	2·3	6·5	1·3	3·1	1·1
6,000	2·3	6·8	1·6	3·7	0·9
3,000	2·3	4·5	2·1	3·7	0·75
1,000	2·15	3·3	3·1	3·5	0·4
500†	1·4	2·05	5·8	3·25	0·45
Aerobic‡	0·65	3·0	0·2	1·0	1·4

* μg. bacteriochlorophyll/μg. phenylalanine incorporated.

† The inoculum for this experiment was diluted sevenfold, the reduction in self-shading occasioned by this more than compensated for the reduction in the light intensity; accordingly, pigment synthesis was inhibited for about 200 min. The differential rate is given for the period after pigment synthesis recommenced.

‡ The pigment content of a photosynthetically grown culture was reduced by a period of aerobic growth (see Sistrom, 1962).

Table 2. *Differential rate of bacteriochlorophyll synthesis in* Rhodopseudomonas spheroides *exposed to a light intensity of* 500 *ft.c.*

Light intensity during growth	Bacteriochlorophyll μg./ml. Initial	Bacteriochlorophyll μg./ml. Final	Bacteriochlorophyll μg./100 μg. protein Initial	Bacteriochlorophyll μg./100 μg. protein Final	Differential rate*
12,000	1·4	6·0	0·85	3·0	0·80
10,000	2·3	6·8	1·3	2·6	0·80
6,000	2·3	6·2	1·6	2·9	0·75
3,000	2·3	4·75	2·1	2·7	0·60
1.000†	2·15	2·4	3·15	2·4	0·30
Aerobic‡	0·65	4·0	0·2	1·2	0·85

* μg. bacteriochlorophyll/μg. phenylalanine incorporated.

† The inoculum for this experiment was diluted; the reduction in self-shading occasioned by this more than compensated for the decrease in light intensity and pigment synthesis was inhibited. The differential rate given is for the period after pigment synthesis recommenced.

‡ See note ‡ in Table 1.

differential rate then declined to a value of 0·6 in the fully pigmented culture. In this experiment ^{35}S-methionine was employed in place of ^{14}C-phenylalanine; this was done to eliminate the possibility that the kinetics of phenylalanine incorporation were in any way responsible for the results.

The differential rate after recommencement of pigment synthesis. The experiments which have just been described were carried out with depigmented cultures, in which the specific pigment content was increasing to the level found in fully pigmented cultures. It was of interest to examine the relation between specific bacteriochlorophyll content and the differential rate, in cultures which initially have an excess of photopigments. This can be done by transferring a culture grown in dim light to bright light. Under these conditions pigment synthesis is repressed while

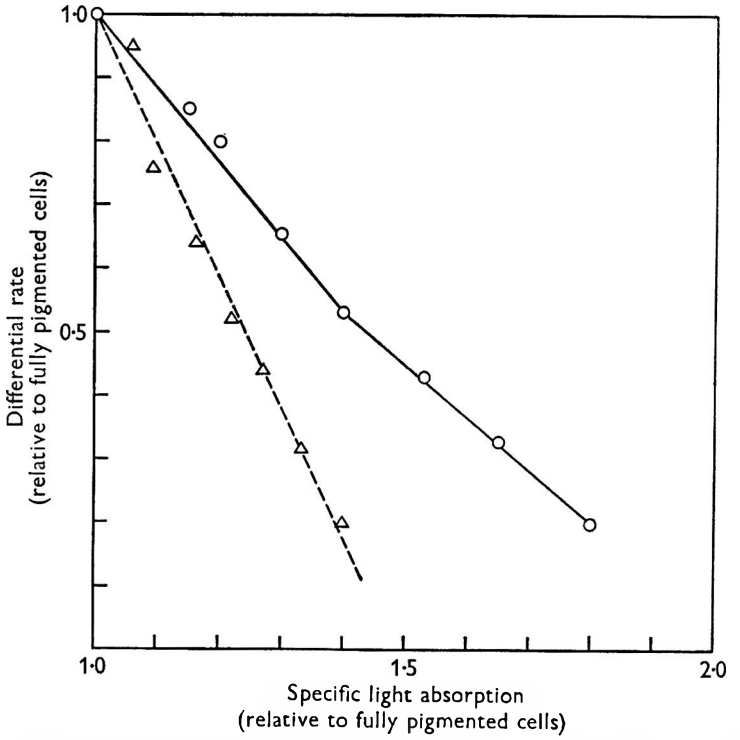

Fig. 6. Differential rate of bacteriochlorophyll synthesis and specific pigment content. Data from two experiments similar to the one described in Fig. 5 have been recalculated. Both the differential rate and the specific bacteriochlorophyll content are expressed relative to the values they finally attain.

growth continues. The specific pigment content therefore decreases until it reaches the value characteristic for that light intensity, at which time pigment synthesis recommences. In these experiments the differential rate was measured during the period just after the recommencement of bacteriochlorophyll synthesis. The results of a typical experiment are shown in Fig. 5. In this experiment the culture was grown in a light intensity of 80 ft.c. and then exposed to one of 500 ft.c. The figure shows the increase of bacteriochlorophyll plotted against the increase of phenylalanine incorporated. It is clear that the differential rate is constantly increasing. During this same time the specific pigment content is still falling, since the differential rate is still less than that for fully pigmented cells. The relation between the differential rate and the specific bacteriochlorophyll content is linear as shown in Fig. 6.

DISCUSSION

The experiments reported here confirm the finding of preferential synthesis of the photopigments in the non-sulphur purple bacteria originally reported by Cohen-Bazire *et al.* (1957). These workers measured growth by turbidity only and the significance of the preferential synthesis they observed was therefore uncertain, since this method would not have detected turnover of protein or other cell components. In the present experiments the differential rate of pigment synthesis was measured relative to the uptake of amino acids into protein rather than to net protein synthesis; protein turnover would not affect these results. In addition, it has been shown directly that there is no turnover of protein during the preferential synthesis of bacteriochlorophyll.

There are two possible interpretations of this preferential synthesis. The first is that all components of the photosynthetic apparatus are synthesized preferentially with respect to the rest of the cell. The second is that the specific pigment content of the photosynthetic apparatus itself increases. At the present time neither interpretation can be ruled out.

These experiments have revealed several facts about the control of pigment synthesis in *Rhodopseudomonas spheroides* which must be taken into account by any hypothesis about the mechanism of this control. (1) Both the specific pigment content and the growth rate change continuously with light intensity; the control of pigment synthesis does not operate so as to keep the growth rate constant. (2) The differential rate of pigment synthesis can remain constant while the specific pigment content is increasing. In other words, under some conditions the differential rate is not proportional to the amount of pigment. (3) When pigment synthesis resumes after an increase in light intensity the differential rate is inversely proportional to specific pigment content.

Only the last of these observations is in accord with the hypothesis originally proposed by Cohen-Bazire *et al.* This said that the control of pigment synthesis operated in such a way as to maintain the concentration of some substance constant and that changes in the concentration of this 'regulator', induced by changes in light intensity, were transient. The observations reported here are easier to interpret on the basis of a model in which the concentration of the 'regulator' does not tend to remain constant and in which a change in the light intensity induces a permanent change in the 'regulator' concentration.

The results shown in Table 1 and 2 show that although the differential rate is independent of specific pigment content in any one culture it does depend upon the initial pigment content when different cultures are compared. The results depicted in Fig. 1 show that it is impossible to obtain cultures with different specific pigment contents which have had the same specific growth rates. It is possible, therefore, that the apparent dependence of the differential rate on initial pigment content is really a dependence on the previous growth rate of the culture. This possibility can be checked by using cultures grown at a fixed rate in a chemostat in different light intensities. Such experiments are in progress.

This work was supported in part by a grant from the National Science Foundation (G-14983) and in part by a grant from the National Council to Combat Blindness (G-232).

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J. gen. Microbiol. (1962), 28, 617–623
With 1 *plate*
Printed in Great Britain

Further Studies on the Antigens of *Paramecium aurelia* with the Aid of Fluorescent Antibodies

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(*Received* 6 *November* 1961)

SUMMARY

A technique for sectioning fixed preparations of *Paramecium aurelia* suitable for subsequent treatment with fluorescent antisera is described. Following application of specific fluorescein and rhodamine conjugates of paramecium antisera to sectioned paramecia, fluorescence was observed both on the surface structures (pellicle and cilia) and in the cytoplasm, but not in the macronucleus. Fluorescence of the cytoplasm was prevented by pretreating the sections with non-homologous, non-fluorescent antiserum. It was concluded that the immobilizing antigens were substances covering only the pellicle and cilia, and that the internal cytoplasmic antigens were relatively invariant in paramecia of diverse immobilization antigen type. Study of transforming organisms showed that new antigen appeared first on the pellicle, and later on the cilia. All the cilia of a given cell bore the same antigen or mixtures of antigen.

INTRODUCTION

In a previous paper (Beale & Kacser, 1957) the effect of treating whole paramecia, both living and fixed, with fluorescein-conjugated antisera was described. The immobilization antigens were then shown to be localized on the surface structures, but the presence or absence of antigens in the interior of the cells could not be established with certainty owing to the possibility that the antibodies had failed to penetrate through the external membranes of the intact organisms. In the present study we describe experiments involving the treatment of sectioned paramecia, whose internal contents are presumably as accessible to applied antisera as the surface structures. We have also now made use of the technique of double staining with both fluorescein and rhodamine conjugates in order to detect the presence of two different antigens in a single preparation and to follow the process of transformation from one antigenic type to another.

METHODS

Culture media. The paramecia were grown in dried grass or lettuce infusions containing *Aerobacter aerogenes*. As a non-nutrient medium for washing the organisms the following solution (which will be referred to as solution MS) was used: 0·013 M-NaCl, 0·0003 M-KCl, 0·003 M-$CaCl_2$ buffered to pH 7 with 0·004 M phosphate. For general methods used in the cultivation of paramecia, see Sonneborn (1950).

Stocks and serotypes of Paramecium aurelia. Stock 168 (variety/syngen 1),

serotypes G and D were used. For testing the specificity of the effect of fluorescent solutions, pairs of conjugating paramecia were prepared, one member of each pair being antigenic type 168G, the other type 168D. Type 168G is stable at 25°, type 168D at temperatures above 30°. To get rapid transformation from 168G to 168D, about 100 ml. of a culture of 168G were placed in a 2 l. flask and 300 ml. of culture medium (preheated to 35°) added. The culture was then incubated at 35° and after about 18 hr. nearly all the organisms had completed the transformation to type 168D.

Preparation of fluorescent serum-conjugates. The antisera were prepared by injecting into rabbits homogenates of whole paramecia, as previously described (Sonneborn, 1950). Conjugation of antisera with fluorescein was at first done by the original method of Coons & Kaplan (1950), later by the modified method of Marshall, Eveland & Smith (1958). Whole sera were used. After conjugation, free dye was removed by passage through a column of De-acidite 'F.F.', buffered to pH 7. The solution was finally dialysed against solution MS to give a final serum dilution of 1/8. This method resulted in a diminution of titre of immobilizing antibody to half the original value, after allowing for dilution.

Conjugation with rhodamine was effected as described by Chadwick, McEntegart & Nairn (1958). Initially whole serum was used but since the resultant preparation gave poor fluorescence, in later work the globulins were precipitated with 40 % ammonium sulphate after conjugation with rhodamine. The precipitated rhodamine-labelled globulins were redissolved in a small volume of solution MS, dialysed against distilled water and solution MS, and finally passed through a column of De-acidite to remove any remaining free rhodamine. The volume was finally adjusted with solution MS to be the same as the original. Loss of antibody titre was again about one-half.

Fixation and sectioning of paramecia. The organisms were concentrated by gentle centrifugation, fixed in 1 % osmic acid in solution MS for 20 min., washed five times in solution MS, and briefly spun down. About 1 ml. of a solution of gelatin dissolved in solution MS was added to the washed pellet; the gelatin solution was prepared by autoclaving a 7 % (w/v) solution at 25 lb. pressure for 20 min. This resulted in partial hydrolysis of the gelatin and gave a solution of barely liquid consistency at 20°. The suspension of paramecia in gelatin was transferred with a pipette to a small plastic tube ($5 \times \frac{1}{2}$ cm.). The PVC ink sacs of Parker pens were found to be excellent for this purpose. After being filled, the sac was placed in a freezing cabinet at −20° with occasional gentle shaking to prevent the paramecia from sinking to the bottom before the contents were solidified. The frozen block was mounted on the chuck of a microtome in a freezing cabinet, the plastic sac stripped off with a razor and the gelatin block trimmed square. The block was sectioned at about 10 μ thickness at a temperature of −8°. The sections were individually unrolled on the knife and transferred to an albuminized slide with a camel hair brush. The sections, after being gently pressed flat on the slide, were left overnight in the freezing cabinet at −8°.

Staining with fluorescent antibody conjugates. The slides were removed from the freezing cabinet and slightly warmed, causing the gelatin to melt and the sections to become well stuck to the slides. Staining and washing was done by carefully pipetting the various solutions on to the slides. The following treatments were

used: (1) treatment with fluorescein conjugate alone, dilution 1/32, for 2 hr. in the cold; (2) treatment with rhodamine conjugate alone, full strength, for 3 hr. in the cold; (3) double staining, first with fluorescein, then rhodamine; (4) any of the above, preceded by treatment with non-fluorescent, non-homologous serum (anti-192X), dilution 1/2, overnight in cold.

After each staining, the slides were washed in solution MS and finally a coverslip was placed on top and ringed with petroleum jelly to prevent evaporation. Fresh mounts gave the best results, but the preparations did not fade for several weeks or even months when kept in the cold and dark.

Fluorescence microscopy. The sections were examined by observing fluorescence under ultraviolet (u.v.) radiation, with a dark ground condenser on the microscope as described previously (Beale & Kacser, 1957). For the greatest magnification ($\times 800$) a special (Zeiss) oil-immersion objective with iris diaphragm was used. Under the conditions adopted, material treated with fluorescein-conjugate fluoresced much more brightly than material treated with rhodamine-conjugate.

Colour photography. High speed ektachrome film ASA 160 was used with exposures of 20 min. or longer. Great difficulty was experienced in getting adequate reproduction of the orange colour of rhodamine, especially in combination with fluorescein.

RESULTS

Localization of antigens

Sections of paramecia when treated with fluorescein or rhodamine conjugates of homologous antisera fluoresced brightly around the pellicle and cilia. This is illustrated in Pl. 1, fig. 1, showing a section through a pair of mating organisms (168G $\times$ 168D), treated with fluorescein-conjugated 168G antiserum. It was found, however, that the cytoplasm of both homologous (168G) and non-homologous (168D) organisms fluoresced in these preparations. The region of the macronucleus was dark. The fluorescence of the cytoplasm was removed by pre-treating the sections with a non-homologous non-fluorescent serum (anti-192X), as shown in Pl. 1, fig. 2. Such pretreatment also improved the specificity of the staining of the surface structures, for when very strong fluorescein-conjugated antisera were applied (in the absence of pretreatment), a faint green fluorescence appeared on sections of non-homologous organisms. The pretreatment eliminated this, leaving only a ghostly bluish 'auto-fluorescence' around the rim of the non-homologous organism (as seen in Pl. 1, fig. 2).

Double staining of mating pairs of paramecia (168G $\times$ 168D) with fluorescein-conjugated 168G antiserum and rhodamine-conjugated 168D antiserum, preceded by treatment with non-fluorescent 192X antiserum, gave preparations showing one member of each pair with green pellicle and cilia, the other with orange pellicle and cilia (Pl. 1, fig. 3). In some pairs it was noticeable that the fluorescence characteristic of one mate extended for a short distance over the other. This was especially marked with the brighter green fluorescence of fluorescein.

These results, in addition to confirming the surface localization of the immobilization antigens, as discussed below, were of value in showing the specificity of the staining procedure, and the feasibility of double-staining of paramecium preparations containing two different antigens in the same cell.

Transformation experiments

The double-staining technique was then used to study the appearance of a new antigen in organisms in the process of transforming from serotype 168G to type 168D. A culture of type 168G was placed at a high temperature, as described in Methods, and at intervals during the process of transformation to 168D samples were taken. Part of each sample was fixed, sectioned and treated successively with 168G fluorescein-conjugated antiserum and 168D rhodamine-conjugated antiserum. In addition parts of some samples were treated with fluorescein only or rhodamine only. Finally, live organisms from each sample were treated with unconjugated 168G antiserum and others with unconjugated 168D antiserum, and the immobilizing effect, when present, noted. These results are summarized in Table 1. It should be stated, however, that as there is some variation in the times for individual organisms to reach a given stage in the transformation process, the times given in Table 1 apply only to an average of the organisms in a given sample.

Table 1. *Stages during transformation of* Paramecium aurelia *from antigenic type 168G to type 168D by growth at* 35°

		Effect of antisera (dil. 1/50) on live organisms				Fluorescence following treatment with antibody conjugates (green, 168G; orange, 168D)	
		168G antiserum		168D antiserum			
Stage	Time of growth at 35° (hr.)	Immobilization time (min.)	Effect after 24 hr. at 20°	Immobilization time (min.)	Effect after 24 hr. at 20°	Pellicle	Cilia
1	0–2	3	Dead	Unaffected	Alive	Green	Green
2	2–3	3		R		Green (+slight orange)	Green
3	7	4		R		Orange (+slight green	Green
*	→						
4	8	6	Alive	R	Dead	Mainly orange	Green (+slight orange)
5	11	35		45		Orange	Green and orange
6	12	R		20		Orange	Mainly orange
7	13–16	R		3		Orange	Orange (+slight green)
8	18–20	Unaffected		3		Orange	Orange

* → indicated approximate time of first fission after beginning of experiment.
R, retarded but not completely immobilized after 2 hr. in serum.

It was found that the organisms passed through a series of intermediate stages showing increasing amounts of 'new' antigen (168D) and decreasing amounts of the old (168G), in individual paramecia. It is of interest to note that at stage 3, after 7 hr. at 35°, the living organisms were capable of being immobilized by 168G antiserum almost as rapidly as at the start, and were only slightly affected by 168D antiserum. This is consistent with the finding from the fluorescence examination that the cilia still contained antigen 168G. At the same stage, however, the pellicle contained predominantly antigen 168D. Thus it is clear that production of 'new' antigen took place first on the pellicle and only later spread to the cilia.

It was also noted that at stage 4 (8 hr.) the cilia still contained such a high proportion of antigen 168G that the paramecia were immobilized by 168G antiserum in a relatively short time (6 min.), but after a further 24 hr. at room temperature in presence of 168G antiserum the same organisms had recovered. Here the process of transformation continued after the paramecia had been immobilized by 168G antiserum. We presume that the 168G-type antigen-antibody complex on the tips of the cilia was eventually shed and replaced by 168D antigen. This is in conformity with the finding described elsewhere (Beale, 1958) that paramecia which have already begun the transformation process carry it through to completion, even though the conditions (in that case a lower temperature) may not favour maintenance of the new serotype (168D).

At intermediate stages when both antigens were present in a single organism, the pellicle was uniform, as regards fluorescence, at different points of the organism; the same applied to the cilia. By careful comparisons of preparations treated with single and double stains, and with observations of a given organism both in u.v. radiation and in visible light, it was possible to establish that a single cilium sometimes contained both antigens. There was no evidence supporting the view that new cilia which developed bore the new antigen and the old cilia bore the old antigen.

DISCUSSION

The present results show that the immobilization antigens of paramecia are localized on the pellicle and cilia only. Other antigens are present within the organisms, as shown by Preer & Preer (1959) by gel diffusion studies with isolated cellular components. Since our antisera were prepared by injection into rabbits of homogenates derived from whole paramecia, antibodies against the internal antigens would be present in the fluorescent antisera used in the present experiments. Hence treatment of sections by such sera resulted in fluorescence in the cytoplasm as well as on the surface structures. The fact that in our earlier studies (Beale & Kacser, 1957) treatment of intact paramecia with fluorescent antisera did not result in any internal fluorescence means that the antibodies had not penetrated through the outer membranes of intact paramecia.

The effect of pre-treatment with non-homologous (192X) antiserum in preventing the internal fluorescence from appearing is interpreted to mean that the internal (cytoplasmic) antigens are relatively invariant in paramecia which bear different immobilization antigens. Hence the 192X antiserum contained antibodies which neutralized the internal antigens of types 168G and 168D, while leaving the immobilization antigens unaffected and able to react subsequently with specific (fluorescent) immobilization antibodies.

The improvement in specificity of the reactions even of the surface structures with fluorescent antisera consequent upon pretreatment with non-homologous antiserum indicates that even the surface structures contained some antigen other than the specific immobilization ones. This effect is, however, relatively slight: only with very concentrated fluorescent antisera was there appreciable staining of the surfaces of non-homologous organisms.

The behaviour of the organisms during antigen transformation indicates that there is a central control of synthesis of antigen over the whole surface. From the

initial appearance of new antigen on the pellicle, it is reasonable to assume that this synthesis occurs there, and that there is a secondary spread of antigen over the cilia. From earlier genetic work (see Beale, 1954, 1957) it is known that the specificity of the immobilization antigens is controlled by certain genes, but that the control in expression of these genes is exercised by cytoplasmic factors. These are the factors which control the development of a particular kind of antigen over the whole surface of the paramecium. It now appears that such cytoplasmic factors cannot be, as once suggested (Beale, 1948), the immobilization antigens themselves, for there is no evidence that the immobilization antigens occur in the cytoplasm. It is therefore now important to try to establish the nature of these cytoplasmic factors. Presumably they consist of some substance or complex occupying a position intermediate between the antigen-determining genes and their products the immobilization antigens, which are now known to be proteins (Preer, 1959; Bishop, 1961). Future work will be directed towards identifying these cytoplasmic factors.

We thank Dr H. Kacser for much help in the earlier stages of this work, Dr R. Alexander for supplying samples of fluorescein and rhodamine preparations, Dr I. Tallan for making preliminary trials of the technique of pretreatment with non-homologous antiserum, and the Parker Pen Co. for kindly supplying the plastic ink sacs. The work was supported by grants from the Medical Research Council for scientific assistance, and from the Royal Society for apparatus.

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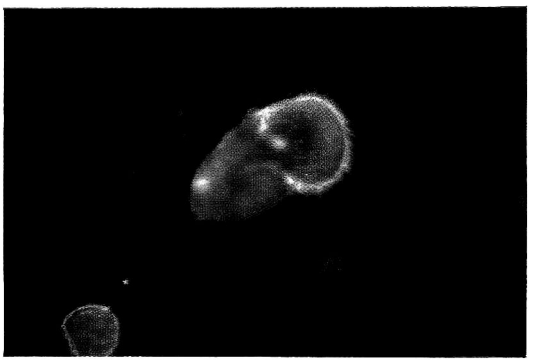

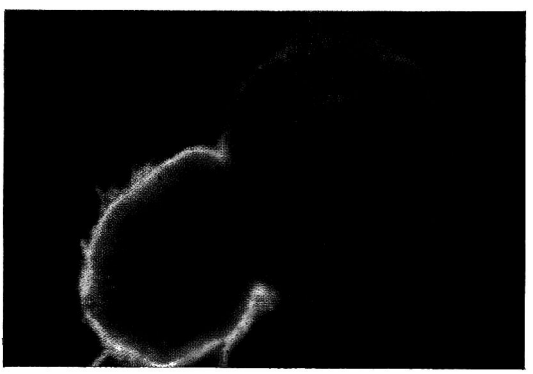

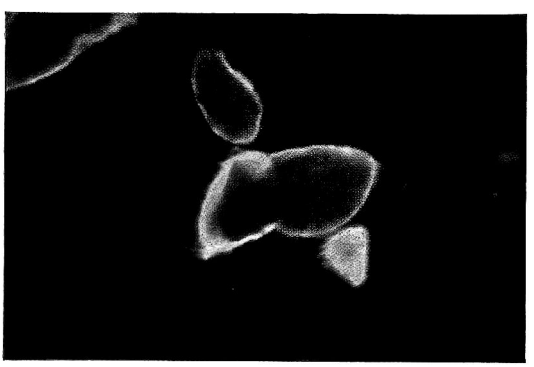

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EXPLANATION OF PLATE 1

Fig. 1. Section through conjugating pair of paramecia, antigenic types 168G and 168D, treated with fluorescein-conjugated 168G antiserum. Note fluorescent pellicle and cilia on conjugant at left (presumed 168G), and fluorescent cytoplasm in both conjugants. ×128.

Fig. 2. As Fig. 1, but pretreated with non-fluorescent 192X antiserum to prevent reaction of cytoplasmic antigens with fluorescent antibody. Only the immobilization antigen, on pellicle and cilia of 168G cell, now shows the green fluorescence of fluorescein. ×320.

Fig. 3. Section through pair of conjugants 168G and 168D, pre-treated with non-fluorescent 192X antiserum, then with fluorescein-conjugated 168G antiserum and finally rhodamine-conjugated 168D antiserum. ×128.

J. gen. Microbiol. (1962), 28, 625–632
With 1 *plate*
Printed in Great Britain

Spontaneous and Ultraviolet Irradiation-Induced Mutants of *Verticillium albo-atrum*

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(*Received* 6 *November* 1961)

SUMMARY

The killing of conidia of *Verticillium albo-atrum* by ultraviolet irradiation followed first-order kinetics; about 0·5 % of the conidia which survived a 97 % lethal dose were nutritionally deficient mutants. This percentage of mutants among survivors was constant for different wild-type isolates for the first-, second- and third-order irradiations. This regularity implies that most conidia exposed at the first irradiations contained a single haploid nucleus. Adenine-requiring mutants formed pionnotes when grown on an agar medium containing low concentrations of adenine, and the extent of pionnotal formation depended on the position at which adenine synthesis had been blocked by the irradiation. 4-Aminoimidazole-5-carboxamide apparently did not act as an extracellular intermediary in adenine synthesis. An acriflavine-resistant mutant arose spontaneously in one of the mutants selected for nutritional deficiency, but mutants resistant to certain other growth inhibitors were not found.

INTRODUCTION

Variation in the soil-borne plant pathogenic fungus *Verticillium albo-atrum* has been recorded by Isaac (1949), Waggoner (1956), Robinson, Larson & Walker (1957) and Van den Ende (1958). Each confirmed the occurrence of the colour variants originally described by the authors of the species (Reinke & Berthold, 1879). Because colour variants alone were insufficient to investigate the mechanism underlying genetic variation in this fungus, further genetic markers were sought by using ultraviolet irradiation. This paper deals with the isolation and characterization of these additional selective markers, and describes the pleiotropic effects of some of the auxotrophic mutations obtained.

METHODS

Four isolates of *Verticillium albo-atrum* were isolated from wilted hop plants at East Malling Research Station, Kent, two from outbreaks of fluctuating wilt (numbered V 1805, V 1809) and two from progressive outbreaks (V 1790, V 1813). Neither the strain V 1809 nor any culture derived from it formed black torulose hyphae, whereas the others produced them abundantly; none formed the microsclerotia characteristic of *Verticillium dahliae* Kleb.

A medium pressure Hanovia XII ultraviolet (u.v.) lamp, emitting about 95 % of its radiation at 2537 Å, was used to induce mutations which were then selected

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for those that blocked the synthesis of essential metabolites. Conidia spread on the surface of a nutritionally complete agar medium (CM; Pontecorvo, Roper, Hemmons, Macdonald & Bufton, 1953) at 400/plate were exposed at 20 cm. from the u.v. source; under these conditions the radiation intensity was about 104 μw./cm^2. From exposures at different time-intervals to determine the irradiation-survival curve, an exposure of 20 sec., giving only 3 % survival, was chosen as a convenient mutagenic dose. The surviving conidia produced viable colonies after 4 days in darkness at 23°. A bulk inoculum of conidia + mycelium was then transferred from each colony that grew from a surviving conidium to CM in plates in which the inocula were arranged in the regular pattern used in colony replications (Pontecorvo *et al.* 1953). These cultures were then replicated from CM to Czapek-Dox agar media on which about 0·5 % of the survivors failed to grow. As CM medium contains hydrolysed yeast nucleic acid and several vitamins, the failure of any strain to grow on Czapek-Dox medium was almost certainly because it could not synthesize an essential metabolite supplied by one of these three mixtures. Accordingly, the strains unable to grow on Czapek-Dox medium alone (auxotrophs) were tested for their capacity to grow when it was supplemented with hydrolysed casein, yeast nucleic acid or a vitamin mixture.

Each auxotroph responded to one of these supplements, thus identifying its nutritional requirements either as an amino acid, organic base or vitamin, depending on which supplement allowed the mutant to grow. Slope cultures of the mutants were then established on CM, and the specific requirement determined by auxanography (Pontecorvo, 1949). Auxanographs were made by mixing 1 ml. of a spore suspension (5×10^6/ml.) of the nutritional mutant in 15 ml. molten Czapek-Dox agar at 42° and immediately pouring this into plates. The appropriate supplements were then placed individually in very small amounts around the periphery of the agar. Areas of growth response soon developed.

Mutants resistant to certain growth inhibitors were also sought. A necessary first step was to assess the concentrations inhibitory to several nutritional mutants of independent origin. To do this the nutritional mutants were grown in the replicating pattern on CM. This template was then used to provide inoculum that was replicated on a range of concentrations of the growth inhibitors incorporated in CM. Attempts to select relatively resistant mutants were then made by spreading dense spore suspensions of the sensitive starting strains on CM containing an inhibitory and therefore selective, concentration of each substance.

RESULTS

Nutritional mutants

Figure 1 shows the properties of conidia of strain V 1790 that survived different doses of u.v. radiation. Each point plotted is the mean survival on eight agar plates each spread with about 400 conidia. The percentage survival is plotted on scale A, and the curve shows the relationship between percentage survival and exposure time. The straight line drawn to represent the relationship between log. percentage survival and exposure was plotted with reference to scale B, and fits the data well. It indicates that the killing of Vertieillium conidia follows first-order kinetics.

The proportion of mutants among survivors after treatment with a mutagen

generally reaches a maximum at mutagenic doses which are over 90 % lethal. Exposures of 20 sec. gave about 3 % survival and the survivors were screened for nutritional mutations by replication and auxanography as already described. Table 1 shows details of the origins of the irradiated conidia and the numbers of u.v. irradiation-induced mutants obtained. The most frequent type of mutant, 13 in all, required vitamins, of which six could not synthesize biotin. All the mutants responding to nucleic acid had a specific requirement for adenine. After some of the strains had been labelled with one nutritional requirement they were re-irradiated

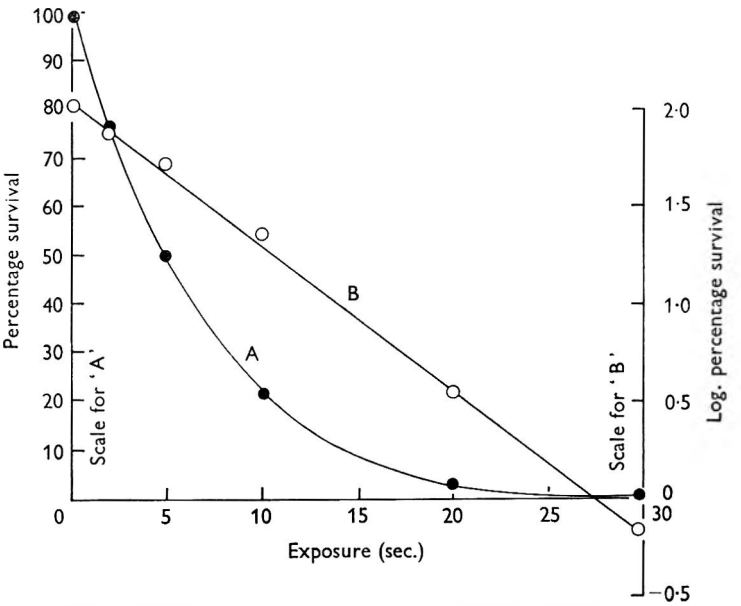


Fig. 1. Survival of conidia of *Verticillium albo-atrum* (strain V1790) to u.v.-irradiation when exposed on a nutritionally complete medium (*CM*). A, plotted on linear scale; B, plotted on log. scale.

Table 1. *Nutritionally deficient mutants of* Verticillium albo-atrum *produced by ultraviolet irradiation*

Serial number of wild-type culture	Survivors (3 % level)	Nutritional requirements of mutants			
		Casein	Nucleic acid	Vitamins	Total
V1790	1006	1	2	2	5
V1813	1140	2	1	3	6
V1809	1528	1	2	5	8
V1805	1575	3	2	3	8
Total	5249	7	7	13	27

to add a second marker (second-order mutants); one double auxotroph was exposed a third time, yielding two third-order mutants. Table 2 shows the proportions of mutants among the first, second and third-order survivors.

The relative ease with which the mutations were detected indicates that the conidia were uninucleate and also that the nuclei were haploid, for nutritional

deficiencies in this fungus are recessive (Hastie, 1962). Too few mutants were detected to justify a statistical treatment of the data, but mutations seemed to be less frequent among survivors of the second and third irradiations. A slight trend in this direction would be expected because there are progressively fewer nutritional requirements detectable, by the methods used, after the later irradiations, as single auxotrophs were exposed at the second irradiation and double auxotrophs at the third.

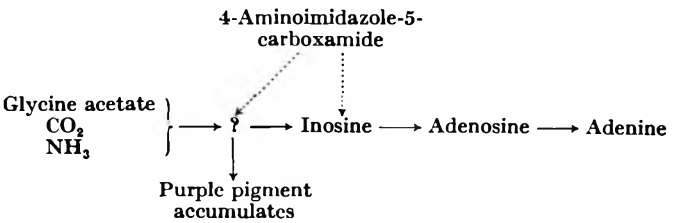
Table 2. *Frequencies of first-, second- and third-order ultraviolet irradiation-induced mutants of* Verticillium albo-atrum

Order of irradiation	Survivors tested	Mutants	Proportion of mutants
First	1911	11	1:174
Second	2913	14	1:208
Third	425	2	1:213
Total	5249	27	1:194

Pleiotropic effect of adenineless mutations

Seven adenineless mutants were recovered. These formed only sparse conidiophores when grown on CM and most of their conidia were produced in pionnotes (slimy nodular masses of conidia; see Pl. 1, fig. 1). The production of conidiophores was increased by increasing the adenine concentration in the medium. Plate 1, fig. 2, shows an adenineless mutant growing on three different concentrations of adenine incorporated in Czapek-Dox agar. No pionnotes were formed at the highest concentration (20 μg./adenine ml.), when the mutant produced abundant conidiophores typical of wild-type strains. Among more than 5000 survivors of irradiation examined on CM, only seven produced pionnotes, and each required adenine for growth. It can be therefore reasonably assumed that the production of pionnotes was a pleiotropic effect of mutations blocking adenine synthesis.

Wagner & Mitchell (1955) suggested the following simplified pathway leading to adenine synthesis in micro-organisms:



Although a more complex pathway exists, this system was used merely to find the stage at which production of pionnotes might be affected.

The darkly coloured adenineless mutant (Pl. 1, fig. 1) accumulated a purple pigment which diffused into the medium, and at low adenine concentrations it produced fewer pionnotes than did any of the other six adenineless mutants recovered. Table 3 shows the results of an auxanographic test to determine whether the position of the blockage to adenine synthesis was correlated with decreased pionnotal production. The mutant B 11/1, which formed fewer pionnotes and accumulated the purple pigment, was blocked immediately preceding synthesis of adenosine; the

other mutants were blocked at earlier positions. Experience with other micro-organisms suggests that only mutants blocked at the steps preceding 4-aminoimidazole-5-carboxamide fail to produce the purple pigment. This may also be true in Verticillium. Presumably there was no response to 4-aminoimidazole-5-carboxamide because it did not act as an extracellular intermediary, rather than as a result of a blockage at a later stage. Fries, Bergstrom & Rottenberg (1949) showed that 4-aminoimidazole-5-carboxamide was used by certain adenineless mutants of *Ophiostoma multiannulatum* when a small amount of adenine was provided to initiate growth. This latter condition was met by the auxanographic technique used here by placing a small amount of adenine HCl at the centre of each auxanograph and the intermediates in adenine synthesis around the edge of the agar medium; none of our adenineless mutants responded to 4-aminoimidazole-5-carboxamide.

Table 3. *Responses of seven adenineless mutants of* Verticillium albo-atrum *in auxanographic test to compare the positions of biochemical blockages to adenine synthesis*

	Supplement			
Mutant	Amino-imidazole-carboxamide	Inosine	Adenosine	Adenine
O2	−	+	+	+
F2	−	+	+	+
C5/3	−	+	+	+
C5/4A2	−	+	+	+
E5	−	+	+	+
G2	−	+	+	+
B11/1	−	−	+	+

+, response; −, no response.

Mutants resistant to growth inhibitors

Some basic dyes, respiratory inhibitors, antibiotics and other fungicidal and fungistatic compounds were used in attempts to select resistant mutants by the methods already described. The following four auxotrophic strains were subjected to tolerance tests: V1790, mutant O1, requiring histidine; V1813, mutant E3/1, requiring pyridoxine + nicotinic acid; V1804, mutant C5/4, requiring methionine + biotin; V1805, mutant F2, requiring adenine. All four strains (Tables 4 and 5) responded similarly to replication at the concentrations tested. Attempts were made to select mutants resistant to some of the substances at the concentrations listed in the tables. The only spontaneous mutants recovered were resistant to acriflavine, and the attempts to recover spontaneous and u.v. irradiation-induced mutations resistant to some of the other substances failed. The eleven acriflavine-resistant mutants were all obtained from the same culture (V1804 mutant C5/4) and were therefore probably not independent mutations, but members of a clone produced as a result of a single mutation. For this reason only one mutant was retained; it was purified by isolating a single conidium. After repeated subcultivation on CM without acriflavine, its survival when exposed to various concentrations of acriflavine was compared with that of the starting strain (V1804, mutant C5/4). Figure 2 shows the two survival curves. Each point plotted is the mean survival on eight

plates of CM containing the different concentrations of acriflavine. The survivors were recorded after incubation for 4 days; later on, the more inhibitory media yielded a few more minute colonies. These continued to grow very slowly and probably arose by enzymatic adaptation. There was never any difficulty in recognizing colonies with a wild-type growth rate.

There is some discrepancy between the tolerance of acriflavine recorded for strain V1084, mutant C5/4 in Table 4 and that given in Fig. 2. The greater tolerance when tested by replicating was probably caused by the greater concentration of the

Table 4. *Tolerance of* Verticillium albo-atrum *mutants to toxic substances incorporated in complete medium*

	Reaction on CM containing toxin at these concns. (μg./ml.)			Selection concn.* (μg./l.)	Spores tested (× 10^6)	Mutants detected
	1	10	50			
Acriflavine	+	+	−	100	180	11
Crystal violet	+	−	−	10, 20	92	0
2-4-Dinitrophenol quinoline-sulphate	+	+	+	50	30	0
Malachite green	+	±	−	100	89	0
Methyl violet	+	+	−	20	92	0
Night blue	+	+	±	.	.	.
Sodium pentachlorophenate	−	−	−	5	30	0

Symbols: +, normal growth rate; ±, reduced growth rate; −, no growth.
* No attempts were made to select for mutants resistant to materials that allowed growth at 50 μg./ml.

Table 5. *Tolerance of* Verticillium albo-atrum *mutants to a range of antibiotics incorporated in complete medium*

	Concns. in CM (μg./ml.)					Selection concn.* (μg./ml.)	No. of spores tested (× 10^6)
	10	25	50	100	250		
Aureomycin	+	+	+	+	+	.	.
Frequentin	±	±	±	−	−	.	.
Fumagillin	+	+	+	+	+	.	.
Gliotoxin	±	−	−	−	−	25	171
Gladiolic acid	+	+	+	+	+	.	.
Griseofulvin	+	+	+	+	+	.	.
Mycophenolic acid	−	−	−	−	−	1, 5, 10	128
Patulin	±	±	±	±	±	.	.
Rimocidin	−	−	−	−	−	1, 5, 10	262·4
Viridin	+	+	+	+	±	.	.

Symbols: +, normal growth rate; ±, reduced growth rate; −, no growth.
* No attempts were made to select for mutants resistant to materials that allowed growth at 50 μg./ml.

inoculum. Each point inoculated by replicating received many conidia, perhaps several thousands, whereas each site inoculated by spreading a spore suspension would usually receive only one conidium. The survival curve for the resistant mutant (Fig. 2) shows that it succumbed to acriflavine only at concentrations

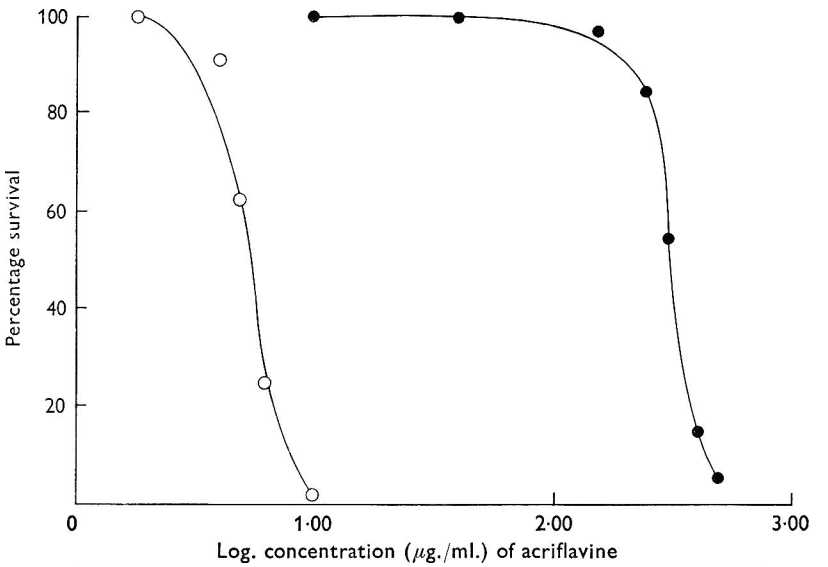


Fig. 2. Survival of un-irradiated conidia of *Verticillium albo-atrum* on complete agar medium containing acriflavine. ○, sensitive strain; ●, resistant mutant.

above 500 μg./ml. This concentration incorporated in CM has been found useful for classifying genetic recombinants as sensitive or resistant in work on the parasexual mechanism of variation in Verticillium (Hastie, 1962).

DISCUSSION

This work was done to provide the genetic markers necessary to detect and elucidate any system of genetic recombination in *Verticillium albo-atrum*. The relative ease with which u.v.-irradiation produced recessive nutritional mutants confirmed that the conidia are uninucleate and was an encouraging sign that genetic analysis would be possible. In multinucleate conidia, a recessive mutation in one nucleus would be phenotypically masked by the dominance of the wild-type alleles in other nuclei. The recessive phenotype could then be detected in this or other imperfect fungi only by taking many monoconidial isolates from the colony produced by each irradiated propagule. Such single-conidial isolations proved to be unnecessary in *V. albo-atrum*, thus supporting the evidence that most of the irradiated spores were uninucleate. This evidence agrees with the cytological observations of at least two authors (Waggoner, 1956; Caroselli, 1957).

The kind of survival curve obtained when the conidia were exposed to u.v.-irradiation also agrees with the idea that most Verticillium conidia are uninucleate. However, it should also be remembered that different curves (multi-hit) have been observed for fungi with uninucleate spores, and the kinetics of the effect is therefore not a reliable indication of nuclear number. Buxton, Last & Nour (1957) found that microconidia of Fusarium were inactivated by u.v.-irradiation according to first-order kinetics, whereas a higher order kinetics governed the inactivation of spores of *Aspergillus niger*. Genetical studies, however, suggest that at least a very high proportion of Aspergillus conidia are uninucleate (Pontecorvo *et al.* 1953).

If the original wild-type isolates were heterogeneous for heritable characters affecting the detection of recessive mutations, the proportion of mutants detected at the later irradiations would have increased greatly. For example, in any original wild-type cultures that may have yielded conidia with both haploid and diploid nuclei, nutritional deficiencies would have been easily detected only in the haploids. Selection of the mutants after the first irradiation would then have provided only haploid strains for the later irradiations, at which there would have been a consequent increase in the proportions of mutants detected.

We thank Dr P. W. Talboys (East Malling Research Station, Kent) for supplying the cultures of *Verticillium albo-atrum* isolated from wilted hop, and members of the Photography Department, Rothamsted Experimental Station, for taking the photographs. One of us (A.C.H.) also thanks the Agricultural Research Council for the award of a Research Studentship, during the tenure of which this work was done.

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EXPLANATION OF PLATE

Fig. 1. Adenineless mutants of *Verticillium albo-atrum* growing on a nutritionally complete medium and showing morphological differences. A, mutant B11/1 accumulating purple pigment in the medium and forming no pionnotes; B, mutant F2, with no purple pigment, but producing pionnotes.

Fig. 2. Adenineless mutant F2 of *Verticillium albo-atrum* growing on Czapek-Dox agar containing three different concentrations of adenine. A, 20 μg./ml.; B, 2·0 μg./ml.; C, 0·2 μg./ml.

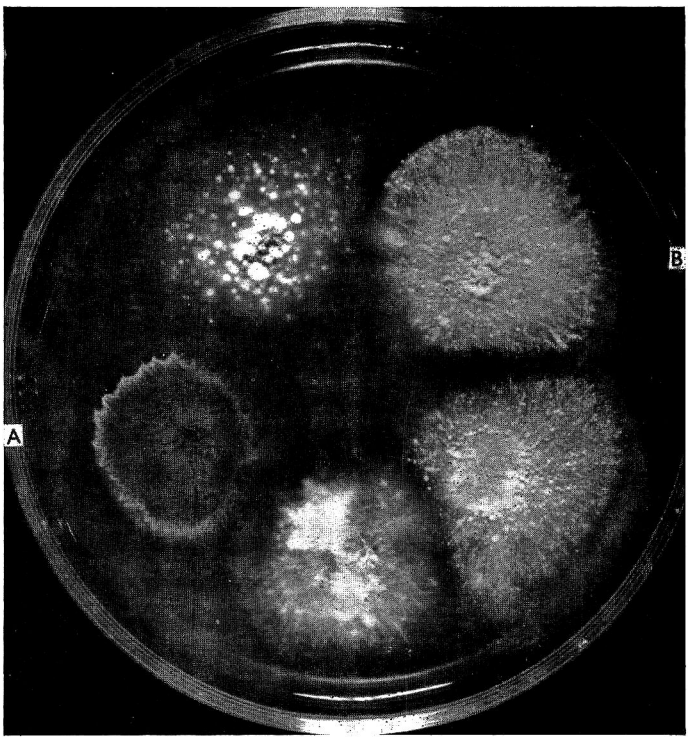

Fig. 1

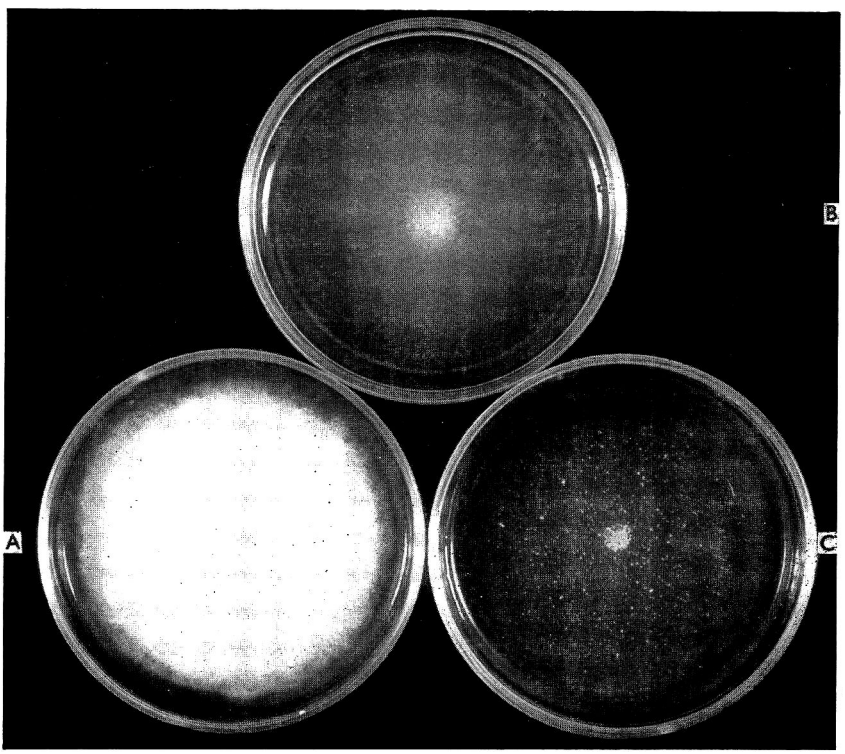

Fig. 2

J. gen. Microbiol. (1962), 28, 633–640
Printed in Great Britain

Experimental Methods in Computer Taxonomy

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(*Received* 6 *November* 1961)

SUMMARY

In the preparation of taxonomic data for computer analysis, some new methods are proposed which compensate for certain inadequacies in present techniques without violating the essential Adansonian axiom that all properties of organisms are of equal significance in the creation of taxa. A modified scoring technique is presented for use with tests wherein two or more alternative responses (none necessarily negative) are possible. The proposed method might well be extended to the scoring of all data; one is never sure of the significance of a 'negative' result, and it could be considered that at least two alternatives of equal value in classification may exist for any determinable property of an organism. It is suggested that computed relationships among organisms might better be expressed in terms of *distance*, which is a logarithmic function of the *similarity* ratio in current use. A new diagnostic parameter, P, is defined as a quantitative estimate of the proportion of organisms in any group (taxon) which possess a given property. Computer programs based on such values could be devised for diagnosis of organisms whose identity is unknown.

INTRODUCTION

It has been proposed (Sneath, 1957*a*, *b*) that electronic computers be employed to analyse taxonomic data in accord with the Adansonian principle that classification depends on estimates of the overall similarity between organisms, based on examination of a large number of equally weighted features. The general validity of this approach to bacterial systematics has since been demonstrated in a number of investigations (e.g. Bojalil & Cerbón, 1961; Colwell & Liston, 1961; Gilardi, Hill, Turri & Silvestri 1960; Hill, 1959; Hill, Turri, Gilardi & Silvestri, 1961; Sneath & Cowan, 1958; Talbot & Sneath, 1960). Despite some manifest shortcomings in procedure, however, most subsequent workers have adhered rather closely to the details of Sneath's original techniques. We now propose some refinements in methodology which may prove useful in extending and strengthening the application of computers to taxonomic problems. The essential axiom of this 'school' of systematics is that all properties of organisms are of equal importance in creating taxa; one may make necessary and convenient modifications in techniques without being guilty either of heretical disregard for the basic principles of Adansonian taxonomy or of lack of respect for the creative impetus provided by Sneath.

METHODOLOGY—A CRITIQUE

For each of the steps involved in the estimation of overall similarity a number of alternatives may exist which are equally valid logically; the more convenient of these methods are chosen in actual practice (Sneath, 1957*b*). On the basis of the experience since accumulated by numerous workers, it may be well to re-apply the criterion of convenience to some of these alternatives. The established method for calculating similarities between pairs of organisms makes use of the expression

$$S = \frac{n_s}{n_s + n_d},$$

where n_s is the number of features possessed by both organisms and n_d is the number of features possessed by one organism but not the other. Each organism is scored as positive (+) or negative (−) for each feature. The symbol (○), for 'no comparison', is used when no data are available. Sneath (1957*b*) used the symbol NC for this purpose. In comparing two organisms, the computer is directed to score (+ +) as a similarity and (+ −) or (− +) as a difference, ignoring (− −) or any combination containing a zero.

However, difficulties arise in scoring certain properties (such as colony morphology) which cannot be considered simply present or absent. In such instances there may be no logical basis for deciding which is the positive trait. It becomes necessary either to make an arbitrary decision to consider, let us say, the smooth colony as positive (in which case two rough strains would not be scored as similar), or to assign two features to colony morphology:

Features	1	2
Strain A—smooth colonies	+	−
Strain B—rough colonies	−	+

A pair of strains with the same colony type would score a single similarity, but strains differing in colony morphology would score *two* dissimilarities, introducing an unwarranted bias into the calculated S values. We have had some success with a slightly different convention, in which the two colony types are scored:

Features	1	2
Strain A—smooth colonies	+	−
Strain B—rough colonies	○	+

Use of the (○) symbol prevents a second dissimilarity being scored. This method is convenient also when there are more than two alternatives, as:

Features	1	2	3	4
Strain A—smooth colonies	+	−	−	−
Strain B—rough colonies (type I)	○	+	−	−
Strain C—rough colonies (type II)	○	○	+	−
Strain D—mucoid colonies	○	○	○	+

It will be seen that a pair of strains with like colony type will score a single similarity, and that a strain of any colony type will score a single dissimilarity in comparison with any organism differing from it in colonial morphology. An individual strain is scored by rule of thumb: place a (+) under the column for the appropriate feature, then place (○) in each column to the left and (−) in each column to the

right. The order in which the features are arranged may be decided arbitrarily, since it will not affect the outcome. Such use of the 'no comparison' (○) category as a part of the scoring system rather than simply to indicate that no data are available was made also by Sneath (1957*b*) in his 'Method C' for scoring quantitative data, although for different reasons. No theoretical justification is offered for the scoring method here proposed; it is quite arbitrary, but justifiable if it is found empirically to yield a logical result.

This technique is also useful for scoring quantitatively the response of organisms to a toxic (physical or chemical) environment. Though the usual procedure has been to score all strains as either sensitive or resistant, then to score only the resistant strains on a quantitative basis, it may be argued that there is no such thing as absolute sensitivity or resistance. Any strain will be inhibited when dosage of a toxic agent is great enough, and any strain will be insensitive to sufficiently small concentrations. Thus a given degree of sensitivity may be considered merely as alternative to all other possible states of sensitivity:

Features	1	2	3
Strain A—insensitive	+	−	−
Strain B—moderately sensitive	○	+	−
Strain C—highly sensitive	○	○	+

The investigator is spared the embarrassment of stating whether resistance or sensitivity is the positive character, since the outcome is the same in either case:

Features	1	2	3
Strain A—insensitive	○	○	+
Strain B—moderately sensitive	○	+	−
Strain C—highly sensitive	+	−	−

We could by similar reasoning extend this argument to include *all* quantitative tests. All we really know about an indole or catalase 'negative' culture is that it does not produce enough indole or catalase to give a positive response in the analytical test we have used. Another test, more or less sensitive than the one used, might have given different results. If quantitative tests are included, failure to produce detectable amounts could well be considered as simply one of the alternatives, rather than being scored as a separate property. Two catalase 'positive' strains would not then be scored as similar unless they produced approximately equal quantities of catalase, but perhaps they should not be.

It would not be well to pursue this viewpoint too far, however, especially if one were scoring quantitative data in rather small increments. An alternative would be to score the qualitative test as one feature, then to score quantitative data for positive strains by the method already proposed, marking negative strains (○) for 'no comparison' in each of the quantitative features. This is similar to the technique presently in use. It has the disadvantage that more comparisons are made between positive than between negative strains, magnifying essentially minor dissimilarities among the former. It is observed in practice that relationships among strains tend to be obscured when too much information of this sort is included. It would seem better to omit questions which cannot legitimately be asked of all organisms being surveyed (e.g. 'how much catalase is produced?' when some strains produce none, 'what is the shape of the leaves?' when not all the organisms studied have leaves).

Such properties are valuable for precise definition of relationships among closely related organisms, and no individual study need be restricted to features applicable to *all* bacteria (Sneath, 1957*b*), but it would perhaps be wise to include in any given survey only tests which apply to all the organisms actually included in that particular study.

Consideration of the hazards encountered when more criteria of similarity are applied to some individuals than to others leads us to re-examine the convention that only 'positive' results are considered as similarities. That is, a test which is scored as negative for both organisms being compared is ignored altogether on the ground that this fact is without significance. As a consequence, since individuals differ in their proportion of positive responses to a given battery of tests, the denominator in the expression

$$S = \frac{n_s}{n_s + n_d}$$

varies for each pair of organisms, and with it varies the statistical significance of individual S values in any survey of the similarities among a group of organisms. This variation is likely to be serious when diverse strains are being studied, or when the battery of tests is not carefully selected to yield approximately the same proportion of positive responses for each strain (which in practice is difficult to attain without bias). Within a relatively homogeneous group of actinomycetes, Gilardi *et al.* (1960) found a disturbing correlation between the percentage of positive tests for any strain and its highest S value. When the same data were analysed using a parameter (M, 'matching coefficient') which permitted comparison of properties negative for both strains (Hill *et al.* 1961), considerably sharper demarcations were obtained between groups. Our own experiments (Beers, Fisher, Megraw & Lockhart 1962) indicate that results are more meaningful when negative similarities are included.

In justification for restricting the scoring of similarities to positive responses, Sneath (1957*b*) stated that the class of negative properties is almost infinite, and that one does not know where to stop. On the other hand, an almost infinite positive class also could be devised. It is no less sensible to consider two bacteria similar because both lack feathers than to consider them similar because both possess cell walls; we must trust to the good judgement of individual investigators to eschew such extremes. The problems encountered in actual practice are likely to be more mundane, if equally troublesome. We have already pointed out that special scoring is necessary for those cases in which there is no logical basis for deciding what should be considered the positive trait. Even for tests in which a 'positive' response appears rather obvious, it is not quite safe on genetic grounds to state that negativity constitutes 'absence'. Although we must score phenotypes, we presume taxa are created ultimately on a genetic basis. When a bacterium fails to ferment lactose, for example, while the failure may be due to the lack of an appropriate genetic determinant, it may equally well result from a modified genetic site causing production of an incomplete or altered enzyme, or from the very positive presence of a modifier gene which inhibits overt expression of fermentative capacity. Hill *et al.* (1961) cited similar arguments in support of their 'matching coefficient'. Even if the view be accepted that similarity should be based only on positive responses, a

recurring dilemma is encountered as to whether positivity is merely illusory for any given test. Rather than to engage in endless debate about how many angels can dance on the heads of each of these pins, it seems better to admit frankly that no compelling theoretical arguments exist either for or against comparing apparently negative results as similarities. We are then free to proceed on the pragmatic basis that we will use whichever scoring convention yields superior results; the available evidence seems to favour permitting comparisons between negatives. The scoring technique already proposed could be used in this way. For example:

Features	1	2
Strain A—produces indole	+	−
Strain B—does not produce indole	O	+

This amounts to saying that for any determinable characteristic of an organism there are at least two alternatives, each of equal value in classification. It would seem at first glance simpler to use only one feature in this example, and merely to instruct the computer to accept pairs of negatives as was done by Hill *et al.* (1961) in defining their M value. But the proposed scoring method would not then be applicable in instances where there are three or more alternatives. As it is, a single uniform scoring convention may be used in all cases, rather than resorting to different scoring methods (Sneath, 1957*b*) for different circumstances. Since the expression for calculation of S remains unchanged, it is possible with this scoring technique to count negative comparisons without altering the computer program.

In fact, however, S (or M) values may not be the most appropriate parameter for expressing relationships among strains. It usually is possible to define groups with fair precision by comparing S values, but relationships within and among groups are not accurately described in this way. The relationships among organisms in nature appear to be organized not as a hierarchy but as a three-dimensional array. (Actually it may be a multidimensional array, but the state of our present knowledge hardly justifies attempting the rather sophisticated mathematics required to depict such an arrangement.) In constructing three-dimensional models of the spatial relationships among enterobacteria, Lysenko & Sneath (1959) found the simple parameter $1-S$ inadequate as a measure of distance between individuals or groups. Needing a distance value that would vary from infinity at $S = 0$ to zero at $S = 1{\cdot}0$, they chose $1/S-1$ and suggested that $\log 1/S$ would serve equally well. Rogers & Tanimoto (1960), in using computer methods for classifying plants, employed $-\log_2 S(=\log_2 1/S)$ as their distance parameter, pointing out that this defines a semimetric space in which there may be two individuals (or groups) related to a third in such a way that they are not necessarily related at all to each other. All such measures have the effect of progressively accentuating the distances between organisms as similarities decrease. In our experiments, we have defined distance as

$$D = \log_2 1/S = \log_2 \frac{(n_s+n_d)}{n_s}.$$

Logarithms to base 2 yield manageable numbers, and are easily handled by digital computers. Finney, Hazlewood & Smith (1955) have prepared convenient tables of logarithms to base 2. We have found D only slightly superior to S as a means of defining groups at high similarities, but it appears to depict intergroup relationships

more clearly. Since D, or some alternative measure of distance, seems to express more meaningfully than similarity ratios the relationships among organisms, it would appear desirable to adopt distance as our primary parameter. Such data should prove increasingly useful for comparative purposes as a catalogue of information gradually is accumulated by various investigators working with divers organisms. Since D can be calculated directly, and should be quite adequate also for sorting organisms into groups, S values need not necessarily even be computed.

As we have proposed changing nearly everything else, it may be wondered why we stated earlier that the principle of equally-weighted features is accepted as axiomatic. Although this viewpoint has been defended adequately already (Sneath, 1957*a*; Rogers & Tanimoto, 1960), some further amplification may be in order. This notion is not palatable to many microbiologists, because they 'know' (from previous experience) that some properties of organisms are indeed more significant than others. But one has only to reflect that the experience on which such judgements are based was itself a somewhat unrefined Adansonian process. All that is really being proposed is that we re-examine, by means of the abundance of data and the greater quantitative accuracy possible from the use of computers, our present concepts about which are the important characteristics. We may discover some whose existence we did not suspect; we shall almost certainly learn that others, presently accepted, are of doubtful validity. In any case, *a priori* assumptions of importance for particular tests are not justifiable. Selection of key characteristics necessarily follows rather than precedes the establishment of taxa. Perhaps much of the present confusion stems from a failure to distinguish the quite different functions of classification and diagnosis. Only after we know what groups actually exist in nature need we be concerned with the properties which are characteristic for a given group, that is, of diagnostic value.

The possibilities for diagnosis in the computer approach have been largely overlooked thus far. Although computer techniques can establish groups, the only means of diagnosing a new isolate as a member of one of these would be to repeat the entire calculation with the new strain included. Otherwise, traditional methods would have to be used; inspection of the data would show the essential characteristics of each group, and a key would be constructed. It would not be at all difficult, however, to let the computer furnish us with a quantitative description of each of the groups. The data for all the strains in any group which had been established could be furnished, and the machine instructed to calculate the proportion of positive responses, within that group, for each feature: $P = n/N$, where n is the number of positive responses for any feature and N is the number of strains in the group. If scoring had been done by the method advocated above, it would be necessary to calculate P for only one of each pair of alternative features (though for each feature in properties with more than two alternatives). The value of P would then be a quantitative description of one property of a group, and a catalogue of P values would constitute a diagnostic description of the group. Very high or low values of P (near one or zero) would indicate key diagnostic characteristics. As with any description of a taxon, such data would be useful only when based on observations of an adequate number of strains. The relationship between P and the number of strains on which it was based would readily yield a statistical estimate of the confidence limits for any alleged property of a group.

One can envisage an ultimate taxonomy in which both objectives of systematics—classification and diagnosis—are satisfied by computer methods. Taxa would be developed and defined by means of D values, and future catalogues might consist of lists of appropriate P values for each taxon. A statement that $P = 0{\cdot}85$ for indole production would be far more helpful than the current 'most strains produce indole'. Further, computer programs could be designed to do the actual labour of diagnosis. An isolate would be subjected to a suitable battery of tests, and—knowing the characteristic P values (and their confidence limits) for each property of various taxa—the computer could furnish not only a diagnosis but a statistical estimate of the reliability of its own findings. The minimum number and kinds of tests for reasonably accurate diagnosis would have been computed beforehand. Such calculations, while a trifle complex, are well within the programming capacity of modern electronic computers. The charms of such a system of diagnosis are readily apparent to anyone who has had the frustrating experience of trying to trace an unknown isolate through even the best of presently available keys.

CONCLUSIONS

It is not our intent to destroy the edifice constructed by many workers on the foundation supplied by Sneath's initial efforts. Only because the structure of this new taxonomy shows itself to be fundamentally sound is it worthwhile to modify it. Any revolutionary development is likely to be somewhat crude in its early stages; experience suggests refinements which could not have been imagined until certain initial steps had been taken. Neither are the techniques suggested here likely to be the ultimate refinement; they may well prove quite naïve in the light of future experience.

The scoring method proposed here has the advantages that it appears to deal more adequately with certain kinds of data, eliminating the need for subjective judgements involving essentially non-resolvable questions, and makes possible the application of a single scoring convention to all tests. Even if an investigator does not agree that similarities should be based on 'negative' results, this convention will be useful in scoring many tests where negativity is not clear cut or quantitative data are involved. We feel quite strongly—for reasons outlined earlier—that quantitative tests, or those dependent on the presence of another property, should be included only when they can be made to apply to all the strains included in a particular study.

We have tried only to indicate that our reasons for proposing these modifications are not altogether illogical. Their true test will be that of experience, and their justification (if any) will be that they yield superior results. In the paper which follows (Beers *et al.* 1962) we have compared the proposed modified methods with other methods, and the results appear encouraging. It is to be hoped that other investigators will test them also. Fortunately the scoring system is such that comparisons will be relatively easy; data need only be re-scored and submitted to the computer program already in use.

There is relatively little practical advantage to be gained at present by using distance rather than similarity as our primary parameter. On theoretical grounds, however, D would appear to be the preferable measure of relationships between

organisms, and its advantages should become more apparent as larger quantities of data become available for comparison.

Our final remarks, concerning the application of computer techniques to diagnosis, are of course premature. Although it may be useful in some instances to compute P values as a means of securing descriptions of groups of organisms encountered in individual studies, there are not nearly enough quantitative data available at present about enough taxa to make feasible any attempts to program a diagnostic scheme. Nevertheless, this must be regarded as a legitimate objective for computer taxonomists. The sooner techniques are devised and adopted for dealing with any unsatisfactory aspects of present scoring schemes, the sooner may we expect to gather enough useful data to make possible the ultimate step.

Journal paper no. J-4212 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project no. 1509.

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A Comparison of Methods for Computer Taxonomy

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(*Received* 6 *November* 1961)

SUMMARY

The systematic relationships among 54 strains of bacteria, representing principally the genera *Achromobacter*, *Aerobacter*, *Alcaligenes*, *Escherichia*, *Mima*, *Pseudomonas*, *Serratia* and *Streptococcus*, were examined by computer methods. Seventy-one properties of these organisms were determined, and the resulting data scored in different ways (according to various proposed techniques) before being submitted to an appropriate computer program for calculation of similarity (S) values. These comparative studies indicated that better division of organisms into mutually similar groups can be achieved when data about properties which may have several alternative expressions are handled in the manner proposed by Beers & Lockhart (1962). The number of comparisons which contribute to individual similarity values should be held constant by adequate treatment of quantitative data and by adoption of scoring methods which permit comparisons between 'negative' properties. It may be useful to employ distance ($D = \log_2 1/S$) rather than similarity as the primary measure of relationships among groups of organisms.

INTRODUCTION

A number of modifications in computer techniques for the analysis of taxonomic data according to the general principles propounded by Sneath (1957*a*, *b*) were suggested by Beers & Lockhart (1962). The present paper reports the results of some comparisons of the various proposed methodologies.

METHODS

The microbial cultures used in this study are listed below. Reference strains obtained from the American Type Culture Collection and the Iowa State University culture collection are designated by the symbols ATCC and ISU, respectively. Cultures 23, 24, 32 and 42 were obtained from the Culture Collection of Entomogenous Bacteria, Prague, Czechoslovakia, through the courtesy of Dr O. Lysenko. Representatives of the tribe Mimeae (cultures 1–5) were secured from Dr Sylvia Cary of the Walter Reed Army Hospital, Washington, D.C. Culture 25 was provided by Dr G. E. Bucher, Entomology Laboratory, Ontario, Canada. The remaining organisms were isolated in this laboratory (Raun, Lockhart & Beers, 1959) from apparently diseased larvae of the European corn borer *Ostrinia nubilalis* (Hübner). These strains (designated as 'isolates') were subjected to appropriate diagnostic tests and found to conform to the descriptions of the indicated species in *Bergey's*

Table 1. *Features employed in computer analyses of relationships among the test organisms*

* Asterisk indicates all features assigned to a property were scored '○' (no comparison) if the preceding property was negative for a given organism.

Property	No. of alternative features assigned for computation no.: I (Fig. 1)	II (Fig. 2)	III (Fig. 3)
1. Cell morphology	3	3	3
2. Motility	1	2	2
3. Colony form	3	3	3
4. Colony edge	4	4	4
5. Smooth colonies	1	2	2
6. Opaque colonies	1	2	2
7. Granular colonies	1	2	2
8. Raised colonies	1	2	2
9. Odour in broth	1	2	5
10. Type of odour	4*	4*	
11. Sediment in broth	1	2	4
12. Type of sediment	3*	3*	
13. Turbidity in broth	1	2	4
14. Amount of turbidity	3*	3*	
15. Pigment production	1	2	5
16. Colour of pigment	4*	4*	
17. Pigment diffusible	1*	2*	Omitted
18. Ammonia production	1	2	2
19. Haemolytic activity	1	2	4
20. Type of haemolysis	3*	3*	
21. Gelatin liquefied	1	2	2
22. Starch hydrolysed	1	2	2
23. Casein hydrolysed	1	2	2
24. Hippuric acid hydrolysed	1	2	2
25. Oxidase produced	1	2	2
26. Catalase produced	1	2	4
27. Amount of catalase	3*	3*	
28. Plasma jelled	1	2	2
29. Indole produced	1	2	2
30. Methyl red test	1	2	2
31. Voges–Proskauer test	1	2	2
32. Nitrate reduced	1	2	2
33. Acid from glucose	1	2	2
34. Gas from glucose	1*	2*	Omitted
Acid from:			
35. Fructose	1	2	2
36. Galactose	1	2	2
37. Xylose	1	2	2
38. Arabinose	1	2	2
39. Maltose	1	2	2
40. Sucrose	1	2	2
41. Lactose	1	2	2
42. Glycerol	1	2	2
43. Mannitol	1	2	2
44. Sorbitol	1	2	2
45. Salicin	1	2	2
46. Litmus milk acid	1	2	2
47. Litmus reduced	1	2	2
48. Milk coagulated	1	2	2
49. Milk peptonized	1	2	2

Table 1 (*cont.*)

Property	No. of alternative features assigned for computation no.: I (Fig. 1)	II (Fig. 2)	III (Fig. 3)
50. Resistant to tellurite	1	2	} 3
51. Degree of tellurite resistance	2*	2*	
52. Resistant to crystal violet	1	2	} 3
53. Degree of crystal violet resistance	2*	2*	
54. Reduction of methylene blue	1	2	2
55. Resistant to penicillin	1	2	} 4
56. Degree of penicillin resistance	3*	3*	
57. Tolerates exposure to 56°	1	2	} 3
58. Extent of tolerance to 56°	2*	2*	
59. Resistant to NaCl	1	2	} 4
60. Degree of resistance to NaCl	3*	3*	
Utilized as sole carbon source:			
61. Glucose	1	2	2
62. Acetate	1	2	2
63. Lactate	1	2	2
64. Succinate	1	2	2
65. Citrate	1	2	2
66. Glycerol	1	2	2
67. Growth at pH 5·5	1	2	2
68. Growth at pH 8·7	1	2	2
69. Growth at 10°	1	2	2
70. Growth at 45°	1	2	2
71. Surface growth in thioglycollate	1	2	2

Manual (1957). A name or epithet enclosed in parentheses indicates that the isolate in question did not agree with any description listed in *Bergey's Manual*, but was judged to resemble most closely the organism designated.

The test organisms were:

1, *Mima polymorpha*, WR 26; 2, *M. polymorpha*, WR 45; 3, *M. polymorpha*, WR 980; 4, *M. polymorpha*, WR 1957.

5, *Herellea* sp., WR 393.

6, *Alcaligenes faecalis*, ISU 2E2; 7, *A. faecalis*, isolate 2C–454.

8, (*Vibrio curveatus*), isolate 50C.

9, *Achromobacter* sp. (*delmarvae*), isolate 2C442A; 10, *Achromobacter* sp. (*eurydice*), isolate 3C442A.

11, *Pseudomonas reptilivora*, isolate B2C437; 12, *P. reptilivora*, isolate I2C437; 13, *P. reptilivora*, isolate 50B; 14, *P. boreopolis*, isolate 1C447; 15, *P. boreopolis*, isolate 2C447; 16, *P. boreopolis*, isolate 1C450; 17, *P. boreopolis*, isolate 3C450.

18, *Escherichia coli*, ATCC 4157 (NCTC 86); 19, *E. coli*, ISU 2B5; 20, *E. coli*, ISU 4157; 21, *E. coli*, ISU 2B6; 22, *E. coli*, isolate 18C.

23, *Aerobacter cloacae*, CCEB 160; 24, *A. cloacae*, CCEB 161; 25, *A. cloacae*, Lister 2163; 26, *A. aerogenes*, isolate 1C3A; 27, *A. aerogenes*, isolate 11; 28, (*Aerobacter* sp.), isolate 1C495; 29, (*Aerobacter* sp.), isolate 2C495.

30, *Proteus vulgaris*, ATCC 6896 (NCTC 4636).

31, *Serratia marcescens*, ATCC 274 (NCTC 1377); 32, *S. marcescens*, CCEB 006; 33, *S. marcescens*, isolate 1C437; 34, *S. marcescens*, isolate 1C442A; 35, *S. marcescens*,

isolate 2C81; 36, *S. marcescens*, isolate 1C81; 37, *S. marcescens*, isolate 1C454; 38, *S. marcescens*, isolate 1C462; 39, *S. marcescens*, isolate 3C462; 40, *S. marcescens*, isolate 1C466; 41, *S. marcescens*, isolate 470.

42, *Streptococcus faecalis*, CCEB 079; 43, *S. faecalis*, isolate 1C237; 44, *S. faecalis*, isolate 2C237; 45, *S. faecalis* var. *liquifaciens*, isolate 3C454; 46, *S. faecalis* var. *liquifaciens*, isolate 2C462; 47, *S. faecalis* var. *liquifaciens*, isolate 2C461; 48, *S. faecalis* var. *liquifaciens*, isolate 2C450; 49, *S. faecalis* var. *liquifaciens*, isolate 1C461; 50, *S. mitis*, isolate B1C437; 51, *S. mitis*, isolate B3C437; 52, *S. mitis*, isolate 2C3A; 53, *S. mitis*, isolate 406A; 54, *S. mitis*, isolate 2C466.

The properties used to determine similarities among these strains are listed in Table 1; all diagnostic tests were performed by conventional methods (*Society of American Bacteriologists*, 1957). Results of each test were scored as positive (+), negative (−), or 'no comparison' (○) according to various scoring conventions. Similarity (S) values for pairs of strains were calculated by means of the expression

$$S = \frac{n_s}{n_s + n_d},$$

where n_s is the number of features positive for both organisms and n_d is the number of features positive for one but not the other. Features negative for both, or any combination containing a zero, were omitted from the calculation.

Table 1 shows the number of alternatives used to designate each property when the scoring methods proposed by Beers & Lockhart (1962) were used. Property number 2 (motility), for example, was assigned a single feature for computation I, but was assigned two alternative features for computations II and III:

I		II and III		
	Feature		Feature	
Property	*A*	Property	*A*	*B*
Motile	+	Motile	+	−
Non-motile	−	Non-motile	○	+

The first property, cell morphology, was scored in each case as three alternative features:

	Feature		
Property	*A*	*B*	*C*
Rods	+	−	−
Cocci	○	+	−
Pleomorphic	○	○	+

Certain quantitative data were handled in a different manner in each computation. For example, properties 26 and 27, having to do with catalase production, were scored as follows:

	Features												
	I				II					III			
Property	*A*	*B*	*C*	*D*	*A*	*B*	*C*	*D*	*E*	*A*	*B*	*C*	*D*
Produces no detectable catalase	−	○	○	○	○	+	○	○	○	+	−	−	−
Slight catalase production	+	+	−	−	+	−	+	−	−	○	+	−	−
Moderate catalase production	+	○	+	−	+	−	○	+	−	○	○	+	−
Pronounced catalase production	+	○	○	+	+	−	○	○	+	○	○	○	+

For computation I, feature *A* was assigned to property 26 and features *B*, *C* and *D* to property 27. For II, features *A* and *B* were assigned to property 26 (scored as two alternatives) and features *C*, *D* and *E* to property 27 (scored as three alternatives). For computation III, properties 26 and 27 were scored together as four alternatives.

Although the calculation of *S* was always the same, use of different scoring conventions assured that in some cases properties 'negative' for both organisms contributed to the value of n_s. We are using the term *feature* here in a slightly different context than did Sneath (1957*b*). Characteristics of organisms are referred to as *properties*. *Features* are used in scoring; a given property may be assigned one, two or more features, according to the scoring method in use.

S values for each pair of strains were calculated by means of the CYCLONE digital computer of Iowa State University, and the strains arranged into groups at various similarity levels by machine sorting followed by visual inspection of the data. Machine programs for computing *S* values and sorting of strains were substantially the same as those employed by Sneath (1957*b*).

RESULTS

In an initial attempt to determine the relationships among the strains listed in Table 1, scoring was done entirely by the method of Sneath (1957*b*); that is, each property was scored as either 'positive' or 'negative', quantitative data were scored in accordance with his method *C* (Sneath, 1957*b*), and characteristics which could have more than two alternative expressions were scored as a series of separate features. Cell morphology, for example, was scored as follows:

	Features		
Property	*A*	*B*	*C*
Rods	+	−	−
Cocci	−	+	−
Pleomorphic	−	−	+

When strains were sorted into groups on the basis of *S* values obtained in this manner, results somewhat similar to those shown in Fig. 1 were obtained. Intragroup similarities were in general quite low, and demarcations between groups were not at all clear. It was necessary, in order to achieve groupings which seemed sensible, to be quite arbitrary in deciding whether certain strains should be assigned to one or another group to which they were almost equally similar (or, rather, dissimilar).

The data were then rescored, treating certain quantitative properties, and those involving three or more alternative expressions, in the manner proposed by Beers & Lockhart (1962); the results obtained are shown in Fig. 1. In this and subsequent figures, horizontal distances indicate degrees of similarity and the horizontal position of any strain in the block diagrams indicates that it is similar to all other strains in that group at the level shown. Vertical positions of strains within a particular block (i.e. group) are of no special significance. Strain numbers correspond to those listed earlier.

The streptococci (strains 42–54) are not included in Fig. 1. In all the computer

analyses performed, these organisms were clearly separated from all other strains, forming a homogeneous group with relatively high intragroup similarity and consisting of two subgroups. Although it is significant that the presence of these data did not interfere with sorting and that the streptococci could be readily separated, the actual values obtained are of no great interest and are omitted from the figures for the sake of simplicity. In computation I, the streptococci formed two subgroups with mean S values of about 0·85, which fused into a single group at $S = 0{\cdot}50$. There was no relationship with any other group at S values above 0·30.

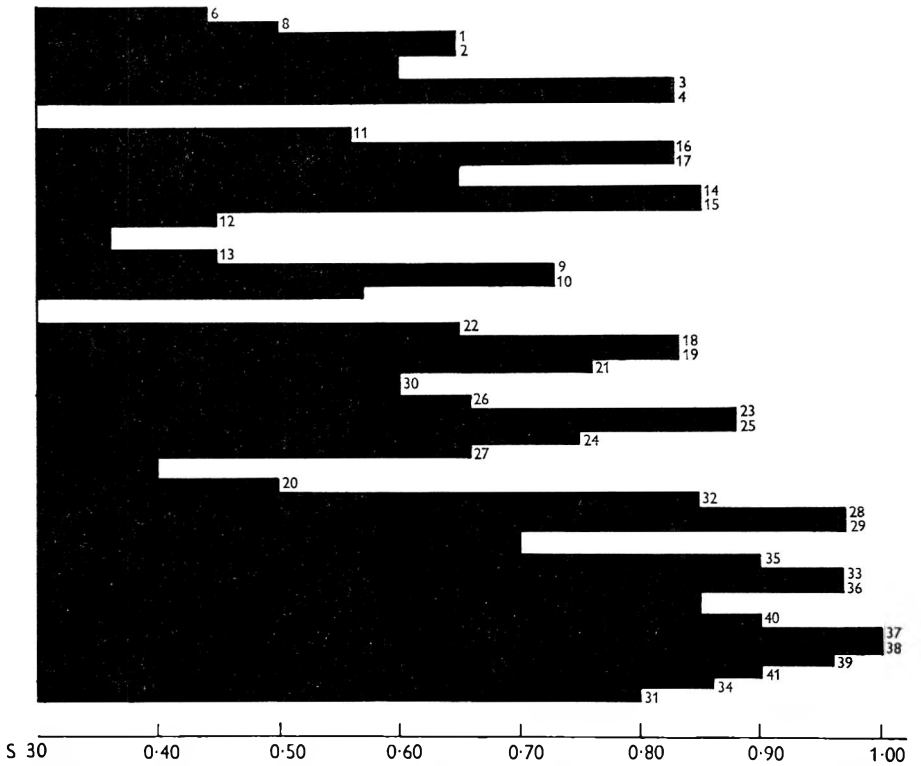

Fig. 1. Similarity relationships among the test organisms as revealed by scoring method I (Table 1).

Although the data summarized in Fig. 1 show in general the groupings one might expect, S values are quite low, with no very high similarities except those between isolated pairs of strains. Intergroup similarities (even between tribes of enterobacteria) are extremely low or not detectable at all. Further, several strains appear to have found their way into the wrong groups altogether. Thus the two strains of *Alcaligenes faecalis* (6 and 7) appear in different groups, one of the Pseudomonas (culture 13) is in the wrong group, and one strain of *Escherichia coli* (20) joins the Serratia group. The single strain of Herellea (5), which is presumed to be closely related to *Mima polymorpha*, does not join this or any other group at any level above $S = 0{\cdot}30$. The presence of culture 8 among the Mima, and cultures 28 and 29 in a subgroup with the 'entomogenous' strain of *Serratia marcescens* (culture 32), are apparently valid relationships, as will be seen later.

When these same data were scored by a method which permits two strains 'negative' for a given property to be considered similar (Beers & Lockhart, 1962), rather different results were obtained. Table 1 indicates the method of scoring (II) used to obtain the groupings shown in Fig. 2. Measured overall, similarities are greater in this case, and groups as well as pairs of strains are now detected at a relatively high similarity level. The two Alcaligenes strains (cultures 6 and 7) now appear in the same group, and the Herellea (culture 5) is in the Mima–Alcaligenes group, where one presumes it should be. Culture 13, along with the two Achromobacter (cultures 9 and 10), has joined the Pseudomonas. However, three strains

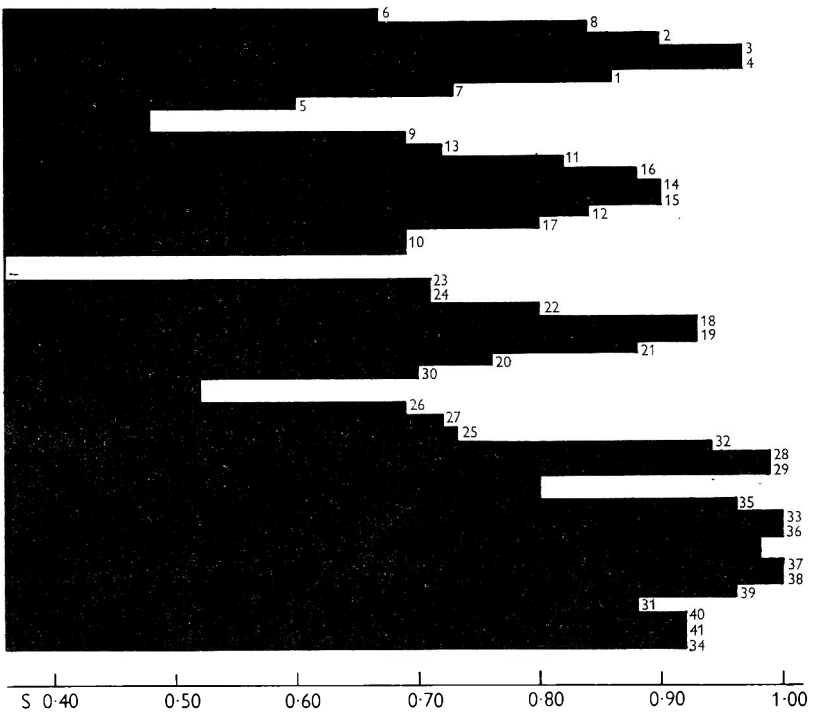

Fig. 2. Similarity relationships among the test organisms as revealed by scoring method II (Table 1).

of Aerobacter (cultures 25, 26 and 27) appear more like Serratia than Escherichia–Aerobacter–Proteus, which now have fused into a single group. Minor differences also occur in the subgroupings among the strains of Serratia (cultures 31–41). The streptococci (cultures 42–54) are omitted from Fig. 2; they formed two subgroups with mean S values of about 0·90, fusing into a single group at $S = 0{\cdot}72$. There was no relationship to other groups in Fig. 2 at any S value above 0·30.

Although the results obtained by method II appear superior in some respects to those shown in Fig. 1, they still leave much to be desired. Even though 'negative' comparisons are used, the total number of features involved in computation of S is not constant (and is thus a source of inconsistency) unless certain classes of quantitative data are handled in a special manner (Beers & Lockhart, 1962). As indicated in Table 1, the coding system was then further modified (III) so that no property

would be excluded because of the result of another test. In properties 26 and 27, for example, failure of a strain to produce detectable amounts of catalase was merely considered one of four possible alternative expressions of its 'catalase property', and any strain (regardless of its catalase activity) scored only a single similarity or a single difference in comparison with any other strain.

The groupings obtained as a result of coding procedure III are shown in Fig. 3. Once again, similarity levels are relatively high, and involve groups rather than pairs of strains. In this instance, however, no strain appears to have been misplaced,

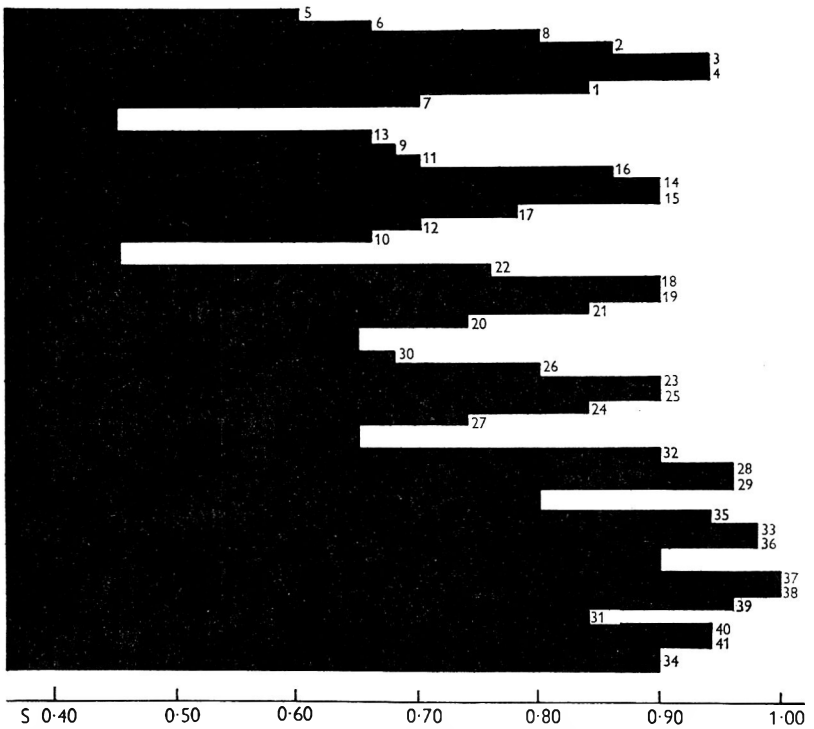

Fig. 3. Similarity relationships among the test organisms as revealed by scoring method III (Table 1).

and intergroup relationships are delineated rather well. The three principal groups—Mimeae–Alcaligenes, Pseudomonas–Achromobacter, and enterobacteria—fuse together at $S = 0{\cdot}45$. Three subgroups of enterobacteria—Escherichia, Aerobacter and Serratia—are well defined at $S = 0{\cdot}65$, with the single strain of Proteus (culture 30) joining in at about the same level. Serratia in turn divides into subgroups at a still higher level of similarity. The streptococci, again omitted from this diagram, consisted of the same two subgroups as before (mean similarity = 0·90), fusing into a single group at $S = 0{\cdot}76$. Once again the streptococci did not fuse with any group shown in Fig. 3 at S values above 0·30. The three isolates which could not be identified (cultures 8, 28 and 29) remained associated with the same groups as before.

It was suggested by Beers & Lockhart (1962) that distance ($D = \log_2 1/S$) rather than similarity might well be adopted as the primary measure of relationships

among organisms. The data from Fig. 3 were converted from S to D values; the resulting diagram is shown in Fig. 4. Use of D tends to make groups appear more homogeneous, by decreasing intragroup differences at high similarities and accentuating the intergroup differences at lower similarities. The distance parameter is only a means of expressing results, of course. Figure 4, being derived from Fig. 3, contains no information which was not already present in the latter.

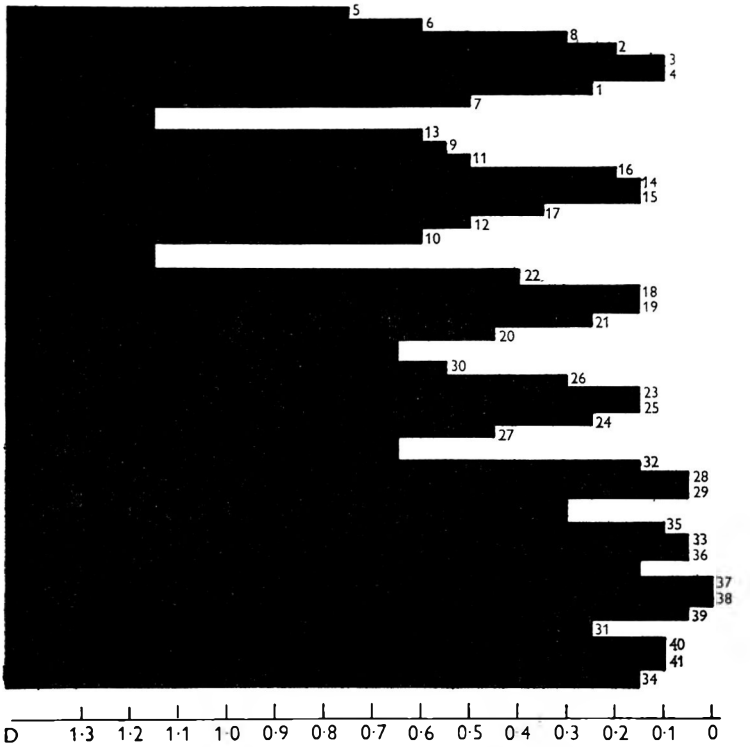

Fig. 4. Relationships among the test organisms, depicted in terms of distance ($D = \log_2 1/S$) rather than similarity. Scoring method III was used.

DISCUSSION

It appears essential that the scoring convention of Beers & Lockhart (1962) be used for properties wherein there is no clear-cut 'negative' response, or where more than two alternatives exist. This procedure was used even in Fig. 1, where Sneath's original scoring methods were otherwise followed meticulously. From this starting-point, it is not difficult to extend the same reasoning to *all* tests, and consider that at least two alternative results (neither necessarily 'negative') exist for any property of an organism which one might determine. The data in Fig. 2, and the results of Hill *et al.* (1961) would indicate that this procedure is both valid and desirable. In the present experiments it must be admitted that some of the differences between Figs. 1 and 2 are rather minor ones. The fact that generally higher similarity values may be obtained is not sufficient in itself to justify use of the proposed scoring system.

It is significant, however, that relatively homogeneous groups of strains, rather than merely isolated pairs of organisms, may thus be distinguished at high similarity levels. This fact considerably simplifies the problem of sorting an array of organisms into mutually similar groups, once S values have been computed. Arranging a random table of S values into groups of similar organisms presents numerous pitfalls. Ideally the computer is directed to sort out all groups of organisms in which each strain is similar to every other strain at a given S-level. In practice, a group may exist composed of, let us say, strains A, C and D. It is quite possible, however, that at a given level of S strain B may be similar to strain A, though not to strains C and D. The computer would designate a group A, B, then reject strains C and D because they were not similar to both organisms already in the 'group'. The group A, C, D would be found in this example if the machine were directed to conduct the search backward, beginning with strain D. In a larger array of organisms, however, where two or more such 'intermediate' strains might show coincidental similarities to some members of a rather large group, such accidents could be avoided only by having the machine conduct a separate search for every possible order in which the strains could be arranged. For an array of 100 organisms, then, it would be necessary to conduct 100 factorial (about 10^{158}) searches, a procedure which would consume infinite amounts of expensive machine time and produce tremendous quantities of duplicate information.

Fortunately, there should exist few individuals like 'strain B' in the above example. If there actually were in nature a continuous spectrum of such organisms intermediate between groups, it would be quite hopeless to attempt to classify micro-organisms by any means. If we are not to abandon systematics altogether, therefore, we must assume that the apparent occurrence of large numbers of such cryptic individuals results from insufficient data or from inadequate methods of computation.

It is obvious that if an extremely small number of features were used for computation of S values, the 'groups' which would appear at various similarity levels would be almost entirely a matter of chance. The low similarity level within groups when certain coding systems are used seems to result from the fact that some S values are, indeed, based on an insufficient number of features. That is, the strains which appear to be intermediate between existing groups are those which give a large proportion of 'negative' responses to the diagnostic tests employed. The information concerning such results may simply be lost, together with any quantitative data which are available only if certain other properties are positive. As a result, some S values may be based in fact on a ridiculously small number of features.

As pointed out by Beers & Lockhart (1962), unbiased selection of an adequately large number of tests which would give approximately equal numbers of positive and negative responses for any appreciable variety of organisms is almost impossible in practice. There would appear to be no alternative but to adopt some scoring method which ensures that the same number of criteria of similarity will be used in computation of all S values. Figure 3, showing the groupings obtained when this was done, would indicate that definitely superior results are thereby obtained. Comparison of Figs. 1–3 shows that such treatment of quantitative data, or of other properties involving more than two alternatives, is quite important. Probably the suggested scoring system should be used for any such data, even by investigators who do not

choose to consider as similarities such things as the fact that neither of two organisms produces indole.

The use of distance (D) rather than similarity (S) values, illustrated in Fig. 4, perhaps offers no important advantage. In some attempts to diagram the three-dimensional relationships among these strains, however, we found it necessary to use D. There at least are sound theoretical reasons for adopting this parameter, and calculation of D rather than S would not be difficult (Beers & Lockhart, 1962). It should be pointed out also that Fig. 4 was obtained simply by converting the S values from Fig. 3. If D values were calculated directly, they would provide a new logical basis for sorting organisms into groups. Intervals on a similarity scale are different from those on the logarithmic D scale. For example, all similarity levels above $S = 0{\cdot}93$ would be included in the groups formed within the interval between $D = 0{\cdot}0$ and $D = 0{\cdot}1$, while the distance within the interval $D = 0{\cdot}9$ to $D = 1{\cdot}0$ would include only the similarity levels between $S = 0{\cdot}50$ and $S = 0{\cdot}54$. Thus for the purpose of sorting organisms into groups, little significance would be attributed to minor differences in similarity at such high levels as $S = 0{\cdot}80$–$1{\cdot}00$ (i.e. subgroups within species; see Figs. 3 and 4), whereas intergroup differences at low levels of similarity ($S = 0{\cdot}50$ or less) would receive added emphasis. Delineation of groups on this basis would perhaps be more valid than the use of similarity values.

Nothing particularly novel or surprising with regard to the relationships among these organisms is to be found in the data presented here. It was our purpose to test and compare various computer techniques rather than to develop new knowledge of the systematics of these particular organisms. It should be pointed out, however, that a suspected relationship between the tribe Mimeae and certain insect pathogens (Beers, Lockhart & Raun, 1959) was not confirmed. Representatives of the Mimeae (cultures 1–5) showed no appreciable similarity either to authentic cultures of *Coccobacillus acridiorum* (= *Aerobacter cloacae*, cultures 23, 24 and 25) and *Bacillus noctuarum* (= *Serratia marcescens*, culture 32), or to any of our isolates. Some of the subgroups found among the various strains of *Serratia marcescens* and among the streptococci (the latter are omitted from the figures) appear to be significant in relation to insect pathogenicity, but such matters are not to the present point and are better discussed in a more appropriate place. Some of our reference strains (cultures 18, 30 and 31) were used in an early survey by Sneath & Cowan (1958). It would appear desirable to include such strains as 'reference points' in studies like these whenever possible, in order to facilitate correlation of the results obtained by different investigators.

Journal paper no. J-4213 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1509. One of us (J. F.) was an undergraduate research participant, National Science Foundation (U.S.).

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Printed in Great Britain

Influence of Calcium and Magnesium on the Growth of Rhizobium

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(*Received* 14 *November* 1961)

SUMMARY

Growth of *Rhizobium trifolii* in a defined medium reflected the supply of Ca^{2+} and Mg^{2+} (subsequently Ca and Mg, respectively) in distinctive fashion. Deficiency of Ca, in the presence of sufficient Mg, caused reduction in growth rate, the level of maximum growth and the proportion of viable cells. Such Ca-deprived cells were markedly swollen and vacuolated. On the other hand, although shortage of Mg (Ca sufficient) was without effect on growth rate down to the lowest concentration at which growth occurred, maximum growth and the proportion of viable organisms were markedly decreased. Mg-deficient organisms were appreciably elongated. Signs of Ca deficiency became apparent at less than 0·025 mM, and Mg deficiency at less than 0·1 mM, most markedly in the range below 0·5 mM. Additionally there was a need for total divalent cations of the order of 0·4–0·6 mM. This could be met by either Ca or Mg provided both were sufficient for their maximum specific effect.

INTRODUCTION

Norris (1958, 1959) reopened the question of the need of Rhizobium for calcium. He was able to maintain a large number of strains in serial subculture in a medium free from detectable calcium; by the same method dependence on magnesium was readily shown. Magnesium seems in fact to be essential for all bacteria that have so far been studied critically, as one would expect from its known role as co-factor, but evidence for dependence on calcium is less common. Such an effect has however been shown for most species and strains examined of Azotobacter (Horner & Burk, 1934; Norris & Jensen, 1957), for Nitrosomonas (Kingma Boltjes, 1935), Bacillus (Brewer *et al.* 1946; Feeney & Garibaldi, 1948), Lactobacillus (Yu & Sinnhuber, 1955), Brucella (McCullough *et al.* 1947) and in some photosynthetic bacteria (Foster, 1944; Hutner, 1946). A marked requirement for calcium has also been ascribed to Rhizobium but, as pointed out by Norris, not all the evidence is valid. However, the stimulation obtained by Pitz (1916) when calcium was added to a defined medium appears to have been real, as do the responses reported by Allyn & Baldwin (1932) and McCalla (1937), the latter in a clay having already adequate magnesium. More recently Ferry, Blachère & Obaton (1959) showed that *Rhizobium meliloti* responded to calcium. Bergersen (1961) also showed responses by this and several other species.

Calcium response and the more striking magnesium effect with the rhizobia of the temperate legumes (*Rhizobium trifolii*, *R. meliloti*) have been demonstrated in this laboratory by Davies, Mullens & Colburn (unpublished data). These observations were based on detailed growth curves obtained under conditions which per-

mitted large populations (about 5×10^9 organisms/ml.) to be attained. Clear responses were established when using a defined medium without special purification. The main trend of our results has been recently reported, particularly the striking cytological abnormalities associated with a deficiency of either calcium or magnesium (Vincent & Colburn, 1961).

Certain points of technique and procedure that need to be observed in investigations of this kind have often been overlooked. These include the following. (i) Readings to be sufficiently frequent to enable growth characteristics to be established. (ii) Other conditions of growth (e.g. nutrients, aeration) to be adequate so that the factor under test will be limiting. (iii) The medium to be chosen so that ions of one charge can be varied without concomitant variations in ions of the opposite charge, except when it has been shown that such variation does not affect growth. (iv) The specific effect of a particular ion to be distinguished from possible non-specific effects associated with the total ionic concentration. (v) Precipitation from the growth medium to be avoided by working within the solubility range of all constituents and, where necessary, by modifying sterilization procedures. The present paper applies these principles in a detailed study of the calcium and magnesium requirements of a strain of *Rhizobium trifolii*. Its chief conclusions have, however, been substantiated with several other strains of this species and with strains of *R. meliloti*.

METHODS

Organism. A non-gummy variant of *Rhizobium trifolii* (SU 298/531) was chosen for detailed study because it maintained more even turbidity in the course of the whole growth curve.

Medium. A defined medium, modified slightly from that of Norris (1958), was able to support good growth without the addition of any other organic material. The constant part of the medium consisted of 0·45 g. $Na_2HPO_4.12H_2O$; 0·029 g. Na_2SO_4, 0·6 g. KNO_3; 0·01 g. $FeCl_3.6H_2O$; 10 g. mannitol; 100 μg. thiamine hydrochloride; 0·5 μg. biotin; in 1 l. twice glass-distilled water. Calcium and magnesium were supplied as chlorides so that the concentration of anion would be unaffected by variation in the relative amounts of the cations. The maximum concentration of divalent cation (1 mM) was such that all salts remained in solution at pH 6, provided the calcium chloride and magnesium chloride solutions were sterilized separately and added to the rest of the medium aseptically. All chemicals, except the vitamins, were of analytical grade. Traces of divalent cations already present in the complete medium were kindly determined by Dr B. Davey by the atomic absorption method. Calcium in the medium containing 0·5 mM magnesium was 0·006 mM (0·25 p.p.m.); magnesium in the medium with 0·5 mM calcium was 0·007 mM (0·16 p.p.m.). Glassware was cleaned with chromic + sulphuric acid, rinsed several times in tap water and then twice with glass-distilled water.

The value pH 6 for the medium was chosen in the first instance because it allowed abundant and rapid growth of the organism whilst avoiding difficulties of solubility at higher concentrations of cations. Several experiments were, however, conducted at other pH levels.

Growth vessels and incubation. Medium (10 ml.) was dispensed into optically matched ⊥ tubes, and aeration obtained by rocking (twenty-six times a minute,

3 in. horizontal displacement) so that the shallow layer of the liquid moved from one end of the ⊥ to the other. Incubation was at 28° in a controlled water bath.

Inoculum. Surface growth at 3 days on yeast mannitol agar, but without $CaCO_3$ was rubbed off in 10 ml. sterile glass-distilled water and adjusted turbidimetrically, to provide about 20×10^6 organisms per ⊥ tube when dispensed as two drops from a standardized dropping pipette. The actual viable count of the inoculum used was determined on each occasion.

Determination of growth. Amounts of growth were determined turbidimetrically in the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with the green (404) filter. Turbidity readings greater than 30 on this intrument departed significantly from a linear relationship to concentration of organism. The readings were adjusted to conform with the extrapolated straight line of a curve constructed from a series of dilutions of a concentrated suspension. Comparisons were made with the total count on a number of occasions and in general the relationship was of the order 1 EEL unit = 50×10^6 organisms/ml. with little difference between young (28 hr.) and old (100 hr.) growth. Growth was however affected by the presence or absence of calcium (e.g. with calcium, 60×10^6/ml./unit; without, 39×10^6/ml.). The smaller value in the absence of calcium reflects the larger average dimensions of the deprived organisms.

Growth rates (r) based on logarithms to base 10, time in hr., were calculated as the line of best fit for readings in the logarithmic phase. Mean generation time can be calculated as $(\log 2)/r$, and has approximated to 3–4 hr. for the complete medium. Specific growth rate (μ) = $2{\cdot}3\,r$. From earlier experiments which had been continued for at least 168 hr., it was apparent that the maximum stationary growth could be determined more conveniently at 96–100 hr. Accurate determination of lag time is difficult in experiments of this nature, except by direct counting, which is time-consuming and imposes such limitations on replication as to be hardly practicable for regular use. Lag period determinations have therefore been limited to special cases.

The values quoted for each treatment are based on three to five, usually four, replicate tubes.

RESULTS

General

The results of one of the earlier experiments, in which the total concentration of divalent cations was kept constant at two values, are briefly summarized in Table 1. Comparison involving calcium deficiency can be made between treatments 1 and 2, 4 and 5 (same total divalent cations) and between 1 and 5 (same magnesium concentration ± calcium). In either case the response to calcium is clear. In this, and in some other experiments, a small amount of growth occurred when no magnesium was added. The results of a similarly planned, but more detailed, experiment are given in Table 2. In this case the characteristics of early growth, before turbidity reached a degree suitable for measurement in the colorimeter, were determined by viable and total counts, each at three times. Examination of the detailed growth curves in this experiment has shown that the logarithmic phase was maintained between 12 and 46 hr. Valid estimates of growth rate could therefore be made by using turbidity data representing the later part of the logarithmic phase. In fact

such rate determinations agreed very well with those based on direct counts at an earlier stage of growth.

All three estimates of the growth rate showed a clear response to calcium. The stationary population (90 hr.) also showed a marked response to this element, but the lag time was unaffected. In this and some other experiments, the tubes without added magnesium did not show any growth.

Table 1. *Response of* Rhizobium trifolii, *strain SU298/531 to calcium and magnesium*

Inoculum = $0{\cdot}12 \times 10^6$ viable organisms/ml.

Treatment no.	Concentration (mM) of Calcium	Concentration (mM) of Magnesium	Growth: Turbidity (EEL units) 40 hr.	Growth: Turbidity (EEL units) 97 hr.	Growth: Organisms $\times 10^{-6}$/ml. 40 hr.	Growth: Organisms $\times 10^{-6}$/ml. 97 hr.
1	0·5	0·5	12·9	109	775	6450
2	0	1·0	4·0	74	156	2900
3	1·0	0	1·1	4	66	240
4	0·25	0·25	9·4	96	565	5750
5	0	0·5	2·3	57	90	2200
6	0·5	0	0·3	4	18	220

Table 2. *Growth constants with and without added calcium and magnesium*

Inoculum = $2{\cdot}5 \times 10^6$ viable organisms/ml.

Concentration (mM) of Calcium	Concentration (mM) of Magnesium	Lag time (hr.)	Growth rate based on: Viable counts 16–23 hr.	Growth rate based on: Total counts 18–29 hr.	Growth rate based on: Turbidity 40–46 hr.	Growth at 90 hr.*
0·25	0·25	12	0·067	0·076	0·070	78 (5600)
0	0·5	12	0·051	0·048	0·059	24 (1150)
0·5	0	No growth detected				

* Turbidity given in EEL units; bracketed values give estimates of total cells in millions, based on factors appropriate to experiment, treatment and age of culture.

Total concentration of cations. Before proceeding to more detailed studies of growth responses with graded doses of calcium and magnesium, it seemed desirable to establish the effect of the total concentration of these ions. The data given in Table 1 (treatments 1 and 4) had already indicated that any effect above 0·5 mM was likely to be small. Table 3 combines the results of two experiments in the range 0·1 to 0·8 mM divalent cations. The stationary population reflected the total concentrations of divalent cations, particularly between 0·1 and 0·2 mM, although the growth rate was unaffected. The total effect was independent of the relative amounts of calcium and magnesium, each at least 0·05 mM.

More detailed attention could therefore be given to lower concentrations of either ion in the region of total cation concentration, 0·4 to 0·6 mM.

Table 3. *Influence of total concentration of divalent cations on growth*

Total divalent cations (mM)	Concentration (mM) of		Growth rate*		Growth at 100 hr.† (EEL units)	
	Ca	Mg	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0·8	0·7	0·1	0·092	—	89	—
	0·1	0·7	0·088	—	83	—
0·6	0·5	0·1	0·084	—	85	—
	0·1	0·5	0·081	—	78	—
0·4	0·35	0·05	—	0·079	—	73
	0·3	0·1	0·084	—	72	—
	0·2	0·2	0·080	—	76	—
	0·1	0·3	0·087	—	75	—
	0·05	0·35	—	0·080	—	73
0·3	0·25	0·05	—	0·074	—	65
	0·05	0·25	—	0·077	—	65
0·2	0·15	0·05	—	0·087	—	52
	0·1	0·1	0·082	—	62	—
	0·1	0·1	—	0·078	—	61
	0·05	0·15	—	0·076	—	55
0·1	0·05	0·05	—	0·066	—	25

* None of the differences in rate reached a 5 % level of significance.
† Differences for significance (5 %): Expt. 1 = 12; Expt. 2 = 9.

Dosage response relationships with calcium and magnesium

Figs. 1 and 2 show detailed growth curves at limiting concentrations of calcium and magnesium, respectively. Marked differences between the growth response to the two ions are immediately apparent.

Effects of calcium. As in earlier experiments, an effect on growth rate could be demonstrated with the lowest concentration of calcium (Table 4). The difference in growth rate was sufficient to explain the spacing of the calcium curves along the time axis without any increase in lag time being involved (see also Table 2). Maximum growth rate occurred at 0·005 mM. Total growth was increased up to 0·025 mM, though most steeply and linearly to 0·005 mM (Fig. 3). The relationship during the linear portion of the dose-response curve can be expressed as: $y = 29 + 9800x$, in which y is growth as EEL units, and x is the mM concentration of added Ca. Quite considerable total growth occurred even without the addition of Ca, but from the nature of the response curve this could well be accounted for by the Ca already known to be present. (See alternate scale on the abscissa of Fig. 3 allowing for 0·006 mM Ca already in the medium.)

Effects of magnesium. With magnesium on the other hand (Fig. 2; Table 5) growth rate and lag time at the lowest concentration (0·001 mM) could not be distinguished from the highest. When, as in Expt. 2, growth occurred without the addition of magnesium, the rate and lag were also normal. Experiments 2 and 3 were put up at the one time to determine whether a tenfold dilution of inoculum would result in the growth rate being decreased at lower values of added magnesium. Experiment 2 ($0{\cdot}2 \times 10^6$ organisms/ml.) showed an extended lag compared with Expt. 3 ($2{\cdot}4 \times 10^6$ organisms/ml.), but the number of organisms in the inoculum

had no effect on the magnesium response. Unfortunately the earlier readings that were needed for the determination of rate in Expt. 3 were not available. However, it was apparent from the similarity of the first readings for all concentrations that the early growth was independent of added magnesium in this experiment also.

Stationary growth data from four experiments plotted against concentration of added magnesium (Fig. 3) showed that a linear relationship ($y = 4{\cdot}9 + 1210x$) was maintained up to 0·05 mM. Again any growth that occurred without added magnesium can be accounted for by the small amount of Mg (0·007 mM) likely to be present in the basal medium.

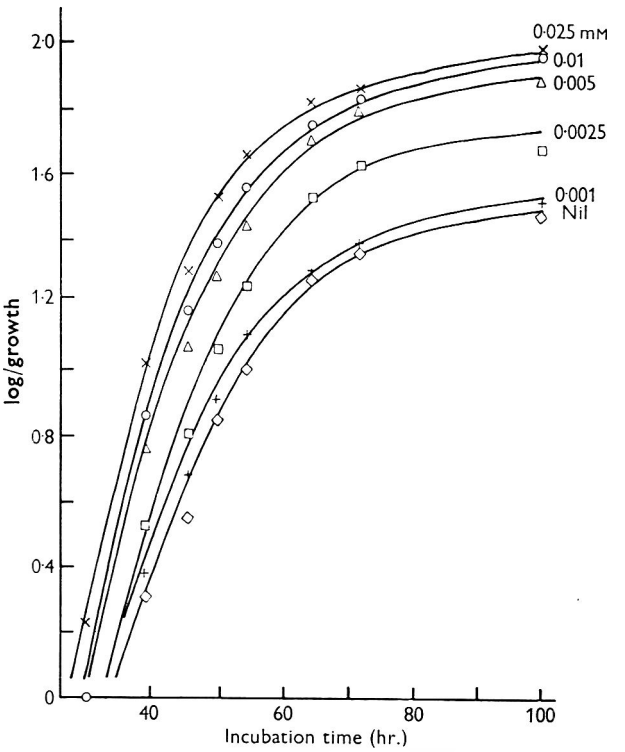

Fig. 1. Growth curves of *Rhizobium trifolii* with varying concentrations of calcium (0·025 mM, × ; 0·01, ○; 0·005, △; 0·0025, □; 0·001, +, nil, ◇). Inoculum: $0{\cdot}54 \times 10^6$ organisms/ml.; log. equivalent turbidity = approx. −2·0. Growth is expressed as turbidity (1 unit = about 50×10^6 organisms/ml.). Curves for concentrations > 0·025 mM are practically identical with that concentration and have been omitted to avoid confusion.

Relationship of pH value to need for calcium and magnesium

Table 6 combines data from two experiments and shows that calcium effects were readily demonstrated from pH 5·5 to 7. There was marked delay in the initiation of growth in one or more of the minus calcium replicates at the lowest pH values (5·5 and 5·8). Otherwise the growth rate response appeared to be independent of pH value. Nor could any consistent interaction be demonstrated between calcium and pH value in respect of total growth. Similarly the pH value in the range 5·5 to 6·6 was without effect on the magnesium response (Table 7).

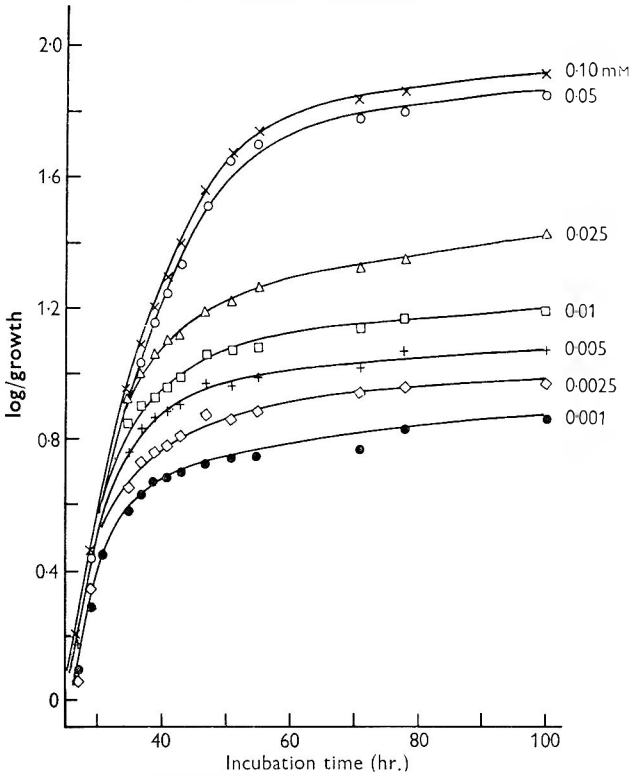

Fig. 2. Growth curves of *Rhizobium trifolii* with varying concentrations of magnesium (0·10 mM, × ; 0·05, ○; 0·025, △; 0·01, □; 0·005, + ; 0·0025, ◇; 0·001, ●). Inoculum: $1{\cdot}3 \times 10^6$ organisms/ml.; log. equivalent turbidity = approx. −1·4. Growth units as for Fig. 1.

Table 4. *Influence of concentration of calcium*

Inoculum: $0{\cdot}5 \times 10^6$ organisms/ml.

Added Ca (mM)*	Growth rate†	Growth at 100 hr. (EEL units)
0·10	0·084	100
0·05	0·085	100
0·025	0·080	99
0·01	0·086	90
0·005	0·082	78
0·0025	0·070	56
0·001	0·052	34
0	0·053	32

* Added to medium containing 0·5 mM Mg.
† Differences for significance: 5 %, 0·017; 1 %, 0·023.

Influence of calcium and magnesium on viability

A deficiency of either calcium or magnesium decreased the proportion of viable organisms. This was found for all stages of growth in the absence of calcium, and at 100 hr. for several concentrations of magnesium (Table 8). The effect of Mg was most striking, the proportion of viable organisms being almost directly related to concentration of magnesium.

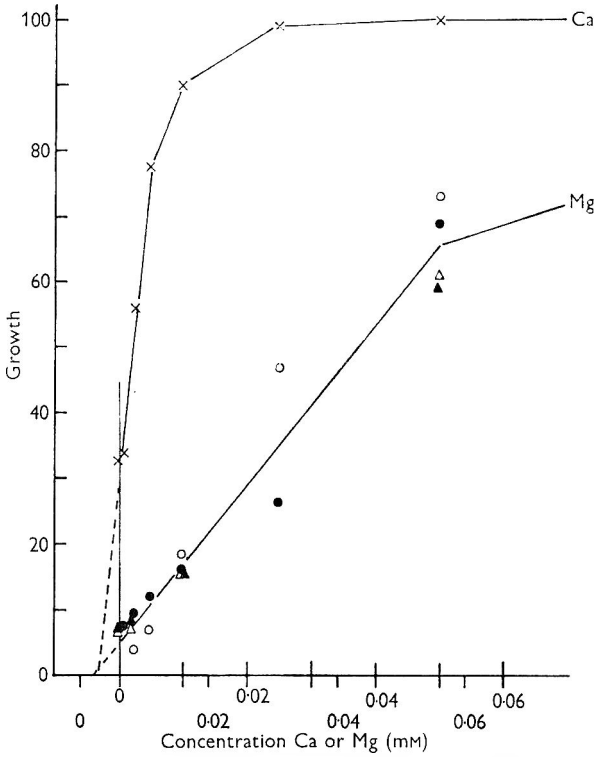

Fig. 3. Stationary growth in relation to concentration of calcium and magnesium. Growth units as for Fig. 1. Abscissa gives the concentration of variable cation (the other in excess): (i) upper set, amount added; (ii) lower set allowing for calculated amount in the basal medium. Mg curve uses points from four experiments as identified by symbols.

Table 5. *Influence of concentration of magnesium*

Experiment: 1 2 3 4
Inoculum: 1·3 0·2 2·4 $0{\cdot}5 \times 10^6$ organisms/ml.

Added Mg (mM)*	Growth rate†		Lag time‡			Growth at 100 hr. (EEL units)			
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3	Expt. 4
0·10	0·092	—	9	—	—	81	—	—	87
0·05	0·099	0·112	9·5	14	11	69	61	59	73
0·025	0·097	—	9	—	—	26	—	—	47
0·01	0·106	0·105	9·5	17	11	16	15·4	15·4	18·5
0·005	0·097	—	9·5	—	—	12	—	—	7·0
0·0025	0·118	—	10·5	—	—	9·5	—	—	3·9
0·002	—	0·107	—	14·5	11	—	7·4	8·1	—
0·001	0·088	—	11	—	—	7·3	—	—	—
0	—	0·102	—	15	12	—	6·6	6·9	—

* Added to medium containing 0·4 or 0·5 mM Ca.
† None of the differences within experiments was significant (5 % probability).
‡ The additional lag time in Expt. 2 reflects smaller inoculum (different starting value allowed for).

Table 6. *Calcium response in relation to pH value*

Total divalent cations = 0·5 mM

pH	Expt.	Growth rate.* Calcium (mM) 0·25	Growth rate.* Calcium (mM) 0	Growth at 100 hr. (EEL units). Calcium (mM) 0·25	Growth at 100 hr. (EEL units). Calcium (mM) 0
5·5	2	0·076	0·060†	94	29†
5·8	1	0·086	n.d.‡	71	52‡
5·9	2	0·078	0·052	96	31
6·1	1	0·091	0·071	73	41
6·3	2	0·075	0·053	91	37
6·5	1	0·083	0·070	72	65
6·6	2	0·075	0·038	88	27
7·0	1	0·081	0·076	70	60

Notes

* Differences for significance in Expt. 2: 5 %, 0·010; 1 %, 0·013. Differences in Expt. 1 did not reach significance, but are in good agreement with the later experiment.

† Based on three of the four replicates. The fourth failed to grow in 100 hr.

‡ Growth too late for reliable determination of rate; maximum growth based on the two of the three tubes that grew.

Table 7. *Magnesium response in relation to pH value*

All treatments contained 0·5 mM Ca.

pH	Growth rate Magnesium (mM) 0·1	Growth rate Magnesium (mM) 0·01	Growth at 96 hr. (EEL units) 0·1	Growth at 96 hr. (EEL units) 0·01
5·5	0·077	0·078	93	10·2
5·9	0·080	0·076	89	11·3
6·2	0·074	0·084	94	12·8
6·6	0·080	0·073	92	10·7

Table 8. *Ratio of viable to total organisms, in relation to calcium and magnesium*

		Time (hr.) 28	52	76	96	100
		Ratio of viable/total count				
(*a*) Calcium* (mM)	0·1	0·9	1·2, 1·1	1·0	0·8	1·0, 0·9
	0	0·5	0·4, 0·2	0·3	0·6	0·3, 0·4
		All at 100 hr.				
(*b*) Magnesium† (mM)	0·1		0·7, 0·8			
	0·01		0·1			
	0·001		0·02			
	0		0·003			

* All tubes contained 0·4 mM Mg.

† All tubes contained 0·5 mM Ca.

DISCUSSION

There is no doubt that a deficiency of either magnesium or calcium decreased the total amount of growth of the strains of *Rhizobium trifolii* and of the several cultures of *R. meliloti* studied. The signs of deficiency in the two cases are however distinctive. Although a relatively large amount of magnesium (0·05–0·1 mM) was required for maximum total growth, the growth rate was unaffected at concentrations at least as low as 0·007 mM. On the basis of total growth, shortage of calcium only became apparent below 0·01 mM, but then the dose response relationship was a very steep one. Below 0·005 mM added Ca, the growth rate was also affected.

These results show the need for continuous growth data, rather than depending on roughly quantitative assessments of growth in serial subculture. The values thus obtained have also to be related to the concentration of the substance concerned. It is not surprising, for example, that Loneragan & Dowling (1958) failed to obtain any response to added calcium in a medium already containing 0·16 mM Ca. Examination of Fig. 3 explains why it is so much easier to demonstrate a deficiency of magnesium than calcium. An equal increment of total growth requires about an eight times greater molar concentration of Mg than of Ca; a large part of the organism's requirement for Ca can obviously be met by a low concentration of Ca as impurity. The small amount of growth that occurred in some experiments without added magnesium could also be attributed to a low concentration of Mg as impurity.

It is also important to pay attention to other signs of nutrient deficiency, e.g. in the present instance decreased viability and abnormal cell morphology. The latter reflects very clearly the element concerned. The grossly swollen and vacuolated cells obtained with a Ca deficiency are the more striking and suggest a loss of rigidity in the cell wall. It might have been expected that this condition would have been reflected in greater osmotic fragility but, although some experiments in which calcium-deficient organisms were exposed to distilled water gave indications of swelling and accelerated rate of loss of viability, the experiments as a whole were inconclusive about this point. Analyses recently carried out in this laboratory by Mrs B. A. Humphrey (personal communication) show that calcium-deprived organisms contained only about one-tenth the amount of calcium as found in normal organisms. Almost all of the calcium of the deficient cells is located in the wall fraction. It seems likely that Ca plays a role in providing a rigid cell wall. This is being investigated.

Closer examination of organisms grown in a medium markedly deficient in magnesium have shown them to be appreciably longer than normal. This elongation is not unlike the condition observed with other bacteria grown in a magnesium-deficient medium and commonly attributed to a failure of normal cell division. This might also explain the very low viability that was found in the present experiments at the lower limiting concentrations of magnesium.

I would like to thank Mrs C. H. Jancey for expert assistance throughout the course of this work. Preliminary experiments were undertaken in this laboratory by Miss Jean Davies, Miss Rosemary Mullens and the late Mr J. Colburn as honours

and post-graduate projects. The work has been supported by the Rural Credits Division of the Commonwealth Reserve Bank, the Research Committee of the University of Sydney and Wheat Industry Research Funds.

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J. gen. Microbiol. (1962), 28, 665–670
Printed in Great Britain

Melanin Biosynthesis by *Streptomyces lavendulae*

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(*Received* 14 *November* 1961)

SUMMARY

Streptomyces lavendulae was studied in a defined medium. When tyrosine was added to the medium a brown-black pigment was synthesized; without tyrosine, only a trace of this pigment was detected. Growth was the same with or without tyrosine. The addition of (2-^{14}C) DL-tyrosine to the medium resulted in synthesis of a radioactive pigment, and tyrosinase activity was demonstrated in extracts of disrupted organisms. Of 15 amino acids added to the medium only tyrosine and tryptophan were used for pigment formation. Pigment production was inhibited by *p*-benzyloxy-phenol, an inhibitor of mammalian melanogenesis. In yeast+glucose medium a pH range of 6·8–8·2 was optimal for pigment synthesis; more pigment was formed at 20° than at 28° in spite of the fact that there was twice as much growth at the latter temperature. The pigment was precipitated from culture media by a method described for precipitating melanin from urine and in every way tested was identical with a synthetic dihydroxyphenylalanine-melanin. The data indicate that the brown-black pigment synthesized by *S. lavendulae* is melanin.

INTRODUCTION

Pigment synthesis by streptomycetes has aroused considerable interest for many years. The brown-black pigment has been referred to as 'melanin', and as 'melanin-like' because of demonstrations of an involvement of tyrosine in its synthesis. In this regard Skinner (1938) observed that pigmentation resulted from the addition of tyrosine to a defined medium upon which he was growing some actinomycetes and Douglas & San Clemente (1956) observed a dark brown pigment in flasks in which mycelial homogenates of *Streptomyces scabies* were mixed with tyrosine, dihydroxyphenylalanine, or both. In attempting to correlate pigment formation and antibiotic synthesis by *S. antibioticus* Sevcik (1957) manometrically determined the presence of phenol oxidase activity, with tyrosine and several other substrates. While these studies suggest that the pigment is melanin, apparently no attempts have been made to isolate and characterize the pigment. *Bergey's Manual* (1957) refers to the pigment as a 'soluble brown pigment'. The present paper reports an attempt to determine whether the brown-black pigment of *S. lavendulae* is actually melanin.

METHODS

Streptomyces lavendulae (no. 3515, Institute of Microbiology, Rutgers University, U.S.A.) was used throughout. Stock cultures of the organism were maintained on yeast+glucose agar, and yeast+glucose liquid medium was used as the seed

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medium as well as for some preliminary observations. A defined medium (GGG) was used for most experiments. It contained (g.): glucose, 5·0; glycine, 10·0; monosodium glutamate, 10·0; K_2HPO_4, 0·5; $MgSO_4.7H_2O$, 0·2; (mg.): $CaCl_2.2H_2O$, 11·0; $FeSO_4.7H_2O$, 15·0; $ZnSO_4.7H_2O$, 13·0; $CuSO_4$, 7·5; distilled water to 1 litre. The medium without glucose (glucose autoclaved separately) was adjusted to about pH 7·5 and then autoclaved at 120° for 15 min. The medium after autoclaving and the addition of glucose was at pH 7·2–7·5. Pigment formation was readily observed because the medium itself was colourless.

Each 100 ml. of medium in a 250 ml. Erlenmeyer flask was seeded with 2·0 ml. washed suspension of organism grown for 18–24 hr. in yeast + glucose liquid medium. Organisms used for inoculation of defined medium were washed by centrifugation and resuspension until two successive washings were colourless; usually four to five washings were adequate. Except for temperature studies, cultures were incubated at 28° on a rotary shaker at 220 rev./min. and were routinely harvested by filtration after one week of incubation. Growth was determined as dry weight.

Relative values of pigment synthesis were obtained by determining % transmission of culture fluid at 540 mμ. using a Bausch and Lomb Spectronic 20 Colorimeter. The pigment was precipitated from culture fluids by a modification of a method described by Blackberg & Wanger (1933) for precipitating melanin from urine. For this culture fluid was first adjusted to pH 7·0 and 1 g. potassium persulphate was added for each 100 ml. of fluid and allowed to stand for 2 hr. with occasional shaking. Then 100 ml. of methanol was added for each 100 ml. of culture fluid and the mixture allowed to stand for 3 days; during this time pigment was precipitated, and was collected and washed by centrifugation. Pigment synthesized under various conditions was compared colorimetrically after dissolving the precipitated pigment in 10·0 ml. M-NaOH.

Synthetic DL-dihydroxyphenylalanine (dopa)-melanin was prepared by the method of Arnow (1938); 20 mg. of DL-dopa were dissolved in 50·0 ml. 0·01 M-NaOH and aerated by bubbling air saturated with water through the solution for 1 hr. The resulting brown-black precipitate was collected and washed by centrifugation.

To prepare cell extracts, washed organism (equiv. about 1·0 g. dry wt.) from an 18 hr. shake culture was suspended in 50·0 ml. 0·1 M-phosphate buffer (pH 6·9) and disrupted at maximum power in a 10 Kc Raytheon sonic oscillator for 3 min., whole organisms were separated from the suspension by centrifugation for 10 min. Tyrosinase activity was determined by a method of Cooper & Brown (1956) in which the cell extracts were added to holes cut in a phosphate-buffered agar plate containing tyrosine, dopa, or both together as substrates. Control plates were run without substrate. A positive reaction resulted in the formation around the hole of a pink ring which changed to brown and then black.

An inhibitor of melanogenesis, *p*-benzyloxy-phenol, was dissolved in methanol. Solutions were prepared on the day of use.

Radioactive pigment was assayed in a liquid scintillation spectrometer (Packard Tri-carb).

RESULTS

The effect of tyrosine on pigment biosynthesis in a defined medium

To determine the effect of tyrosine on pigment synthesis by *Streptomyces lavendulae* various concentrations of tyrosine were tested in the defined medium described above. An increase in tyrosine concentration did not result in an increase or decrease in yield of organism nor did it have an effect on the pH value of the culture (Table 1). The principle effect of the tyrosine was on the amount of pigment synthesized: as the tyrosine concentration increased, the amount of pigment increased. To substantiate further the role of tyrosine in the formation of pigment, (2-^{14}C) DL-tyrosine was added to the defined medium, resulting in the synthesis of a radioactive pigment.

Table 1. *Effect of tyrosine on pigment synthesis by* Streptomyces lavendulae *in a defined medium*

Tyrosine concentration (g./l.)	Resuspended pigment (% transmission at 540 mμ)*	Yield of organism (mg. dry wt.)*	pH value of culture†
Control (no tyrosine)	80	201	8·4
	83	165	8·3
0·1	76	152	8·4
	68	162	8·5
0·2	52	151	8·5
	52	171	8·5
0·3	39	147	8·5
	36	149	8·5
0·4	12	168	8·5
	16	166	8·5

* Values are those of duplicate flasks run at the same time.
† Duplicate flasks; after incubation for 1 week at 28°.

Demonstration of tyrosinase activity

The presence of a tyrosinase in *Streptomyces lavendulae* was shown by the tyrosinase assay described. At 20°–24° the extract of sonically disrupted organisms began to oxidize dopa within 3 min. as evidenced by the appearance of a pink ring around the extract-containing hole; this ring became dark brown to black within 1 hr. Autoxidation of dopa was not the cause of the colour change; if it were, the entire plate would have changed to the same colour. Tyrosine alone was oxidized by the extract but at a slower rate than dopa since the oxidation became apparent in 30 min. Catalytic concentrations of dopa added to the tyrosine did not shorten this period. All controls were negative. At 37° only dopa was oxidized by the extract. Removal of copper by adding sodium diethyldithiocarbamate to a system containing dopa + cell extract prevented a positive tyrosinase reaction.

Inhibition of pigment biosynthesis

p-Benzyloxy-phenol is an inhibitor of melanogenesis (Lerner & Fitzpatrick, 1950; Peck & Sobotka, 1941). To investigate the effect of this inhibitor on *Streptomyces lavendulae* different concentrations of it were added to a complete medium

containing 0·3 g. tyrosine/l. With increasing concentration of inhibitor there was a decrease in the amount of pigment synthesized (Table 2). However, cell synthesis appeared to be slightly stimulated by these concentrations of inhibitor. Concentrations of $1{\cdot}0 \times 10^{-4}$ M-*p*-benzyloxy-phenol inhibited cell synthesis as well as pigment synthesis. The pH values were only slightly lower in the media containing *p*-benzyloxy-phenol. The effect of this compound was an inhibition of pigment synthesis without inhibition of cell synthesis.

Table 2. *Inhibition of* Streptomyces lavendulae *pigment synthesis by* p-*benzyloxy-phenol in defined medium containing* 0·3 *g. tyrosine/l.*

p-benzyloxy-phenol $\times 10^{-5}$ M	Resuspended pigment (% transmission at 540 mμ)*	Yield of organisms (mg. dry wt.)*	pH value of culture†
3·0	94	190	8·2
	89	180	8·3
2·5	89	237	8·3
	81	204	8·5
2·0	91	205	8·4
	90	218	8·4
1·5	79	160	8·4
	85	175	8·6
1·0	72	160	8·5
	80	174	8·7
0·5	57	178	8·4
	56	178	8·5
Control A‡	60	107	8·8
	54	149	8·7
Control B	97	102	8·9
	94	95	8·9

* Values are those of duplicate flasks run at the same time.
† Duplicate flasks; after incubation for 1 week at 28°.
‡ Control A: no *p*-benzyloxy-phenol.
Control B: no *p*-benzyloxy-phenol, no tyrosine.

Comparison of the pigment from Streptomyces lavendulae *with a synthetic dopa-melanin*

A comparison of the brown-black pigment synthesized by *Streptomyces lavendulae* and a sample of synthetic dopa-melanin showed them to be identical in several chemical and physical properties (Table 3). Their solubilities were the same, they reacted to oxidation and reduction in the same way, were precipitated by the same techniques, and neither exhibited any characteristic absorption peaks.

Effect of amino acids other than tyrosine on pigment biosynthesis

To examine the possibility that amino acids other than tyrosine have an effect on pigment biosynthesis by *Streptomyces lavendulae*, the following amino acids were added singly to the defined medium at 0·4 g./l.: DL-alanine; L-arginine; DL-serine; DL-lysine monohydrochloride; DL-phenylalanine; sodium aspartate; L-histidine monohydrochloride; DL-leucine; L-proline; DL-threonine; DL-valine; L-tryptophan.

Glycine and sodium glutamate were present as components of the synthetic medium. Of the fifteen amino acids tested only tyrosine and tryptophan were used for pigment synthesis. The pigment which resulted from tryptophan utilization was identical with the tyrosine pigment in all of the properties listed in Table 3. Phenylalanine, reported to be an inhibitor of melanogenesis (Lerner & Case, 1959), actually stimulated pigment synthesis in the presence of tyrosine.

Table 3. *Comparison of pigment of* Streptomyces lavendulae *with a synthetic dopa-melanin*

Property	Pigment of *S. lavendulae*	Dopa-melanin
Solubility in water:	Insoluble	Insoluble
Solubility in M-NaOH:	Soluble	Soluble
Colour:	Brown-black	Brown-black
Appearance in water:	Evenly dispersed floc	Evenly dispersed floc
Precipitation by Blackberg–Wanger technique:	Precipitated	Precipitated
Precipitation from NaOH by HCl	Precipitated	Precipitated
Formation of black precipitate with $FeCl_3$ which dissolves with excess $FeCl_3$	+	+
Reduction by sodium dithionite and re-oxidation by potassium ferricyanide	Black → tan Tan → black	Black → tan Tan → black
Absorption spectrum (350–650 mμ)	No characteristic peaks	No characteristic peaks

The effect of pH value and temperature on pigment biosynthesis

Yeast+glucose liquid medium adjusted to eleven different values from pH 5·2 to 8·5 was inoculated with *Streptomyces lavendulae*. The range pH 6·8–8·2 was most favourable for pigment synthesis. Yeast+glucose liquid medium seeded with *S. lavendulae* and incubated at 37°, 28° and 20° yielded more pigment at 20° than at 28°, in spite of the fact that there was only one half as much yield of organism. At 37° the yield of organism was equal to that obtained at 20°, but only a trace of pigment could be detected.

DISCUSSION

The influence of tyrosine on pigment production by *Streptomyces lavendulae* is like melanin formation in other cells (Raper, 1927; Mason, 1948; Lerner, 1953). Although the disappearance of tyrosine from the medium was not followed it is probable that some tyrosine also was used for growth in spite of the fact that no net increase was observed in amount of organism dry weight. However, the tyrosine appeared to be preferentially utilized for pigment synthesis. A qualitative observation showed its incorporation into the pigment when (2-^{14}C) DL-tyrosine was supplied in the medium. The utilization of tryptophan for pigment synthesis is not surprising because tryptophan is a precursor of kynurenine and anthranilic acid which can be metabolized to catechol. It is possible that melanin was synthesized by this route with catechol as substrate for tyrosinase. The tryptophan pigment had physical and chemical characteristics identical with the tyrosine pigment and with synthetic dopa-melanin. Conditions of synthesis, inhibition, and the physical and chemical

characteristics of the precipitated pigment correspond to those known for dopa-melanin. Because of these data it is submitted that the brown-black pigment of *S. lavendulae* is in fact melanin.

The authors wish to thank Dr Ruth E. Gordon, Institute of Microbiology, Rutgers University for the cultures of *Streptomyces lavendulae*. This investigation was supported in part by P.H.S. grant E-3119 for the National Institutes of Health, U.S.A.

One of the authors, J.R.M., was a trainee of U.S.P.H.S. Grant 2E–183.

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J. gen. Microbiol. (1962), 28, 671–687
Printed in Great Britain

Transmission of Colicinogeny between Strains of *Salmonella typhimurium* Grown Together

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(*Received* 17 *November* 1961)

SUMMARY

Ability to produce colicines I, E1, E2, K or B was transferred to *Salmonella typhimurium* strain LT2 by growth in broth with suitable colicinogenic strains of *Escherichia coli* or *Shigella sonnei*. When LT2 (*colI*), i.e. carrying the colicine I factor, or LT2 (*colB*) were grown overnight in broth with LT2 *col*⁻ (non-colicinogenic), *c.* 50 % of the latter became colicinogenic; LT2 (*colE2*) and LT2 (*colK*) did not transmit; LT2 (*colE1*) transmitted to only *c.* 0·1 % of the acceptor population. But LT2 carrying either *colI* or *colB* in addition to *colE2*, *colK* or *colE1*, transmitted both factors.

When overnight broth cultures of LT2 (*colI*) and LT2 *col*⁻ were mixed and incubated *c.* 40 % of the latter acquired *colI* by 20 hr. (when the viable count had doubled); but only *c.* 0·02 % acquired *colI* in 3 hr. The low initial transfer results from the fact that in a stock culture of LT2 (*colI*) only *c.* 1/5000 bacteria are 'competent donors', able to transmit *colI*. The later large increase in the proportion of colicinogenic bacteria probably results from 'epidemic spread' of the *colI* factor amongst the acceptor population, initiated by the few acceptor bacteria which originally receive it. It is supposed that most bacteria which have just acquired *colI* become competent donors. In a doubly colicinogenic strain most competent donors transmit both colicine factors.

Aeration by shaking during incubation interfered with transmission of colicinogeny, probably by abolishing the prolonged phase of slow growth of unaerated cultures. Growth in the presence of acriflavine did not 'cure' LT2 (*colI*) or LT2 (*colI*) (*colE2*) of colicinogeny, nor of ability to transmit.

LT2 (*colE1*) and LT2 (*colE2*) supported the epidemic spread of *colI* or *colB* about as well as did LT2 *col*⁻; but in LT2 (*colK*) the spread of *colI* was greatly reduced and that of *colB* somewhat reduced. The prior presence in an acceptor strain of one of the readily transmissible factors, *colI* or *colB*, did not interfere with the epidemic spread of the other. But LT2 (*colI*) did not become a competent donor on accepting *colE2* and, by inference, *colI* from LT2 (*colI*) (*colE2*).

INTRODUCTION

Colicines are antibiotics produced by some strains of Enterobacteriaceae and active on others; colicinogeny, i.e. the ability to produce a colicine, is a stable heritable property of many Escherichia and Shigella strains (for review see Fredericq, 1957). It is a character of special interest to the bacterial geneticist; for

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some colicinogenic strains of *Escherichia coli* and *Shigella* sp. when grown in broth with non-colicinogenic strains transmit their colicinogenic property to some cells of the latter (Fredericq, 1954*a*; Hamon, 1956). Colicinogeny could not be transmitted by culture filtrates. It is inferred that the character is transmitted from one bacterium to another by the transfer of a genetic determinant, during some kind of conjugation. We refer below to the postulated genetic determinant for colicinogeny as a colicine factor, indicating the colicine concerned by the symbol assigned to it by Fredericq. Thus; colicine factor I or, for short, *colI*, *colE2*, etc.

Experiments on the transmission of a colicine factor 'ER' (also termed 'K30', and falling in group E1; see Fredericq (1957)) between F^+ and F^- sublines of *Escherichia coli* strain K12 showed that only F^+ strains transmitted colicinogeny; that all recombinants from crosses of F^+ non-colicinogenic and F^- colicinogenic parents, and many from crosses of F^+ colicinogenic and F^- non-colicinogenic parents, were colicinogenic; and that in the latter cross there was no detectable linkage between colicinogeny and any other character which was segregating (Fredericq & Betz-Bareau, 1953*a*,*b*; Fredericq, 1954*b*). It was inferred that colicinogeny resulted from possession of a non-chromosomal factor, transmissible during conjugation mediated by the *F* agent. However, experiments on the inheritance of the same colicine factor ('K30') in crosses of *Hfr* with F^- sublines of *E. coli* K12 (Alfoldi, Jacob & Wollman, 1957; Alfoldi, Jacob, Wollman & Maze, 1958) showed that the colicinogenic or non-colicinogenic character could be mapped at a particular point on the bacterial chromosome. Thus it seems that a colicine factor may be either located on the bacterial chromosome, being then transmissible only when the relevant portion of chromosome enters another bacterium; or may occupy some non-chromosomal site, and be transmissible by conjugation even in the absence of chromosomal transfer. Jacob & Wollman (1958) defined as 'episomes' those non-essential heritable agents of bacteria which could be acquired or lost and having two alternative states; either on the chromosome, multiplying *pari passu* with it; or not on the chromosome, multiplying autonomously, sometimes more rapidly than the chromosome. The *F* factor of *E. coli*, the colicine factor 'K30' of *E. coli* and certain temperate phages (or prophages) of *E. coli* were episomes as thus defined (Jacob, Schaeffer & Wollman, 1960).

When the present work was begun, no genetic recombination by cell conjugation, mediated by the *F* factor or otherwise, had been reported in Salmonella or between Salmonella and Escherichia (but see Miyake & Demerec, 1959; Baron, Carey & Spilman, 1959; Zinder, 1960). We therefore investigated the transmission of colicinogeny between Salmonella strains grown together, a phenomenon observed by Hamon & Stocker (unpublished) and probably resulting from cell conjugation, hoping that fuller knowledge of this process would enable us to obtain recombination of the chromosomal genes of Salmonella by conjugation. This expectation has been realized (Ozeki & Howarth, 1961; Smith & Stocker, 1962; see also Stocker, 1960). In the present paper we describe the preparation of colicinogenic derivatives from nutritionally exacting sublines of *Salmonella typhimurium* strain LT2 and observations on the ability of such sublines to transmit their colicinogenic property to other LT2 sublines when the strains are grown together. Standard strains of *Escherichia coli* and *Shigella sonnei* producing known colicines, supplied by Professor P. Fredericq, were used as primary sources of various colicine factors; the process

of transmission from strains of these genera to *S. typhimurium* has not been investigated in detail and, in particular, we have not tested the *F* character of the donor strains.

We were for long unable to obtain transmission of colicinogeny to a substantial proportion of the cells of a non-colicinogenic LT2 strain by contact with a colicinogenic strain for short periods; the present paper therefore concerns results obtained when the colicinogenic and non-colicinogenic LT2 strains were incubated together in broth for some hours. We later found (Stocker, Smith & Ozeki, in preparation) that *Salmonella typhimurium* cultures which have just acquired the ability to produce colicine I transmit this character to a high proportion of the bacteria of a non-colicinogenic strain in a short time, because a high proportion of the cells of such newly infected cultures can conjugate; we state this conclusion now because it helps in the interpretation of the results described below.

Salmonella typhimurium strain LT2 does not produce any detectable colicine; and it is resistant to all the colicines we have used, viz. I, E1, E2, B and K.

METHODS

Media. Nutrient broth was made from a tryptic digest of beef. The minimal medium contained: K_2HPO_4, 10·5 g.; KH_2PO_4, 4·5 g.; $MgSO_4$, 0·05 g.; $(NH_4)_2SO_4$, 1 g.; sodium citrate, 0·47 g.; glucose, 2 g.; water, 1000 ml. The concentrations of nutritional supplements were as described by Lederberg (1950). Streptomycin was used at 1 mg./ml. These media contained 1·5% agar for plate culture, and 0·35% for soft agar. Bacterial strains were kept on Dorset egg slopes at laboratory temperature.

Bacterial strains. Table 1a shows the strains of *Escherichia coli* and *Shigella*, colicinogenic for known type(s) of colicine, from which various colicinogenic properties were transferred to strains of *Salmonella typhimurium*.

Table 1b lists the auxotrophic derivatives of *Salmonella typhimurium* strain LT2 used. A cysteine-exacting mutant, *cysD–36*, was used in many experiments, because it is very stable, reversion to prototrophy having never been observed (Clowes, 1958). Mutants resistant to streptomycin were isolated from these stocks. Some naturally colicinogenic strains of *S. typhimurium* were also used. Among strains supplied by Dr E. S. Anderson, of the Enteric Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9, 41 strains of 280 tested were found to be colicinogenic (Stocker, Ozeki & Anderson, unpublished). Strains LT7 and LT22 (Zinder & Lederberg, 1952), widely used in experiments on the genetics of *S. typhimurium*, were also observed to be colicinogenic, producing colicine I.

Table 1*c* records the colicine-sensitive *Escherichia coli* strains, and their derivatives, used in testing for the production of colicines, i.e. colicine-indicator strains. For the most part these were colicine-resistant mutants of sensitive strains, either obtained from Professor P. Fredericq or isolated by us from colonies appearing in colicine inhibition zones. The E group of colicines are defined by the resistance to all E colicines and to phage BF 23 of mutants obtained from sensitive strains by selection with any E colicine or with phage BF 23; within this group colicine E1 is recognized by the specific immunity to colicine E1 of strains made colicinogenic for colicine E1; and colicine E2 is similarly recognized by the specific immunity to

colicine E2 of derivatives producing colicine E2 (Fredericq, 1956). We confirmed Fredericq's observation on the resistance pattern of *E. coli* K12 lines made colicinogenic for E1 or E2, and used strain CL136, a K12 stock producing colicine E1, as an indicator sensitive to E2 but resistant to E1. We also confirmed Fredericq's observation that mutants selected for resistance to E1 are resistant to E2; but in stocks derived from *E. coli* strain ϕ of Gratia (1925) we found that mutants selected for resistance to E2 were still sensitive to E1, though less so than originally, and we used two such mutants as differential indicators.

Table 1. *Bacterial strains used*

(*a*) Standard colicinogenic strains, used as source of *col* factors: received from Professor P. Fredericq, Liège

Strain	Colicines produced
Shigella sonnei P9	I and E2
Escherichia coli K12–30	E1
Escherichia coli K77	B
Escherichia coli K49	K

(*b*) *Salmonella typhimurium* strain LT2, auxotrophic mutants

Strain no.	Character	Reference
cysD–36	requires cysteine	Clowes (1958)
cysC–7	requires cysteine	Clowes (1958)
athC–5	requires adenine and thiamine	Yura (1956)

Mutants of these strains resistant to streptomycin (1 mg./ml.) are indicated by *str-r* added to strain no. Colicinogenic derivatives are indicated by addition in parentheses of symbol for colicine agent concerned, e.g. *cysC*–7 (*colI*) indicates a derivative of strain *cysC*–7 producing colicine I.

(*c*) Colicine indicator strains

Strain no.	Sensitivity to colicine I	E1	E2	B	K	Streptomycin sensitivity (1 mg./ml.)	Origin
CL 18	S	S	S	S	S	S	*E. coli* ϕ of Gratia (1925)
CL104	S	S	S	S	S	R	*str-r* mutant of CL18
CL147	R	S	S	r	S	S	Colicine I-res. mutant of CL18
CL148	S	S	R	S	S	S	Colicine E2-res. mutant of CL18
CL150	R	S	S	r	S	R	Colicine I-res. mutant of CL104
CL151	S	S	R	S	S	R	Colicine E2-res. mutant of CL104
CL152	R	S	R	r	S	R	Colicine I-res. mutant of CL151
				—I—			
CL 56	S	S	S	S	S	S	*E. coli* K12–W677, *thr*, *leu*, *thi*
CL135	S	S	S	R	S	S	*E. coli* K12–W1177/V*
CL136	S	R	S	S	S	R	*E. coli* K12–30*, carries *colE1*
CL184	R	R	S	r	S	R	Colicine I-res. mutant of CL136
CL185	S	R	R	S	S	R	Colicine E2-res. mutant of CL136
CL142	S	S	S	S	S	R	*E. coli* K12-Row*
CL143	R	S	S	S	S	R	*E. coli* K12-Row/V*
CL144	r	S	S	R	S	R	*E. coli* K12-Row/B*
CL145	S	R	R	S	S	R	*E. coli* K12-Row/E*
CL146	S	S	S	S	R	S	*E. coli* K12-Row/K*

S = sensitive; r = partly resistant; R = completely resistant. * = strains received from Professor P. Fredericq, of Liège, designated as shown.

Culture methods. Cultures were grown at 37°. Broth cultures were usually grown, without aeration, in loosely capped containers; in some experiments 5 ml. cultures in 20 ml. bottles (loosely capped) were grown on a shaker (throw 9 cm., 100 strokes/min.).

Test for the production of colicine. To test for the production of colicine by colonies grown either from single bacteria or from stab inocula, the bacteria were killed by chloroform vapour and the plate covered with a layer of soft agar seeded with *c.* 10^8 bacteria of an indicator strain; after overnight incubation colonies of colicinogenic bacteria were surrounded by clear zones. In experiments involving two or more colicines, the colicine or colicines produced were identified by the use of a set of test plates, each layered with a different indicator, resistant to one or more colicines.

Preparation of colicinogenic derivatives of Salmonella typhimurium *LT2 by contact with standard colicinogenic* Escherichia *and* Shigella *strains*

Sublines of strain LT2 producing each of the colicines I, B, E1, E2 or K, or combinations of them, were obtained by growing strain LT2 in mixed culture with appropriate 'donor' strains of *Escherichia coli* or *Shigella* sp. producing these colicines: 10 ml. broth was inoculated with about equal volumes (0·1 ml; or a loopful) of broth cultures of the colicinogenic (donor) strain and a subline of strain LT2, generally one marked by nutritional requirements, etc. After overnight incubation, colicinogenic clones of the LT2 component of the culture were isolated, the procedure used varying according to the proportion of the LT2 bacteria which had acquired colicinogeny. When this proportion was high, the overnight mixed culture was streaked on nutrient agar and Salmonella colonies, recognizable by their smoothness, were picked and tested for colicinogeny. When the proportion was low the mixed culture was so treated that only the Salmonella component would grow when the mixture was plated; either the donor bacteria were killed by exposure to a suitable colicine (a chloroform-killed broth culture of a colicinogenic strain); or the mixture was plated on a medium, e.g. streptomycin agar, on which only the Salmonella strain could grow. Rare colicinogenic colonies amongst a large number of non-colicinogenic ones were then detected and isolated in one of the following ways: (i) Replica plates (Lederberg & Lederberg, 1952) were tested for the presence of colicinogenic colonies; colonies corresponding to colicinogenic ones were then picked from the master plate. (ii) A first soft-agar layer inoculated with a suitable number of bacteria from the mixed culture was covered by a second uninoculated layer and after incubation a further layer containing indicator bacteria was added; after further incubation colicinogenic colonies underlying the centres of inhibition zones were picked (Fredericq, 1954*a*). (iii) When the proportion of Salmonella made colicinogenic was very small an inoculum of up to 10^7 Salmonella was plated on a medium which would support only a very limited growth of the Salmonella, for instance unsupplemented minimal medium when the acceptor strain (*cysD–36*) required cysteine; after incubation the plate (not chloroformed) was covered with a layer of soft minimal agar supplemented with threonine, leucine and thiamine, and containing indicator bacteria (CL56) requiring these factors. After overnight incubation the indicator bacteria (but not the cysteine-exacting Salmonella) had grown and tiny inhibition zones were observed in the confluent growth; a small piece of agar containing a zone was cut out and suspended in broth, and from this (by

repeating the same procedure, if necessary) the colicinogenic Salmonella clone could be isolated.

Singly colicinogenic derivatives of strain LT2. The standard colicine-producing strains used as donors of colicinogeny and the approximate proportion of LT2 cells which in a typical experiment became colicinogenic during overnight mixed culture are recorded in Table 2. Factors I and E2 were both obtained from *Shigella sonnei* strain P9, which produces both these colicines. After overnight culture of an LT2 subline with strain P9 about 50% of the LT2 cells had acquired *colI*, and about 5% *colI* and *colE2* together, but no detectable proportion had acquired factor *colE2* alone (see Ozeki & Stocker, 1958). However, a subline of LT2 with the E2 factor of strain P9, but not the I factor, was obtained, apparently by the spontaneous loss of *colI* from an LT2 stock given *colI* and *colE2* simultaneously from strain P9. It was later found that LT2 strains with factor E2 from strain P9 but without factor I could be regularly obtained either (i) by transduction, using phage PLT22 grown on an LT2 subline carrying *colI* and *colE2* (Ozeki & Stocker, 1958); or (ii) by interrupted mating of such a strain with LT2 *col*$^-$; these results will be described in subsequent papers. Strain LT2 carrying *colE2*, but not *colI* derived from *Sh. sonnei* P9 behaved similarly, regardless of how it had been obtained.

Table 2. *Transfer of colicine factors from standard colicinogenic strains of* Escherichia coli *and* Shigella sonnei *to sublines of* Salmonella typhimurium *strain LT2 by mixed culture*

Donor of colicinogeny		Acceptor of colicinogeny		
Strain	Colicines produced	Strain	Selected by	Acceptor colonies colicinogenic
Shigella sonnei P9	I & E2	LT2 *cysD–36 str-r*	Streptomycin	*c.* 50% (I only) *c.* 5% (I and E2)
E. coli K12–30	E1	LT2 *cysD–36*	Colicine K	*c.* 10%
E. coli K77	B	LT2 *athC–5 str-r*	Streptomycin	*c.* 20%
E. coli K49	K	LT2 *cysC–7 str-r*	Streptomycin	*c.* 0·01%

10 ml. broth was inoculated with loopfuls of broth cultures of the standard colicinogenic strain (donor) and of a subline of strain LT2 (acceptor). After overnight incubation the acceptor strain was selected and the proportion of its colonies which were colicinogenic was measured. Other LT2 sublines, and other methods of selection, have also been used, with similar results.

Doubly colicinogenic derivatives of strain LT2. To obtain doubly colicinogenic derivatives we used as acceptor strain an LT2 subline carrying one colicine factor, and incubated it in broth with a donor strain (*Escherichia coli*, *Shigella sonnei* or LT2) carrying some other factor. With one exception (Table 3*c*) singly colicinogenic LT2 sublines accept a second, different, colicine factor in the same way that non-colicinogenic LT2 does. LT2 (*colI*) (*colE2*), a doubly colicinogenic LT2 derivative used in many experiments, was prepared by several methods: (i) in a single step by growth in mixed culture with a donor strain carrying both factors; (ii) by transduction of *colE2* by phage PLT22 from LT2 (*colI*) (*colE2*) into a stock already carrying *colI*; (iii) by transmitting *colI* by mixed culture to stocks carrying *colE2* only, obtained either by transduction of *colE2* into a non-colicinogenic stock, or by the spontaneous loss of *colI* from a stock carrying *colI* and *colE2*.

Test for transmission of colicine factors between LT2 sublines

To test for the transmission of colicinogeny between LT2 sublines we used the technique described above, of overnight incubation of broth inoculated with equal numbers of bacteria of the donor and acceptor strains, in this case genetically labelled LT2 sublines. In most experiments we used a streptomycin-sensitive donor and a resistant acceptor, plated the mixed culture on streptomycin agar and tested for colicinogenic acceptor bacteria by the methods described above. In the case of colicine K no streptomycin-sensitive subline carrying *colK* was available for use as donor; but as the donor strain (*cysC–7 str-r* (*colK*)) was nutritionally exacting, LT2 wild-type was used as acceptor, and was selected by two serial passages of the mixed culture in liquid minimal medium.

RESULTS

The colicinogenic derivatives of *Salmonella typhimurium* LT2 obtained by contact with standard colicinogenic donor strains (see Methods) were indistinguishable from the strains from which they had acquired colicinogeny, in respect both of their range of activity and of the appearance of the inhibition zones they produced. That is, the colicine produced by the derivative seems to be identical with that produced by the original strain.

A method for detecting the production of colicine by individual bacteria of these colicinogenic LT2 sublines has been described elsewhere (Ozeki, Stocker & de Margerie, 1959), together with observations on the proportion of the bacteria of such strains which liberated colicine, spontaneously or after ultraviolet irradiation. The colicinogenic LT2 derivatives varied in their stability. Sublines producing colicine I were stable, even on prolonged storage at room temperature on Dorset egg slopes; on a single occasion, however, a variant producing only colicine E2 was found in a stock initially producing both I and E2. Derivatives carrying *colE1* or *colE2* were also in general stable. Colicinogeny for colicines B and K was less stable; for when Dorset egg stock slopes of LT2 lines carrying *colB* or *colK*, kept at room temperature for a year or more, were streaked out, only 10–50 % and *c.* 0·2 %, respectively, of the colonies were colicinogenic.

When a culture of an F^+ strain of *Escherichia coli* is incubated in a sub-lethal concentration of an acridine dye or a cobalt salt, many of the bacteria lose the *F* factor and become F^- (Hirota, 1956; Hirota & Iijima, 1957). We therefore tested acriflavine for effect on the *colI* and *colE2* agents in *Salmonella typhimurium* LT2. Strains *cysD–36* (*colI*) and *cysD–36* (*colI*) (*colE2*) were grown in broth with acriflavine, 80 μg/ml.; after 24 hr. at 37° the count of viable bacteria had increased from *c.* 10^6/ml. to *c.* 7×10^7/ml.; all of several hundred colonies tested retained their colicinogenic character unchanged. All of 20 clones isolated from the acriflavine-treated *cysD–36* (*colI*) culture transmitted colicinogeny in the same way as the untreated parent strain.

Singly colicinogenic LT2 sublines were tested for their ability to transmit colicinogeny to non-colicinogenic sublines during overnight mixed cultured in broth. Table 3*a* records the results of a typical experiment; similar results were obtained when the donor or acceptor lines carried other genetic markers, and when other methods of selection of the acceptor strain were used. It appeared that in strain LT2

colicine factors I and B are readily transmissible by mixed culture; that factors E2 and K are not transmitted to a detectable extent; and that factor E1 is transmitted at a very low rate. All of 20 'wild' colicinogenic strains of *Salmonella typhimurium* found to produce colicine I readily transmitted colicinogeny to LT2 *cysD–36 str-r*; whereas none of 12 'wild' strains found to produce colicine E2 transmitted colicinogeny to a detectable extent.

Table 3. *Transfer of colicine factors between LT2 strains during overnight mixed growth*

(*a*) Singly colicinogenic donor and non-colicinogenic acceptor strains.
(*b*) Doubly colicinogenic donor and non-colicinogenic acceptor strains.
(*c*) Colicinogenic donor and colicinogenic acceptor strains.

			State of acceptor bacteria after mixed growth			
			Producing indicated colicine			Non-colicinogenic
	Donor strain	Acceptor strain				
(*a*)	*cysD–36* (*colI*)	*cysD–36 str-r*	I, 50 %			50 %
	cysD–36 (*colB*)	*cysD–36 str-r*	B, 20 %			80 %
	cysD–36 (*col E1*)	*cysD–36 str-r*	E1, 0·1 %			99·9 %
	cysD–36 (*col E2*)	*cysD–36 str-r*	E2, < 0·001 %			100 %
	cysC–7 str-r (*colK*)	LT2 (wild-type)	*K, < 0·001 %			100 %
(*b*)	*cysD–36* (*colI*) (*colB*)	*cysD–36 str-r*	I, 3·5 %	B, 5·5 %	I & B, 55 %	36 %
	cysD–36 (*colI*) (*colE1*)	*cysD–36 str-r*	I, 43 %	E1, 0 %	I & E1, 5 %	52 %
	cysD–36 (*colI*) (*colE2*)	*cysD–36 str-r*	I, 55 %	E2, 0 %	I & E2, 22 %	23 %
	LT2 w.t.†(*colI*)(*colK*)	*cysD–36 str-r*	I, 55 %	K, 0 %	I & K, 1 %	44 %
(*c*)	*athC–5* (*colI*)	*cysD–36 str-r* (*colB*)	I & B, 33 %	B, 67 %		
	athC–5 (*colI*)	*athC–5 str-r* (*colE1*)	I & E1, 50 %	E1, 50 %		
	athC–5 (*colI*)	*cysC–7 str-r* (*colE2*)	I & E2, 40 %	E2, 60 %		
	athC–5 (*colI*)	*cysC–7 str-r* (*colK*)	I & K, 0·4 %	K, 99·6 %		
	athC–5 (*colB*)	*cysC–7 str-r* (*colI*)	B & I, 35 %	I, 65 %		
	athC–5 (*colB*)	*athC–5 str-r* (*colE1*)	B & E1, 29 %	E1, 71 %		
	athC–5 (*colB*)	*cysC–7 str-r* (*colE2*)	B & E2, 58 %	E2, 42 %		
	athC–5 (*colB*)	*cysC–7 str-r* (*colK*)	B & K, 8 %	K, 92 %		

Loopfuls of overnight broth cultures of acceptor and donor strains were inoculated together into 10 ml. broth. After 18 hr. incubation the acceptor bacteria were selected on streptomycin agar and scored for colicinogeny with appropriate indicator strains.

* As the donor strain, *cysC–7 str-r* (*colK*) was streptomycin-resistant, but auxotrophic, the prototrophic acceptor bacteria were selected on minimal medium.

† Wild-type.

The *F* agent can be transmitted by contact from F+ strains of *Escherichia coli* to *Salmonella typhimurium* (Zinder, 1960); and one of our standard colicinogenic strains, *E. coli* K12–30, used as source of the *colE1* factor, was known to be F+ (we have not investigated the *F* character of the other standard colicinogenic strains; see Table 2). We therefore considered the possibility that our colicinogenic derivatives of LT2 had become F+ as well as colicinogenic. However, they did not show the kind of fertility found by Zinder (1960) in F+ derivatives of LT2; and all were sensitive to phage SP6 which does not attack LT2 lines carrying the *F* agent (Zinder, 1961). We conclude that none of our colicinogenic LT2 derivatives carries the *F* agent.

Though factors E1, E2 and K are scarcely, if at all, transmitted by singly colicinogenic donor strains they are readily transmitted by donor strains which carry,

in addition, one of the readily transmissible factors, I or B (see Table 3*b*). Most of the experiments described in this paper concern factors I and E2, as representatives of the classes of factors which are, respectively, transmissible and non-transmissible by singly colicinogenic donor strains.

Transfer of the colI *factor*

When *cysD–36* (*colI*) (donor) and *cysD–36 str-rcol*$^-$ (acceptor) were grown together in broth (inoculum *c.* 10^5 bacteria/ml. of each strain), more than 50 % of the acceptor population became colicinogenic during overnight incubation, by which time the total population was *c.* 10^9 bacteria/ml. Undiluted broth cultures of the same donor and acceptor strains, grown overnight without aeration, were mixed in equal parts and re-incubated, without addition of fresh broth; after 3 hr. only *c.* 0·02 % of the acceptor bacteria were colicinogenic, and after 6 hr. *c.* 0·1 %; but after 20 hr. incubation the proportion of colicinogenic acceptor bacteria had increased to *c.* 50 %. No such transfer of colicinogeny occurred when the viable donor culture was replaced by either (1) a bacterium-free culture filtrate, or (2) the pasteurized (60°, 45 min.) supernatant of a centrifuged culture, or (3), a culture killed by shaking with chloroform. We conclude that transmission of colicinogeny between *Salmonella typhimurium* sublines grown together requires contact of 'live' cells, presumably because it occurs by conjugation, as has been inferred in other genera.

The transfer of colicinogeny during 20 hr. incubation of an undiluted mixture of unaerated overnight broth cultures of donor and acceptor strains might be thought to indicate that transmission of the *colI* factor was occurring under conditions where there was no bacterial growth. However, in our broth, incubated at 37° without aeration by shaking, the growth of strain LT2 slows down when the count reaches $1-2\times10^8$/ml., and after overnight incubation there are about 10^9 viable bacteria/ml.; slow growth continues on longer incubation, the number of viable bacteria increasing to *c.* 2×10^9/ml. in a further 24 hr. Thus the transmission of colicinogeny to *c.* 50 % of the acceptor bacteria took place in conditions permitting slow bacterial growth.

If a broth culture of strain LT2 is aerated by shaking during incubation the number of viable bacteria increases rapidly until it reaches $4–6\times10^9$/ml. and then growth ceases. When aerated 18 hr. cultures of donor and acceptor strains were mixed, without dilution, very few of the acceptor bacteria became colicinogenic during 20 hr. further incubation, with or without shaking of the mixture. The almost complete absence of transmission of the *colI* factor under these conditions presumably results from the environmental conditions which are preventing bacterial growth (probably lack of any available energy source).

Shaking of a mixture of overnight unaerated cultures of donor and acceptor strains greatly reduced the proportion of acceptor bacteria which became colicinogenic during 20 hr. incubation; e.g. to 0·8 %, compared with 45 % in an unshaken control. As stated above nearly all the acceptor bacteria which acquire *colI* in an unshaken mixture do so between the sixth and twentieth hour of incubation, during which time there is slow bacterial growth. In the shaken mixture by contrast growth would have ceased by the sixth hour of incubation and presumably no transmission of colicinogeny could occur thereafter.

We therefore conclude that all the observed inhibitory effects of shaking on the transfer of *colI* can be accounted for by the effects of aeration by shaking on bacterial growth, without postulating any direct effect, e.g. on pair formation.

Proportion of competent donors in overnight broth cultures of LT2 (colI)

The very small proportion of acceptor bacteria which acquired the *colI* factor during the first few hours of incubation of a mixture of undiluted overnight broth cultures of donor and acceptor strains suggested that perhaps only a small fraction of the bacteria in a broth culture of an LT2 line carrying *colI* are 'competent donors', able to transmit *colI* (even though all bacteria in such a culture carry the factor). This hypothesis was tested, and the proportion of competent donors estimated, as follows: (i) graded numbers (e.g. 300, 3000 or 30,000) of bacteria of strain *cysD–36* (*colI*) in 0·2 ml. of saline were added to 0·2 ml. volumes of an overnight broth culture of a streptomycin-resistant acceptor strain; (ii) after either 15 min. or 20 hr. incubation 10 ml. of streptomycin broth were added to each tube, to prevent further growth of the streptomycin-sensitive donor bacteria; (iii) after overnight incubation each tube was tested for the presence of colicinogenic bacteria of the streptomycin-resistant acceptor strain. In such an experiment (Table 4) the final test for

Table 4. *Estimation of proportion of competent donors in a broth culture of strain* cysD–36 (colI) *by the dilution method*

No. of bacteria of donor strain added	No. of tubes giving positive or negative test for presence of streptomycin-resistant colicinogenic bacteria after incubation in streptomycin broth added at			
	15 min.		20 hr.	
	+	−	+	−
3×10^4	12	0	12	0
3×10^3	4	8	5	7
3×10^2	0	12	1	11

0·2 ml. volumes of saline dilutions of an overnight unshaken broth culture of the donor strain *cysD–36* (*colI*) were mixed with 0·2 ml. volumes of a similar culture of strain *cysD–36 str-r*, 24 tubes being set up for each size of inoculum. After 15 min. incubation 10 ml. streptomycin broth was added to half the tubes of each set; the remainder were similarly treated after 20 hr. After 24 hr. at 37° all tubes were streaked on streptomycin-agar plates, which after incubation were layered with a streptomycin-resistant indicator; reconstruction experiments showed that this final test would detect as few as 0·01 % of streptomycin-resistant colicinogenic bacteria.

colicine production was either completely negative or strongly positive, the latter indicating that a large fraction of the acceptor bacteria had become colicinogenic; and the distribution of positive tubes was the same whether 15 min. or 20 hr. had been allowed, without streptomycin, for transmission from the donor to the acceptor strain. We infer that positive tubes received, in the inoculum of donor bacteria, at least one competent donor bacterium, able to transmit colicinogeny, and that negative tubes received none. The mean number of effective donor bacteria inoculated per tube, calculated by the Poisson series from the proportion of negative tubes (15/24), is *c.* 0·48; as each tube received *c.* 3000 bacteria the proportion of

effective donors in the broth culture of strain *cysD–36* (*colI*) was *c.* 1/6000. The proportion of competent donors was also estimated by a different method. Samples of an overnight broth culture of a streptomycin-resistant acceptor strain were innoculated with graded numbers of bacteria of a streptomycin-sensitive donor strain. After 15 min. at 37°, samples (*c.* 10^6 acceptor bacteria/plate) were plated on streptomycin agar, and after incubation over-layered with a streptomycin-resistant indicator. The number of colicine inhibition zones produced indicated the number of streptomycin-resistant acceptor bacteria made colicinogenic, and hence the number of effective donor bacteria. The calculated proportion of effective donor bacteria was 1/5000 to 1/7800 (Table 5).

Table 5. *Estimation of proportion of competent donors in a broth culture of strain* cysD–36 (colI) *by plating method*

	No. of bacteria inoculated/plate		
Donor strain *cysD–36* (*colI*)	Recipient strain *athC–5 str-r*	No. of colicine zones/plate*	No. of donor bacteria/zone
$1{\cdot}5 \times 10^6$	*c.* 10^6	192	$7{\cdot}3 \times 10^3$
$3{\cdot}2 \times 10^5$	*c.* 10^6	51	$6{\cdot}3 \times 10^3$
$1{\cdot}3 \times 10^5$	*c.* 10^6	24	$5{\cdot}4 \times 10^3$
$2{\cdot}9 \times 10^4$	*c.* 10^6	4	$7{\cdot}3 \times 10^3$
$1{\cdot}5 \times 10^4$	*c.* 10^6	3	$5{\cdot}0 \times 10^3$

1 ml. volumes of an overnight unshaken broth culture of the streptomycin-resistant recipient strain were seeded with graded numbers of bacteria from a similar culture of the streptomycin-sensitive donor strain. After 15 min. at 37° 0·1 ml. volumes of 1/100 dilutions were spread on streptomycin-agar plates; after 15 hr. incubation these were tested with a streptomycin-resistant indicator (CL104).

* Average of three plates.

The large number of acceptor bacteria which on long incubation become colicinogenic, even in tubes receiving only one competent donor, do so, we believe, as a result of the serial transmission of the *colI* factor amongst the acceptor bacteria. To estimate the number of acceptor bacteria which become colicinogenic as a result of the inoculation of a single effective donor bacterium, graded numbers of bacteria of a donor strain were added to 1 ml. volumes of an overnight culture of a streptomycin-resistant acceptor strain and the tubes were re-incubated, without the addition of fresh broth; after 20 hr. (by which time there was only a *c.* twofold increase in the number of bacteria) the number of colicinogenic acceptor bacteria in each original tube was estimated by subculturing serial decimal dilutions of each tube in streptomycin broth and testing each subculture after incubation for the presence of streptomycin-resistant colicinogenic bacteria, as above. This experiment showed that original tubes which had received *c.* 3000 bacteria of the donor strain contained, 20 hr. later either (i) <10 and presumably no colicinogenic acceptor bacteria or (ii) $>10^5$. The positive tubes had, by inference, received one (or a very few) effective donors. It is unlikely that the one effective donor in the inoculum had mated, successively, with 10^5 acceptor bacteria during the 20 hr. incubation; the epidemic spread of the *colI* factor amongst the acceptor population seems the only plausible explanation of these results.

Transfer of the colE2, colK, colE1 *and* colB *factors*

Singly colicinogenic LT2 sublines carrying either *colE2* or *colK* did not transmit their colicinogenic properties to any detectable extent to non-colicinogenic LT2 during overnight growth in broth (Table 3*a*); transfer of colicinogeny to $1/10^5$ of the acceptor population would have been detected. LT2 sublines carrying only *colE1* transmitted colicinogeny to *c.* 0·1 % of an acceptor population during overnight mixed culture. LT2 sublines carrying only *colB* transferred colicinogeny to *c.* 20 % of an acceptor population during overnight mixed culture, we assume as a result of the epidemic spread of the *colB* factor amongst the acceptor population.

Although singly colicinogenic LT2 (*colE2*) strains did not transmit *colE2*, overnight incubation of a doubly colicinogenic LT2 (*colI*) (*colE2*) strain with a non-colicinogenic acceptor resulted in the acquisition of both factors by some (e.g. 22 %) acceptor bacteria and of *colI* alone by a larger fraction (e.g. 55 %; see Table 3*b*); acceptor bacteria which had acquired *colE2* only were not detected (cf. transmission of *colI*, and of *colI* and *colE2* together, by *Shigella sonnei* P9 to LT2, Table 2). LT2 (*colI*) (*colE2*) stocks behaved alike in transmission tests, regardless of whether they had acquired the two factors simultaneously, or successively, in either order (see Strains, in 'Methods'). All the 12 'wild' *Salmonella typhimurium* strains producing colicine E2 transmitted *colE2*, with *colI*, after the latter factor had been introduced into them; a clone of *Sh. sonnei* P9 which had lost the transmitting ability of the doubly colicinogenic parent strain was found to have lost the ability to produce colicine I; ability to transmit was regained when factor *colI* was re-introduced.

Thus LT2 and several other strains of *Salmonella typhimurium* and *Shigella sonnei* strain P9 when carrying only *colE2* do not transmit this factor, but when carrying both *colE2* and *colI* transmit both factors.

The carriage of *colI* by a donor strain likewise resulted in some transmission of *colK*, not transmitted by singly colicinogenic strains, and facilitated the transmission of *colE1* (Table 3*b*). In the case of the latter *c.* 5 % of an acceptor population acquired *colE1* (with *colI*) during overnight growth with a doubly colicinogenic donor, as against *c.* 0·1 % when the donor carried *colE1* only.

From analogy with the transmission of *colI* we infer that most of the acceptor bacteria which acquire *colE2* (or *colK* or *colE1*), together with *colI*, during overnight growth with a doubly colicinogenic donor strain do so through the epidemic spread amongst the acceptor population of the two factors in association. In an experiment similar to that recorded in Table 4 the proportion of competent donor cells in a broth culture of *cysD–36* (*colI*) (*colE2*) was measured; as in *cysD–36* (*colI*) this proportion was only one in several thousand. In this experiment the tubes inferred to have received only one (or a very few) competent donor cells of *cysD–36* (*colI*) (*colE2*) gave rise to streptomycin-resistant (acceptor) cultures producing colicine E2 as well as colicine I; this suggests that in the doubly colicinogenic strain all or nearly all competent donor cells can transmit both factors. If many acceptor bacteria which have just acquired both *colI* and *colE2* (or *colK* or *colE1*) from such competent donors themselves become competent donors of both factors, epidemic spread of the two factors in association would be expected. The larger number of acceptor bacteria which acquire *colI* alone, instead of *colI* and *colE2*, would be accounted for if during conjugation *colI* is sometimes transmitted without *colE2*.

A few experiments indicated that the presence of *colB* in a donor strain facilitated the transfer of *colE2*, *colK* and *colE1* in the same way that the presence of *colI* did, though not to quite the same extent.

As singly colicinogenic donor strains carrying either *colI* or *colB* transmitted colicinogeny to a high proportion of acceptor bacteria during overnight mixed culture (Table 3*a*), we could not use a comparison of the transmitting ability of singly and doubly colicinogenic donor strains as a test for the ability of one of these factors to assist the transfer of the other. However, when a donor strain carrying both *colI* and *colB* was incubated with a non-colicinogenic acceptor more than half the acceptor bacteria acquired both factors, while 36% acquired neither, a distribution incompatible with the hypothesis of independent transmission of the two factors (Table 3*b*). This suggests that competent donor bacteria carrying two transmissible factors resemble competent donors carrying one transmissible and one 'non-transmissible' factor, in that they usually transmit both factors.

Transfer of colI *and* colB *factors to colicinogenic acceptor strains*

When an acceptor strain already producing colicine E2 or E1 was incubated overnight with a donor strain producing colicine I (or B), a high proportion of the acceptor bacteria acquired the ability to produce colicine I (or B), just as they did when the acceptor strain was non-colicinogenic (Table 3*c*); and the acquisition of *colI* or *colB* did not result in loss of the colicine factor already carried by the acceptor strain. When a 'donor' strain carrying *colI* was incubated with an 'acceptor' carrying *colB*, or vice versa, many bacteria of the 'acceptor' strain acquired the colicine factor carried by the 'donor', becoming doubly colicinogenic (Table 3*c*). Thus for most pairs of colicine factors tested the prior presence in an acceptor strain of one factor failed to prevent the epidemic spread, in this acceptor, of some other, transmissible, factor, introduced by a donor strain.

However, when an acceptor strain carrying *colK* was incubated overnight with LT2 (*colI*) or LT2 (*colB*) only 0·4 and 8%, respectively, of the acceptor bacteria acquired *colI* or *colB*, as against *c.* 50% and *c.* 20%, respectively, when the acceptor was *col*$^-$. This limited spread of *colI* in LT2 (*colK*) has been observed in several experiments.

We could not directly test whether the prior presence of one *colI* factor in an acceptor strain would prevent the epidemic spread in this strain of some other *colI* factor, introduced by a donor strain, because all the *colI* agents we examined were indistinguishable. But as LT2 (*colI*) (*colE2*) transmits both factors to many bacteria of a non-colicinogenic acceptor strain (Table 3*b*), presumably through the epidemic spread of the two factors in association, we could test whether *colE2* was transmitted in this way when the acceptor strain already carried *colI*. Strain *cysD–36* (*colI*) (*colE2*) was used as donor, and either *cysD–36 str-r col*$^-$ or *cysD–36 str-r* (*colI*) as acceptor; after overnight incubation of broth inoculated with *c.* 10^7 bacteria/ml. of donor and of acceptor strains, *c.* 15% of the non-colicinogenic acceptor population, but no detectable proportion (and thus certainly less than 0·01%) of the acceptor population carrying *colI* had acquired *colE2*. This inhibition by the prior presence of *colI* of the acquisition of *colE2* from a donor carrying *colI* and *colE2* suggests that there is no epidemic spread of *colI* and *colE2* together in a population already carrying *colI*, and so presumably no spread of *colI* in such a population.

DISCUSSION

The determinants for the production of five distinct colicines were thus transferred from *Escherichia coli* or *Shigella sonnei* into *Salmonella typhimurium* strain LT2, wherein each caused the production of a colicine indistinguishable by its spectrum of activity from that produced by the donor strains. Colicines E1 and E2, produced either by the original donor strains or by the LT2 derivatives, were distinguishable from each other not only by their lack of activity on indicator strains themselves producing the homologous colicine (Fredericq, 1956) but also by mutants resistant to colicine E2 but still sensitive to E1.

In the case of factors *colI*, *colE1* and *colE2* the newly introduced factors were never or almost never lost by the LT2 derivatives; but on storage at room temperature for many months non-colicinogenic bacteria become predominant in initially pure colicinogenic LT2 cultures carrying *colK* or *colB*. The reason for this difference in stability is unknown. Growth in the presence of acriflavine did not cause loss of *colI* or *colE2*. Perhaps the stability of these two factors in *Salmonella typhimurium* LT2 during storage and when exposed to acriflavine during growth results from their integration into the bacterial chromosome; for acriflavine treatment, though it 'cures' F^+ *Escherichia coli* strains of their autonomous F agent, does not affect the integrated *F* agent of *Hfr* strains (Hirota, 1960).

The factors differed in their transmissibility by singly colicinogenic *Salmonella typhimurium* strains; *colE2* and *colK* were not transmitted at all, *colI* and *colB* on prolonged incubation were acquired by a high proportion of the cells of a non-colicinogenic acceptor, and *colE1* was of intermediate transmissibility. The ability of the source strain of *Escherichia coli* carrying the *colK* agent to transmit it to *S. typhimurium* may reflect an inherent difference in the behaviour of this factor in the two host species, or may result from the presence of some assisting factor, for instance the *F* agent, in the colicinogenic *E. coli* strain.

Only a small fraction (10^{-3} to 10^{-4}) of the bacteria in a broth culture of an LT2 strain carrying *colI* actually transmits the factor; and it appears that during long incubation of a mixed culture, *colI* spreads 'epidemically' in the acceptor population. The phenomenon of epidemic spread of *colI* (or *colB*) amongst non-colicinogenic populations can be explained by postulating that many or all bacteria newly infected by either (or both) these factors become 'effective donors', able to transmit. Experimental proof of the correctness of this postulate was later obtained (Stocker, Smith & Ozeki, in preparation). Transmission to a high proportion of an acceptor population, and so, by inference, epidemic spread, took place only when conditions were such that there was slow bacterial growth, at high bacterial concentrations, for some hours. Aeration of mixed cultures interfered with transmission apparently because of the effect of aeration on the 'growth cycle' of a broth culture, rather than any direct effect on the ability of colicinogenic bacteria to transmit their agent. This effect of aeration is therefore probably unrelated to its effect in producing an F^- (i.e. non-fertile) phenotype in F^+ strains of *Escherichia coli* K12 (Cavalli, Lederberg & Lederberg, 1953).

The factors not transmitted at all by singly colicinogenic LT2 strains, viz. *colE2* and *colK*, and the poorly transmissible *colE1*, were transmitted, with the transmissible factor concerned, to many acceptor bacteria by doubly colicinogenic donor

strains carrying *colI* or *colB* as well as *colE2*, *colK* or *colE1*. These observations, and the data on the transmission of both their colicine factors by individual effective donors in doubly colicinogenic strains, suggest that when a bacterium becomes an effective donor, either spontaneously in an established colicinogenic strain carrying *colI* or *colB* or as a result of newly acquiring one of these factors, it transmits all the colicine factors it possesses.

During prolonged incubation the transmissible factor *colI* (or *colB*) was acquired by a high proportion of the bacteria of acceptor strains already carrying some colicine factor other than that of the donor strain; except in the case where the donor carried *colI* and the acceptor *colK* (Table 3*c*). One may infer that, with this one exception, bacteria already carrying some other factor are susceptible to the epidemic spread of *colI* or *colB*; and therefore that a bacterium carrying some other factor can accept *colI* or *colB* and then becomes an effective donor. On the other hand, *colE2* was not transmitted from LT2 (*colI*) (*colE2*) to a high proportion of an acceptor carrying *colI*. One may probably infer that a bacterium already carrying *colI* does not become an effective donor when it accepts *colE2*, and presumably *colI* also. Thus a bacterium already carrying *colI* apparently does not become an effective donor when it acquires an additional *colI* factor.

The epidemic spread of *colI* initiated by a single effective donor may involve $> 10^5$ acceptor bacteria, during a period in which the number of bacteria is only doubled. Under these conditions *colI* is clearly multiplying faster than the bacteria carrying it, and so may be said to be multiplying autonomously, by definition a property of an episome in its non-integrated state. By the same argument applied to epidemic spread initiated by doubly colicinogenic donors, factors *colE2*, *colK* and *colE1* are likewise capable of autonomous multiplication, at a rate faster than their hosts. The present data do not show whether colicinogenic bacteria which are not effective donors (all bacteria in strains carrying only *colE2* or *colK*; and nearly all in strains carrying *colI* or *colB*) are those in which the colicine factor(s) are integrated into the host chromosome; for their failure to transmit their factor(s) may result merely from failure to pair with acceptor bacteria.

The readily transmissible factors *colI* and *colB* in some ways resemble the *F* factor of *Escherichia coli*. Thus the presence of *colI* or *colB* in LT2 enables it to transmit the otherwise non-transmissible *colE2* and *colK*; and F^+ but not F^- sublines of *E. coli* K12 transmit colicine factors ER (= *colE1*) and S_2 (= *colE2*) (Fredericq, 1954*b*). Furthermore, under some conditions the presence of the *colI* agent in a line of *S. typhimurium* results in conjugation and chromosomal recombination (Ozeki & Howarth, 1961; Smith & Stocker, 1962; see also Clowes, 1961). These similarities in behaviour of the *colI* agent in *Salmonella typhimurium* and the F agent in *E. coli* probably arise from the ability of each of these episomes to confer on its host the capacity to conjugate. It is likely that the large majority of the population carrying *colI* or *colB* are not 'competent donors' because the agent, though still present, does not confer ability to conjugate. It may be that the non-transmissibility (in the absence of assistance) of the *colE2* and *colK* factors in *S. typhimurium* is due to their inability to confer this ability to conjugate on even a small fraction of bacteria carrying them.

Most of the work described formed part of a London Ph.D. thesis of one of us (H.O.) We thank Professor P. Fredericq, and Dr N. D. Zinder for providing bacterial strains and phages.

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J. gen. Microbiol. (1962), 28, 689–699
With 1 plate
Printed in Great Britain

Light-Stimulated Polysaccharide and Protein Synthesis by Synchronized, Single Generations of *Blastocladiella emersonii*

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(*Received* 21 *November* 1961)

SUMMARY

Improved methods are described for growing about 10^8 to 10^9 synchronized single generations of ordinary-colourless (OC) cells of the aquatic fungus *Blastocladiella emersonii* in submerged liquid 1200 ml. cultures at 24°. The stimulatory effect of white light on the synthesis of the internal pool of soluble protein and soluble polysaccharide, and on the dry wt./cell during ontogeny was assessed. Soluble protein was characterized by fractionation on DEAE-cellulose into a number of components: the quantities/cell were affected differentially by illumination. Light caused a sharp decrease in total glucose-6-phosphate dehydrogenase activity/cell during the last stages of growth, when the light-induced formation of soluble polysaccharide/cell was most pronounced.

INTRODUCTION

An ordinary-colourless (OC) plant of the aquatic phycomycete *Blastocladiella emersonii* (Cantino & Hyatt, 1953) begins its life history as a uninucleate uniflagellate spore. After a swimming stage, the flagellum is retracted, the single nucleus divides, and the cell begins to enlarge. Successive nuclear divisions and a gradual increase in size culminate in the formation of a two-celled plant. The prominent terminal cell is globose and contains hundreds of nuclei. Each nucleus becomes surrounded by a membrane, and the uninucleate units of protoplasm thus formed are subsequently liberated from the parent cell as uniflagellate spores; from these, a second generation is begun. The small subterminal cell (which bears the rhizoids) is delimited by a septum during ontogeny. Its shape and size is greatly affected by environmental conditions and in our cultures is so decreased in size that it becomes almost invisible; in any event, it is essentially devoid of contents. Thus, it is justifiable to consider the development of *B. emersonii* in terms of the growth and differentiation of a single cell (Cantino, 1961).

A population of swimming spores when placed in a liquid medium grow into mature blastocladiellas. These first-generation OC cells discharge spores which develop into second-generation cells, and these, in turn, produce a third, a fourth, etc., generation in similar fashion. About 6 years ago an interesting phenomenon involving such 'multiple' generation cultures was discovered (Cantino & Horenstein, 1956); they yielded much more dry weight when grown in the light than when grown in the dark. Subsequently, but again with multiple generation cultures, it

was found (Cantino & Horenstein, 1956; Cantino, 1959) that: (*a*) white light induced the organism to consume glucose and CO_2 more rapidly; (*b*) the degree of light stimulation was affected by the concentration of exogenous glucose; (*c*) the relative quantities of CO_2-fixation products, nitrogenous constituents, and sulphur-containing compounds in the cell were altered by exposure of the organism to light. Later, with populations grown for only one generation, it was found that light accelerated the rate of reproduction of nuclei and the synthesis of deoxyribonucleic acid (DNA; Turian & Cantino, 1959) in young cells, that it depressed the rate of glycine uptake by such cells (Cantino & Turian, 1961), and that the latter phenomenon required exogenous CO_2. With older cells, light accelerated uptake of glycine, incorporation of labelled carbon from glycine-2-^{14}C into DNA, and net synthesis of DNA/cell (Cantino & Turian, 1961). Also illumination extended the generation time of the cell (provided CO_2 was present). The effective wavelengths were in the blue end of the spectrum, and the requirement for light could be by-passed by providing the cells with equimolar quantities of exogenous succinate and glyoxylate (Cantino & Horenstein, 1959). These observations, particularly those dealing with glycine uptake, made it clear that the qualitative and quantitative expressions of the light effect in *Blastocladiella emersonii* depended on the age of the cell. We devised a means of growing the organism so that it could be examined at all stages in its development—i.e. synchronized single-generation cultures. With such cultures certain aspects of enzyme synthesis during ontogeny have already been established (McCurdy & Cantino, 1960; Lovett & Cantino, 1960, 1961; Cantino, 1961). For OC cells in particular, this technique has now been refined to a point where, in spite of their extensive system of tapering rhizoids, up to 10^9 cells/1200 ml. of medium are made to develop un-entangled and evenly distributed in submerged liquid culture as individuals of uniform dimensions. Even the light-induced extended generation time, exhibited by plants grown on solid media (Cantino & Horenstein, 1959), is reproduced in liquid culture. With these cultures, we have begun to examine the quantitative and qualitative aspects of 'lumisynthesis' (light-induced increase in dry wt. of a micro-organism devoid of chlorophyll; McCurdy & Cantino, 1960) in *B. emersonii*; some results are here reported.

METHODS

Culture methods. Uniform spore inocula were obtained by flooding with water 14 cm. diam. Petri plates of Difco PYG agar bearing thousands of mature OC plants on the agar surface (McCurdy & Cantino, 1960). The resulting suspension of swimming spores was filtered through filter paper (No. 500; E. H. Sargent Co., Chicago, Illinois, U.S.A.) to remove mature cells dislodged from the agar; it generally contained 5×10^5 to 5×10^6 spores/ml. Population densities of spore inocula were estimated turbidimetrically (10^5 cells/ml. = optical density (OD) of 1·0 units on a Klett–Summerson colorimeter with a 420 mμ filter). The viable spore count was also always determined accurately by transferring measured samples of the suspension to plates of PYG medium at 22°, and counting the number of mature 1st generation plants produced.

We support the pleas (e.g. Herbert, 1961) for the use of chemically defined media in studies of micro-organisms. However, the defined medium (Barner &

Cantino, 1952) at present available, while adequate for propagating multiple-generation cultures, is not suitable for single-generation cultures. Therefore, Difco PYG broth was used for growing such cultures; while not a chemically defined medium, it supports vigorous growth and a reproducible pattern of development. Media (6·6 g. PYG/1200 ml. 5×10^{-3}M-phosphate buffer, pH 6·7) were dispensed in 2 l. Erlenmeyer flasks fitted with two aeration tubes, autoclaved (35 min., 120°), inoculated with spores (final conc., 10^7 to 3×10^8 spores/flask), placed in a water bath (24° ± 0·05°) and aerated vigorously (about 5 l. air/min.). Cultures were either illuminated from below with fluorescent ('cool white'; KEN-RAD brand, U.S.A.) bulbs, yielding 500 f.c. at the bottom surface of the vessels, or they were kept dark by covering with aluminium foil. They were sampled at intervals for size measurements and photography (Leitz Ortholux, Kodak Panatomic X). Populations were harvested on filter paper with suction and washed with about 500 ml. water/g. wet wt. organism, care being taken to keep the cells wet throughout the procedure. Finally, surface water was removed by vacuum filtration, the cells sucked for about 5 min., and then frozen. The generation time of a population was defined as that time at which 5% of the cells had produced discharge papillae.

Analytical methods. Known numbers of cells were disrupted in glass homogenizers (about 1 g. wet wt. organism/2 ml. water) at about 0° for 10 min. Samples of the whole homogenate were dried to constant weight at 75° *in vacuo*, and the dry wt./cell thus found. Other samples were centrifuged at 16,000 *g* (5 min., 2–4°) when trichloroacetic acid (TCA) extractions for polysaccharide studies (see later) were to be involved, and at 22,000 *g* (20 min., 10°) for all other work. The latter supernatant fluids were dialysed for 20 hr., with one change, against 500 ml. 10^{-2}M-'Tris-HCl' buffer, pH 7·4.

The approximate nucleic acid content was estimated from 260 and 280 mμ absorption data, by using a Beckman DU spectrophotometer (method of Warburg and Christian; Layne, 1957).

Soluble protein assays were made turbidimetrically with TCA (Stadtman, Novelli & Lipman, 1951) on dialysed supernatant fluids.

The dialysed protein pool was further fractionated on DEAE-cellulose (Eastman; Distillation Products Ind., Rochester, N.Y., U.S.A.). After removing fines by repeated suspension in water and decantation, the DEAE-cellulose was washed successively with acetone, acetone plus water (1 + 1 by vol.), M-NaCl, and water, and transferred to a water-jacketed tube (coolant, 10°) to yield a column of DEAE-cellulose (1 cm. diam. × 12 cm. high) placed over an automatic fraction collector (Rinco Instr. Co., Greenville, Ill., U.S.A.). Protein solutions (generally 5–15 ml.) were fed to the column (protected by a glass-wool plug) by gravity flow, followed by 16 ml. of 0·005M-NaCl. An additional 34 ml. of 0·005M-NaCl were placed in a 50 ml. reservoir, arranged at the top of the chromatography tube so that the solvent fell dropwise (about 1·7 ml./min.) on to the 10 ml. head of liquid above the DEAE-cellulose at the same rate as fluid left the column. Once the 0·005M-NaCl in the 50 ml. reservoir was exhausted, 50 ml. of 0·0055M-NaCl were introduced. This in turn was replaced in succession by 50 ml. of each of the following NaCl solutions (M): 0·007, 0·01, 0·035, 0·05, 0·065, 0·09, 0·125, 0·175, 0·4, 0·5, 0·65, and 1·0. Thus, the 10 ml. liquid head over the DEAE-cellulose was maintained throughout the procedure, and it served to introduce an element of gradient elution into an other-

wise essentially step-wise procedure. This empirical approach was selected after repeated attempts with direct gradient elution and other step-wise procedures failed to yield satisfactory results. The fractionation patterns were highly reproducible, and even the smallest peaks (e.g. as in Fig. 6) were repeatedly obtained. Protein in the fractions was estimated from absorption data at 260 and 280 mμ (Layne, 1957).

Blastocladiella polysaccharide. The isolation of polysaccharide (modified to include two successive TCA extractions), its hydrolysis (cf. Hassid & Abraham, 1957), and its assay with glucose oxidase ('Glucostat' reagent of Worthington Biochem. Corp., Freehold, N.J., U.S.A.) have been described (Cantino & Goldstein, 1961). Methods for its purification have been improved by a slight modification. After the initial extraction and precipitation of polysaccharide, care was used in removing TCA-insoluble contaminants which precipitate with the polysaccharide. The polysaccharide was redissolved in water and centrifuged (1600 *g*), the supernatant fluid brought to 5 % TCA, and the polysaccharide reprecipitated with an equal volume of 95 % ethanol; this process was repeated. The polysaccharide was washed successively with 48, 95 and 100 % ethanol, followed by two extractions with diethyl ether. The resulting product yielded about 98 % glucose on hydrolysis.

Glucose-6-phosphate dehydrogenase. The dialysed supernatant fluids remaining after centrifugation at 22,000 *g* were used. Reaction mixtures contained: about 0·2 mg. protein, 20 μmole $MgCl_2$, 30 μmole 'Tris-HCl' buffer, pH 7·4, 30 μmole glucose-6-phosphate, 0·6 μmole triphosphopyridine nucleotide, and water to 3·0 ml. After addition of enzyme, the change in OD at 340 mμ was followed for 10 min., during which time the rates were linear. Endogenous activity was essentially zero.

RESULTS

Growth patterns for synchronized cultures

Synchronized OC cells at different stages in development are shown in Pl. 1. Note the point of attachment of rhizoids; the second basal cell to which rhizoids are usually attached when *Blastocladiella emersonii* is grown on solid media or on solid substrata submerged in liquid media, is almost non-existent (see Pl. 1, bottom left). The changes in log vol./cell (dark-grown) and log dry wt./cell (light grown) are shown in Figs. 1 and 2. The growth pattern appears to be essentially exponential; the reason for the break in the dry wt. curve at 70 % of the generation time remains to be clarified.

Effects of illumination on cellular constituents

In PYG liquid medium at 24°, the generation time of a dark-grown cell was 16 hr.; for a light-grown cell, 17·5 hr. To facilitate comparisons and interpretations of the effect of light, the parameters associated with growth have been related to the generation time of the cell.

Dry wt./cell. The increase in dry wt./cell was greater in the light than in the dark; it became most readily detectable once the cell had progressed beyond 40 % of its generation time. At maturity, the final dry wt./light-grown cell was about 51 % greater than that for a dark-grown cell (Fig. 3).

Nucleic acid/cell. The nucleic acid in the dialysed supernatant fluid fraction after

centrifugation at 22,000 *g* increased more rapidly in the light than in the dark and the final quantity in a mature light-grown cell was 28 % higher than that in a dark-grown cell (Fig. 4).

Soluble protein/cell. The protein found in dialysed supernatant fluid after centrifugation at 22,000 *g* was also greatly increased by exposure of the cell to illumination (Fig. 5); the final soluble protein/mature light-grown cell was about 37 % greater than that of its dark-grown counterpart. Fractionation of this protein pool on DEAE-cellulose (Fig. 6) revealed over twenty components; even the minor ones were detectable with great reproducibility.

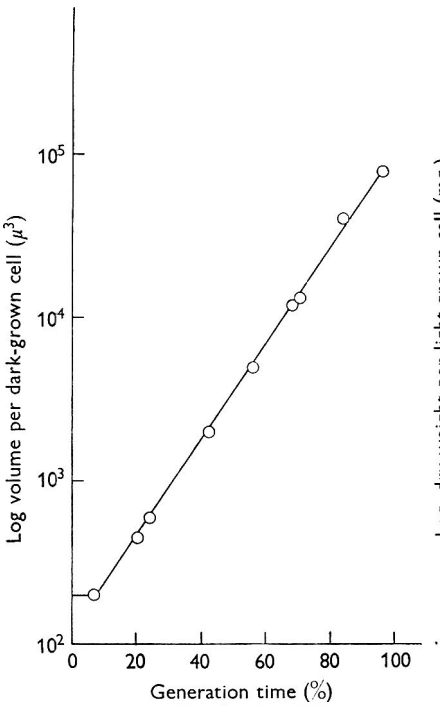

Fig. 1

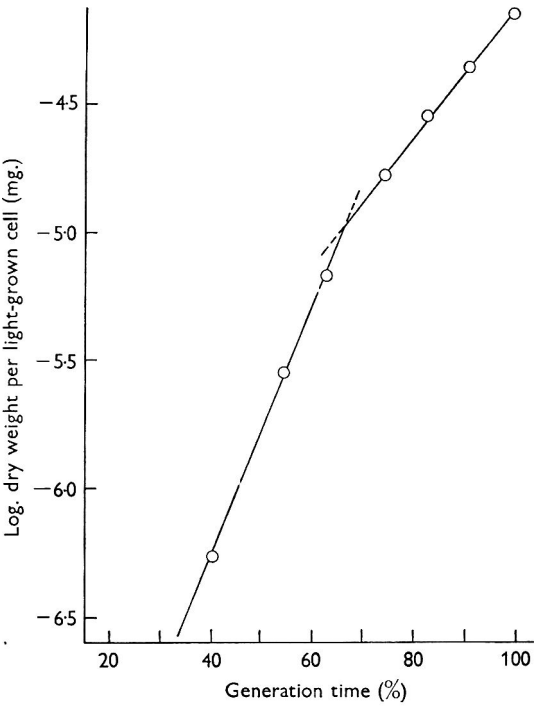

Fig. 2

Fig. 1. The log-vol./OC cell during growth in the dark, medium PYG, 24°. Note lag of about 1½ hr.; it results from a pre-germination swimming stage for the spores.

Fig. 2. The log-dry wt./OC cell during growth in 500 f.c. white light, medium PYG, 24°.

The patterns (Fig. 6) for soluble proteins in mature light-grown and dark-grown cells were similar but not identical. After fractionation the total protein/cell recovered from the light-grown organism was 57 % greater than that recovered from the dark-grown organism (Table 1; note that the increase in total unfractionated protein/cell shown in Fig. 5 is 37 %; this apparent discrepancy was due to the easily coagulable protein—P_{F2} in footnote, Table 1—which was not increased by light to the same degree). The overall light-induced increase in fractionated protein appears to be due to the major component in fractions 6, 9, 9*a*, and 24, for which the average increase was 48 %. However, light also induced differential changes in the minor protein components (Table 1); for example, some fractions (e.g. 11–14, 15–17, 18–19)

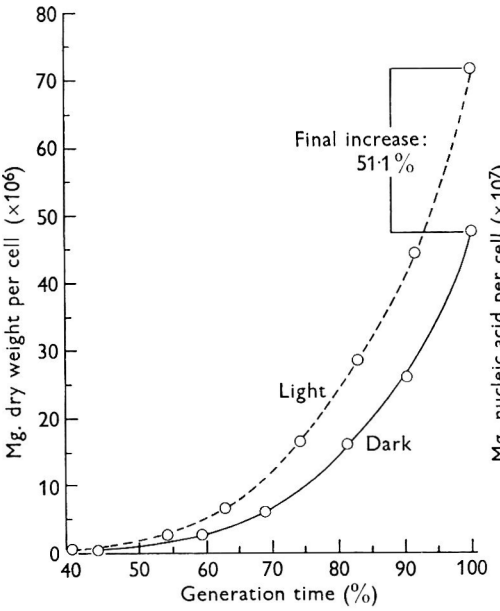

Fig. 3

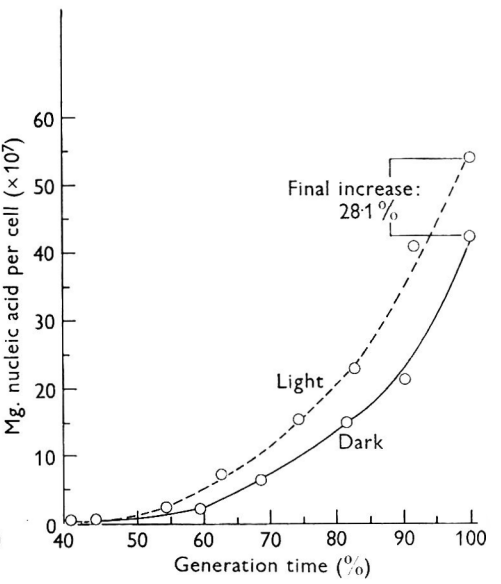

Fig. 4

Fig. 3. The dry-wt./OC cell during growth in the dark and 500 f.c. white light, medium PYG, 24°.

Fig. 4. The nucleic acid/OC cell during growth in the dark and 500 f.c. white light, medium PYG, 24°.

Table 1. *Protein fractions recovered after DEAE-cellulose fractionations from light- and dark-grown ordinary-colourless (OC) cells of* Blastocladiella emersonii

Protein fraction	Organisms light-grown	Organisms dark-grown	% increase in light
	mg./cell at generation time (× 10^8)		
6	174·9	93·5	87*
9–9*a*	222·5	179·0	24*
11–14	55·8	23·3	141
15–17	35·3	19·6	80
18–19	66·1	32·7	102
21–22	71·5	39·2	83
24	About 100	75·7	About 32*
Total protein recovered (= P_{F1})	726†	463†	

* These three major fractions account for over 70 % of the protein recovered (P_{F1}); the average % increase induced by light for these three fractions is 48 %, which compares favourably with the overall, light-induced increase (37 %; see Fig. 5) in total soluble protein (= P_T) from direct analysis before fractionation.

† The P_{F1} represents about 36 % (38·2 and 33·4 % for light-grown and dark-grown cells, respectively) of the P_T originally present in the 22,000 *g* supernatant fluid of the cell. About 60 % of the P_T occurred in an easily coagulable portion (P_{F2}), removed by a single freezing and thawing of the dialysed supernatant fluid; the rest of it consisted of fraction 1 (see Fig. 6) which was not estimated accurately because of the presence of a large quantity of nucleic acid in the first part of this band.

increased 141, 80 and 102 %, respectively, in the light, whereas others (e.g. fractions 4 and 5) changed very little.

Polysaccharide/cell. The stimulatory effect of light was most strikingly displayed by the data on the polysaccharide pool (Fig. 7). In the light, polysaccharide/cell reached a final concentration almost double that in the dark-grown cell.

Glucose-6-phosphate dehydrogenase/cell. The specific activity (see Fig. 8) of this enzyme was lower in the light-grown cells than in dark-grown ones throughout the

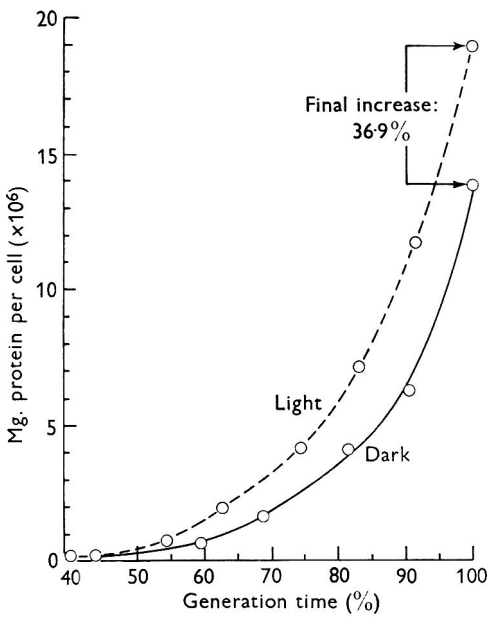

Fig. 5

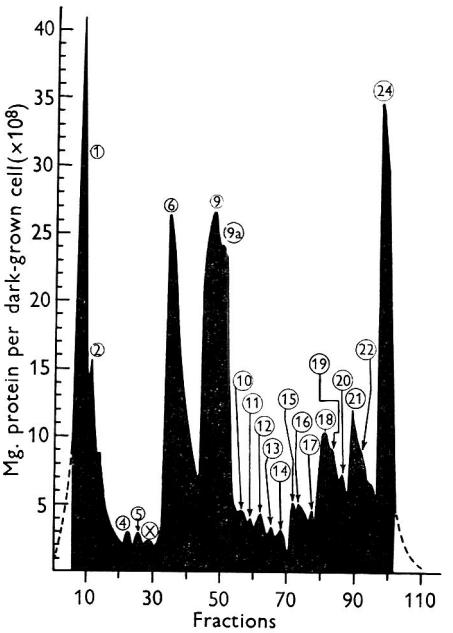

Fig. 6

Fig. 5. The soluble protein/OC cell during growth in the dark and 500 f.c. white light, medium PYG, 24°.

Fig. 6. The pattern obtained by fractionating the soluble protein of dark-grown cells on DEAE-cellulose (text for details). Each fraction was 5·2 ml.

last half of the generation time. But the total units of enzyme/cell was higher in the light-grown cells up to about 80 % of the generation time (Fig. 9); this value then levelled off abruptly while, in the dark, it continued to rise until the cell was mature.

DISCUSSION

The main purpose of this report was to document the stimulatory effect of light upon gross growth parameters during development of the carotene-free OC cell of *Blastocladiella emersonii*. With cell populations grown synchronously for one generation, the dry weight, soluble protein, non-sedimentable (22,000 *g*) nucleic acid, and soluble polysaccharide per cell increased more rapidly when the organism was exposed to 500 f.c. of white light than when it was grown in the dark; the final increases for mature cells were 51·1, 36·9, 28·1 and 73·8 %, respectively.

Attempts (summarized in Cantino & Turian, 1961) have been made to integrate

previous observations on light-stimulated CO_2-fixation, glucose uptake, nucleic acid production, glycine uptake, and the need for CO_2 in some of these phenomena, into a working hypothesis about the biochemical mechanism for lumisynthesis in *Blastocladiella emersonii*. Now light-induced synthesis of polysaccharide will also have to be included.

In part because of a peculiar effect of light on the oxidation of glucose-6-phosphate *in vitro* (Cantino & Horenstein, 1959), it occurred to us that, *in vivo*, light might have inhibited a pathway for glucose degradation, thus shunting metabolism toward manufacture of polysaccharide. The fact that the specific activity of glucose-6-phosphate dehydrogenase was highest in dark-grown cells (from 55% of their

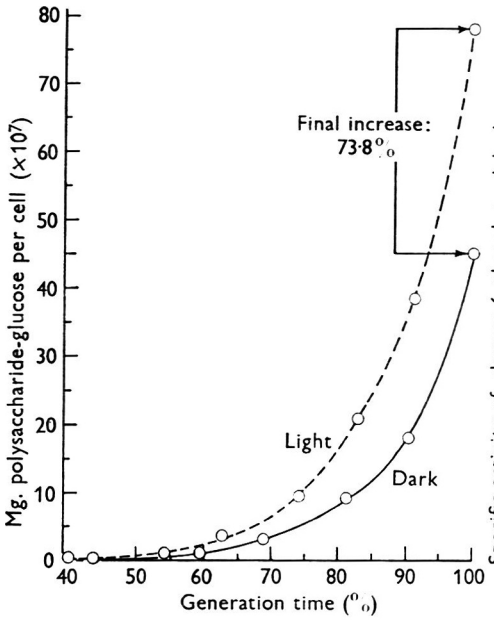

Fig. 7

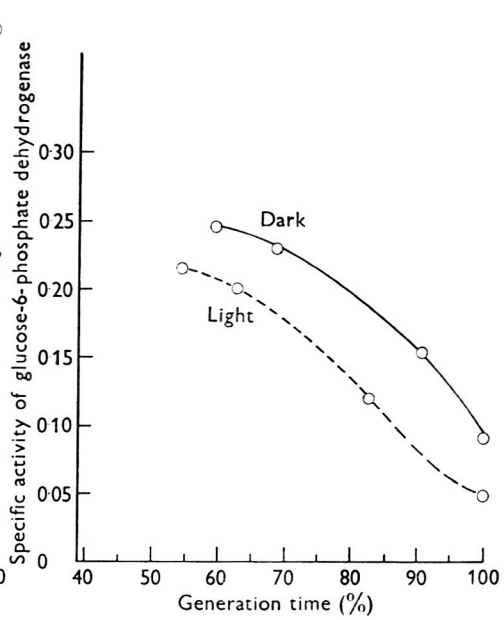

Fig. 8

Fig. 7. The polysaccharide (as polysaccharide-glucose)/OC cell during growth in the dark and 500 f.c. white light, medium PYG, 24°.

Fig. 8. The specific activity (optical density change × min^{-1} × mg. $protein^{-1}$; rates linear in first 10 min.) of glucose-6-phosphate dehydrogenase in OC cells during growth in the dark and 500 f.c. white light, medium PYG, 24°.

generation time to maturity) is consistent with the hypothesis. But when, instead of specific activities, total units of enzyme activity/cell are examined, a different interpretation becomes possible. At 80% of the generation time, a sharp light effect is suddenly manifest. If the observed degrees of enzyme activity reflect quantities of enzyme protein, then light causes net synthesis of the dehydrogenase to stop at 80% of the cell's generation time, whereas in the dark synthesis of the enzyme continues until the cell is mature. Thus, as the cell approaches maturity, synthesis of the dehydrogenase and synthesis of polysaccharide appear to be reciprocally related, with cessation of synthesis of an enzyme which carries glucose off along

an oxidative pathway occurring at that time in the ontogeny of a light-grown cell when net synthesis of polysaccharide becomes most pronounced.

Whatever the ultimate biochemical explanation, however, the light-induced increase in CO_2 fixation and polysaccharide production as well as soluble protein and dry weight in an organism devoid of both chlorophyll and coloured carotenoids is noteworthy.

No other studies of the fungi (for example, see summaries compiled by Marsh, Taylor & Bassler, 1959) have provided evidence for light-induced increases in total

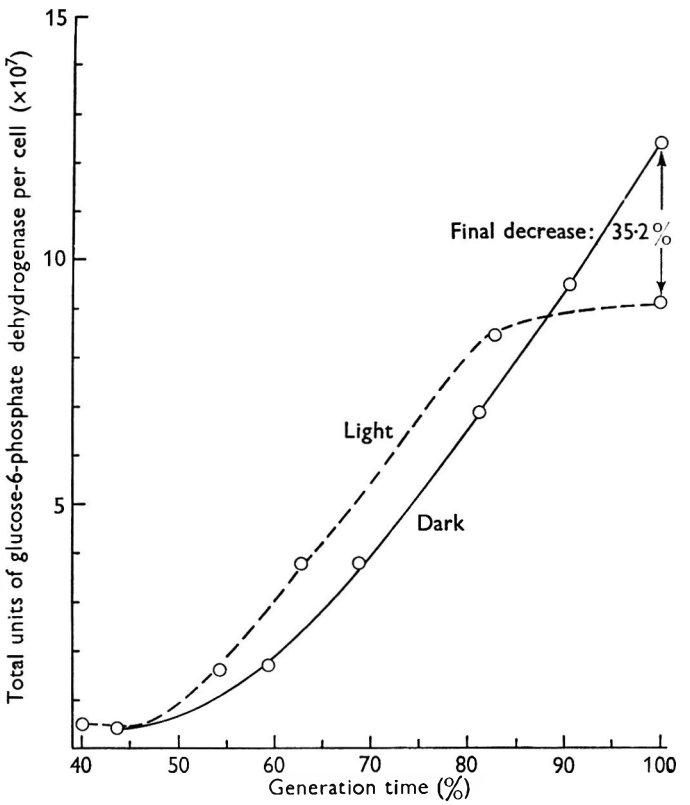

Fig. 9. The total units (specific activity × mg. protein/cell) of glucose-6-phosphate dehydrogenase/cell during growth in the dark and 500 f.c. white light, medium PYG, 24°.

fungal mass, nor bulk constituents therein. In part this may be because a septate filamentous fungus, by its very nature and because of the usual methods used for its propagation, does not lend itself readily to studies of this kind. A mass of mycelium, started from a few reproductive units and grown for days on solid or in liquid media, is a mixture of cells at many stages of physiological and chronological age. If the end in view is an understanding of the biology of the individual organism itself, then clearly the filamentous fungus is inherently a difficult creature to deal with, and to hurdle the obstacles that it offers will tax the ingenuity of the experimental biologist. Synchronized cultures of a filamentous fungus—albeit even of a

very small portion of its 'generation time'—would help tremendously to achieve solutions to many problems in mycology now largely unapproachable.

Many non-filamentous aquatic fungi, although they present idiosyncrasies of their own (Emerson, 1950, 1955, 1958; Cantino & Turian, 1959; Sparrow, 1960; Cantino, 1961), can probably be grown synchronously in reasonably large quantity for a single generation. With *Blastocladiella emersonii*, heavy inocula of newly formed spores are derived from an immediately preceding and essentially simultaneous division of thousands of multinucleate protoplasts: newly formed cells of the sort obtainable from bacteria, for example, with special selection procedures (Abbo & Pardee, 1960). Furthermore, synchronized growth does not depend upon an environmental shock, and there seems no question of creating artifacts derived therefrom (e.g. see Williamson & Scopes, 1961). Because the synchronized Blastocladiella cultures involve only one generation, no complications are introduced by death and lysis. It seems likely that most non-filamentous water fungi can be grown in this fashion.

This work was supported by a U.S. Public Health Service fellowship (GF-14,225) from the Division of General Medical Sciences, Public Health Service, to author A.G., and by research grants from the National Institutes of Health and the National Science Foundation, U.S.A., to author E.C.C.

This is paper no. 61-5 from the Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan, U.S.A.

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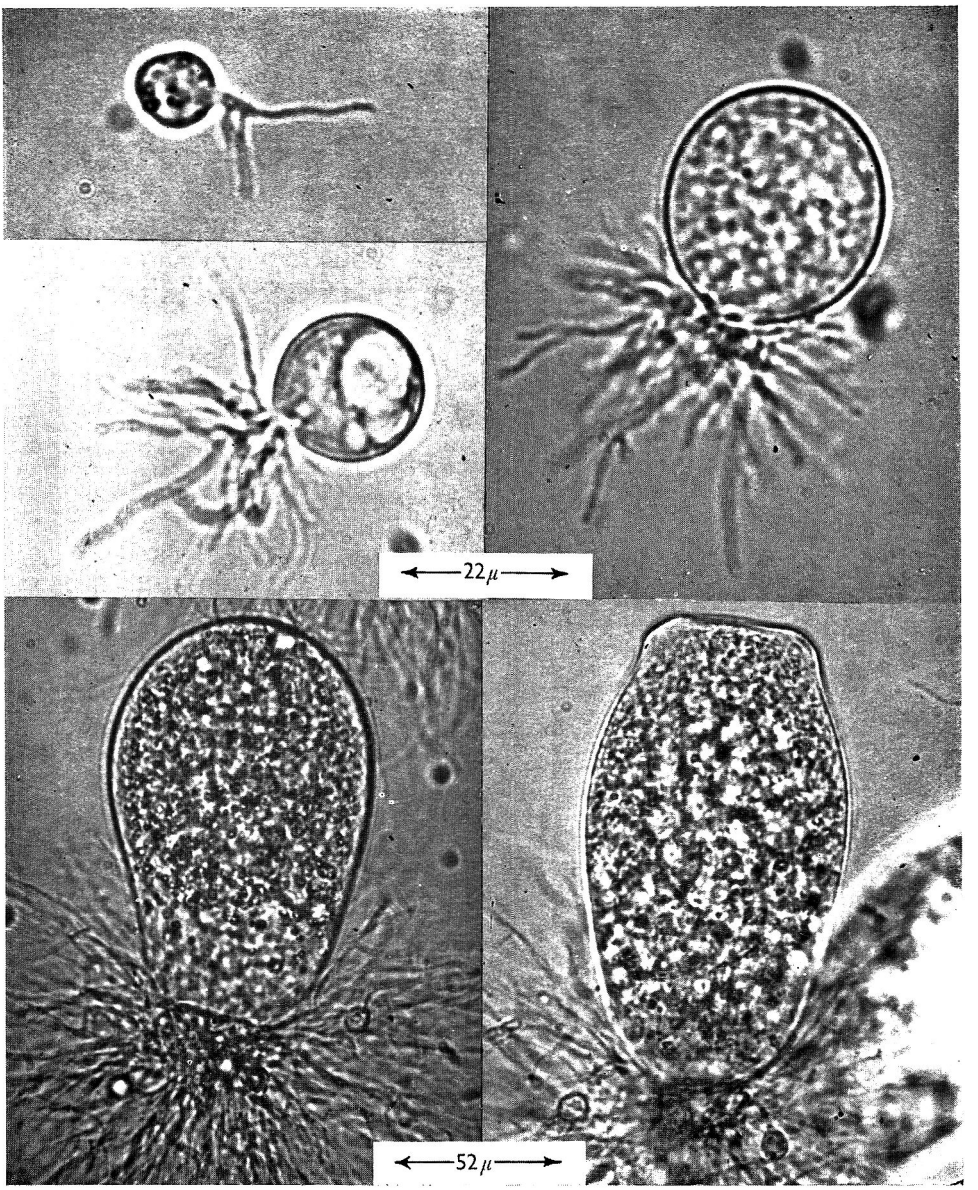

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EXPLANATION OF PLATE

The appearance of ordinary-colourless (OC) cells at different stages in their generation time (in 500 f.c. white light, medium PYG, 24°). Top: 3, 8 and 10 hr.; bottom left and right: 17 and 17·5 hr. The three upper figures are reproduced at ×2·3 magnification of the lower figures. Note the barely visible basal stalk in the nearly mature 17 hr. plant, and the newly formed pair of exit papillae in the mature 17·5 hr. plant.

J. gen. Microbiol. (1962), **28**, 701–709
Printed in Great Britain

The Influence of the Growth Medium on the Demonstration of a Group D Antigen in Faecal Streptococci

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(*Received* 24 *November* 1961)

SUMMARY

When grown under certain conditions some group D streptococci did not yield HCl extracts which would react serologically with group D antisera. By varying the composition of the medium, it was shown that the optimum yield of group D antigen was obtained when the organisms were grown in an unbuffered medium containing 5–10 g. glucose/l. where the ultimate pH of the culture was 4·0 to 4·2. In a buffered medium containing a low glucose concentration the final pH value of the culture remained above 6·0 and poor yields of group D antigen in the acid extract were obtained with *Streptococcus faecium*, *S. durans*, and *S. bovis*.

INTRODUCTION

It has frequently been reported that it was difficult to demonstrate the group D antigen/antibody reaction in organisms which on other grounds would be considered to belong in the Lancefield serological group D. Although *Streptococcus faecalis* and its variants *liquefaciens* and *zymogenes* have been shown to be in serological group D with comparative ease (Lancefield, 1933; Lancefield, 1937; Sherman, 1938; Smith, Niven & Sherman, 1938) many difficulties have been experienced with strains of *S. faecium*, *S. durans* and *S. bovis* (Sherman, 1938; Skadhauge, 1950; MacPherson, 1953; Higginbottom & Wheater, 1954; Buttiaux, 1955; Morelis & Colobert, 1958). This has often been attributed to the lack of a sufficiently potent grouping serum; but Shattock (1949 and personal communication) stressed the importance of the nature of the medium in which the organisms are grown for preparing vaccines and the antigen extracts. In the present paper, the effect of the medium on production of group antigen by various faecal streptococci has been studied.

METHODS

Media. Difco-Todd Hewitt Broth and a modified Todd Hewitt which contained (g./l.): proteose peptone (Difco), 10; glucose, 2; sodium chloride, 2; desiccated heart (Oxoid), 100; disodium hydrogen phosphate, 0·40; sodium bicarbonate, 2; adjusted to pH 7·6. This medium was distributed in 50 ml. lots in centrifuge tubes and sterilized by steaming on 3 successive days. *Glucose Lemco broth* (Shattock, 1949). This medium contained (g./l): peptone (Evans), 10; Lab-Lemco, 10; glucose, 10; sodium chloride, 5; adjusted to pH 7·2. This medium (without glucose) was distributed in 47·5 ml. lots in centrifuge tubes and auto-

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claved at 120° for 15 min. After cooling, 2·5 ml. of a 200 g./l. glucose solution (sterilized separately at 115° for 10 min.) were added to each flask.

Modifications of the above media. For all the media, the constituents except the glucose were dissolved together and adjusted to pH 7·2 before autoclaving. This basal medium was autoclaved at 120° for 15 min. The glucose was prepared and sterilized as above and added to the basal medium after autoclaving to the required concentration.

Preparation of inoculum. All the organisms were grown in brain heart infusion broth (Difco) for 18 hr. at 37°, about 0·025 ml. of culture then being used as inoculum.

Enumeration of organisms. By using an EEL (Evans Electroselenium Ltd.) nephelometer, curves were made to relate total microscopic counts to nephelometer readings. For example, with *Streptococcus faecalis* grown in Todd Hewitt medium or glucose Lemco medium a nephelometer reading of 40 corresponded to about 3×10^9 organisms/ml. However, with a strain of *S. faecium*, the organisms growing in the glucose Lemco medium were slightly larger so that total numbers were less for the same optical density value than when grown in Todd Hewitt medium. It was decided to relate yield of group D antigen to amount of cell material rather than the total number of organisms. Only the optical density measured as above is recorded in Results.

Strains tested. The *Streptococcus bovis* strains used were isolates from sheep and cattle of different serological types (Medrek & Barnes, 1962*a*). *S. durans* 98D and H2 and *S. faecium* S748 were provided by Dr M. E. Sharpe. *S. faecalis* var. *zymogenes* H69D5 and C1, *S. faecalis* var. *liquefaciens* D10, D15 and D76 and *S. durans* C3 were provided by Dr S. D. Elliott. *S. faecalis* var. *liquefaciens* 50, 3·5 k, 75 and 120 and *S. faecalis* 782 and 567 were provided by Dr K. Skadhauge. *S. faecium* TM/C/80 was isolated from a cow. The other strains of *S. faecium* P3, P7, P/4/16 and N/H2 together with unclassified strains P/14/10, P/16/5, P/17/8 and CH12 were isolated from pigs (Barnes & Ingram, 1955) and were of various serological types (Sharpe & Fewins, 1960).

Serological methods

Group D sera. (1) A streptococcus grouping serum (Lancefield Group D) from Burroughs Wellcome and Co. (2) A grouping serum prepared in the laboratory by the method of Shattock (1949). *Streptococcus durans* 98D was used for the preparation of the vaccine. The serum obtained contained group D antibodies as shown by testing against extracts of known strains of different serological types and was found to be specific.

Precipitin tests. Preparation of HCl extracts. HCl extracts were prepared by the method of Lancefield (1933) by extracting the centrifuged organisms from 50 ml. of 48 hr. cultures incubated at 37° with 1·5 ml. of 0·05N-HCl in saline. This extract was about pH 2·0 before neutralization. Some extracts which did not react with a group D serum were precipitated by ethanol (fraction A; Shattock, 1949) to concentrate any group D substance present.

Precipitin test. Extracts were layered on sera in small glass tubes (internal diameter 3·0 mm.) and examined for ring formation against diffuse light at intervals up to 30 min. When a precipitin ring formed within 0–2 min. the reaction was

recorded as + + +; at 2–5 min. + +; at 5–10 min. +; at 10–20 min. ±; at 20–30 min. tr. When no precipitin ring formed within 30 min. the reaction was recorded as negative.

RESULTS

Faecal streptococci isolated from sheep and cattle were identified biochemically as *Streptococcus faecalis*, *S. faecium*, *S. durans* or *S. bovis* (Medrek & Barnes 1962*b*). When some of these organisms were grown in Todd Hewitt medium and tested for the serological group D reaction, it was found that the *S. bovis* strains did not react with any of the grouping sera used; similar extracts of *S. faecalis* gave good reactions. When the same *S. bovis* strains were grown in glucose Lemco medium positive reactions were obtained.

Extracts were then compared from the following strains grown in Todd Hewitt medium and glucose Lemco medium: *S. faecalis* (N83), *S. durans* (98D), *S. faecium* (TM/C/80) and *S. bovis* (TM/S/5, TM/S/19, TM/S/105, and TM/C/36). It was found that only the extract of *S. faecalis* gave a significant reaction when grown in the Todd Hewitt medium. Even concentrating the extracts (Shattock, 1949) prepared from the other strains grown in the Todd Hewitt medium did not give extracts which gave significant reactions. It thus appeared that insignificant amounts of group D substance were being formed by *S. durans*, *S. faecium* and *S. bovis* when these were grown in the Todd Hewitt medium, while significant amounts were formed in glucose Lemco medium. To establish this influence of the growth medium 32 other strains of group D were grown in Todd Hewitt (Difco) medium and glucose Lemco medium. Optical density values and the final pH values were determined and the serological group reaction tested with two different grouping sera (one a commercial preparation; the other, 98D serum, prepared in this laboratory; see above).

The results are summarized in Table 1. It will be seen that *Streptococcus faecalis* as well as its variants *liquefaciens* and *zymogenes* gave strong group reactions when grown in Todd Hewitt or glucose Lemco medium, whereas *S. faecium*, *S. durans* and the unclassified strains, gave strong group reactions only when the organisms were grown in glucose Lemco medium. Furthermore, the result was the same whether the commercial D anti-serum or the 98D antiserum was used. The total amount of growth (as indicated by turbidity) was greater in the glucose Lemco medium than in the Todd Hewitt medium. But the differences between the two media (generally less than twofold) were not sufficient to account for the very marked difference in the group reactions shown with all the strains (other than *S. faecalis* and variants) tested. It will be noted that the pH value remained above 6·0 in the Todd Hewitt medium but decreased to pH 4·0–4·2 in the glucose Lemco medium. To explain these differences obtained with organisms grown in the two media, the influence of the different media constituents was examined.

Effect of changing the composition of the medium

A comparison of the composition of the Todd Hewitt medium with the glucose Lemco medium showed that these differed in several respects, in particular, in the peptone, the concentration of glucose and the buffer. Media were prepared at the same time with Proteose peptone (Difco) or Evans peptone 10 g./l., with glucose 2 g. or 10 g./l., and with or without buffer (disodium hydrogen phosphate

Table 1. *A summary of the influence of the growth medium on the demonstration of a group D reaction by individual species and unclassified strains of faecal streptococci*

		Growth medium							
		Todd Hewitt				Glucose Lemco			
Group D species	Number of strains	Group reaction (1)*	Group reaction (2)	Optical density†	Final pH	Group reaction (1)	Group reaction (2)	Optical density†	Final pH
S. faecalis	2	+ + +	+ + +	56	6·4	+ + +	+ + +	76	4·0
S. faecalis var. *zymogenes*	2	+ + +	+ + +	48	6·6	+ + +	+ + +	71	4·0
S. faecalis var. *liquefaciens*	7	+ + +	+ + +	41	6·6	+ + +	+ + +	78	4·0
S. faecium	5	− ±	− ±	36	6·7	+ +	+ +	82	4·2
S. durans	3	−	−	33	6·6	+ + +	+ + +	69	4·0
S. bovis	9	− ±	−	nt.	nt.	+ + +	+ + +	nt.	nt.
Unclassified	4	−	−	37	6·6	+ +	+ +	82	4·0

* (1) 98D and (2) Burroughs Wellcome group D sera.
† Nephelometer readings.
nt. = not tested

0·4 g./l. and sodium bicarbonate 0·2 g./l.). Thus, eight media with different mixtures of these components were prepared. Throughout sodium chloride was at (g./l.) 5; Lab–Lemco at 10; initial pH 7·2 (before autoclaving). Each of the eight media was inoculated with 0·025 ml. of an 18 hr. culture of the required organism. After incubation for 48 hr. at 37° the cultures were shaken to obtain uniform turbidity and the optical density values recorded. The cultures were centrifuged and the final pH value determined colorimetrically on the supernatant fluids. The results obtained are shown in Table 2. When growth with proteose peptone and Evans peptone were compared the only difference was a slightly lower pH value with proteose

Table 2. *The influence of some media constituents on the demonstration of the serological group D reaction*

Glucose conc. (g./l.)	Buffer	Peptone*	Group reaction†	Optical density‡	Final pH
			S. faecalis N83		
2	–	A	+ + +	55	5·0
		B	+ + +	55	4·7
2	+	A	+ + +	80	6·9
		B	+ + +	64	6·6
10	–	A	+ + +	80	3·9
		B	+ + +	80	3·9
10	+	A	+ + +	> 100	4·0
		B	+ + +	> 100	4·0
			S. faecium S748		
2	–	A	+ +	63	5·1
		B	+ +	55	4·9
2	+	A	±	80	7·1
		B	–	23	7·0
10	–	A	+ + +	80	3·9
		B	+ + +	80	3·9
10	+	A	+ + +	> 100	4·0
		B	+ + +	> 100	4·0
			S. durans 98D		
2	–	A	+ +	47	5·0
		B	+	55	4·8
2	+	A	±	55	7·0
		B	–	39	6·8
10	–	A	+ + +	80	4·1
		B	+ + +	80	4·0
10	+	A	+ + +	> 100	4·2
		B	+ + +	> 100	4·2
			S. bovis TM/S/105		
2	–	A	±	39	4·8
		B	±	80	4·6
2	+	A	–	> 100	7·0
		B	–	80	7·0
10	–	A	+ + +	80	4·1
		B	+ + +	80	4·1
10	+	A	+ + +	> 100	4·1
		B	+ + +	> 100	4·1

* A, Evans peptone; B, proteose peptone.
† 98D grouping serum used.
‡ Nephelometer value.

Table 3. *The influence of the glucose concentration on the demonstration of the serological group D reaction*

Glucose conc. (g./l.)	Group D species								
	S. faecalis N83			*S. faecium* S748			*S. durans* 98D		
	Group reaction*	Optical density†	Final pH	Group reaction*	Optical density†	Final pH	Group reaction*	Optical density†	Final pH
0	+	5	7·0	−	6	7·1	−	4	7·0
2	+ + +	49	5·1	+	42	5·1	+	40	5·1
5	+ + +	83	4·0	+ +	80	3·9	+ + +	71	4·1
10	+ + +	82	4·0	+ +	80	3·9	+ + +	69	4·2
20	+ +	80	4·0	+ +	76	3·9	+ +	69	4·2
50	+ +	73	4·0	+	69	3·9	+	55	4·3

* 98D gronp D serum used. † Nephelometer readings.

peptone. The effect on the yield of group D antigen was similar in all the strains tested. *Streptococcus faecalis* gave a strong group reaction with all the mixtures studied, irrespective of glucose concentration or final pH value. This contrasted with the results obtained with the three other species (*S. faecium*, *S. durans* and *S. bovis*). All the latter behaved similarly in that negative or dubious group reactions resulted when the organisms were grown with glucose 2 g./l. and buffer (Todd Hewitt medium). In parallel with the negative or doubtful group reactions, the final pH value attained in the medium remained at about pH 7. With glucose (2 g./l.) and no buffer in the medium, the final pH value varied from 4·6 to 5·1, and the serological group D reactions were somewhat improved for all the strains which gave negative or dubious reactions when grown in a medium containing glucose 2 g./l. with buffer. For glucose 10 g./l. with or without buffer, strong group D reactions occurred in every case and the final pH value was low: 3·9–4·2.

Thus, except for *S. faecalis* which gave a strong group reaction under all conditions studied, the other three species gave strong reactions only when low final pH values were attained.

Effect of varying the glucose concentration in Lemco medium

Glucose Lemco medium was thus confirmed as the optimal growth medium for detecting the presence of group D antigen. To determine the most effective concentration of glucose for use in this medium, it was prepared to contain 2, 5, 10, 20 and 50 g. glucose/l. These media were tested with *Streptococcus faecalis* N83, *S. faecium* S748 and *S. durans* 98D. The results (Table 3) showed that the best serological group reactions were obtained when the organisms were grown in medium containing 5–10 g. glucose/l. The results with glucose at 2 g./l. confirmed the results shown in Table 2; in the absence of glucose growth was poor. Although there was little difference in the total amount of growth with 2–10 g. glucose/l. in every case the serological group D reaction was less with the higher glucose concentrations.

DISCUSSION

Two media commonly used for grouping faecal streptococci have been compared. Whilst the group D antigen could be demonstrated in acid extracts prepared from strains of *Streptococcus faecalis* and variants *zymogenes* and *liquefaciens* grown in either Todd Hewitt medium or glucose Lemco medium, strains of the other species failed to give a good serological group D reaction when grown in the Todd Hewitt broth. No explanation can be offered for this difference; however, *S. faecalis* has been shown to differ biochemically in many respects, even from the closely related *S. faecium* (Deibel, Evans & Niven, 1958).

With *Streptococcus faecium*, *S. durans* and *S. bovis* strong serological group D reactions occurred only in parallel with the attainment of a low final pH in the growth medium. Shattock (1949) drew attention to the importance of using a particular brand of peptone but in these experiments no difference was found between the two peptones used. Nevertheless, it is conceivable that the result could be influenced if a peptone of higher buffering power were used together with a marginal glucose concentration.

The explanation for the differences in the serological group D reaction of the

extracts does not depend on the amount of growth obtained in the different media. For example, in Table 1 a difference in the turbidity measurements of 36 and 82 for *S. faecium* growing in the Todd Hewitt and glucose Lemco media represents about a twofold difference in cell mass but the extract showing a + + group reaction had to be diluted six times before even a + reaction was recorded.

It is possible that the group antigen is formed in the Todd Hewitt broth by these organisms but is liberated into the 'metabolic liquid'. Hobson & MacPherson (1954) demonstrated the presence of group D antigen in the 'metabolic liquid' of a rumen streptococcus, whilst the HCl extract prepared from the cells gave a negative reaction. Elliott (1960) has shown that unlike the group antigens of streptococci groups A, C, E and G, the group D antigen does not form part of the cell wall and can be readily extracted from the cells at a pH of between 7·0 and 9·0. However, three of the strains he used were *Streptococcus faecalis* or variants and the same strains (D76, C1 and D10) were tested above (Table 2) and were shown to produce a good group reaction in Todd Hewitt broth. It may be that *S. faecalis* produces a greater amount of group antigen per cell so that even if some is eluted at an alkaline pH there will be sufficient remaining with the cell to obtain a good group reaction. It is believed that *S. faecalis*, unlike the other species, produces neither capsules nor 'slime'. The group D antigen has been shown by Elliott (1960) to contain a large amount of glucose. Bailey & Oxford (1959) have shown that the capsular polysaccharide of *S. bovis* contains glucose whilst the extracellular slime produced by *S. bovis* is a dextran. It is possible that with *S. bovis*, when the glucose in the medium is limiting, capsular material or dextran may be produced rather than group D substance.

Whilst the interpretation of the above results is difficult without further investigation, the practical implications are clear. Many of the weak or negative results reported for the group D streptococci can possibly be attributed to the use of the wrong type of medium for growing the organisms. Todd Hewitt broth is widely and successfully used for the grouping of the other streptococci (in particular those of group A). If a negative group reaction is obtained with the acid extracts prepared from a streptococcus grown in this medium and the organism is suspected from biochemical tests to belong to one of the species within group D, then it would be wise to repeat the test growing the organism in either glucose Lemco medium or in the Todd Hewitt medium with an increased concentration of glucose.

The authors are indebted to the Department of Animal Pathology, University of Cambridge for providing animal house facilities for the preparation of the grouping sera. This investigation was supported in part by a pre-doctoral fellowship (EF-9792) awarded to T.F.M. from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

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J. gen. Microbiol. (1962), 28, 711–719
Printed in Great Britain

Conjugal Transfer of Immunity to Phage λ Multiplication in *Escherichia coli* K-12

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SUMMARY

The transfer of a cytoplasmic component from λ-lysogenic F^+ to non-lysogenic F^- cells of *Escherichia coli* K-12 was demonstrated; similar transfer from λ-lysogenic Hfr to non-lysogenic F^- cells was not detected. The component transferred confers on the recipient cell temporary immunity against maturation of the bacteriophage λ. The relevance of these observations to the mechanism of conjugation is discussed.

INTRODUCTION

The demonstration of zygotic induction (Jacob & Wollman, 1954) and studies on non-inducible mutants of bacteriophage λ (Jacob & Campbell, 1959) have led to the idea of the existence of a specific cytoplasmic repressor, capable of maintaining either prophage or superinfecting phage λ in a non-vegetative condition. Work by Bertani (1956) and Bertani & Six (1958) suggested in the case of prophage P2, that immunity of lysogenic bacteria was probably due to a physiologically active prophage product, i.e. a repressor. The demonstration of the transfer of this repressor would substantiate its existence and reveal the occurrence of cytoplasmic transfer in some of the conjugal systems of *Escherichia coli* K-12. There have been previous reports from work with Hfr × F − crosses, based on the inability to detect the transfer of phage genomes and of the enzyme β-galactosidase, that cytoplasmic transfer does not occur (Jacob & Campbell, 1959; Pardee, Jacob & Monod, 1959). The results of experiments reported here support this conclusion but demonstrate the occurrence of cytoplasmic transfer in $F^+ \times F^-$ crosses, the term cytoplasmic being used to denote anything of a non-chromosomal location.

METHODS

Media. Nutrient broth was prepared from Oxoid no. 2 powder at 25 g./l. and adjusted to pH 7·4.

Nutrient agar was prepared by solidifying nutrient broth with agar powder (Davis Gelatine (N.Z.) Ltd., Christchurch, New Zealand) at 12 g./l.; 'λ agar' was prepared by solidifying nutrient broth with agar powder at 8 g./l.; 'top agar' was prepared by solidifying nutrient broth with agar powder at 5 g./l.

Minimal agar was described by Tatum & Lederberg (1947) except that asparagine was omitted and the medium solidified with agar powder at 15 g./l.

Organisms. All of the strains used in this work were derivatives of *Escherichia coli* K-12. Characteristics of the strains used, and the order in which the initial markers of the various Hfr strains are transferred, are shown in Table 1.

Wild-type λ phage suspensions were prepared by ultraviolet (u.v.) induction of *Escherichia coli* K-12 strain Y10. The final titre was about 2×10^{10} plaque-forming particles (p.f.p.)/ml. Coliphage T6 was prepared by infection of *E. coli* B. Suspensions of this phage, containing about 5×10^{10} p.f.p./ml. were employed in some experiments to kill the donor strains.

Table 1. *Strains of* Escherichia coli *K-12 used*

Strain	Genotype	Order of transfer of loci
P678 F^-	$T^-L^-B_1^-gal_b^-\ S^r\lambda^r$	
C600 F^-	$T^-L^-B_1\ S^rT_6^r\lambda^s$	
C600 F^+ (lysogenic)	$T^-L^-B_1^-\ S^sT_6^s\lambda^r(\lambda^+)$	
C600 F^+	$T^-L^-B_1^-\ S^sT_6^s\lambda^r$	
*P14 F^+ (lysogenic)	$T^-L^-B_1^-lac^-\ T_6^sS^s\lambda^r(\lambda d_{14}^+)$	
*Hfr P4X (lysogenic)	$M^-gal^+S^s(\lambda^+)$	*O pro L T B_1 M*
A′ Hfr (lysogenic)	Prototrophic $gal^+S^s(\lambda^+)$	*O T L pro lac gal* λ

Code:

pro⁻	requirement for L-proline
T⁻	requirement for L-threonine
L⁻	requirement for L-leucine
B⁻	requirement for thiamin
M⁻	requirement for methionine
gal_b^-	inability to ferment galactose
lac⁻	inability to ferment lactose
λ^s	adsorbs bacteriophage λ
λ^r	does not adsorb bacteriophage λ
(λ^+)	lysogenic for bacteriophage λ
(λd_{14}^+)	lysogenic for defective bacteriophage λ

S^s or S^r sensitivity or resistance to 200 μg. streptomycin/ml. respectively.
T_6^r or T_6^s resistance or sensitivity to coliphage T6 respectively.
* Strains kindly provided by Dr F. Jacob.

Assays. The following technique was used throughout these experiments for the detection and assay of λ infective centres. Plates containing 40 ml. of 'λ agar' were used, after drying with lids removed for 30 min. at 37°. The plates were maintained closed at 37° until the overlayers containing the infective centres were ready to pour. The overlayers were prepared by mixing the unknown sample containing infective centres with about 10^8 exponentially growing indicator organisms (C600 $S^rT_6^r\lambda^sF^-$) and 3 ml. of top agar. Where necessary 200 μg. streptomycin/ml. was added to both the λ agar and the top agar.

Population densities of bacterial cultures were determined with the aid of a Petroff–Hausser counting chamber.

Testing for F infection. Separated areas, about 7–8 mm. diameter on a nutrient agar plate were spread with re-isolated clones, and reference F^+ and F^- clones. Following incubation of these masterplates at 37° for 3 hr. the growth was replica plated (Lederberg & Lederberg, 1952) on to a lawn of a suitable auxotrophic F^- strain prepared on minimal agar supplemented with D-glucose (2 g./l.) and thiamin (5 μg./ml.). These plates were incubated at 37° for 24–36 hr., when areas corresponding to F^+ strains on the masterplates show about 20 recombinant colonies.

The same clones were scored for λ lysogeny by replicating growth from a second set of masterplates (carrying reference lysogenic and non-lysogenic clones) on λ agar seeded with indicator bacteria. The plates were incubated for 3 hr. at 37°

and then irradiated by u.v. for 30 sec. at 50 cm. from a Honovia, 15 W. low-pressure u.v. lamp. After irradiation, the plates were incubated for a further 18 hr. in the dark at 37°. With this technique lysogenic clones were surrounded by a narrow band of lysis of the indicator strain.

For the conjugation experiments all cultures were grown for 105 min. in broth at 37° after 1/11 dilution of an overnight culture. Matings were also performed in broth.

RESULTS

Zygotic induction occurs under certain conditions when an inducible prophage is injected from a lysogenic Hfr cell into a sensitive F^- cell. This selects against the recovery of Hfr genetic markers closely linked to the site of the prophage, because of the greater probability of their joint injection. Thus the recovery of recombinants carrying these markers is reduced with increasing frequency of zygotic induction. A useful marker closely linked to the site of the λ prophage is one which controls the fermentation of galactose, namely gal_b.

Cytoplasmic transfer by F^+ cells

To prevent direct infection of recipient organisms by free λ phage, which is present in cultures of lysogenic bacteria, a recipient which does not adsorb the phage was used, namely P678$\lambda^r F^-$. Parallel mixtures were made from a culture of this strain with the three donor strains, C600$S^s F^+$ (non-lysogenic); C600$S^s(\lambda^+)$;

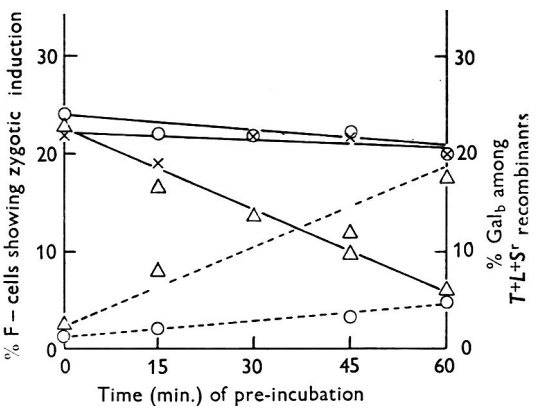

Fig. 1. Three cultures of *Escherichia coli* K-12 strain P678F^- were prepared in broth at 37° containing about 2×10^8 organisms/ml. To one was added about 2×10^8 C600 $S^s F^+$ (non-lysogenic), to the second about 2×10^8 C600$(\lambda^+)S^s F^+$ (lysogenic) and to the third about 2×10^8 Hfr P4X$(\lambda^+)S^s$ (lysogenic). At times indicated on the abscissa, samples were removed from the cultures, agitated vigorously to separate pairs, and added to about 2×10^8 A'Hfr organisms/ml. at 37°. After a further incubation for 45 min., samples were removed and appropriate dilutions plated on minimal agar supplemented with glucose thiamin and streptomycin to assay for $T^+L^+S^r$ recombinants formed. Parallel samples were plated with indicator strain, C600$S^r T_6^r \lambda^s F^-$ on agar supplemented with streptomycin to assay for λ infectious centres. The $T^+L^+S^r$ recombinants were analysed for ability to ferment galactose (inheritance of gal_b^+ from A'Hfr) and for lysogeny. ○ = pre-incubation with C600 $S^s F^+$; × = pre-incubation with HfrP4X$(\lambda^+)S^s$; △ = pre-incubation with C600 $(\lambda^+)F^+$; – – –, gal_b^+ inheritance; ——, zygotic induction (λ plaques as % P678 F^- bacteria plated.).

and Hfr P4X $S^s(\lambda^+)$. This particular Hfr strain carries λ prophage near to the distal extremity of the chromosome and it is not therefore transferred to F^- cells during the time of the experiment. At intervals after preparing the three mixtures, samples were removed from each and added to separate samples of strain A'Hfr to give about equal numbers of A'Hfr and F^- organisms. These mixtures were left for a further 45 min. at 37° to permit transfer of the λ prophage, effected by A'Hfr 28 min. after making contact with an F^- organism. When the latter is not lysogenic for λ, there is a very high probability that zygotic induction will occur. This frequency of zygotic induction was determined by plating suitable dilutions of each of the three mixtures with excess indicator bacteria ($C600S^rT_6^r\lambda^sF^-$).

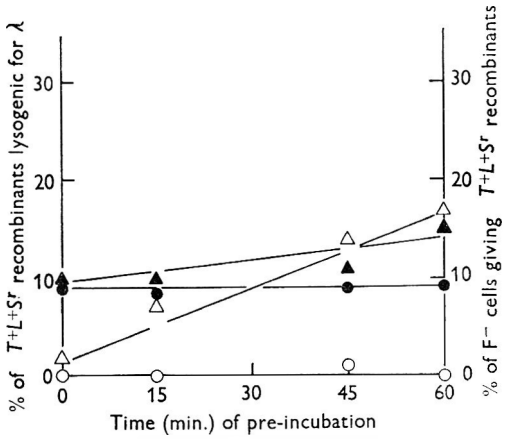

Fig. 2. For experimental conditions see legend to Fig. 1. ● = pre-incubation with $C600S^sF^+$, % of F− cells giving $T^+L^+S^r$ recombinants; ▲ = pre-incubation with $C600S^s(\lambda^-)F^+$, % of F− cells giving $T^+L^+S^r$ recombinants; ○ = pre-incubation with $C600S^sF^+$, % of $T^+L^+S^r$ recombinants lysogenic for λ; △ = preincubation with $C600S^s(\lambda^-)$, % of $T^+L^+S^r$ recombinants lysogenic for λ.

From Fig. 1 it can be seen that where pre-incubation was with a lysogenic F^+ strain, the frequency of zygotic induction was decreased from 23% of F^- cells to 6% after 60 min. pre-incubation. In the case of pre-incubation with a non-lysogenic F^+ or lysogenic Hfr the frequency of zygotic induction was only decreased by 3–5% over the same period.

Besides assaying for zygotic induction, parallel samples of the three mixtures were plated on minimal agar supplemented with streptomycin and thiamin to select for $T^+L^+S^r$ recombinants. These were subsequently tested for the inheritance of gal_b^+ and λ prophage from A'Hfr. The latter part of the analysis cannot be applied to samples involving pre-incubation with Hfr P4X, since this gives rise to large numbers of recombinants which cannot be distinguished from those due to A'Hfr. Figure 1 shows that the recovery of gal_b^+ increased from 2 to 18% following pre-incubation for 60 min. with a lysogenic F^+; pre-incubation with a non-lysogenic F^+ for the same period produced a very small effect. Pre-incubation of lysogenic F^+ cells with non-lysogenic F^- cells resulted in an increase, from 10 to 15%, of the F − cells which yielded $T^+L^+S^r$ recombinants (Fig. 2). Over the same period, the proportion of these recombinants which were lysogenic for λ increased from

3 to 17% (Fig. 2). Pre-incubation with a non-lysogenic F+ showed no effect. The decrease in the frequency of zygotic induction can be explained in two ways. Either the pre-incubation with a lysogenic F+ in some way prevents the A'Hfr from injecting its genetic material together with the prophage, or the pre-incubation allows passage of the repressor of vegetative growth of λ. Recovery of the gal_b markers (Fig. 1) amongst the $T^+L^+S^r$ recombinants isolated, following pre-incubation with a lysogenic F+ increases, thus ruling out the first possibility. Thus one concludes that an F+ lysogenic cell transfers λ immunity when it conjugates with an F− cell whereas an Hfr lysogenic cell does not do so to a detectable extent. The fact that zygotic induction is observed in Hfr × F− crosses, in itself indicates an absence of transfer of λ immunity.

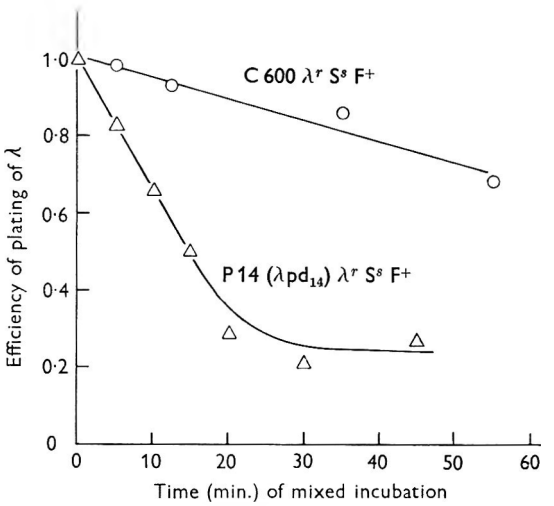

Fig. 3. Two mixed cultures of *Escherichia coli* K-12 in broth at 37° were prepared containing about 2×10^8 organisms $C600S^rT_6^r\lambda^sF^-$/ml. and about 2×10^8 organisms of either $P14S^sT_6^s\lambda^rF^+$ or $C600S^sT_6^s\lambda^rF^+$/ml. At intervals after mixing (abscissa), samples were removed to a mixture of coliphage T6 and wild-type λ phage, such that the input multiplicity of λ for the F− strain was about 0·5. These mixtures were agitated vigorously and then incubated for 15 min. at 37°. Suitable dilutions were plated, with excess $C600S^rT_6^r\lambda^sF^-$ organisms as indicator, on λ agar supplemented with streptomycin, to assay for λ infectious centres. The efficiency of plating (ordinate) is given by (no. of infectious centres at time T/unit sample) ÷ (no. of infectious centres at time 0/unit sample).

Transfer of repressor with challenge by free λ particles

The immunity of lysogenic bacteria to lysis by superinfecting phage has been attributed to the cytoplasmic repressor (Jacob & Campbell, 1959). Accordingly, the ability of passively immunized cells (i.e. cells immunized by conjugal transfer of the repressor material) to suppress the plaque-forming ability of the wild-type λ particles was determined. Broth cultures of normal lysogenic cells in the middle of the exponential growth phase contain about 10^6 infective λ particles/ml. Obviously such a culture cannot be used to transfer repressor substance to λ-sensitive cells. For this reason an F+ strain carrying a defective prophage (λpd_{14}^+) was used as the immunizing strain. Broth cultures of this organism have only

about 10 p.f.p./ml. The parent cultures were mixed, and at intervals samples removed to a mixture of coliphages T6 and λ. The input multiplicities of the two phages were 100 and 0·5, respectively, the coliphage T6 being used to kill the donor strain and the λ phage to determine the immunity of the recipient organisms. Figure 3 shows the efficiency of plating of λ on $C600T_6^rS^r\lambda^sF-$ which has been incubated for varying periods of time (abscissa), either with P14 $(\lambda d_{14}^+)T_6^sS^s\lambda^rF^+$ or $C600T_6^sS^s\lambda^rF^+$ (i.e. a lysogenic or non-lysogenic F^+). Pre-incubation for 45 min. with the lysogenic F^+ resulted in protection of about 75% of the recipient cells, whereas pre-incubation with a non-lysogenic F^+ strain for the same period protected only about 25%. The protection afforded by the latter may have been due to steric blocking of λ receptor sites on the surface of the F^- cells by attached F^+ bacteria. It was noted during the course of these experiments that there was a background of indistinct phage plaques whenever coliphage T6 was added at high multiplicity (about 100 particles/F^+ organism) to an $F^+T_6^s \times F^-T_6^r$ mixture. Similar samples of T6, when plated on F^- cells, show very few plaques, presumably formed by host range mutants. The plaques due to λ which had to be read against the above-mentioned background could be distinguished by their typical morphology.

Table 2. *The independent transfer of F factor and immunity to λ multiplication*

Parallel mixtures of the two *Escherichia coli* K-12 donor strains $P14(\lambda d_{14}^+)\lambda^rF^+$ (lysogenic), and $C600\lambda^rF^+$ (non-lysogenic) with $C600T_6^rS^r\lambda^s$ F^-, containing 2×10^8 organisms/ml. of the donor parent and 10^8/ml. of the recipient were made in broth. At the times shown samples were removed to a mixture of coliphage T6 (multiplicity of 100) and λ (input multiplicity of 0·5) and incubated at 37° for 15 min. Appropriate dilutions were plated on nutrient agar containing 200 μg. streptomycin/ml. to re-isolate the recipient strain. These were examined for lysogeny, which is taken as an indication of a temporarily immune cell at the time of infection with λ, and for *F* character. Percentages of re-isolated F^- clones having acquired *F* and/or lysogeny are given.

Time (min.) after mixing donor and recipient organisms	Donor strain $P14(\lambda d_{14}^+)\lambda^rF^+$ Characters of re-isolated recipient clones (%)				Donor strain $C600\lambda^rF^+$ Characters of re-isolated recipient clones (%)			
	$F^+\lambda^-$	$F^-\lambda^+$	$F^+\lambda^+$	$F^-\lambda^-$	$F^+\lambda^-$	$F^-\lambda^+$	$F^+\lambda^+$	$F^-\lambda^-$
0	4	1	0	95	3	0	0	97
10	14	16	2	68	10	3	1	86
20	28	18	1	53	23	6	1	70
30	31	20	6	43	38	9	0	53
40	30	25	7	38	42	4	1	53

Transfer of the fertility factor F *and of repressor as distinct events*

The ability of F^+ cells to convert F^- cells, by contact, to F^+ has been established for a considerable time (Cavalli, Lederberg & Lederberg, 1953; Hayes, 1953). The high efficiency with which *F* is transferred suggested the possibility of its simultaneous transfer with the repressor substance. To test this, parallel mixtures of P14 $(\lambda d_{14}^+)T_6^sS^s\lambda^rF^+$ (lysogenic) cells or $C600T_6^sS^s\lambda^rF^+$ (non-lysogenic) cells with $C600T_6^rS^r\lambda^sF^-$ cells were prepared. At intervals, samples were treated with coliphage T6 (to destroy the donor cells) and λ added at an input multiplicity of 0·5. Samples of suitable dilutions were plated on streptomycin agar to re-isolate the recipient strain. These were subsequently tested for *F* character and lysogeny (as an indication of immunity transfer). The results shown in Table 2 suggest that

fertility factor *F* and λ immunity are transferred independently, the recovery of 7 % $F^+(\lambda^+)$ clones at 40 min. being close to the expected value, 7·5 % (calculated as the product of independently acquired factors). The results are complicated, however, by the fact that unmated F^- cells will either be destroyed by lytic infection of λ or be scored as $F^-\lambda^-$. Similarly, a proportion of those cells which at the time of infection by λ have acquired *F* but not λ immunity, will also be lysed.

DISCUSSION

The immunity of lysogenic bacteria to related superinfecting bacteriophages has been equated with regulation of enzyme synthesis (Jacob, Perrin, Sanchez & Monod, 1960). The demonstration that a cytoplasmic factor, i.e. non-chromosomal, can be transferred from F^+ cells to F^- cells to produce immunity of the F^- cell verifies the existence of the repressor substance. Further evidence (Fisher, to be published) suggests that the immunity is of a temporary nature. Although in this paper the repressor has been used as a tool to demonstrate cytoplasmic transfer it is obvious that new experimental vistas, in the direction of regulation of protein synthesis, are opened up by the ability to isolate the repressor from its site of synthesis. Experiments have been started with this end in view. The fact that there is at least partial mutual exclusion of cytoplasmic and chromosomal transfer in the *Escherichia coli* K-12 conjugation system is of interest from the point of view of the mechanism of transfer. Whether or not the alternatives, i.e. cytoplasmic or chromosomal transfer in $F^+ \times F^-$ and Hfr $\times F^-$ crosses, respectively, have a common mechanism or if each has a separate mechanism is not known. The transfer of genetic material associated with the fertility factor *F'*, reported by Adelberg & Burns (1960), may argue in favour of a common mechanism for transfer. This would lead to the suggestion that the mere possession of *F* by a cell enables it to perform the preliminary steps in its role as donor and the exclusion of either cytoplasmic or chromosomal transfer would be decided by other factors. The proposed circular chromosome in *E. coli* K-12 (Jacob & Wollman, 1958) may be a restraint on transfer of chromosomal material in $F^+ \times F^-$ crosses. The evidence for its linear nature in Hfr strains allows one to suggest that in Hfr $\times F^-$ crosses, the chromosome from the donor, once it has begun to enter the intercellular bridge, effectively blocks the tube and prevents cytoplasmic transfer. There exists, of course, the possibility of cytoplasmic transfer in Hfr $\times F^-$ crosses occurring before chromosome movement starts. This might explain the early transfer of *F'* factors in Hfr $\times F^-$ crosses.

The transfer of cytoplasm in $F^+ \times F^-$ crosses may explain the observations of Borek & Ryan (1958) on cross-induction where conjugation of a previously u.v.-irradiated non-lysogenic F^+ culture with a lysogenic F^- culture led to the induction of the prophage carried by the F^- cell. In addition, Borek & Ryan (1960) showed that there was cross-induction to a lesser extent in Hfr $\times F^-$ crosses. If this involves cytoplasmic transfer it may be confined to Hfr/F^- pairs which have not initiated chromosome movement.

Work on the transfer of colicin factors (Fredericq & Betz-Bareau, 1953; Alfoldi, Jacob & Wollman, 1957; Alfoldi, Jacob, Wollman & Mazé, 1958) shows that F^+ strains transmit colicin factor *E*1 to about 70 % of F^- cells in mixed cultures. The

degree of transfer of colicin *E*1 effected by Hfr strains to F^- cells, however, varies from 1 to 80% (Alfoldi *et al.* 1958) according to the particular Hfr strain used. Alfoldi *et al.* (1958) suggested that colicinogenic factors can exist as cytoplasmic or chromosomal entities. If this be true, the low rate of transfer of colicin factor *E*1 effected by certain Hfr strain, might be due to lack of cytoplasmic transfer in $Hfr \times F^-$ crosses. In connexion with $F^+ \times F^-$ crosses, Drs R. C. Clowes and D. Hild (personal communication) have concluded from preliminary experiments that there is independent transfer of three entities namely the *E*1 colicinogenic factor, *F* factor and λ immunity, from F^+ to F^- cells. In the case of colicinogeny for *E*1, the transfer appears to be only from F^+ to F^- cells, confirming the observations of Alfoldi *et al.* (1957).

Jacob, Schaeffer & Wollman (1960) have stated that the *F* factor of *Escherichia coli* K-12 cannot exist in an autonomous state in Hfr cells. However, the fact that there is little or no cytoplasmic transmission by Hfr cells precludes any direct test of this. Consequently, without experimental test, it cannot be held that chromosomal attachment of *F* in Hfr cells is the result of, or causes the establishment of an immunity mechanism for *F*, comparable to that established for λ phage. Recent experiments by Scaife & Gross (1962) have indicated that an *F'lac* factor introduced into an Hfr cell by infection cannot multiply autonomously. This observation supports the suggestion of Jacob, Schaeffer & Wollman (1960) regarding the establishment of an immunity mechanism for *F*.

My thanks are due to friends and colleagues who have made constructive suggestions during this work and preparation of the paper.

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J. gen. Microbiol. (1962), 28, 721–732
Printed in Great Britain

The Amino Acid Metabolism of *Microsporum canis*

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SUMMARY

Microsporum canis utilized several single amino acids as nitrogen source during growth on a defined medium; ammonia was also used but not nitrate or methionine. Several extractants for releasing cytoplasmic contents were examined. With acetic acid (100 g./l.) as extractant the content of α-amino nitrogen, pentose, ammonia and phosphate in the extracts was studied. A marked increase in orthophosphate with age of the mycelium was found. The free amino acid pool was examined chromatographically, qualitatively and quantitatively, during growth. Glutamic acid, glutamine, alanine and proline were the principal components of this pool at all stages of growth; proline formed 40 % of this fraction at the end of the logarithmic phase of growth. Phenylalanine and tyrosine were present only in trace amounts; tryptophan was not detected in the free amino acid fraction. These three amino acids were present in mycelial protein. Glucosamine was present in increasing amounts in mycelial hydrolysates as the mould aged.

INTRODUCTION

The principal free amino acids in a number of dermatophytes have been reported (Chattaway, Toothill & Barlow, 1961); during that work it was noted that the concentration of these amino acids varied with the time at which the mycelium was harvested from batch cultures. A quantitative study of the free amino acid pool has now been made of samples taken at intervals during growth of batch cultures of *Microsporum canis* with glutamic acid as nitrogen source. This was correlated with the utilization of substrates and with the amino acid composition of the mycelium. The growth characteristics of this organism were examined with defined media containing other sources of nitrogen.

METHODS

Maintenance and growth of organism. The organism, *Microsporum canis* strain JMcA, used in this work was obtained from Dr Jacqueline Walker (formerly of the London School of Hygiene and Tropical Medicine). The chemically defined medium used in all growth experiments was that described by Chattaway *et al.* (1961) with replacement as required of glutamic acid by different nitrogen sources; the total N content was adjusted to be the same in each medium.

The organism was maintained in stock culture on Sabouraud's broth at room temperature (16°–21°) and was subcultured every 4–6 weeks. Inoculations for experiments were carried out by washing a culture grown on Sabouraud's broth, three times with 20–30 ml. portions of sterile water and finally homogenizing with

20 ml. water for 30–60 sec, by using the homogenizer of Moore & Mason (1951); 1 ml. portions of the resulting homogenate constituted the inoculum.

Growth curves. These were constructed by inoculating eighteen 250 ml. conical flasks containing 20 ml. of appropriate medium with 1·0 ml. of homogenate. These cultures were incubated at 16°–21°. At suitable intervals three flasks were removed, the mycelium harvested and its dry weight (24 hr. at 105°) determined. The culture filtrates from the three flasks were pooled and diluted with distilled water to the original volume of 60 ml. and subsequently examined for amino acid content, ammonia, reducing sugars and pH value. When nitrate was the source of nitrogen this also was estimated.

Chromatographic techniques. The solvent systems used in the two-dimensional paper chromatography and the composition of the ninhydrin spray were as used by Chattaway *et al.* (1961). The quantitative separation of the amino acids was carried out by ion-exchange column chromatography (Moore, Spackman & Stein, 1958). The eluant from the column was collected with a Locarte Fraction Collector (Locarte Co., 24, Emperor's Gate, London, S.W. 7) and the fractions estimated by the method of Moore & Stein (1954).

Preparation of mycelium. Organisms were extracted by immersion in extractant for a suitable period after harvesting mycelium on a sintered glass crucible and washing three times with water. The mycelium for hydrolysis and quantitative study of mycelial protein was obtained after extraction of cytoplasmic contents by immersion in acetic acid (100 g./l.) for 4 hr.; the residue was freeze-dried. The dry material was then crushed in a mortar and extracted at room temperature (about 20°) with chloroform + methanol (2 + 1 by vol., Wren & Mitchell, 1959) thus removing lipid to decrease the amount of humin formed during the subsequent hydrolysis (Block & Weiss, 1956). The lipid-free residue was freeze-dried; this material is referred to as 'prepared mycelium'. Acid hydrolysis was effected by refluxing this prepared mycelium with a large excess of 6 N-hydrochloric acid (Block & Weiss, 1956), for 24 hr., excess acid being removed by distillation *in vacuo*. Alkaline hydrolysis of the material was effected by refluxing 150 mg. with 20 ml. barium hydroxide (140 g./l.) solution for 18 hr. Excess barium was precipitated by gaseous carbon dioxide (Block & Weiss, 1956) and the solution taken to dryness. The residue was dissolved in aqueous *iso*-propanol (10 vol. + water 90 vol.).

Analytical determinations. Dry weight. The mycelium was harvested on a tared Whatman Filter paper no. 1, 3·0 cm. diam., dried for 24 hr. at 105° and re-weighed. In all the growth curves shown the dry weight values are means of triplicate determinations.

Chemical estimations. Inorganic phosphorus was estimated according to Fiske & SubbaRow (1925), pentose by the method of Mejbaum (1939), α-amino nitrogen according to the method of Moore & Stein (1954) with suitable correction for ammonia. Reducing sugars were estimated according to Somogyi (1952), tryptophan according to Gooder & Happold (1954) and indole by the method of Turner (1961). Ammonia was determined by the method of Seligson & Seligson (1951) except that a rotor was not used and diffusions were carried out overnight. Nitrate was estimated by the method of Noll (1945) as modified by Mr J. Hume (Chemistry Department, University of Leeds). For this with a sample containing up to 70 μg. nitrate-N/5·0 ml., 10·0 ml. of a freshly prepared solution of brucine (80 mg./100 ml.

concentrated sulphuric acid) was added; this is allowed to stand for 3–10 min., then 10·0 ml. distilled water added and the container immersed in cold water for 15 min. The final solution was read in the Unicam Spectrophotometer SP. 600 at 410 mμ.

Chemicals. Unless otherwise stated all chemicals used in this work were of Analytical Reagent grade.

RESULTS

Growth of the organism

A knowledge of the growth characteristics of this strain of *Microsporum canis* was required in order that later results could be correlated with the 'age' of the mycelium. Therefore growth studies were carried out by using the chemically

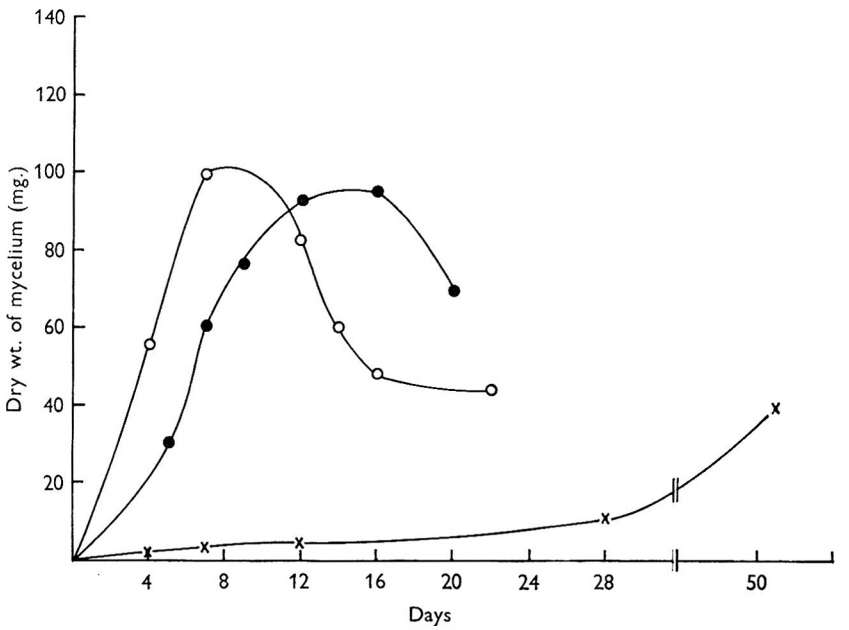

Fig. 1. Growth curves of *Microsporum canis* JMcA grown in a chemically defined medium containing, as nitrogen source, glutamic acid (●—●), arginine (○—○) or methionine (×—×).

defined medium containing different single amino acids, as nitrogen source. The amino acids selected were: alanine, arginine, aspartic and glutamic acids and their respective amides, leucine, proline, methionine. The growth curve with glutamic acid as nitrogen source (Fig. 1) was typical of all the above amino acids with the exception of arginine and methionine; growth was more rapid with arginine, while methionine was a poor nitrogen source.

Table 1 shows the utilization of carbon and nitrogen sources, production of ammonia and pH changes when the above amino acids were the nitrogen sources. It can be seen that the rapid utilization of arginine, as suggested by the rapid increase in the dry weight, was confirmed and was accompanied by a rapid utilization of glucose. Of the remaining amino acids examined the rate of disappearance from the medium was similar and paralleled the rate of removal of glucose.

Table 1. *The growth of* Microsporum canis *strain JMcA; utilization of carbon and nitrogen sources, production of ammonia and* pH *changes occurring in a chemically defined medium, containing different amino acids*

Starting values of glucose were 55·6 μM./ml.

Source of nitrogen	Amino acid					Glucose				Ammonia				pH			
	Incubation periods (days)																
	0	4	8	12	18	4	8	12	18	4	8	12	18	4	8	12	18
	Medium components (expressed in μM./ml. culture filtrate)																
Alanine	44·4	36·0	13·5	10·1	2·3	49·4	37·9	17·8	0	3·9	8·7	7·6	7·6	7·2	7·5	7·6	7·6
Arginine	11·1	3·3	0·5	0·5	0·5	26·6	0	0	0	1·8	4·1	9·1	15·1	6·3	6·4	7·7	7·6
Asparagine	22·2	13·6	10·6	9·9	2·3	50·0	47·2	46·0	16·7	4·4	8·4	9·5	3·5	7·0	7·4	7·4	6·8
Aspartic acid	44·4	28·5	16·6	13·5	7·6	45·5	36·5	27·2	10·0	3·9	3·7	3·4	1·8	7·9	8·3	8·4	8·8
Glutamine	22·2	15·8	6·8	4·1	0	45·5	37·2	19·5	0	15·5	15·3	9·6	4·8	7·1	7·4	7·5	7·5
Glutamic acid	44·4	20·4	5·5	2·0	1·4	39·0	14·4	0	0	3·5	3·7	2·8	4·1	7·9	8·1	8·4	8·8
Leucine	44·4	36·0	19·8	0·8	0·8	54·5	47·2	30·6	0	1·3	4·8	6·1	2·6	6·7	6·7	6·8	7·3
Methionine	44·4	43·0	42·4	40·4	—	51·8	51·8	51·8	—	2·0	5·2	5·8	—	6·5	6·6	6·5	—
Proline	44·4	36·6	24·3	7·0	3·5	51·2	39·5	11·1	2·2	0	0	0	0·9	6·7	6·7	6·9	7·3

These results seem to be in agreement with those of other workers who have carried out this type of study with dermatophytes (reviewed by Stockdale, 1953) and with the genus *Microsporum* in particular (Johnson & Grimm, 1951; Bereston, Robinson & Williams, 1958), although Johnson & Grimm did find that methionine was as good a source of nitrogen as glutamic acid.

The high ammonia concentration found during growth on arginine after the maximum dry-weight value had been achieved suggests the possible development of arginase and urease activity in the later stages of growth. Urease activity has been recorded in dermatophytes by Tate (1929) and Thompson (1955). Growth on the dicarboxylic amino acids yielded pH values of the medium higher than would be accounted for by ammonia production and suggests the formation of other basic substances.

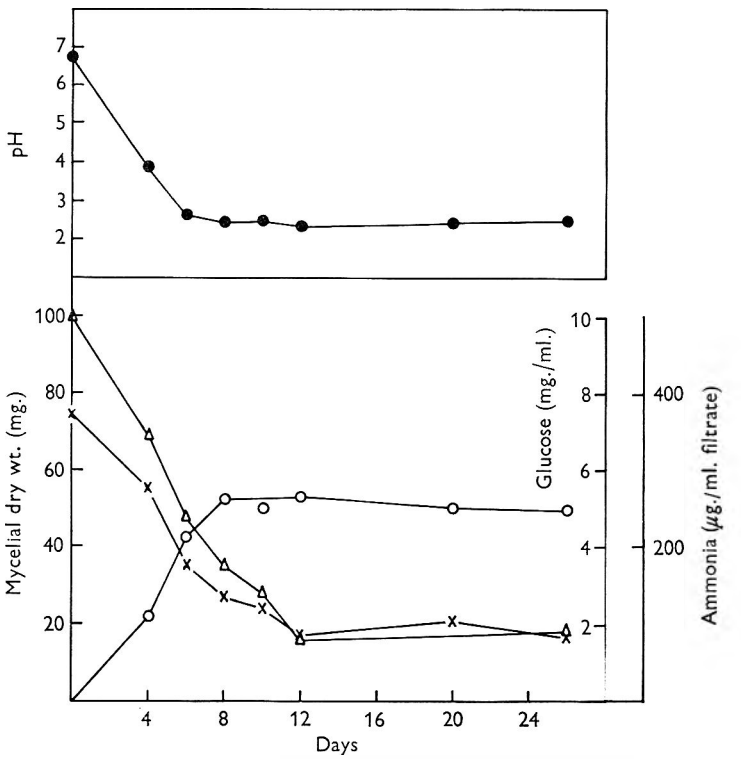

Fig. 2. Increases in dry weight (○—○), and changes in the culture medium when *Microsporum canis* JMcA was grown on a defined medium with ammonium nitrate as nitrogen source. Ammonia, ×—×; glucose, △—△; pH value ●—●.

Growth of *Microsporum canis* JMcA with ammonium nitrate as nitrogen source showed the ability of this organism to utilize ammonia; the nitrate concentration remained constant (Fig. 2). The culture was still viable, as shown by subculture on to Sabouraud medium, after 21 days in a medium of pH 2·5; this pH decrease was due to preferential utilization of ammonia. Morton & MacMillan (1954) found with *Scopulariopsis brevicaulis* that the non-utilization of nitrate in media containing ammonium nitrate was due to residual ammonia. However, attempts to grow

M. canis on a medium containing nitrate as sole nitrogen source were unsuccessful, although Tate (1929) and Johnson & Grimm (1951) reported the growth of some dermatophytes on such a medium. Two points are worth considering: (i) the finding of Fujii (1957) that nitrate utilization occurs with pleomorphic strains only, thus representing a gain in synthetic ability; (ii) the finding of Cochrane (1958) that *Streptomyces* spp. only metabolized nitrate when the mycelium was pre-grown with other N sources.

Intracellular components

Comparison of extractants. Several methods have been used to obtain cytoplasmic contents, including the use of the Mickle shaker (Gale, 1947), organic solvents (Work, 1949) and protein precipitants (Hancock, 1958). Our reasons for using acetic acid (100 g./l.) as extractant for these moulds were given by Chattaway *et al.* (1961). During the present work it became apparent that acetic acid was extracting cytoplasmic contents from the moulds. Thus the ultraviolet (u.v.) spectra of the extracts showed a maximum absorption at 260 mμ, indicative of cytoplasmic material (Salton, 1951); also similar amino acid patterns are shown by material obtained by other extraction methods (see below), several of which cause the release of intracellular amino acids from other micro-organisms. A comparison was therefore made between the efficiency of acetic acid (100 g./l. water), water alone (both at 20° and at 100°), aqueous ethanol (75 vol. ethanol + 25 vol. water) and by cetyltrimethylammonium bromide (CTAB; 100 μg./ml.). The release of inorganic phosphate, pentoses, ammonia and α-amino nitrogen was measured. The results with aqueous acetic acid (100 g./l.) over a period of 0–20 hr. showed that the extraction period of 4 hr. originally used (Chattaway *et al.* 1961) was sufficient for the maximum release of all the components measured; repetition with other extractants showed a similar relationship between the rates at which other cytoplasmic components were released. The rates of release and amounts of components liberated by CTAB were different from those obtained by using other extractants, although the rate of release was slower in the early stages a greater amount was released after 7·5 hr. CTAB is known to be active in removing material attached to bacterial cell walls; for this reason it has been avoided by many workers in the preparation of cell-wall material (Professor J. Baddiley, personal communication). Therefore it was rejected as an extractant for cytoplasmic components in the present work, although extracts obtained using CTAB contained a greater number of amino acids than were seen with any other extractant. Water at about 20°, as one would expect, removed the smallest amount of amino acids; water at 100° appears to be a rather harsh method, amides, for example, being readily hydrolysed under these conditions; the absence of glutamine was noted only when water at 100° was used as extractant. Because relatively little is known about the different actions of these methods of obtaining cytoplasmic contents, or to what extent amino acids exist free within the cell, we felt justified in continuing to use acetic acid (100 g./l.) as extractant.

Differences of cytoplasmic content with age of mycelium

The release, by aqueous acetic acid (100 g./l.), of the various intracellular components was examined for mycelium harvested at different times; the results are shown in Fig. 3. It can be seen that the free α-amino nitrogen had a maximum

value when the dry weight of organism was maximal. The pentose concentration remained relatively constant but the inorganic phosphate and ammonia values increased steadily as the time of harvesting the crop of mycelium increased. The sharp increase in inorganic phosphate after the end of the log phase may be a manifestation of autolysis or of utilization of polyphosphate following the exhaustion of glucose in the medium. Alkaline phosphatases have been shown to be present in two dermatophytes (*Microsporum canis*, by Chattaway, Thompson & Barlow, 1954; *Trichophyton rubrum*, Nickerson, 1951).

The clear differences in the concentration of total α-amino nitrogen was examined. Extracts of *Microsporum canis* from mycelium harvested from 3 to 29 days, after suitable concentration was examined quantitatively for amounts of amino acids

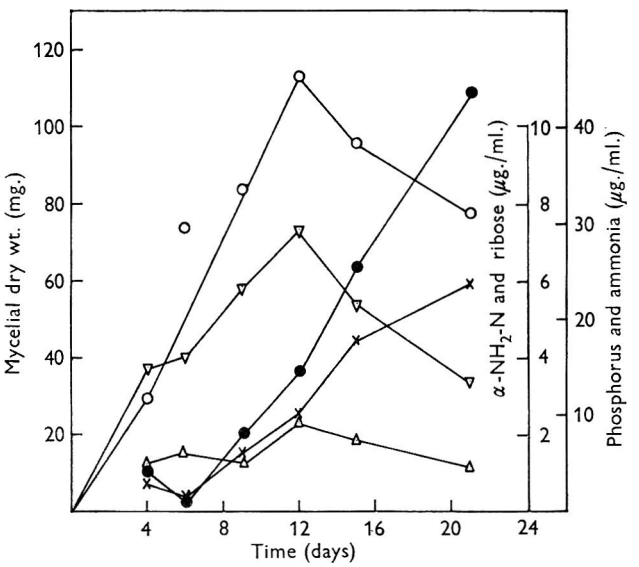

Fig. 3. The release of cytoplasmic components from *Microsporum canis* JMcA, harvested at different times, by acetic acid (100 g./l. water) at 20° for 4 hr. The concentrations of the components are expressed as μg./mg. dry weight mycelium. The growth curve (○—○) is as mg. dry-weight mycelium; phosphorus, ●—●; ammonia, ×—×; α-amino nitrogen, ▽—▽; pentose, △—△.

by ion-exchange chromatography. The results of these analyses are given in Table 2. The 'glutamine' column represents glutamine + serine + threonine, because glutamine was in such high concentration that difficulty was experienced in resolving it from the two amino acids.

The results shown in Table 2 confirm those shown in Fig. 3; the maximum concentration of the pool amino acids was observed towards the end of the log phase of growth. It can be seen from these results that most of the compounds which were previously reported (Chattaway *et al.* 1961) were present in the highest concentrations. The relatively low concentrations of the other acids together with the rather high concentrations required to detect many of them on paper chromatograms (Block, Durrum, & Zweig, 1958) presumably accounts for the only occasional

detection of these amino acids as compared with the frequent occurrence of glutamine, glutamic acid and aspartic acid, proline and alanine. The high concentration of proline in the amino acid pool is striking. Only occasionally were tyrosine and phenylalanine observed in this quantitative survey; even at their highest concentrations they were present only at 0·05–0·20 μg./mg. mycelium. Tryptophan was never detected.

Another feature of the results shown in Table 2 is the steadily increasing free ammonia that was extracted; a particularly sharp rise being observed after the log phase; this is in agreement with the data given in Fig. 3.

Table 2. *The intracellular amino acids present in the mycelium of* M. canis *JMcA at various stages of growth: results expressed as μg. amino acid/mg. dry mycelium*

'Glutamine' = glutamine + serine + threonine since the high level of glutamine made separation impracticable.

Age of pad (days) ...	3	6	9	11	13	17	22	28
Dry weight (mg./pad) ...	10·1	45·4	65·4	98·5	108·8	116·0	91·8	78·0
Amino acids								
Aspartic acid	0·29	0·35	0·85	0·41	0·33	1·11	0·32	0·14
'Glutamine'	—	2·06	9·25	8·70	5·15	7·15	4·97	Nil
Glutamic acid	—	6·60	7·29	14·6	6·96	8·55	1·03	0·66
Proline	8·70	1·59	2·12	3·60	19·40	9·95	1·44	0·68
Glycine	0·38	0·07	0·74	0·44	0·43	0·43	0·11	0·05
Alanine	2·17	1·77	9·55	2·80	6·88	6·76	1·22	0·81
Cystine	2·37	0·35	7·20	0·49	3·43	1·40	0·40	0·46
Valine	0·82	0·83	0·21	0·71	1·55	0·12	0·11	0·10
Methionine	1·43	0·30	1·60	0·51	0·60	0·60	0·08	0·14
Leucine/isoleucine	Nil	0·21	2·71	0·59	0·70	0·73	0·11	0·11
Lysine	1·61	0·62	2·60	0·77	1·50	1·56	0·16	0·21
Histidine	Nil	0·18	0·97	0·29	0·54	0·88	0·28	0·30
Ammonia	0·57	0·99	0·68	0·38	1·22	5·01	9·34	8·58
Arginine	0·87	0·30	2·50	0·39	1·39	1·62	Nil	Nil

Table 3. *The amino acid composition of the prepared mycelium of* M. canis *JMcA. Results expressed as mg. amino acid/*100 *mg. prepared mycelium*

Amino acids	11-day-old mycelium	30-day-old mycelium
Alanine	1·17	0·15
Arginine	1·47	0·17
Aspartic acid	1·83	0·26
Cystine	2·07	0·54
Glucosamine	5·67	14·34
Glutamic acid	1·98	0·27
Glycine	1·00	0·20
Histidine	0·67	0·24
Leucine/isoleucine	1·49	0·27
Lysine	2·60	0·71
Methionine	1·14	0·25
Phenylalanine	0·99	0·11
Proline	0·82	0·32
Serine	0·88	0·31
Threonine	0·95	0·31
Tryptophan	0·25	0·05
Tyrosine	1·56	0·22
Valine	0·26	0·02

The amino acid composition of prepared mycelium

Samples of mycelium grown for 11 and 30 days on the glutamic acid medium were hydrolysed as described. The hydrolysates were analysed quantitatively for amino acids. This was done to see whether a correlation existed between the amino acid concentrations in the pool and the concentration of the corresponding amino acid in the mycelial protein. The results of these analyses are presented in Table 3.

Variation of the components of the amino acid pool with the nitrogen source of the medium

Similar patterns of amino acids were observed in extracts when single amino acids or ammonium salts served as the nitrogen source, non-detection of certain amino acids being possibly explicable by low concentrations rather than complete absence (Table 4). The most complete picture was obtained with Proteose peptone as nitrogen source in the Sabouraud medium; the occurrence of hydroxyproline

Table 4. *Intracellular free amino acids present in the mycelium of* M. canis *JMcA grown on different nitrogen sources*

A record of qualitative examination of paper chromatograms of acetic acid (100 g./l.water) extracts of mycelium.

Nitrogen source in the medium ...	Glu.	Glu-NH_2	Ala.	Pro.	Leu.	Asp.	Asp-NH_2	Arg.	Ammonium $-SO_4$	Ammonium $-NO_3$	Tar-trate	Sabou-raud broth
Amino acids found												
Alanine	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	−	+	−	−	−	+	+	+	+
Aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+
γ-Aminobutyric acid	−	+	−	+	−	−	−	−	+	+	+	+
Cystine	+	+	+	+	+	+	+	−	+	+	+	+
Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+
Glutamine	+	+	+	+	+	+	−	−	+	+	+	+
Glycine	+	+	−	+	+	+	+	+	+	+	+	+
Histidine	−	+	+	+	+	+	−	−	−	−	+	+
Hydroxyproline	−	−	−	−	−	−	−	−	−	−	−	+
Leucine	+	+	−	−	−	−	−	+	+	+	+	−
Lysine	+	+	+	+	+	+	+	+	−	−	+	+
Methionine/valine	+	+	−	+	+	+	+	+	+	+	+	+
Ornithine	−	−	−	−	−	−	−	+	−	−	−	−
Proline	+	+	+	+	+	+	+	+	+	+	+	+
Serine	+	+	−	+	+	+	+	+	+	+	+	+
Threonine	+	+	+	−	−	+	+	+	+	+	+	+
Tyrosine	+	+	−	−	−	−	−	−	−	−	−	−

in these extracts is notable. This seems to be the first report of the presence of this compound in extracts of dermatophytes, although Hoare (1955) has detected it in aqueous ethanol extracts of *Sarcina lutea.*

The occurrence of ornithine in the extract from mycelium grown on arginine is not surprising because of the importance of this amino acid in the urea cycle, the operation of which is suggested by the high concentrations of ammonia found in these cultures (Table 1). The almost complete absence from the chromatograms of tyrosine and the absence of phenylalanine and tryptophan was noticeable.

DISCUSSION

The qualitative differences in the free amino acid pool with changes in nitrogen source in *Microsporum canis* (Table 4) were in keeping with the results of Simonart & Chow (1953) with *Aspergillus oryzae*. They found that glutamic acid, alanine, aspartic acid, glutamine and glycine were present in organisms grown on all nitrogen sources, that growth on arginine gave ornithine in the pool and that a complex nitrogen source yielded a more comprehensive collection of amino acids in the pool. That this last finding is not always the case was shown by Fujii & Yoshimura (1957) who found more amino acids in extracts of *Trichophyton mentagrophytes* when this organism was grown on a defined medium containing glutamic acid than when it was grown in Sabouraud medium; Okazaki & Tamemasa (1953) only detected six amino acids during the growth of *T. asteroides* on the latter medium. γ-Amino butyric acid was found in all extracts from growth on ammonium salts where the pH value of the medium decreased to small values during growth, but only rarely from growth on amino acids. Simonart & Chow (1954) found that *A. oryzae* only formed γ-amino butyric acid when the medium was below pH 4. However, growth of *Microsporum canis* on Sabouraud medium became alkaline, but γ-aminobutyric acid was readily detected in the amino acid pool.

The quantitative study of the free amino acids during the growth of *Microsporum canis* with glutamic acid (Table 2) confirms the qualitative studies in that proline, alanine, glutamic acid and glutamine were found to be the most abundant components; the general picture was of increasing concentrations of free amino acids during the logarithmic phase of growth and a rapid decrease thereafter. This pattern is like that recorded by Pillai & Srinivasan (1956) for *Aspergillus flavus*. Proline, alanine and glutamine are all closely related metabolically to glutamic acid; this may explain the high concentration observed. These four amino acids comprised 80–90% of the free amino acids, except that after 6 and 28 days growth the value was 60%. After 13 days of growth proline alone comprised 40% of the free amino acid fraction; this may indicate some particular function for this amino acid. The yellow pigment characteristic of many strains of *Microsporum canis* was present in much greater amounts in *M. canis* strain JMcA when this was grown with proline as sole nitrogen source than when it was grown on any other single amino acid. The high concentration of glutamine may reflect its utilization as a source of glucosamine-6-phosphate (Leloir & Cardini, 1953), a precursor of chitin. Chitin is a major component of the mycelial cell wall of dermatophytes (Blank, 1953); its increased concentration in ageing mycelium is reflected in the three-fold increase in glucosamine seen in the hydrolysate of 30-day mycelium as compared with that of 11-day mycelium (Table 3).

The amino acids found in hydrolysed mycelium of *Microsporum canis* are similar to those found for numerous other micro-organisms and resemble those found by Hare (1953) in 8 dermatophytes, except that Hare did not detect tryptophan. This was present (Table 3) in low concentration in *M. canis* stain JMcA. Neither tryptophan, tyrosine or phenylalanine were found in the free amino acid fractions although all three were present in the mycelial protein; these amino acids must be utilized rapidly by *M. canis* following synthesis and thus were not present in detectable concentrations in the free state.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its thirty-fourth General Meeting in the Royal Institution, London, on Monday, Tuesday and Wednesday, 9, 10 *and* 11 *April* 1962. *The following communications were made*:

COMMUNICATIONS

Some Physical and Chemical Properties of Wound Tumour Virus. By L. M. Black, G. J. Hills and R. Markham (*Agricultural Research Council, Virus Research Unit, Cambridge*)

The wound tumour virus is a large plant virus of interest because it can multiply in its insect vector. The virus has been examined in the ultracentrifuge using turnip crinkle virus as an internal control for the elimination of viscosity and density corrections and the sedimentation coefficient of the virus is about 510S. The purified preparations have an absorption spectrum with 260/240 and 260/280 mμ absorption ratios of about 1·16 and 1·55, respectively. We have examined the nucleic acid obtained from purified preparation of the virus and find that this is ribonucleic acid. Preliminary measurements of the base composition of this virus have been made and show no unusual proportions that might suggest pairing of bases. Recent interpretations of electron micrographs by Bils and Hall showing wound tumour virus negatively stained with phosphotungstate are partially confirmed in our similar micrographs. This virus appears hexagonal in outline with a membranous structure supporting many surface subunits which we have resolved as helical structures. Some of the subunits which appear to have been stretched look like minute corkscrews. They appear to have a hollow centre when viewed either parallel or at right-angles to their long axis. Rarely, the supporting membrane is seen free of other components but free surface subunits are common in the background. Many empty particles are encountered.

This work was partly supported by a U.S. Public Health Service grant.

Some Relations of Hydrogen Bonding in the Poliovirus Particle to Growth of the Virus. By P. D. Cooper (*M.R.C. Virus Culture Laboratory, Carshalton, Surrey*)

The temperatures at which growth is optimal may differ considerably for different strains of poliovirus type 1. These findings, and others (Lwoff, A. & Lwoff, M. (1961), *Ann. Inst. Pasteur*, **101**, 469) involving reagents which alter the magnitude of hydrogen bonding, have suggested (Lwoff, A. & Lwoff, M. (1961), *Nature, Lond.* **192**, 477) that the growth of poliovirus includes cycles composed of the breaking and reformation of certain hydrogen bonds. The existence of different thermal growth requirements among poliovirus strains is postulated to reflect cyclic changes during growth in hydrogen bonds of numbers or strengths which differ according

to the thermal growth requirements, such that weaker bonding results in inhibition of growth at higher temperatures.

Concentrated urea solutions have been shown to cause a progressive damage to the poliovirus particle, which probably results from simple rupture of hydrogen bonds (Cooper, P. D. (1962), *Virology*, **16**, 485). Such damage culminates in the abrupt release of the RNA from the particle in a soluble and sometimes infectious form, while the protein remains sedimentable. The inactivation curve has the shape characteristic of a multistep reaction.

This paper reports that treatment of poliovirus strains of different thermal growth requirements with 7·2 M urea at 37° produces a family of inactivation curves of similar shape, but differing in that strains which will grow at higher temperatures are less readily inactivated by urea. A similar family of inactivation curves is obtained if the bonding of the system is modified by incorporating D_2O into the particles or into the urea solutions, by varying the temperature or by varying the urea concentrations. Therefore, the concept that the different thermal growth requirements of the different strains reflects bonding of different strengths is concluded to be confirmed. It is also concluded that these differences in bonding are reflected in the particles themselves.

Cell-wall Precursors in *Escherichia coli* 26-26. By M. D. Lilly (*Department of Biochemistry, University College London*)

The lysine-requiring mutant of *Escherichia coli* 26-26 was grown in a medium containing glucose, inorganic salts and limiting amounts of lysine. Exhaustion of the lysine resulted in the accumulation of lipomucoprotein, diaminopimelic acid and nucleotides in the growth medium (Meadow, P. M. (1958), *J. gen. Microbiol.* **18**, iii).

The nucleotides accumulating in the growth medium were concentrated by absorption and elution from charcoal and separated on a Dowex-1 × 10 column using a formic acid elution gradient. They included cytidine diphosphate ribitol and cytidine diphosphate glycerol.

The trichloracetic acid-soluble nucleotides from bacteria grown in various lysine concentrations were separated on a Dowex-1 × 2 column using a hydrochloric acid and calcium chloride elution gradient. Several uridine diphospho-acetylmuramic acid peptides were found in extracts of lysine-deficient bacteria. Two of these contained diaminopimelic acid, alanine and glutamic acid. Another was shown to contain serine and glutamic acid.

Determination of the amino acid composition of the isolated mucopeptide from the cell wall of this organism gave values similar to those obtained in other strains by J. Mandelstam ((1961), *Nature, Lond.* **189**, 855) except that aspartic acid was present in significant quantities.

Extraction of isolated cell walls with cold 10% trichloracetic acid removed a material which was fractionated by ethanol precipitation. The ethanol-insoluble material was hydrolysed and the components identified by paper chromatography. It appeared to be a polymer containing ribitol, glucose, phosphate and all the amino acids found in the mucopeptide.

It is suggested that the cell walls of *E. coli* 26-26 contain a mucopeptide component in which two peptides are present, one containing diaminopimelic acid and the other lysine, and that a ribitol teichoic acid may be linked in some way to this mucopeptide.

I am indebted to the D.S.I.R. for a Postgraduate Studentship.

Mutants with Impaired Respiration in *Staphylococcus afermentans*. By G. F. Gause (*Institute of Antibiotics, Academy of Medical Sciences, Moscow*)

Mutants with small colonies and impaired respiration were induced by 5-fluorouracil in the cultures of *Staphylococcus afermentans*, NCTC 7503. The consumption of oxygen in colourless mutants attained 66–77 %, and in mutants with orange colonies 68–77 % of normal values. Inhibitors of protein synthesis (chloramphenicol, tetracycline, chlortetracycline), inhibitors of cell-wall synthesis (penicillin G, phenoxymethylpenicillin), as well as an inhibitor of RNA formation (actinomycin C) inhibited the growth of the parent and mutant staphylococci to the same degree. On the other hand, inhibitors which affected DNA—namely, degranol [1,6-bis-(*β*-chloroethylamino)-1,6-deoxy-*d*-mannitol], myleran-mannitol (1,6-dimethanesulphonyl-*d*-mannitol) and mitomycin C selectively inhibited the growth of mutant staphylococci with impaired respiration. Mitomycin C induced irreversible depolymerization of DNA in bacterial cells; colourless mutants were 2·7–3·5 times, and mutants with orange colonies 33–45 times more vulnerable to its action than the parent culture. Differences were also found in the DNA–protein relationship of normal bacteria and of mutants. The extraction of DNA from bacterial cells by Schmidt and Thannhauser procedure, as modified by A. S. Spirin & A. N. Belozersky (1957, *Biokhimia* (*Moscow*), **22**, 744), showed that the capacity of DNA for extraction from a complex with proteins was decreased in colourless mutants by 2·8–2·9 times, and in mutants with orange colonies by 5·0–7·3 times. This points to the increased firmness of DNA-protein bonds in the cells of mutants, which accompanies their vulnerability to the action of mitomycin C. Some differences in the DNA-protein binding in normal and tumour cells were recently recorded (Kirby, K. S. (1961), *Progr. exp. Tumour Res.* **2**, 291).

The Swelling of Bacterial Spores during Germination and Outgrowth. By A. D. Hitchins and G. W. Gould (*Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford*)

It is well known that after germinating, bacterial spores swell prior to the emergence and growth of new vegetative cells. However, although the processes of germination and outgrowth have been intensively studied morphologically and biochemically, swelling of growing spores has received little attention. We have therefore studied the changes in volume of spores during germination and outgrowth.

Swelling was followed by measuring changes in the packed cell volume (p.c.v.) of suspensions of growing *Bacillus* spores and by photomicrographic measurements of the single cells. Three stages of swelling were recognized: (1) germination swelling which involved a rapid increase of *c.* 20 % in p.c.v. followed by (2) pre-emergence swelling of *c.* 100 % increase in p.c.v., and (3) post-emergence swelling during growth of the new vegetative cell.

Pre-emergence swelling was affected by aeration. This was suspected when it was noticed that the extent of this swelling was inversely dependent on the spore concentration in poorly aerated media. Furthermore, with a given spore concentration the amount of pre-emergence swelling was greater with vigorous than with poor aeration. Pre-emergence swelling was also inhibited by nisin, which is known to prevent emergence from the spore coat whilst not affecting germination. In contrast to pre-emergence swelling, germination swelling was not reduced by increasing the spore concentration or by poor aeration, and occurred even when the medium was bubbled with nitrogen or contained nisin. During germination swelling the increase in spore breadth exceeded the increase in spore length.

These results indicate that the germination swelling observed is an inseparable part of the germination process, and is due to uptake of water for rehydration of the spore. This process is probably not dependent on exogenous oxygen or energy sources whereas post-germination (pre-emergence) swelling requires an external source of energy and involves synthesis of new cell material.

The Relation Between Protein and Bacteriochlorophyll Synthesis in *Rhodopseudomonas spheroides*. By M. J. Bull and J. Lascelles (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The photosynthetic pigments, bacteriochlorophyll and carotenoids, are associated with subcellular particles, termed chromatophores (Schachmann, H. V., Pardee, A. B. & Stanier, R. Y. (1952), *Arch. Biochem. Biophys.* **38**, 245). Previous work showed that bacteriochlorophyll synthesis by suspensions of organisms requires a N-source and is inhibited by amino acid and purine analogues (Lascelles, J. (1959), *Biochem. J.* **72**, 508).

Various analogues which are known to effect protein synthesis, e.g. ethionine, 8-azaguanine, 5-fluorouracil, also inhibit formation of bacteriochlorophyll by cultures of *Rhodopseudomonas spheroides* growing photosynthetically; in fact pigment synthesis is more sensitive to inhibition by these analogues than is overall protein synthesis.

When suspensions of organisms, initially low in pigment, are incubated with low aeration, they synthesize bacteriochlorophyll. Under these conditions incorporation of [$^{14}C_1$]phenylalanine into the particulate protein fraction of the organisms is twice as great as that into the soluble fraction. Incorporation occurs at the same rate into each fraction when organisms are growing exponentially.

Enzymes associated with bacteriochlorophyll synthesis (e.g. δ-aminolaevulic acid (ALA) synthetase) increase rapidly during adaptation of cells to form the photosynthetic pigments. Analogues prevent synthesis of this enzyme, as does the omission of histidine or adenine from the medium of a mutant requiring these nutrients. If the organisms are incubated with low aeration for one hour the level of ALA synthetase reaches a maximum, yet bacteriochlorophyll synthesis is diminished by subsequent addition of analogues, or by transfer of wild type organisms to N-poor medium or of the mutant to deficient medium. Under most of these conditions the level of ALA synthetase decreases, obscuring the interpretation of the effect on pigment synthesis. However, in the case of organisms transferred to N-poor medium, the level of ALA synthetase remains unaltered while

bacteriochlorophyll synthesis is greatly diminished compared to control organisms transferred to fresh complete medium.

The balance of evidence at present favours the hypothesis that there is an obligatory link between the synthesis of bacteriochlorophyll and synthesis or turnover of particulate protein.

Hydrogen Sulphide Formation by Pantothenate-Deficient Yeast. By T. WAINWRIGHT (*Chemist's Laboratory, Arthur Guinness Son and Co. (Dublin) Ltd., Dublin*)

Many yeasts require pantothenate or β-alanine for growth. All such strains tested (62) form H_2S from sulphate or sulphite when grown with suboptimal pantothenate. With adequate pantothenate no sulphide is detected. Pantothenate deficient yeast produces no H_2S when grown with methionine, cysteine, homocysteine or glutathione as sulphur source. These compounds do not stop the formation of sulphide from sulphate or sulphite.

Non-growing suspensions in potassium phosphate buffer yield H_2S in amounts proportional to the glucose and sulphate or sulphite concentrations. Up to 65% of the sulphur is recovered as sulphide. Added pantothenate suppresses sulphide formation. The smaller amounts of H_2S formed from compounds such as cysteine are not affected by pantothenate.

The reaction occurs aerobically or anaerobically. Glucose is metabolized mainly to alcohol and CO_2. Fluoride, arsenate and dinitrophenol inhibit. About 1 m-mole glucose is metabolized per μmole H_2S formed.

Extracts from pantothenate-starved yeasts rapidly form sulphide from sulphite by a reaction requiring TPNH (Wainwright, T. (1961), *Biochem. J.* **80**, 27P). Added pantothenate or coenzyme A has no effect; extracts from pantothenate-rich yeasts are equally active.

Pantothenate probably affects H_2S formation relatively indirectly. Methionine was reported to prevent H_2S formation by pantothenate deficient Saké yeast (Kodaira, R., Ito, Y. & Uemura, T. (1958). *Nippon Nôgei-Kagaku Kaishi*, **32**, 49; Kodaira, R. & Uemura, T. (1960), *Kôso Kagaku Shinpojiumu*, **14**, 133). In the present work no added sulphur compound reduced H_2S formation. This suggests that it is not produced as a result of a blocked biosynthetic reaction. Sulphide utilization is not inhibited and deficient cells can use it as the sulphur source for growth.

Possibly the deficient yeast makes more use of pathways involving TPN for hydrogen transfer, the TPNH formed being oxidized by the reduction of sulphite.

A Succinyl Derivative of Homoserine as a Precursor of Methionine in *Escherichia coli*. By R. J. ROWBURY (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The formation of cystathionine (a precursor of methionine) from homoserine and cysteine by extracts of *Escherichia coli* has recently been reported (Rowbury, R. J. (1961), *Biochem. J.* **81**, 42P). The reaction requires succinate, glucose and ATP, and

is increased by CoA. Two steps have been shown: (*a*) the formation of a stable intermediate (X) from homoserine and succinate followed by (*b*) condensation of X with cysteine to form cystathionine (Rowbury,R. J. (1962), *Biochem. J.* **82**, 24P). The intermediate is separable from homoserine and succinate by chromatography on paper or Dowex-50 resin and is ninhydrin positive; hydrolysis in N-HCl for 2 hr. at 105° yields succinate and homoserine.

In the presence of homoserine, [$^{14}C_{2,3}$]succinate was incorporated into the amino acid fraction and paper chromatography showed that the radioactive region coincided with that of the new ninhydrin positive compound; after hydrolysis (as above) radioactivity coincided with the succinic acid fraction. The extent of incorporation was compatible with X being formed from equimolar amounts of homoserine and succinate.

The intermediate is stable to heat (100°, 2 min.) and N-HCl (5 min. at 20°) but is destroyed by mild alkali (pH 11, 5 min. at 20°). The presence of a free amino group and a succinyl residue, and the alkali lability, suggest that X is probably an *O*-succinyl derivative of homoserine. The partial requirement for CoA suggests that succinyl CoA is the actual reactant; the enzyme should be named homoserine trans-succinylase.

N-Acyl derivatives of amino acids are intermediates in other biosynthetic pathways, e.g. lysine (Gilvarg, C. (1960), *Fed. Proc.* **19**, 948), but the present observations appear to be the first case in which an acylation activates a condensing group of an amino acid.

A Taxonomic Study of the Genus *Micrococcus*. By M. Kocur and T. Martinec. *Department of Microbiology, Faculty of Sciences, University of J. E. Purkyně, Brno, Czechoslovakia*)

Attempting to contribute to the simplification of the classification of cocci we have studied from the nomenclatural and taxonomic points of view more than 300 strains of micrococci. We have revised most of hitherto valid species of micrococci and have suggested a key for the identification of individual species. Some present taxonomic papers classify a great number of species in the afore-mentioned genus, for example, N. A. Krasilnikov (1949, *Opredělitěl baktěrij i aktinomycetov.* Moskva) quotes sixty species, and Hucker & Breed (1957) sixteen species in the genus *Micrococcus*. In contrast to these authors we recommend that there should be placed six species and two varieties in the genus *Micrococcus* at present. They are the following species and varieties: *Micrococcus luteus*, *M. luteus* var. *cryophilus*, *M. roseus*, *M. denitrificans*, *M. denitrificans* var. *halodenitrificans*, *M. litoralis*, *M. conglomeratus* and *M. varians*.

In accordance with other authors' data we have ascertained that micrococci show a great variability of properties which makes their classification more difficult. We have ascertained, for example, during our studies of aerobic species of the genus *Sarcina*, that the characteristic grouping of cells in packets is not a stable character. This character, therefore, is not suitable for the differentiation of micrococci in the genera *Sarcina* and *Micrococcus*. It has also been proved that the pigmentation of most species of micrococci is variable and is therefore not suitable—with some exceptions—for the purpose of differentiation.

As follows from our results, it is possible to classify micrococci in individual species and it is not necessary to classify them in groups, as some authors do. However, the classification of micrococci must not be continually complicated by acknowledging formal and insignificant differences between species and by uncritical descriptions of new species.

Electrophoretic Analysis of Bacterial Esterase Systems—an Aid to Taxonomy.

By JOHN R. NORRIS (*Department of Bacteriology, University of Glasgow*)

The esterases of *Bacillus cereus*, *B. megaterium*, *B. subtilis*, *B. licheniformis* and *B. pumilus* were studied by starch gel electrophoresis using α-naphthyl acetate as the substrate. Several different esterases were detected in ultra-sonic disintegrates of vegetative cells and there was a close similarity between the esterase patterns of various strains within the different species. One esterase band was present in the patterns from each of the small-celled species but was absent from the large-celled species. The taxonomic implications of these findings were discussed.

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