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The Effect of Bacteriophage λ on Host Cell Reactivation in *Escherichia coli* K12

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SUMMARY

Host cell reactivation (hcr) of phage T1 by two strains of *Escherichia coli* K12, Hfr C (λ^+) *thy*$^-$ and Hfr B1 (λ^-) *thy*$^-$, was studied. Strain Hfr C showed decreased ability to reactivate ultraviolet (u.v.)-irradiated phage T1, as predicted from its behaviour in mating experiments after thymine starvation. Thymine starvation of host Hfr B1 bacteria resembled u.v. irradiation in decreasing hcr, but thymine starvation of Hfr C increased hcr, apparently as a result of λ induction. The presence of λ prophage in *E. coli* K12 was found to decrease hcr.

INTRODUCTION

In examining the effects of intranuclear ^{32}P decay in *Escherichia coli* K12, Jacob & Wollman (1958) were able to infer the presence of chromosome breaks in Hfr nuclei by examining the frequency with which various genetic markers were transferred to F^- recipients. Donor bacteria in which ^{32}P disintegration had occurred showed an altered gradient of transfer: the frequency of transfer of genetic markers distal to the chromosomal origin relative to the transfer of markers near to the origin was lower with ^{32}P-damaged donor bacteria than with normal bacteria. The extent to which transfer of distal markers was reduced was proportional to their distance from the origin and to the fraction of ^{32}P which had distintegrated at the time of transfer. This reduction of the frequency of transfer of distal markers was called 'marker inactivation' and was thought to be due to interruptions in the transfer process resulting from breaks in the donor chromosome.

A recent examination of the effects of thymine starvation on the gradient of genetic transfer by *Escherichia coli* K12 strains Hfr B1 *thy*$^-$ and Hfr C *thy*$^-$ revealed that the strains differ in their response (Hart, 1966). 'Marker inactivation' was obtained in experiments in which Hfr B1 *thy*$^-$ was starved of thymine before mating but the gradient of transfer by Hfr C *thy*$^-$ in similar experiments was unaffected. It was suggested that single-strand gaps in the chromosome might be developed as a result of the excision of primary genetic lesions accompanying thymine starvation. In the absence of thymine these gaps would not be closed by synthesis of new DNA and a blockage of chromosome transfer similar to that which occurs after intranuclear ^{32}P decay (Jacob & Wollman, 1958) might result. In a strain unable to modify the primary genetic lesions chromosome transfer would occur with normal kinetics as in donor bacteria irradiated with ultraviolet (u.v.) (Jacob & Wollman, 1958).

Because of the numerous observations already made on the similarities between the effects of thymine starvation and u.v. irradiation (Coughlin & Adelberg, 1956; Gallant

& Suskind, 1961; Rassmussen & Painter, 1963; and Drs K. A. Stacey, N. D. Symonds & C. Atkinson, private communication) it was thought likely that any difference between the capacity of the two strains to modify genetic lesions might be detected in their ability to repair u.v. radiation damage. In the present investigation a comparison of the u.v. sensitivity and dark repair capacity of the two strains has been made and the effect of thymine starvation on the activity of the repair systems has been observed.

Escherichia coli K12 strain Hfr C *thy*$^-$ is lysogenic for phage λ, whereas strain Hfr B1 *thy*$^-$ is λ^- λ-resistant and it was therefore anticipated that the Hfr C strain would show greater sensitivity to direct u.v. irradiation. However, since the induced bacteria were thought to make no contribution to the recombination data obtained previously (in mating experiments with thymine-starved Hfr C) there remained the possibility that the sensitivity of the lysogenic strain might be compounded of the lethal effect of λ induction and a deficiency in dark repair mechanisms. It is not possible to estimate the lethal effect of induction from the numbers of infective centres in an induced population since these are also subject to the lethal action of the inducing agent. The efficiency of dark repair was therefore estimated by examining the extent of host cell reactivation (Garen & Zinder, 1955; Harm, 1963) of u.v.-irradiated T1 bacteriophage.

METHODS

Organisms and media. The bacterial strains, media and culture methods used were as described previously (Hart, 1966). *Escherichia coli* K12 strains Hfr H(λ^+) and Hfr H(λ^-), which were kindly supplied by Dr W. Hayes, and strains AB1157 (u.v. resistant—*uvr*$^+$) and AB1886 (*uvr*$^-$) (Howard-Flanders, Simpson & Theriot, 1964), which were kindly supplied by Dr K. A. Stacey, were also used. *Escherichia coli* B was obtained from the National Collection of Industrial Bacteria.

Ultraviolet irradiation. Bacteria and phage were suspended in buffer (3 g. KH_2PO_4; 7 g. Na_2HPO_4 anhy.; 4 g. NaCl; 0·2 g. $MgSO_4.7H_2O$; distilled water to 1 l.) and irradiated at 10 cm. distance from a Hanovia Chromatolite u.v. lamp.

Bacteriophage techniques were those of Adams (1959). Pre-absorption of phage to bacteria was done in T1 absorption medium with 0·002 M-KCN according to the method of Sauerbier (1961). Experiments were performed by the light of a green safelight to avoid photo-reactivation.

RESULTS

Ultraviolet sensitivity and host cell reactivation of Escherichia coli K12 strains Hfr C (λ^+) and Hfr B 1 (λ^-)

Figure 1*a* shows the sensitivity of the two strains to direct u.v. irradiation. In Fig. 1*b* the survival of u.v.-irradiated phage T1 plated on the two different strains is plotted against the dose of u.v. radiation. The phage was much more susceptible to small doses of u.v. radiation when plated on strain Hfr C (λ^+) than when plated on strain Hfr B1 (λ^-), but additional irradiation gave the same additional kill, regardless of the plating bacterium used. For comparison the survival of irradiated phage T1 on *Escherichia coli* B was tested and found to be identical with that on strain Hfr B1. Strain Hfr C (λ^+) was thus less able to compensate for u.v. damage to phage T1 and this may account for the absence of marker inactivation in mating experiments after

thymine starvation of this strain. However, the increased sensitivity of phage T1 on strain Hfr C is clearly not the result of a simple loss of uvr activity, which causes an increase in the rate constant for the decline in the log fraction of phage T1 surviving (Howard-Flanders *et al.* 1962). Further investigation will be necessary before the difference can be attributed to an excision defect.

Effect of thymine starvation of host bacteria on the survival of u.v.-irradiated phage T1

If the products of thymine starvation are subject to the activity of u.v. repair systems, then thymine starvation of host bacteria might resemble u.v. irradiation (Garen & Zinder, 1955; Tessman & Ozaki, 1957) in decreasing the ability of the host

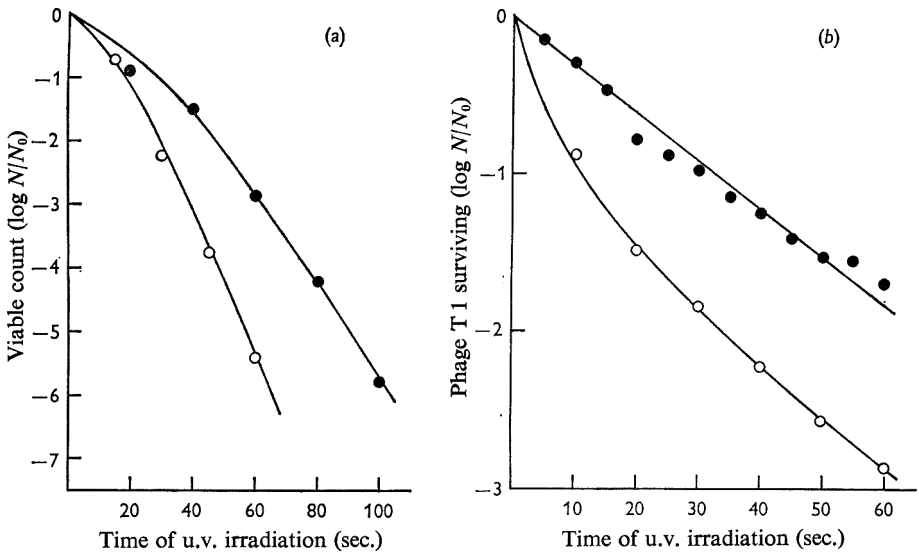

Fig. 1. (*a*) Ultraviolet irradiation: survival curves of *Escherichia coli* K12 strains Hfr B1 (λ^-) *thy*$^-$, ●—●; Hfr C (λ^+) *thy*$^-$, ○—○. (*b*) Ultraviolet radiation survival of phage T1 plated on the two strains. Symbols as in (*a*).

Table 1. *Effect of thymine starvation on the ability of E. coli K12 strain Hfr B1 thy*$^-$ *to support growth of irradiated T1 bacteriophage*

	Viable phage particles/ml.			
	Normal T1		T1 irradiated 60 sec.	
Plating bacteria	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Log phase Hfr B1	$1{\cdot}3 \times 10^7$	$1{\cdot}4 \times 10^6$	$2{\cdot}5 \times 10^5$	$1{\cdot}3 \times 10^4$
Hfr B1 starved of thymine 180 min.	$4{\cdot}5 \times 10^6$	$7{\cdot}0 \times 10^5$	$1{\cdot}9 \times 10^4$	$6{\cdot}2 \times 10^2$
Ratio: *thy*$^-$/*thy*$^+$	0·35	0·50	0·08	0·05

to repair u.v.-induced lesions in u.v.-irradiated T1 phage. Table 1 shows the results of two experiments in which normal and irradiated phage T1 was pre-absorbed on normal strain Hfr B1 *thy*$^-$ bacteria and on Hfr B1 *thy*$^-$ bacteria starved of thymine for 180 min. and then plated in the normal way. In both experiments a loss in bacterial

viability of more than two log units occurred after 180 min. of thymine starvation. Thymine-starved host bacteria showed a marked decrease in their ability to support the multiplication of irradiated phage T1, whereas a comparatively small effect of thymine starvation was noted when bacteria were infected with un-irradiated T1 phage. This result was confirmed by subjecting phage T1 to different degrees of u.v. irradiation and pre-absorbing to host bacteria previously thymine starved for 180 min. The results of several experiments are collected in Fig. 2*a* and *b*. Figure 2*a* shows the survival of irradiated phage T1 on thymine-starved and normal *Escherichia coli* K12 Hfr B1 *thy*⁻ bacteria. Figure 2*b* is constructed from the results of identical experiments with strain Hfr C *thy*⁻. The results with Hfr B1 were analogous to those obtained with

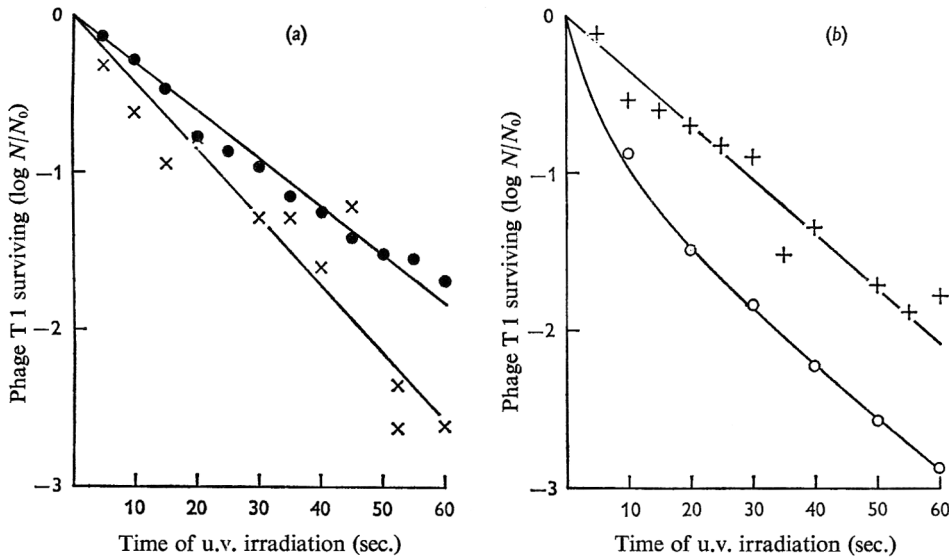

Fig. 2. (*a*) Ultraviolet irradiation: survival of phage T1 plated on *E. coli* K12 Hfr B1 (λ^-) *thy*⁻ without thymine starvation, ●—●; or pre-absorbed on the same strain starved of thymine for 180 min., ×—×. The difference between the slopes is statistically significant at the 5% level. (*b*) As (*a*) but with *E. coli* K12 strain Hfr C (λ^+) *thy*⁻ without thymine starvation, ○—○; and with thymine stavation, +—+.

u.v. irradiation: thymine starvation of the host bacteria decreased the plating efficiency of irradiated phage as compared with plating on unstarved bacteria. It had been anticipated that strain Hfr C would show a smaller or nil effect under these conditions. The increase in the ability of this strain to repair T1 damage which occurred on thymine starvation was entirely unexpected and suggested that phage λ might be affecting repair mechanisms. The induction of λ which occurs as a result of thymine starvation had apparently allowed multiplication of u.v.-radiation-damaged phage T1 particles which were normally excluded from multiplication in the un-induced host. The survival of u.v.-irradiated phage T1 on thymine-starved Hfr C bacteria was comparable to that found on normal strain Hfr B 1.

Comparison of survival of u.v.-irradiated phage T 1 plated on Escherichia coli K 12 strains lysogenic and non-lysogenic for phage λ

The effect of λ prophage in decreasing the efficiency of plating of u.v.-irradiated phage T 1 was confirmed by using *Escherichia coli* strains Hfr H (λ^+) and Hfr H (λ^-) and a strain of Hfr C *thy*$^-$ which had been cured of λ by selection after a heavy dose of u.v. radiation; the results are shown in Fig. 3*a* and *b*. The differences noted between λ^+ and λ^- strains were of the same order as those observed between Hfr C (λ^+) and Hfr B 1 (λ^-) (Fig. 1*b*) and it is clear that the presence of λ prophage in the host had a marked effect on the survival of irradiated phage T 1. *Escherichia coli* strain Hfr C appeared to give a greater overall sensitivity than did strains Hfr H, Hfr B 1 and AB 1157, which were also tested.

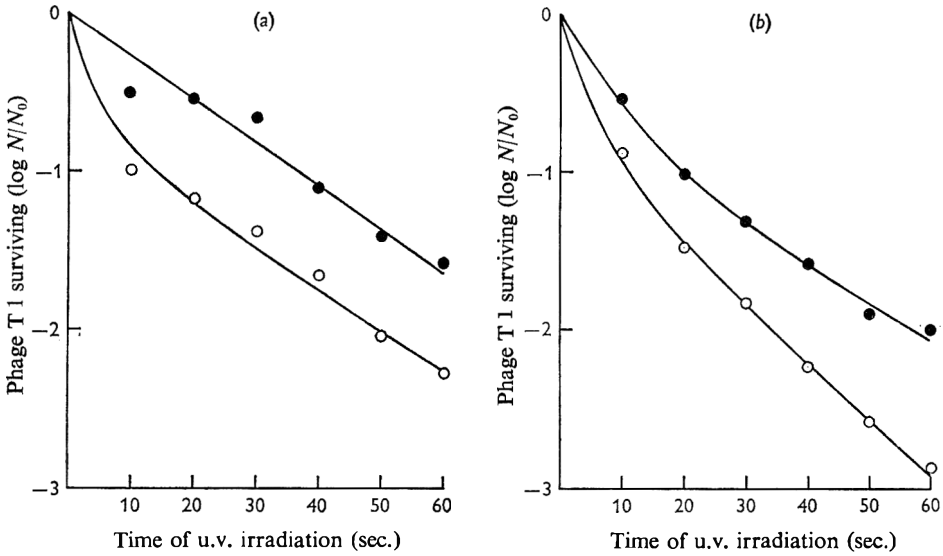

Fig. 3. (*a*) Ultraviolet irradiation: survival of phage T 1 plated on *E. coli* K 12 strain Hfr H (λ^+), ○—○; on strain Hfr H (λ^-), ●—●. (*b*) Ultraviolet irradiation; survival of phage T 1 plated on *E. coli* K 12 strain Hfr C (λ^+) *thy*$^-$, ○—○; on the cured strain Hfr C (λ^-) *thy*$^-$, ●—●.

DISCUSSION

The present results go some way towards confirming the suggestion that thymine starvation of *Escherichia coli* strains resembles u.v. irradiation, causing genetic damage of a kind which can be modified by dark repair systems. If the increased sensitivity of irradiated phage T 1 on λ^+ strains of *E. coli* can be shown to be due to decreased ability to excise damaged DNA, then the production of single-strand gaps by excision in the absence of DNA synthesis would provide an adequate explanation of marker inactivation observed in thymine-starved Hfr B 1 (λ^-) and of its absence in Hfr C (λ^+) (Hart, 1966).

The influence of λ prophage on dark repair of irradiated phage T 1 might be a side effect resulting from the presence of the λ episome on the bacterial chromosome: phage λ is closely linked with the *uvr*B gene (Howard-Flanders *et al.* 1964).

Alternatively, it might be directly connected with the mechanisms of λ immunity and prophage maintenance.

The probability that thymine starvation results in excision places this starvation in the company of other agents also known to be inducers of λ prophage, namely ultraviolet radiation (Setlow & Carrier, 1961), mitomycin C and nitrogen mustard (Boyce & Howard-Flanders, 1964) and X-rays (Howard-Flanders *et al.* 1962). Induction of λ might occur by excision of the prophage from its point of attachment on the bacterial chromosome. Inducing agents, all of which are known to have the property of causing genetic damage, may share the ability to de-repress a bacterial excision system maintained at a lesser degree of activity by the λ repressor. The inducibility of repair systems in general is supported by the existence of 'u.v. reactivation' (Harm, 1963).

It is also possible to speculate on the involvement of similar mechanisms in λ immunity. The increased sensitivity to u.v. irradiation of phage T1 plated on λ^+ strains of *Escherichia coli* differed from the uniform increased sensitivity obtained by plating on a *uvr*$^-$ mutant such as AB1886 (Howard-Flanders *et al.* 1962). In the λ^+ strains, a small dose of u.v. radiation results in the rapid development of strain sensitivity in a large fraction of the phage particles, but at larger doses the size of the sensitive fraction remains constant (Fig. 3*a*) and subsequent decline in the log fraction surviving occurs at the same rate on both strains. This would suggest that low doses of u.v. radiation may alter the DNA of some of the phage T1 particles so that they are incapable of multiplying in λ^+ cytoplasm but can do so in λ^- cytoplasm or in induced (thymine-starved) λ^+ cytoplasm. In fact, the λ^+ bacterium may be 'immune' to the u.v.-modified phage T1, and the mechanism which prevents multiplication of superinfecting λ might likewise prevent multiplication of u.v.-damaged phage T1. A bacterial excision system could thus be responsible for: (*a*) removing the prophage from its attachment site and allowing its replication; (*b*) removing from exogenous λ some modified portion of DNA which prevents replication. Both of these functions might be controlled by the λ repressor in λ^+ bacteria. At the present stage the evidence is not sufficiently detailed to allow adequate discussion of this model, which is compatible with most of the information at present available on the behaviour of λ and its mutants. However, it predicts that a class of u.v.-sensitive bacterial mutants should exist which when λ^- behave as λ-resistant to all except λ_v mutants, and when λ^+ behave as *ind*$^-$. These would be defective in the bacterial gene which codes for the excision enzyme. Mattern, van Winden & Rorsch (1965), who examined a number of *hcr*$^-$ mutants of *E. coli*, found that they could be lysogenized and were more sensitive to induction by u.v. irradiation. These results are not compatible with the idea of excision playing an essential role in λ induction and immunity, and the exact mechanism of the λ-induced decrease in host bacterium reactivation will have to be determined before its importance can be assessed.

The author thanks Mr M. H. L. Green for helpful discussions of these problems and Mr R. N. Laverty for technical assistance.

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Interaction Between Ultraviolet Light and γ-Radiation Damage in the Induction of Mutants of *Escherichia coli*: the Response in Strains with Normal and Reduced Ability to Repair Ultraviolet Damage

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SUMMARY

The exposure of tryptophan-requiring *Escherichia coli* B/r WP2 to u.v.- and γ-irradiation (in either order) results in more revertants than would be expected if the two treatments were independent. The excess mutants ('interaction mutants') were produced in increasing number by increasing doses of u.v.- or γ-radiation. Evidence is presented that the interaction occurs post-irradiation. Although the excision-repair and mutation frequency decline systems for u.v. damage are not involved in the interaction itself (since a strain deficient in these systems, *E. coli* WP2 hcr$^-$, is no less efficient at producing interaction mutants than *E. coli* B/r WP2) the u.v. component involved in interaction is sensitive to one or both of these systems as is shown by the very much lower u.v. doses at which interaction occurs in *E. coli* WP2 hcr$^-$.

INTRODUCTION

It has been recently demonstrated that synergistic interactions occur between ultraviolet light (u.v.) and X-rays for lethal damage in some strains of *Escherichia coli* (Haynes, 1964). In those strains lacking certain repair mechanisms or in those possessing them but in which the repair processes have been suppressed, this interaction does not occur (Haynes, 1964; Baptist, Haynes & Uretz, 1966). On the basis of these results it has been postulated that synergism between u.v.- and X-rays involves repair processes. No comparable effort has been made to demonstrate such an interaction in terms of mutational damage. Though the early work of Swanson (1952) on *Aspergillus terreus* claimed to show such an effect, later work on *A. nidulans* has failed to substantiate this claim (Arlett, to be published). Doneson & Shankel (1964) were also unable to demonstrate interaction between u.v.- and γ-radiation in the production of high-level streptomycin-resistant mutants of *E. coli*.

It is well recognized that repair of mutational lesions occurs, particularly in reversion to prototrophy, and so a similarity to the responses obtained for lethal damage might be predicted. In the experiments reported here the aim was first to determine

whether a synergistic interaction occurred, and if it did, to examine the role of repair mechanisms in the process. This has been done by comparing the responses of strains possessing and lacking certain repair mechanisms.

METHODS

The bacterial strains used were the tryptophan requiring *Escherichia coli* B/r WP2 (subsequently referred to as the hcr$^+$ strain) and a mutant form of this strain which is deficient for certain dark repair activities *E. coli* WP2 hcr$^-$ (subsequently hcr$^-$). The hcr$^-$ strain is unable to perform host cell reactivation of bacteriophage, is more sensitive than the hcr$^+$ strain to both the lethal and mutagenic actions of u.v. (Hill, 1965; Ashwood-Smith & Bridges, 1966), has a reduced ability to excise thymine dimers from its DNA (Setlow, quoted by Hill, 1965) and exhibits slower mutation frequency decline of u.v.-induced mutants in minimal medium (Munson & Bridges, 1966*b*). (We are grateful to Dr Ruth Hill for providing us with this strain). Logarithmic phase bacteria were used for all experiments. These were grown with aeration at 37° in a glucose salts medium (M medium of Haas & Doudney, 1957) supplemented with 10 μg./ml. tryptophan. After reaching a suitable population density ($1–3 \times 10^8$/ml.) the bacteria were spun down and resuspended in buffer salts solution (M medium minus glucose) at 4°; thereafter the bacteria were maintained at this temperature until plated. Counts of viable and mutant organisms were made on the same medium (M enriched with 0·75 μg./ml. tryptophan and solidified with 1·5% agar) following incubation for 48 hr at 37°. All manipulations were done in yellow light or under low light intensities to prevent photoreactivation.

Experiments were performed independently in both laboratories and some inconsistencies in radiation response were noted. These may have been due to slight differences either in biological technique or in the spectra of the two lamps. Each interaction experiment was therefore carried out with a complete set of internal controls thus obviating the need to compare results obtained at different times and in different places.

Sources of radiation

(*a*) *Ultraviolet—low pressure mercury vapour*

(1) A 30 W. Phillips TUV lamp giving an incident dose rate of 45 ergs mm^{-2} sec.$^{-1}$ at 75 cm., the target to lamp distance used.

(2) A Vitreosil (England) lamp in the form of an incomplete circle giving 3·29 ergs mm.$^{-2}$ sec.$^{-1}$ at 90 cm.

(*b*) *γ-ray—^{60}Co*

(1) A 4000 Ci 'Hot Spot' (UKAEA, Harwell) giving a dose rate of 17 krad. min.$^{-1}$.

(2) Combination of two 250 Ci sources giving a dose rate of 5 krad. min.$^{-1}$.

RESULTS

(1) *Ultraviolet followed by γ-radiation*

(*a*) *Hcr$^+$ strain*

Typical responses to u.v.- and to γ-radiation in terms of survival and of mutation to prototrophy are shown in Fig. 1. The u.v. responses represent those obtained in the dark, and the γ results, those obtained following vigorous bubbling of the cells with

oxygen both before and during irradiation. Within the range of γ doses the mutational response was linear, whereas for u.v. mutation induction the yield increased in proportion to the square of the dose.

To test whether a synergistic interaction occurred cells were first exposed to a low dose of u.v. and then to a range of γ doses. Other samples were given either the u.v. dose alone or the range of γ doses alone.

In different experiments the time interval between doses varied from a few minutes to over an hour but there was no significant difference in the responses obtained. A low u.v. dose had little effect on the survival characteristics of the cells, but enhanced

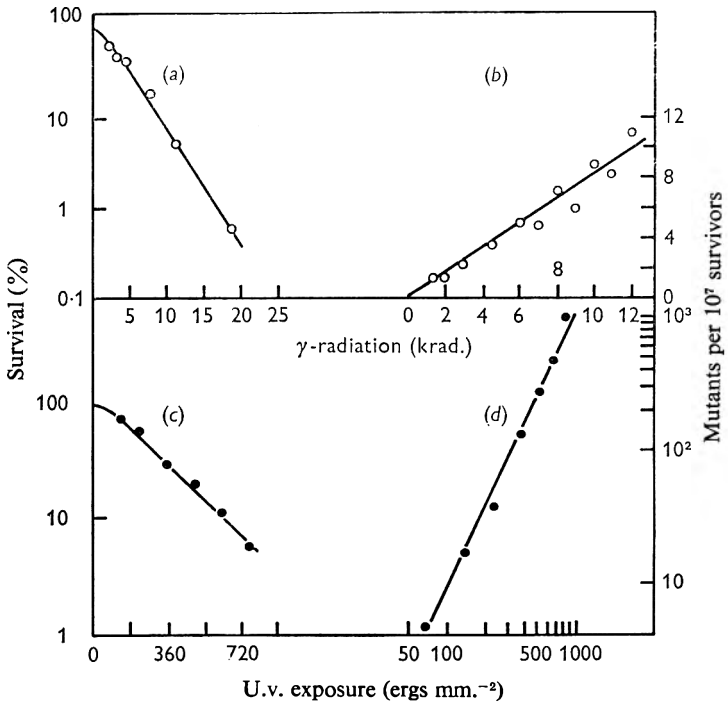

Fig. 1. (*a* and *b*). Lethal and mutagenic action of γ-radiation on *E. coli* WP2 hcr⁺. (*c* and *d*) Lethal and mutagenic action of u.v. on *E. coli* WP2 hcr⁺. Single representative experiments.

the yield of mutations induced by later γ doses. This enhancement can be best demonstrated by plotting the number of interaction mutants produced for different doses of γ radiation after a constant u.v. dose. The results of a typical experiment are given in Fig. 2. For the purpose of this paper the frequency of interaction mutants per survivor is defined as the number of mutants per survivor induced by a combined u.v.–γ treatment less the number of mutants per survivor induced by each treatment given separately to similar bacterial suspensions. In the experiment shown the frequency of these interaction mutants increased linearly with dose, though in other experiments there was some indication of non-linearity.

(*b*) *Hcr⁻ strain*

Typical response curves for u.v.- and γ-radiation alone are shown in Fig. 3. It can be seen that, in contrast to its sensitivity to u.v., the hcr⁻ strain does not differ signifi-

cantly from the hcr+ in its sensitivity to γ-radiation under these conditions, confirming the observations of Bridges & Munson (1966). Results of interaction experiments with the hcr⁻ strain were very similar to those with the hcr+ strain except that comparable interaction was obtained at very much lower u.v. doses (Fig. 4).

A feature of the hcr⁻ results is that an appreciable number of interaction mutants could be obtained following doses of radiation where the lethal effect was small. This makes it possible to eliminate the possibility that 'interaction mutants' are artifacts arising from the selection by γ-radiation of u.v. induced revertants possessing abnormally high resistance to the lethal action of γ-radiation.

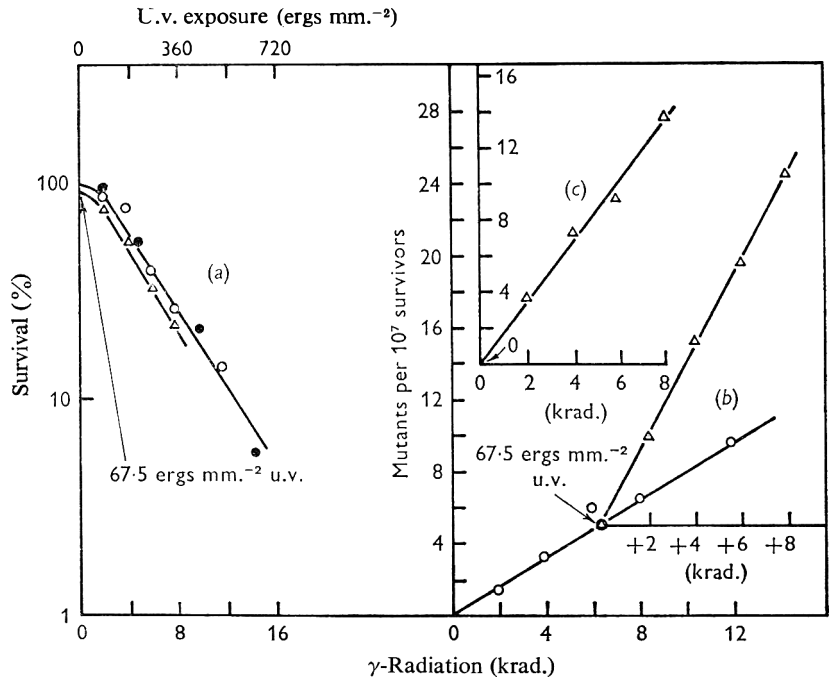

Fig. 2. (*a*) Lethal effects of γ- (○), u.v.- (●) and combined u.v.- and γ- (△) radiations on *E. coli* WP2 hcr+. (*b*) Mutagenic effect of γ- (○) and of combined u.v.- and γ- (△) radiations on *E. coli* WP2 hcr+. (*c*) Yield of interaction mutants by a range of γ-radiation doses following a dose of 67·5 ergs mm.⁻² u.v. to *E, coli* WP2 hcr+.

(*c*) *Dependence on preliminary u.v. dose*

With both the hcr+ and hcr⁻ strains a larger u.v. pretreatment enhanced markedly the frequency of interaction mutants. With the hcr+, a treatment with 500 ergs mm.⁻² sec.⁻¹ reduced survival to 20% of the unirradiated control, and after this pretreatment the survival curve obtained with a range of γ doses had an increased slope. This confirms the observations of Haynes (1964). Under his conditions no shoulder was obtained on the γ-radiation curve. Under our conditions the shoulder would appear to be due to the presence of several segregating targets within the bacteria (Munson & Bridges, 1966*a*) and not to a dose-dependent repair system as postulated by Haynes (1964) to explain his u.v.-survival curve. If the γ-radiation targets were chromosomal, the reduction of the shoulder on the γ-survival curve by low doses of u.v. would be expected.

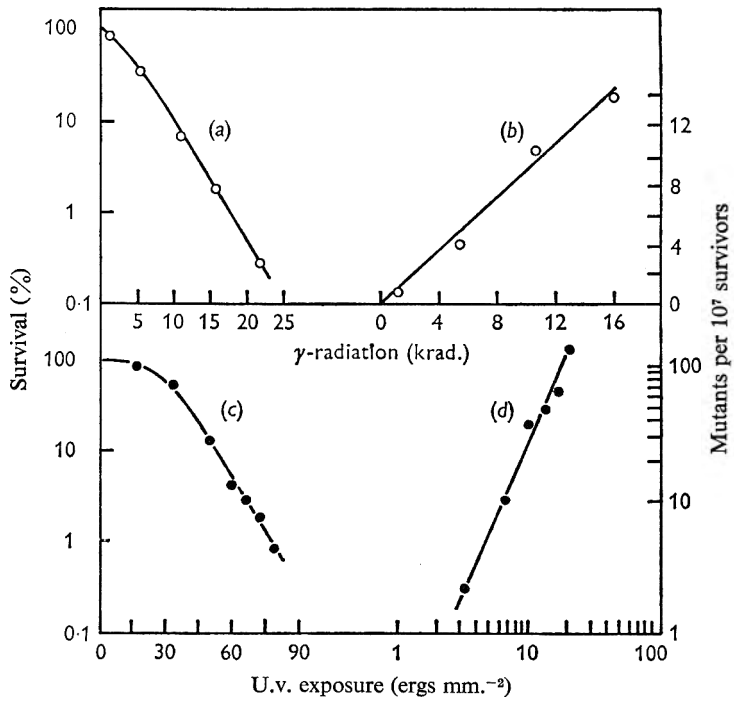

Fig. 3. (*a* and *b*) Lethal and mutagenic action of γ-radiation on *E. coli* WP2 hcr⁻. (*c* and *d*) Lethal and mutagenic action of u.v.-radiation on *E. coli* WP2 hcr⁻. Single representative experiments.

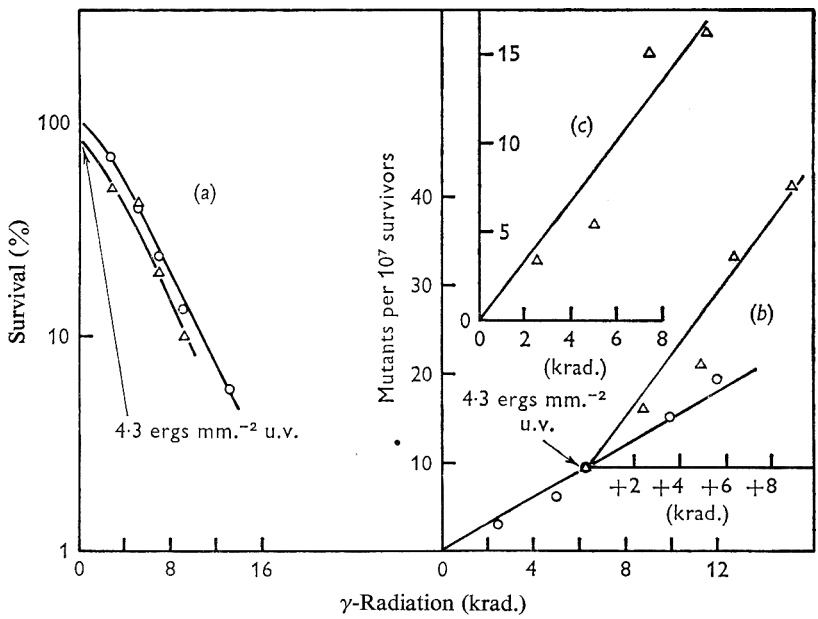

Fig. 4. (*a*) Lethal effects of γ- (○) and combined u.v.- and γ- (△) radiations on *E. coli* WP2 hcr⁻. (*b*) Mutagenic effects of γ- (○) and of combined u.v.- and γ- (△) radiations on *E. coli* WP2 hcr⁻. (*c*) Yield of interaction mutants by a range of γ-radiation doses following a dose of 4·3 ergs mm.⁻² u.v. to *E. coli* WP2 hcr⁻.

The frequency of interaction mutants induced by a series of γ-doses following high or low u.v. doses is shown in Fig. 5. The frequency appeared not to be proportional to the dose.

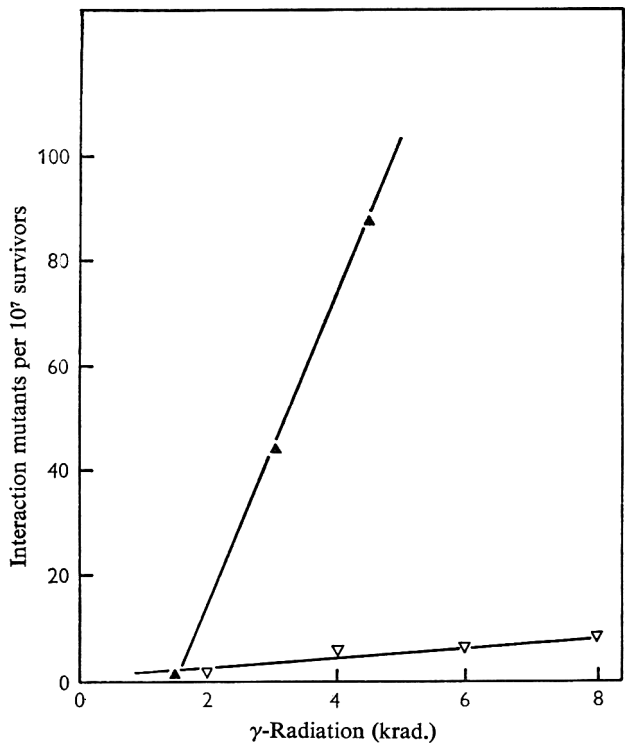

Fig. 5. Yield of interaction mutants by a range of γ-radiation doses following a u.v. dose of 67·5 (▽) and 495 (▲) ergs mm.$^{-2}$ to *E. coli* WP2 hcr^{+}.

Table 1. *Frequency of induced prototrophs following γ-radiation alone, u.v. alone or u.v. followed by γ-radiation*

Treatment		Total no. of prototrophic colonies scored	Surviving fraction	Induced prototrophs per 10^7 survivors	Interaction mutants per 10^7 survivors
u.v. (ergs mm.$^{-2}$)	γ (krad.)				
0	0	31	1·00	—	—
0	2	190	0·852	1·6	—
0	4	306	0·674	3·5	—
0	6	317	0·394	6·2	—
0	8	233	0·262	6·5	—
0	12	204	0·150	9·8	—
Number of bacteria plated per treatment = $1{\cdot}18 \times 10^9$.					
0	0	10	1·00	—	—
67·5	0	63	0·926	5·2	—
67·5	2	891	0·761	10·5	3·7
67·5	4	973	0·545	16·0	7·3
67·5	6	762	0·332	20·6	9·2
67·5	8	645	0·225	25·6	13·9
Number of bacteria plated per treatment = $1{\cdot}10 \times 10^9$.					

When the production of interaction mutants by a series of u.v. doses each followed by the same γ-dose was compared with the production of mutants by the u.v. dose alone, it was apparent that the hcr⁻ strain is roughly twenty times more sensitive to u.v. than the hcr⁺ strain for both u.v. and interaction mutants.

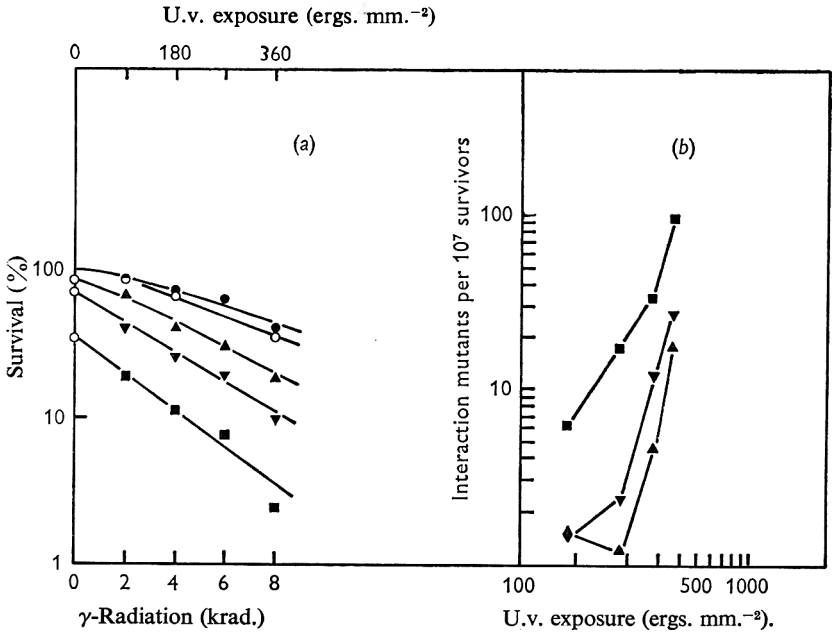

Fig. 6. (*a*) Lethal effects of γ- (○), u.v.- (●), and combined γ- (▲, 2 krad.; ▼, 4 krad.; ■, 8 krad.) and u.v.- radiations on *E. coli* WP2 hcr⁺. (*b*) Yield of interaction mutants in *E. coli* WP2 hcr⁺. γ pre-doses 2 krad. (▲), 4 krad. (▼) and 8 krad. (■).

(2) *γ-followed by ultraviolet-irradiation*

The effect of reversing the order of the two irradiations was examined using the hcr⁺ strain. Samples were exposed to 2, 4, or 8 krad. of γ-radiation and then to a range of u.v. doses. The shoulder of the u.v.-survival curve became smaller with increasing γ dose and disappeared after 8 krad. (Fig. 6). A synergistic interaction was also evident for the induced mutants (Fig. 6). The yield of interaction mutants clearly increased in a non-linear manner with respect to both the u.v. doses and the preliminary γ dose. Other experiments indicated that the frequency of interaction mutants obtained with any combination of u.v. and γ doses was similar irrespective of the order in which they were given.

Finally the effect of varying the time interval between the γ- and u.v.-irradiation treatments was examined. Organisms were pretreated with 8 krad. of γ-radiation then held in the usual buffer at 4° for intervals of 5–120 min. prior to exposure to 230 or 360 ergs mm^{-2} u.v. There was no significant effect of interval between treatments on the response (Table 2).

One can summarize the main features of the results of the present experiments as follows:

(*a*) Pretreatment of cells of the hcr⁺ and hcr⁻ strains with low doses of u.v. resulted in an enhancement of the yield of mutations induced by a later dose of γ-radiation.

(*b*) Much lower doses of u.v. were required to induce this synergistic interaction in the case of the hcr$^-$ than of the hcr$^+$ strain.

(*c*) The higher the pretreatment dose the greater the frequency of interaction mutants.

(*d*) There was no effect of the time interval between treatments under the holding conditions used.

(*e*) When hcr$^+$ cells were pretreated with γ-radiation prior to exposure to u.v. there was also an enhanced yield of mutations similar in magnitude to that when the u.v. exposure was given first. Again the interval between the treatments did not affect the frequency of interaction mutants.

Table 2. *Effect of interval of time between exposure to γ-radiation and u.v. on the induction of prototrophs. The bacteria were held at 4° in a buffered salts solution between treatments*

Treatments		Interval between	Spontaneous	Total no. of prototrophic		Total no. of bacteria	Induced prototrophs
γ (krad.)	u.v. (ergs mm.$^{-2}$)	treatments (min.)	plate mutants	colonies scored	Surviving fraction	plated ($\times 10^9$)	per 10^7 survivors
8	0	At start of experiment	21	238	0·313	1·08	6·4
		At finish of experiment	21	224	0·306	1·08	6·1
0	360	At start of experiment	16	1723	0·397	1·24	35
		At finish of experiment	15	1590	0·350	1·06	43
8	270	5	11	668	0·118	0·90	62
		30	21	659	0·090	1·08	66
		60	21	785	0·104	1·08	68
		120	21	804	0·120	1·06	61
8	360	5	11	748	0·061	0·90	135
		30	15	891	0·063	1·06	131
		60	15	1018	0·074	1·06	128
		120	15	1035	0·059	1·06	164

DISCUSSION

One possible interpretation of the data involves the assumption that the first irradiation sensitizes the DNA itself such that the second irradiation induces a higher yield of mutational lesions. But as the order of irradiations is immaterial it seems more likely that the interaction takes place after the second irradiation has been given. The fact that holding the cells at 4° for different periods between irradiations had no effect on the yield of interaction mutants is not inconsistent with the concept that the events which resulted in the enhanced yield of mutations took place at the time when the cells were returned to 37°. If this is so, two alternative interpretations may be made: (*a*) that the synergistic interaction is due to accelerated rates of fixation (and therefore diminished possibility of loss of mutants) following combined treatments, or (*b*) that it is associated with an inhibition or inactivation of repair processes. The first of these alternatives seems untenable. Fixation is generally considered to be associated with the onset of DNA synthesis (Lieb, 1960; Weatherwax & Landman, 1960; Witkin, 1961)

and there is no reason to assume that this would occur more rapidly after a combined than after a single treatment. Furthermore, the onset of DNA synthesis after irradiation is delayed to a far greater extent in hcr$^-$ in than hcr$^+$ strains (Setlow, 1964) and so the onset of fixation might be similarly delayed in the former. The synergistic interaction would therefore seem to be associated with a decrease in repair of premutational damage after irradiation. This is supported by the observation that interaction was observed after much lower doses with the hcr$^-$ than the hcr$^+$ strain, the difference between these strains lying not in their inherent sensitivity to damage but in their capacity for repairing this damage (Hill, 1965).

Bridges (1966) has recently proposed a model for the induction of prototrophic mutations by u.v., in which he seeks to interpret the second-order dependence on dose in terms of a requirement for two events. The first of these (event A) is not of itself a mutational lesion, but it can interfere with the repair of the mutational event (B). The linear and low level of response obtained with X- or γ- radiation could in these terms be attributed to a limitation of the production of 'type A' events; u.v. pretreatment would then have the effect of removing this limitation. Following a γ-irradiation the yield of mutations could be enhanced possibly by the additional frequency of 'type B' events or the production of other ('type A') lesions which interfere with u.v.-induced 'type B' events. However, the specific events which Bridges has described could not be induced by γ-radiation. 'A' is a 'thymine-dimer' type of lesion and 'B' is characterized e.g. by its ability to be repaired by the mutation frequency decline system and by its susceptibility to acriflavine; neither of these affects γ-induced mutational lesions (Kada, Brun & Marcovich, 1960; Bridges & Munson, 1964; Munson & Bridges, 1966*b*). Thus although this specific interpretation in terms of 'A' and 'B' events cannot be made, the concept of an interference at the level of repair is a useful one in the context of the present results.

The next feature to consider is the type of repair process that could be interfered with. One may interpret the results either as indicating that the u.v. treatment enhances the yield of γ-induced mutations, or that the range of γ doses allows an expression of an increasing number of mutations induced by the u.v. doses. In other words, the interaction mutants could be either basically γ- or u.v.-induced. Although the present results do not bear on this, indications that the induction of interaction mutants has the properties of u.v. mutagenesis are presented in the accompanying paper (Bridges, Munson, Arlett & Davies, 1967). The fact that u.v.–γ interaction occurs in both the hcr$^-$ and hcr$^+$ strains indicates that the excision-repair and mutation frequency decline processes (which are carried out with reduced efficiency by the hcr$^-$ strain) are not involved in the actual interaction phenomenon. It is clear, however, that the component of u.v. damage which is involved is subject to the excision-repair and/or mutation frequency decline processes since the hcr$^-$ strain yields interaction-mutants at very much lower doses of u.v. than the hcr$^+$. This is presumably because more of the u.v. lesions involved in interaction are present for the necessary period of time after irradiation in the hcr$^-$ than in the hcr$^+$ strain.

It is difficult to invoke a mechanism to explain the nature of the interference in repair capacity. The responses obtained seem to be similar to those observed by Haynes (1964) who studied the lethal effects of combined u.v.-and X-irradiations. He interpreted the synergistic interaction as being due to the fact that the probability of repair of one potentially lethal lesion in DNA is reduced by the presence of other

defects within some given distance of it. Equally one can assume that the interference is due to there being some steps in common in the repair systems for both u.v. and γ lesions.

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Interaction between Ultraviolet Light and γ-Radiation Damage in the Induction of Mutants of *Escherichia coli*: the Effect of Some Modifying Treatments

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SUMMARY

The effect of various modifying agents has been studied on the synergistic interaction of u.v.- and γ-radiation in the induction of prototrophic revertants of tryptophan-requiring *Escherichia coli* B/r WP2. The component of γ-radiation damage involved in the interaction shows a large effect of oxygen during irradiation (dose modifying factor of up to 3) and is thus more like lethal than mutational damage. The interaction mutants respond to various post-irradiation treatments in the same way as u.v.-induced, but not γ-induced, mutants, i.e. they are photoreactivable, their frequency is increased by acriflavine and nutrient broth after irradiation and they are susceptible to mutation frequency decline in minimal medium. It is postulated that damage induced in DNA by γ-radiation inhibits the repair of premutational lesions induced by u.v. thus increasing the yield of u.v.-induced mutants. The possible nature of the system involved in this repair (which is not that responsible for the excision of thymine dimers from DNA) is discussed.

INTRODUCTION

In the preceding paper (Davies, Arlett, Munson & Bridges, 1967) we have shown that more prototrophic revertants of the tryptophan requiring strain *Escherichia coli* WP2 are induced by combined treatments with ultraviolet light (u.v.) and γ-radiation than would be expected if the effect of the treatments were merely additive. The additional mutants, which we have termed interaction mutants, have been shown to arise by the interaction of u.v.- and γ-radiation damage during post-irradiation development. For the purpose of this paper the frequency of interaction mutants per survivor is defined as the number of mutants per survivor induced by a combined u.v.-γ treatment less the number of mutants per survivor induced by each treatment given separately to similar bacterial suspensions. In this paper we record the effect on the yield of interaction mutants of various modifying treatments which are known to affect either the initial amount of γ-radiation damage (e.g. oxygen) or the cellular repair processes affecting u.v. damage (e.g. photoreactivating light, acriflavine, nutrient broth). The results indicate that the interaction mutants behave as though they are u.v.-induced.

METHODS

Escherichia coli B/r WP2 was used for most experiments; where stated confirmatory experiments were carried out with *E. coli* WP2 hcr⁻ (Hill) which is deficient in certain dark repair processes.

Microbiological techniques and details of the irradiation sources have been given elsewhere (Davies *et al.* 1967). Glucose-salts medium enriched with 0·75 μg./ml. tryptophan and solidified with 1·5% agar was used for all experiments except those involving mutation frequency decline when enrichment was made with 2·5% Oxoid nutrient broth instead of tryptophan.

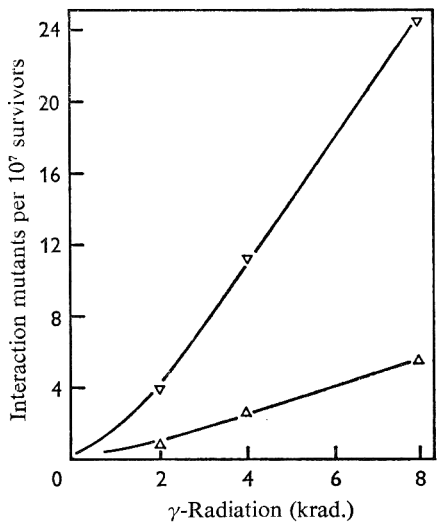

Fig. 1

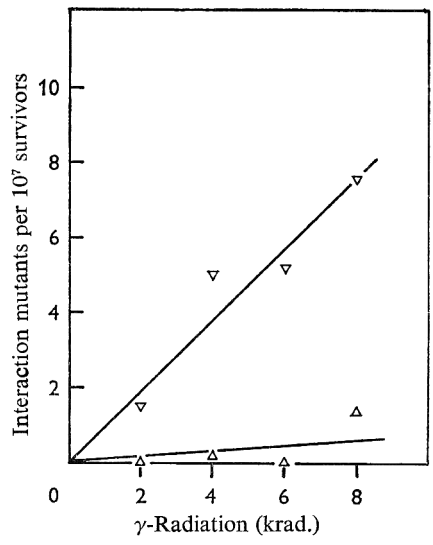

Fig. 2

Fig. 1. Effect of presence (▽) or absence (△) of oxygen during γ-irradiation on the production of interaction mutants after 67·5 ergs mm.$^{-2}$ u.v.

Fig. 2. Effect of photoreactivation after 45 ergs mm.$^{-2}$ u.v. on subsequent yield of interaction mutants with γ-radiation. ▽, no photoreactivation; △, with photoreactivation.

The source of photoreactivating light was a 500 W photoflood lamp. Suspensions of bacteria in the glucose-salts medium were placed for 20 min. at 10 cm. from the lamp in a water-bath maintained at 15°. Non-photoreactivated controls were wrapped in aluminium foil during an identical treatment.

Acriflavine was obtained from General Biochemicals.

RESULTS

Effect of oxygen

When a dose of u.v. was followed by a range of γ doses given with either oxygen or 'oxygen-free' nitrogen bubbling through the suspension, interaction mutants in *Escherichia coli* B/r WP2 were induced as shown in Fig. 1. The slope of the response curve under oxygenated conditions varied from 1·5 to 3 times that under anoxic conditions in four different experiments.

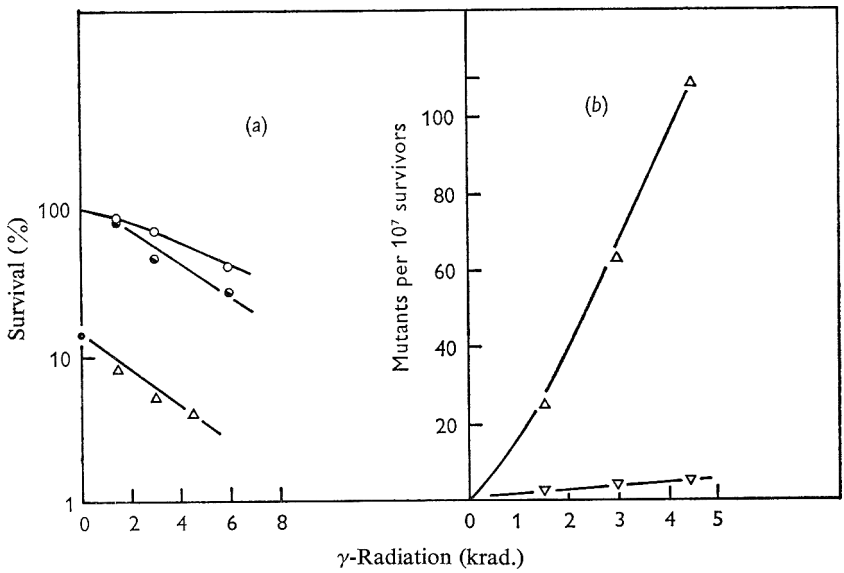

Fig. 3. (*a*) Effect of 3 μg./ml. acriflavine in the plating medium and u.v. on the lethal action of γ-radiation on *Escherichia coli*. ○, no acriflavine; ◒, acriflavine; ●, 67·5 ergs mm.$^{-2}$ u.v. on acriflavine plates; △, 67·5 ergs mm.$^{-2}$ u.v. followed by γ-radiation, on acriflavine plates. (*b*) Yield of interaction mutants by 67·5 ergs mm.$^{-2}$ u.v. followed by a range of doses of γ-radiation. ▽, no acriflavine; △, 3 μg./ml. acriflavine in plating medium.

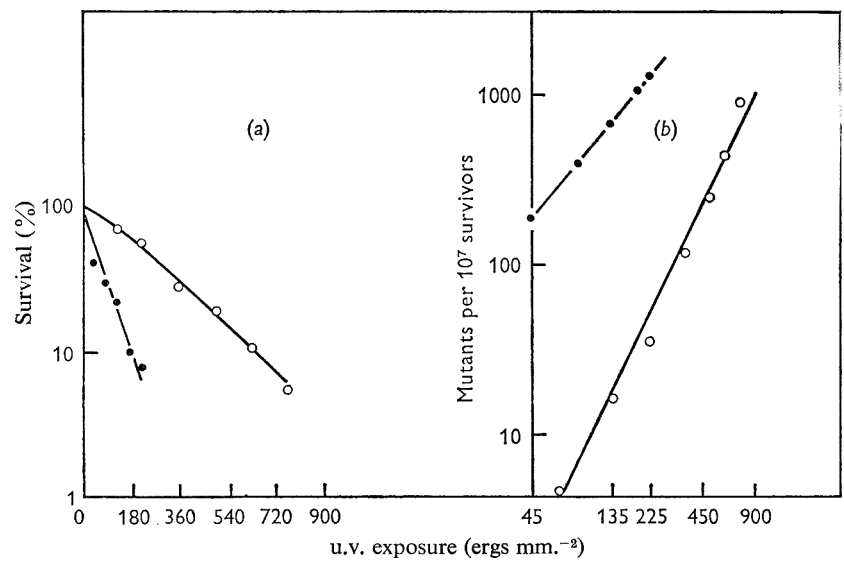

Fig. 4. (*a*) Lethal effect of u.v. on *Escherichia coli* plated on medium with (●) and without (○) 3 μg./ml. acriflavine. (*b*) Yield of u.v.-induced mutants plated on medium with (●) and without (○) 3 μg./ml. acriflavine.

Effect of photoreactivating light

When a photoreactivating treatment was given between a u.v. dose and a γ-radiation dose, the rate of induction of interaction mutants in *Escherichia coli* B/r WP2 was reduced by a factor of about 8 (Fig. 2). This also happened with *E. coli* WP2 hcr⁻ where, in addition, it was shown that the photoreactivating treatment was effective even when given after the γ-irradiation.

Effect of acriflavine

The addition of acriflavine to the plating medium is known to enhance the lethal and mutagenic action of u.v. (Witkin, 1963) but not the mutagenic action of γ-radiation (Bridges & Munson, 1964). Our experiments, using 3 μg./ml. acriflavine,

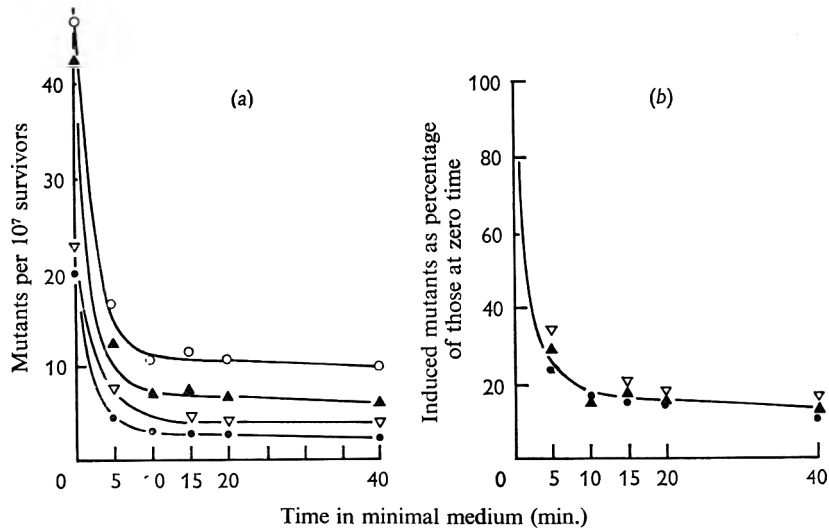

Fig. 5. Decline of mutation frequency with time as revealed on broth-enriched plates when bacteria are held in glucose salts medium at 37° after irradiation with 195 ergs mm.$^{-2}$ and/or 4 krad. γ-radiation. ○, total induced mutants after u.v. and γ-irradiation; ▲, total induced mutants after u.v. and γ-irradiation less those induced by γ-radiation alone; ●, mutants induced by u.v. alone; ▽, interaction mutants, i.e. total mutants induced by combined u.v.- and γ-irradiation less those induced by u.v. and γ-radiation alone. In (*a*) absolute mutation frequencies are plotted, in (*b*) these have been plotted as a percentage of their values immediately after irradiation.

confirm this and also shown an enhancement of the lethal action of γ-radiation (Fig. 3*a*) similar to that observed by Alper (1963) with *E. coli* B/H. The induction of interaction mutants was enhanced by acriflavine (Fig. 3*b*) to roughly the same extent as the induction of u.v. mutants (Fig. 4).

Mutation frequency decline

It is a well-established fact, dating from the observations of Witkin (1956), that more u.v.-induced mutations appear on glucose+salts plates supplemented with a little nutrient broth than on plates supplemented with tryptophan alone. The amino acids in the broth in some way stabilize the premutational lesions so that more of the latter

are 'fixed'. If u.v.-exposed bacteria are incubated at 37° in minimal medium before plating on plates enriched with broth, the number of mutants appearing rapidly falls until by about 40 min. it is the same as that on minimal plates supplemented with tryptophan alone. This process has been termed 'mutation frequency decline' (Doudney & Haas, 1958); it does not occur with mutations induced by ionizing radiation which are unaffected by the presence of broth (Kada, Brun & Marcovich, 1960; Munson & Bridges, 1966). In Fig. 5*a* we show mutation frequency decline of all the mutants induced by a combined u.v. and subsequent γ-radiation treatment, of u.v. mutants alone, and of interaction mutants. It is clear that interaction mutants are stabilized on plates enriched with nutrient broth immediately after irradiation, and in the absence of broth show the phenomenon of mutation frequency decline. In Fig. 5*b* curves for u.v. mutants, interaction mutants, and u.v. and interaction mutants together, have been re-plotted as a percentage of their value at zero time. Neither the rates of mutation frequency decline nor the ratios of final to initial frequencies are significantly different for the three curves.

DISCUSSION

The oxygen effect experiments may throw some light on the component of γ-radiation damage which is involved in this interaction between u.v. and ionizing radiation damage. The oxygen enhancement ratio for lethality under the conditions of these experiments is about 3 and for mutation induction about 1·3 (Bridges & Munson, 1964). For the production of interaction mutants it varied between 1·5 and 3 which might indicate that straightforward γ-radiation-induced mutations are not involved in the interaction but possibly the same sort of damage which is involved in lethality.

The fact that the u.v. component involved in interaction is photoreactivable does not necessarily imply that thymine dimers are involved since Witkin (1964) has shown that u.v.-mutagenesis may be photoreactivable even in strains known to lack the photoreactivating (dimer-splitting) enzyme. In such a case photoreactivation appears to involve the same indirect mechanism (similar to that involved in photo-protection) which can remove lethal damage (probably including thymine dimers) under certain conditions (Kondo & Jagger, 1966; Kondo & Kato, 1966). The sensitivity of the u.v.-interaction component to photoreactivation after both the u.v. and γ doses have been given is, of course, a further demonstration that interaction occurs during the post-irradiation period, as is also the post-irradiation sensitivity to acriflavine. The enhancing effect of acriflavine on u.v. mutagenesis is believed to be due to an inhibition of excision-repair and/or mutation frequency decline and the enhanced yield of interaction mutants on acriflavine plates confirms the conclusion of Davies *et al.* (1967) that the u.v.-interaction component is also sensitive to these processes.

A direct demonstration that interaction mutants are subject to mutation frequency decline (and thus behave like mutants induced by u.v. alone but not γ-radiation alone) comes from the experiment shown in Fig. 5. It is clear that the proportion of interaction mutants to u.v.-induced mutants is the same in samples plated immediately after irradiation on plates enriched with nutrient broth (which provides partial stabilization against mutation frequency decline) as on those plated after 40 min. in minimal medium (when mutation frequency decline is complete). In addition the rate of decline is the same for interaction as for u.v.-induced mutants.

The slope of a mutation frequency decline curve depends both on the rates of removal and of fixation of premutational damage. The experiment shown in Fig. 5 makes it unlikely that interaction mutants result from the interference by γ-radiation with the rates of either of these processes.

So far then it appears that the γ-component of the damage involved in interaction may be more like lethal than mutational damage, and that the u.v.-component behaves like u.v. mutational damage. The most reasonable interpretation of these results is that damage induced in DNA by γ-radiation inhibits the repair of premutational lesions induced by u.v.

It is not possible at present to state the precise nature of the γ-radiation component of damage. There is evidence that both *Escherichia coli* B/r WP2 and *E. coli* WP2 hcr$^-$ can repair a large proportion of lethal ionizing radiation damage, including possibly single strand breaks (Bridges & Munson, 1966, McGrath & Williams, 1966), but it is not known whether the lesions which are ultimately lethal are unrepaired single strand breaks or of a further unidentified type.

There is general agreement that pyrimidine dimers are involved in the induction of mutations by u.v. alone, whether as the sole type of lesion (Hill, 1965; Kondo & Kato, 1966) or in conjunction with further unidentified lesions (Witkin, 1964, 1966; Doudney, 1963, 1966; Bridges, 1966). One of the strongest arguments against pyrimidine dimers being the only lesions involved is the fact that the removal of premutational lesions by the mutation frequency decline system is severely inhibited by the presence of nutrient broth, whereas the removal of thymine dimers is stimulated (Setlow & Carrier, 1964).

If we are to explain our mutation interaction results in a way similar to that used by Haynes (1964) to explain his lethal interaction results, we must do so in terms of a repair system able to remove premutational lesions induced by u.v. and inhibited by γ-radiation lesions (which might imply an ability to repair certain γ-radiation lesions). So far two candidates appear as possible contenders for the role of this hypothetical repair system.

The first has been described in terms of a low temperature mutation loss (l.t.m.l.). When minimal plates enriched with tryptophan are incubated at temperatures below about 16° after irradiation, fewer mutants appear compared with plates incubated at 37°, whether after γ-radiation (Bridges & Munson, 1964) or u.v. (Munson & Bridges, 1966).

While this l.t.m.l. might seem at first sight unlikely to be associated with u.v.-γ interaction at 37°, closer examination shows that this need not be so. The phenomenon of l.t.m.l. has been ascribed to a differential temperature effect on the rates of repair and fixation of premutational lesions (Bridges & Munson, 1964), but since at temperatures above about 22° the rates of both repair and fixation apparently share the same dependence upon temperature it follows that the repair may still go on to an appreciable extent at 37° although we have no means of detecting it. L.T.M.L. is also a suitable candidate for the interaction effect as it occurs in both hcr$^+$ and hcr$^-$ strains to a similar extent and does not appear to be related to excision repair or mutation frequency decline.

The second presumed repair system for u.v. damage (K-reactivation) has been described by Kneser (1965, 1966) as being present in wild-type strains of *Escherichia coli* K12 and B/r, absent in 'recombination-less' K12 and inhibited in *E. coli* B and

Bs-1. Since it is present in both the hcr$^+$ and hcr$^-$ strains which we have used we cannot tell whether it is involved in our interaction phenomenon and, although there is evidence that it operates on both u.v. and γ-radiation lethal damage, it may act on non-genetic components of radiation damage. In some early work, Demerec & Latarjet (1946) showed that the rate of mutation induction to phage resistance was the same in *E. coli* B and B/r which indicates that the premutational damage involved there was not amenable to K-reactivation. The same conclusion has been reached by Dr E. M. Witkin (personal communication) for reversion to prototrophy. It is possible that K-reactivation is the system involved in the u.v.-γ lethal interaction observed by Haynes (1966) since a similar interaction occurs with *E. coli* WP2 hcr$^-$ which possesses K-reactivation (Bridges & Munson, unpublished observation) but not *E. coli* Bs-1 (Haynes, 1964) which does not. It would obviously be desirable to examine whether synergistic interaction for mutation induction is also absent in *E. coli* Bs-1, as this would give an indication of the possible role of K-reactivation in the mutant interaction phenomenon.

If, however, neither l.t.m.l. nor K-reactivation is involved in interaction of mutational damage, it would presumably be necessary to postulate a further repair system.

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Note added in proof

An interaction between u.v. and X-irradiation has recently been reported by Kada, Doudney & Haas (1966) (*Mutation Res.* **3**, 118) in *E. coli* B/r WP 2. Interaction occurred only when there was a period of incubation at 37° between the two irradiations and appeared to depend on RNA and protein synthesis. Furthermore, minimal plates without enrichment were used so that most mutants (both u.v. and X-ray induced) would not be expressed. We consider that the interaction observed under these conditions is probably concerned with the expression of mutations in the absence of tryptophan and is a quite different phenomenon from that which we have examined.

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Citrate-Induced Citrate Production and Light-Induced Growth of *Blastocladiella emersonii*

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SUMMARY

Exogenous citrate induced the growing thin-walled ordinary colourless form (OC cell) of the water fungus *Blastocladiella emersonii* to produce more citric acid and, simultaneously, to re-utilize lactic acid liberated previously in one large wave of activity. In the absence of added citrate, release of citric acid was not detectable at the end of the cell's generation time, while production and re-utilization of lactic acid did occur, but in very small amounts and in a series of successive waves. An increase in the generation time of an OC cell, previously known to be induced by visible light, was also induced by exogenous citrate in the dark, provided environmental conditions were favourable. Furthermore, increasing amounts of exogenous CO_2 increasingly decreased the dark generation time; this inhibition was annulled by light. Some effects of gaseous (CO_2/O_2) and ionic (Na^+/K^+) balance are also described.

INTRODUCTION

Light-stimulated growth of the ordinary colourless organism (OC cell) of *Blastocladiella emersonii* was first detected with multiple generation cultures in peptone + yeast-extract + glucose (PYG) medium (Cantino & Horenstein, 1956). It was later shown (Cantino & Horenstein, 1959) with synchronized single-generation cultures (SSGC) grown on PYG agar media, whereon it was in part reflected in a bicarbonate and/or CO_2 dependent prolongation of the cell's generation time (GT). Finally, both accelerated growth and the GT effect were achieved (Goldstein & Cantino, 1962) with SSGC in liquid Difco PYG medium supplemented with 5×10^{-3} M-phosphate (pH 6·7; PYG-P). However, the liquid medium which yielded the best synchronized cultures, i.e. in which uniform cell suspensions were maintained most consistently with the least effort, was one designed for *B. britannica* (Horenstein & Cantino, 1964) and modified for use with *B. emersonii*: namely a Difco PYG medium made up in $1{\cdot}1 \times 10^{-2}$ M-Na_2HPO_4 and 3×10^{-3} M-citric acid (pH 6·6; PYG-PC). But in spite of its advantages, we had difficulty in demonstrating light-induced prolongations of *B. emersonii* generation time on this medium; the light effects were slight at best and not always reproducible. The purpose of the present report is to show that there was no light effect in PYG-PC medium because citrate substituted for visible light as an inducer of the extended generation time, although modification of the gaseous and ionic balance did permit it to occur even in the presence of citrate. Pertinent terminology, background history, and cultural procedures applicable to this water

fungus are covered in reviews (Cantino & Lovett, 1964; Cantino, 1966; Lovett, 1967); past studies of the effects of light on *B. emersonii* were summarized recently (Cantino, 1965).

METHODS

All synchronized single-generation cultures of OC cells of *Blastocladiella emersonii* were started with spores and propagated in white light (500 ft.c.) and darkness, at 24°, according to Goldstein & Cantino (1962) in either peptone + yeast-extract + phosphate medium (PYG-P) or in a similar medium + citrate (PYG-PC), but with numerous modifications as described below. Citric acid was determined by Stern's (1957) method; lactic acid, according to Ryan (1958).

RESULTS

Comparison of medium without citrate (PYG-P) and with citrate (PYG-PC)

Uninoculated PYG-P and PYG-PC, autoclaved and non-autoclaved, were illuminated (500 and 2000 ft.c.) for 18 hr; ultraviolet (u.v.) and visible absorption spectra were taken thereon. Other samples and untreated controls were chromatographed in several solvent systems; papers were examined under u.v. radiation and developed with ninhydrin, ammoniacal $AgNO_3$ and other reagents. No significant chemical differences (other than in citric acid) between the two media were detected which were judged to be important factors in, or which provided additional clues about the reason for, the differences in response of *Blastocladiella emersonii* to light in these two media.

Comparison of metabolic products in PYG-P and PYG-PC media

Blastocladiella emersonii was grown in PYG-P and PYG-PC media. Citric acid was not detected in the PYG-P at generation time (GT), while in PYG-PC medium citrate actually increased (Fig. 1); its final concentration in light-grown and dark-grown cultures was about the same. Thus, the presence of exogenous citric acid induced *B. emersonii* to make more of it. Large quantities of lactic acid were also liberated but were re-utilized during the period when citricacid was released (Fig. 2). In PYG-P medium, small amounts of lactic acid were produced and then consumed again, but in at least six successive waves of activity (Fig. 2); by the end of one generation time, little lactate had accumulated.

Effect of aeration rate and O_2/CO_2 balance

Some fifty experiments, in which the aeration rates varied between 1600 and 4200 ml./min., led us to conclude that light-induced increases in generation time were small or negligible at low flow rates (i.e. 1500 ml./min.) but greater at higher flow rates (i.e. 3400 ml./min.; for example, see controls, shaded *vs.* unshaded bars, Fig. 3). At this higher aeration rate cultures were grown under elevated O_2 tensions. The results showed that increased O_2 (*a*), did not affect the generation time of dark grown cells (Fig. 3, controls, shaded, *vs.* 8% O_2, shaded); (*b*), at best, slightly augmented the effect of light on the generation time; (*c*), combined with the removal of CO_2 from the air stream, induced an increase in the dark generation time; i.e. the generation time was maximum and about the same for both light-grown and dark-grown organisms.

Effect of Na^+ concentration

The generation time was affected by exogenous Na^+ (Fig. 4); increasing exogenous Na^+ from $1{\cdot}6 \times 10^{-2}$ M to $2{\cdot}2 \times 10^{-2}$ M prolonged the generation time by about 4 hr. Below this range, progressive decreases in Na^+ concentration caused organisms to adhere to the glass vessels; above this range toxicity symptoms appeared.

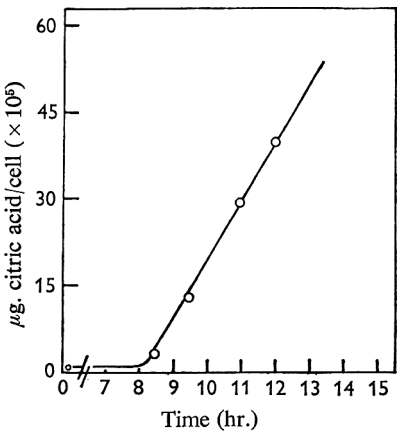

Fig. 1

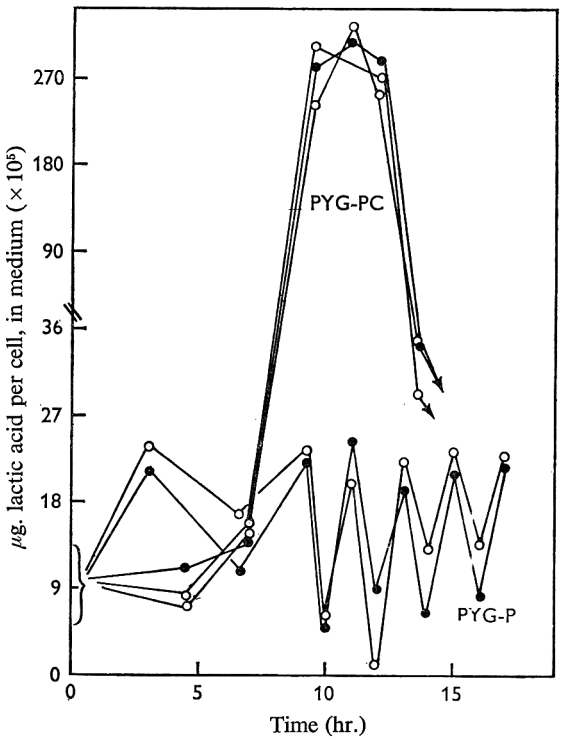

Fig. 2

Fig. 1. Accumulation of citric acid in a SSGC of OC cells in PYG-PC, pH 6·6. Aeration, 3600 ml./min.; vol., 600 ml.; population density, $3{\cdot}1 \times 10^5$/ml. Each point is an average derived from analyses of light- and dark-grown cultures in duplicate.

Fig. 2. Production and re-utilization of lactic acid in SSGC of OC cells in PYG-P and PYG-PC, both pH 6·6. Aeration, 3600 ml./min.: vol. 600 ml.; population density, $3{\cdot}1 \times 10^5$/ml. for PYG-PC and $2{\cdot}3 \times 10^5$/ml. for PYG-P; open circles, light-grown; shaded circles, dark-grown. Similar results for the lactic acid content of spent PYG-PC were published previously (Cantino, 1965); new and more extensive data are shown here for comparison with the analyses of PYG-P.

Effect of cationic balance

The generation time was also affected by exogenous K^+. For example, with phosphate buffer provided as a mixture of Na and K salts, 8×10^{-3} M for each cation, light induced increases of generation time of 120 min and more. However, the OC cells did not discharge their spores. This effect was obtained when $Na^+ : K^+$ ratios reached unity, and often with ratio value up to 3; in such cultures, the final pH values were no different from the controls containing only Na^+. Replacement of all the Na^+ with K^+ (i.e. $1{\cdot}6 \times 10^{-2}$ M) yielded OC cells which were highly vacuolate and grew poorly; they

were still very small and did not discharge spores even after 28 hr (roughly double the normal generation time). Replacement of Na^+ with NH_4^+ yielded spherical organisms which grew at more nearly normal rates; but again, these were highly vacuolate and not capable of spore discharge after 28 hr.

Effect of CO_2 tension

Blastocladiella emersonii was grown in PYG-PC medium, and the CO_2 in the air stream was varied between 0·03% and 27% (v/v). At one generation time, cell shapes were judged subjectively and estimated quantitatively in terms of the ratio cell width: cell length, and cell volumes calculated therefrom. Differences in generation time between light-grown and dark-grown cultures were recorded. Mature OC cells grown

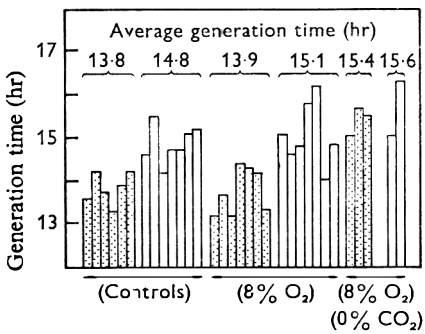

Fig. 3

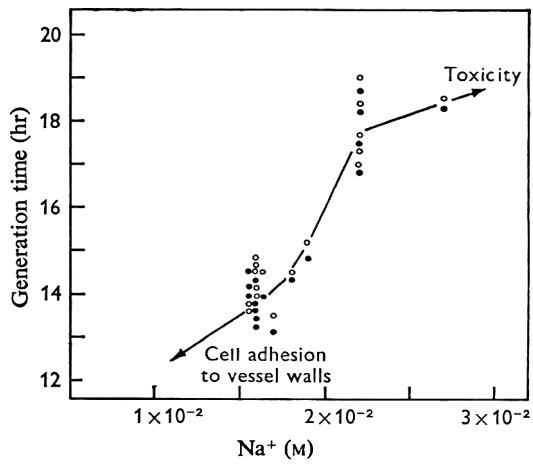

Fig. 4

Fig. 3. Effects of O_2/CO_2 balance on the GT and the light-induced increases in GT in SSGC of OC cells in PYG-PC, pH 6·6, using reduced levels of Na_2HPO_4 ($0·8 \times 10^{-2}$ M; i.e. Na^+, $1·6 \times 10^{-2}$ M) and citric acid ($2·2 \times 10^{-3}$ M). Each bar represents a separate 600 ml. culture. Aeration, 3400 ml./min.; population densities varied between 1·1 and $1·5 \times 10^5$/ml. in the different experiments; unshaded, light-grown; shaded, dark-grown.

Fig. 4. Effect of exogenous Na^+ concentration on the GT in SSGC of OC cells in 600 ml. PYG-PC, pH 6·5–6·6. Each point represents a separate culture. Open circles, light-grown; shaded circles, dark-grown. The Na^+ level was varied by adjusting the concentration of Na_2HPO_4 from $0·8 \times 10^{-2}$ to $1·35 \times 10^{-2}$ M, and citrate from $2·2 \times 10^{-3}$ to 3×10^{-3} M. Separate control experiments established that the changes in concentrations of phosphate and citrate were not responsible for alterations in GT. Variations in GT at $1·6 \times 10^{-2}$ and $2·2 \times 10^{-2}$ M Na^+ (two main clusters of points on graph) were due mainly to the four different flow rates of the aeration stream (1500, 2400, 2800, and 3400 ml./min.) and the two population densities ($1·33 \times 10^5$ and $2·6 \times 10^5$ cells/ml.) used in these experiments. *Additional note:* a serious bottleneck which prevents taking full advantage of a SSGC is the tenacity with which OC cells can sometimes adhere to the glass wall of a culture vessel. Very many experiments, all involving full scale cultures, were done to identify and thereby to circumvent some of the causes involved. These included treatment of glass surface with various agents, and alterations (in various combinations) of pH, aeration rates, spore population densities, and concentrations of HCO_3^-, total phosphate, HPO_4^{2-}, total citrate, $citrate^{3-}$, Na^+ and K^+. We concluded (*a*) that the stickiness of cells was seldom if ever due to incorrect concentrations of HPO_4^{2-}, HCO_3^-, or $citrate^{3-}$, to pH *per se*, or to an incorrect balance between total phosphate and total citrate, or between Na^+ and K^+; and (*b*), that insufficient amounts of total phosphate and/or total Na^+ were most likely responsible.

in the dark under 6·7% and 13% (v/v) CO_2 were elongated and only about 1/3 normal size; this inhibitory effect of CO_2 on volume was overcome by illumination at 6·7% (v/v) CO_2 but not at 13% (Table 1). Increases in CO_2 up to 13% led to roughly proportional light-stimulated increases in generation time over the values for dark controls; however, the generation times of all light-grown organisms was essentially the same. Thus, the capacity of CO_2 to yield light-induced increases in generation time on citrate media was actually due to CO_2-induced decreases in the generation time of dark-grown organisms, these decreases being overcome by illumination.

Table 1. *Effect of % CO_2 in the air stream on the volume, shape, and GT of OC cells grown in light and darkness on PYG-PC*

SSGC volumes were 600 ml.; aeration, 1500 ml./min.; population density, $1{\cdot}17 \times 10^5$/ml.; initial pH, 6·5–6·6 in all cultures; final pH, 6·5–6·6 for 0·03% and 0·5% CO_2 6·4–6·5 for 6·7% CO_2, and 6·3–6·4 for 13% CO_2. Dark-grown controls without CO_2, when adjusted to pH 6·3 and 6·4, did not exhibit decreased GT. Attempts to increase the CO_2% in the air stream up to *ca.* 27% failed due to harmful decreases in pH to 6·0 and below at GT. Slight increases in phosphate buffer failed to prevent the pH from dropping too far. Further increases in Na_2HPO_4 to more than $2{\cdot}5 \times 10^{-2}$ M, with or without increases in citric acid to 5×10^{-3} M, caused spores to disintegrate (see Fig. 4 and text section on cationic balance). Note that controls in this sort of experiment are particularly critical; the GT depends in part upon aeration rate, population density, Na^+ concentration, etc., various combinations of which can lead to a GT as high as 19 hr or as low as 14 hr.

	Light			Dark		
% CO_2	Volume (μ^3)	Width:length	GT (hr)	Volume (μ^3)	Width:length	GT (hr)
13·0	$6{\cdot}2 \times 10^4$	0·62	17·0	$5{\cdot}9 \times 10^4$	0·68	15·3
6·7	$22{\cdot}6 \times 10^4$	0·50	16·9	$6{\cdot}0 \times 10^4$	0·66	15·8
0·5	$20{\cdot}0 \times 10^4$	(*ca.* 0·8)	17·1	$19{\cdot}0 \times 10^4$	(*ca.* 0·8)	16·7
0·03	$18{\cdot}8 \times 10^4$	0·82	17·1	$18{\cdot}3 \times 10^4$	0·84	16·9

Table 2. *Comparison of GT and volume of OC cells grown in light and darkness in PYG-P and PYG-PC*

Growth on PYG-P: values shown are averages for two experiments, each one involving light- and dark-grown cultures in duplicate; population densities were $0{\cdot}9 \times 10^5$ ml. and $2{\cdot}5 \times 10^5$/ml.; all other details were as described by Goldstein & Cantino (1962). Growth on PYG-PC: average values are shown for six different experiments, all cultures in duplicate; population density, $1{\cdot}3 \times 10^5$/ml.; aeration rate, 1500 ml./min.; initial and final pH, 6·55–6·6 in all instances.

	Medium			
	PYG-P		PYG-PC	
	Light	Dark	Light	Dark
GT (hr)	17·5	16·0	17·2	17·1
Cell volume (μ^3)	$19{\cdot}3 \times 10^4$	$8{\cdot}0 \times 10^4$	$18{\cdot}2 \times 10^4$	$18{\cdot}3 \times 10^4$

Lastly, the generation time and the volume at one generation time of organisms grown without CO_2 supplements in light and darkness on PYG-PC medium were compared with organisms similarly grown on PYG-P medium (Table 2). In PYG-P medium, light-induced increases (about 90 min.) in generation time were associated with about twofold light-induced increases in cell volume to about 19×10^4 μ^3 (see Goldstein & Cantino, 1962, for details about this effect). In PYG-PC medium, dark-

grown organisms displayed the same generation time and volume as those grown in the light in either PYG-PC or PYG-P media, i.e. with or without added citrate. Thus, given suitable CO_2 and Na^+ concentrations, aeration rates, and population densities, exogenous citrate effectively substituted for white light as an inducer of increased size and an extended generation time of an OC cell.

DISCUSSION

Production and re-utilization of lactate in PYG-P

Although our studies dealt primarily with the growth of *Blastocladiella emersonii* in PYG-PC, production and re-utilization of lactate in PYG-P is noteworthy. Its rhythmic appearance and disappearance in successive waves must reflect some endogenous cyclic process during exponential growth of an OC cell. We suspect that a succession of synchronous nuclear divisions is the cause. The motile spore of this fungus is uninucleate; at maturity, the OC cell derived therefrom can produce a few hundred to perhaps 2000 such spores, depending upon its size at GT. Assuming that a germling becomes binculeate by 3 hr (Turian & Cantino, 1959), that the doubling time for nuclei lies between one and 2 hr, and that nuclear divisions during exponential growth are synchronous, some 8 to 12 replications would suffice to generate the required number of nuclei for the next generation of spores. If metabolism of lactic acid were associated with mitosis, production and re-utilization of lactate in 8 to 12 successive waves might occur; in the present study, 8 waves were detected. But judging from the fact (Turian & Cantino, 1959) that the nuclear doubling time in an OC germling in PYG is *ca.* 2 hr in the dark but more like 1·2 hr in the light, and assuming that such differential rates of nuclear reproduction continue throughout ontogeny, more 'lactic acid waves' should have been seen in light-grown cultures than in dark-grown ones. Although the results (Fig. 2) do not show this, analyses were only made at hourly intervals between 9 hr and GT, and even less frequently during the earlier stages in development. A systematic approach with analyses for lactic acid at much more frequent intervals throughout the ontogeny of light- and dark-grown cells should help determine if this notion has validity.

The relation between citrate-induced and light-induced increases in GT of an OC cell

In 1956 (for all references to *Blastocladiella emersonii* in this discussion, see Cantino, 1965), we found that a mixture of OC cells of various ages, pre-grown in multiple generation culture and then resuspended in $H^{14}CO_3^-$, consumed more ^{14}C in the light than in the dark and simultaneously accumulated a greater intracellular pool of succinate-^{14}C. From this and other work, it was hypothesized that light-stimulated growth of an OC cell, and the light-induced CO_2-dependent extension of its GT, may have been due to increased reductive carboxylation of α-ketoglutarate to isocitrate and its cleavage to succinate and glyoxylate. Some *in vivo* corroboration came from demonstrations that an equimolar mixture of exogenous succinate and glyoxylate did, in fact, substitute for white light by inducing an extended GT of a dark-grown cell. We have now shown that exogenous citrate can also substitute for light by inducing prolongation of the cell's GT, and that—unlike the HCO_3^-/CO_2 dependent light induction *per se*—CO_2 is no longer necessary when citrate is provided directly. Assum-

ing that citrate gives rise to isocitrate within the cell, this observation lends new support for our belief that the light-induced extension of the GT of *B. emersonii* stems directly or indirectly from a light-induced acceleration of the sequence: α-keto-glutarate + CO_2 → isocitrate → succinate + glyoxylate.

In previous reports, we discussed possible connexions between this reaction sequence and other demonstrable effects of light upon Blastocladiella: effects upon its nuclear reproduction and the need for thymidine; upon its capacity for glycine uptake and the latter's relation to exogenous CO_2, intracellular isocitratase activity, and DNA production; upon its genesis of protein and polysaccharide during growth, etc. Assuming that the *in vivo* effect of citrate and of the combination succinate plus glyoxylate have a common basis, there is no need to cover this ground again for it is discussed fully elsewhere.

However our work with citrate does raise unexpected questions for which no clear-cut answers are available. For example, if citric acid substitutes for light by functioning *in vivo* as the progenitor of succinate and glyoxylate, why does exogenous CO_2 *depress* the GT in the dark when citrate is present? Does CO_2 perhaps inhibit entry of lactate via pyruvate into the tricarboxylic acid cycle, or affect decarboxylations therein, or accelerate formation of citrulline via synthesis of carbamyl phosphate? A case could be made for all these possibilities (not from comparative metabolic maps but from what has been established about Blastocladiella's physiology). Why does exogenous citrate induce accumulation of lactic acid, while little if any appears in its absence, only to have it re-utilized just before sporogenesis? And why does exogenous citrate bring about release of *additional* citrate at this stage in ontogeny? What is the relation of these things to other events associated, in particular, with the terminal stages of ontogeny when an OC cell approaches the end of its development but remains capable of additional light-stimulated growth; events such as the light-induced depression of its D-glucose-6-phosphate: NADP oxidoreductase; the light-induced change in its intracellular distribution of haemoprotein; the sharp rise in its L-glutamine amidohydrolase, associated with rapid incorporation of glutamic acid into protein but almost no equilibration with its amino acid pool; and its very large increase in L-ornithine carbamoyl transferase, decrease in L-arginine ureohydrolase, and rapid uptake of arginine for protein synthesis? And finally, with Wyatt's (1964) provocative discussion of cations and metabolism in mind—especially K^+ activated, Na^+ inhibited pathways from phospho-enol-pyruvate and acetate to the Krebs cycle—are the effects of Na^+/K^+ balances on the GT related to cationic effects upon activity of the OC cell's tricarboxylic acid cycle? It is tempting to speculate about the interrelationships which must exist among the reactions known to occur in Blastocladiella. We shall, however, control this urge in favour of returning to the bench for further work.

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A Comparison of Two Methods for Detecting Attack on Glucose by Pseudomonads and Achromobacters

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SUMMARY

One hundred and forty-eight Gram-negative bacteria, mostly pseudomonads and achromobacters, were tested for glucose utilization by detection of acid production and by an enzymic method for detecting residual glucose. The enzyme test showed that some bacteria which produced little or no acid in glucose media (e.g. *Pseudomonas maltophilia*, *Pseudomonas* groups III and IV of Shewan, Hobbs & Hodgkiss, 1960) did remove glucose from the media. Representatives of the genus *Comamonas* did not produce acid from glucose or remove it.

INTRODUCTION

Hugh & Leifson (1953) used acid production to show whether bacteria attacked carbohydrates fermentatively, oxidatively or not at all (oxidation versus fermentation test; O/F test). The test has been modified for certain types of bacteria (Baird-Parker, 1963) or to give greater sensitivity (Board & Holding, 1960; Scholefield, 1962). In assessment of the reliability of their O/F test, Board & Holding (1960) found that only strains which produced acid showed oxygen uptake when glucose was used as substrate in the Warburg apparatus. However, in cases where it is difficult to decide whether any acid has been produced from glucose, a simpler alternative method for detecting attack on glucose is required. This note describes the application of such a method to several non-fermentative Gram-negative bacteria. It consists of incubating bacteria in a glucose-containing medium and using a simple enzymic method (glucose oxidase + peroxidase + *o*-tolidine) to determine whether any glucose remains after incubation.

METHODS

Strains. Various Gram-negative bacteria, mostly pseudomonads and achromobacters, were obtained from the National Collection of Type Cultures, the National Collection of Industrial Bacteria, the National Collection of Marine Bacteria, various members of the Pseudomonas Working Party and the author's own collection. They were maintained on heart infusion agar (Difco) slopes in screw-capped bottles.

Inoculation and incubation of tests. Liquid media were inoculated with a 2 mm. loop and semi-solid media by stabbing with a straight wire. Nutrient broth cultures incubated at 25° for 24 hr, or in the case of slow-growing strains for 48 hr, were used for the inocula. Results were recorded after incubation at 25° for 1 day and 7 days.

Oxidation versus fermentation (O/F) tests which use acid production to indicate attack on glucose. The general procedure followed was that described by Hugh & Leifson (1953) but it was not necessary to incubate a duplicate set of tubes under a

seal. The basal medium used here had the following composition: peptone (Evans), 2·0 g.; NaCl, 5·0 g.; K_2HPO_4, 0·3 g.; agar No. 3 (Oxoid), 3·0 g.; 1% (w/v) aqueous solution of bromothymol blue, 8 ml.; in 1 l. demineralized water; pH 7·2. When required, glucose (10%, w/v, filter-sterilized solution) was added at the rate of 10 ml. solution/100 ml. molten basal medium. Each strain was inoculated into 1 tube of the basal medium + glucose and 1 tube of the basal medium alone. Tests were also made with: the medium described above but with Casitone (Difco) in place of Evans peptone; the Difco O/F basal medium; the O/F medium described by Hugh & Ryschenkow (1961); the O/F (2) medium of Scholefield (1962).

Enzymic method for demonstrating glucose disappearance. 'Clinistix' reagent strips (Miles Laboratories, Stoke Poges, Buckinghamshire), which are sensitive to 0·01% (w/v) glucose, are strips of paper impregnated with glucose oxidase (an enzyme specific for glucose; Keilin & Hartree, 1948) + peroxidase + *o*-tolidine. From glucose, glucose oxidase produces acid and hydrogen peroxide, and this hydrogen peroxide in the presence of peroxidase then oxidizes the *o*-tolidine to a blue compound.

Two kinds of false readings may be expected when these reagent strips are used to detect glucose in cultures of bacteria: (i) reducing substances may inhibit development of colour on the strip so that glucose, though present, is not detected; (ii) any hydrogen peroxide present will give a blue colour on the strip, whether or not glucose is present.

To use this enzymic method for detecting ability to attack glucose the following procedure was adopted. A liquid medium similar to the Hugh & Leifson (1953) medium but lacking agar and bromothymol blue was prepared. It was called MHL medium and it consisted of Evans peptone, 2·0 g.; NaCl, 5·0 g.; K_2HPO_4, 0·3 g.; in 1 l. demineralized water; pH 7·2. This medium was dispensed in 5 ml. amounts in 25 × 150 mm. tubes and when required glucose as a filter-sterilized solution was added to a final concentration of 0·05% (w/v). For each strain a tube of MHL medium + glucose (+G medium) and a tube of MHL medium alone (−G medium) were inoculated and incubated in a sloped position for 7 days. After 1 day and 7 days a loopful of each culture was put on a Clinistix reagent strip which was examined after 1 min. When, at 7 days, no blue colour appeared with a +G culture, glucose (0·05%, w/v) was added, and the culture immediately retested. No colour on the strip after this would indicate that inhibitors of the test were present. Four results are theoretically possible from examining two tubes in this way.

(1) Absence of colour from strip treated with +G and −G cultures would indicate one of three alternatives: (*a*) that glucose had disappeared from the +G culture and an inhibitor for the development of colour on the strip had not been produced by the culture; (*b*) that glucose remained but an inhibitor had been produced (*c*) that glucose had disppeared from the +G culture and an inhibitor had been produced. The possibility that an inhibitor had been produced can be eliminated by adding glucose to the +G cultures and retesting, as described above. On no occasion was an inhibitor detected and so with the organisms used in this work it was not necessary to distinguish between alternatives (*b*) and (*c*).

(2) Colour on strips impregnated with +G culture but not on those impregnated with −G culture would indicate that glucose remained in the +G culture after incubation and that H_2O_2 production was not interfering with the test.

(3) Colour on strips impregnated with +G culture and with −G culture would

indicate that production of H_2O_2 was interfering with the test. In no case did this occur. It is possible that inhibition of catalase by acids might allow H_2O_2 to accumulate more in a glucose-containing culture, so making valueless the use of a −G culture to control this possibility. In doubtful cases the following method was used to confirm that H_2O_2 production was not interfering with the test. To 1 ml. culture grown in −G medium was added 0·05 ml. '10 vol.' H_2O_2, sufficient, to make the culture give an intense reaction with the strips. To this, and to 1 ml. of culture in +G medium, was added 0·1 ml. iodine solution (iodine, 0·3 g.; potassium iodide, 0·6 g.; distilled water, 90 ml.) and after heating in boiling water for 5 min. both cultures were tested with Clinistix strips. The procedure removed any peroxide present, as indicated by the −G culture giving no blue reaction, so that any blue reaction with the +G culture indicated that glucose was in fact still present after incubation.

(4) Colour on strips impregnated with −G culture but not on those impregnated with +G culture. This result is unlikely and did not occur.

The enzymic method for detecting glucose utilization was also tested with two other media. These were nutrient broth (%, w/v; Evans peptone, 0·5; Lab. Lemco, 0·5; NaCl, 0·5; in distilled water; pH 7·2) and MBH medium which was the basal medium of Board & Holding (1960) but without agar (%, w/v: $NH_4H_2PO_4$, 0·05; K_2HPO_4, 0·05; Difco yeast extract, 0·05; mineral supplement; in distilled water; pH 7·2).

RESULTS

One hundred and forty-eight Gram-negative bacteria, mostly pseudomonads and achromobacters from a variety of habitats, were examined for ability to produce acid from glucose, by using various oxidation versus fermentation (O/F) tests, and for ability to remove glucose from liquid cultures by using the enzyme strips. Most strains gave comparable results in these tests (Table 1). Sixty-two strains produced

Table 1. *Comparison of results obtained by two methods for detecting attack on glucose by various Gram-negative bacteria, mostly pseudomonads and achromobacters*

No. of strains tested	Attack detected by showing that cultures produced acid in various oxidation *v.* fermentation test media + glucose (1 %). Reaction after incubation for 7 days at 25°	Attack detected by showing with an enzyme reagent strip that after 7 days at 25° glucose had been removed from:	
		Cultures in MHL medium* + glucose (0·05 %)	Cultures in nutrient broth + glucose (0·05 %)
62	Acid	Glucose removed	Glucose removed
2	Acid	Glucose removed	Glucose remained
59	Alkaline	Glucose remained	Glucose remained
25	Slight acid or alkaline or no change	Glucose removed	Glucose removed

* MHL medium was similar to the medium of Hugh & Leifson (1953) but lacked agar and bromothymol blue.

acid from glucose in all varieties of the O/F test and removed glucose from cultures in +G MHL medium and in +G nutrient broth. Not all strains grew well in MBH medium but all those which did do so removed glucose from the +G cultures. Attack

on glucose was detected after 24 hr more frequently by the enzyme test that by the O/F test. Two strains produced acid in O/F tests and removed glucose from +G MHL medium, but did not remove glucose from +G nutrient broth or grow in +G MBH medium. Fifty-nine strains did not produce acid from glucose in any varieties of the O/F test (in all cases an alkaline reaction developed) or remove glucose from +G cultures.

Table 2. *Strains which removed glucose from liquid media, as shown by an enzyme test, but which produced little or no acid from glucose in Hugh & Leifson glucose medium*

Pseudomonas maltophilia (designated type strain of the species by Hugh & Ryschenkow, 1961) ATCC 13637, NCTC 10257; supplied by Dr R. Hugh, no. 810–2
P. maltophilia ATCC 13636; supplied by Dr R. Hugh, no. 560
P. maltophilia ATCC 13843; supplied by Dr R. Hugh, no. 611
P. nigrifaciens NCIB 8614
P. oleovorans NCIB 6576
P. putrefaciens NCIB 8615
'Brown' organisms; supplied by Dr Margaret Thornley, nos. EB/F 44/1, EB/F 44/15, EB/F 44/84, EB/F 44/148
Gram negative bacteria from cheese; supplied by Dr M. Elizabeth Sharpe, nos. GN 39, GN 48, GN 49, GN 50, GN 52, GN 53
Pseudomonas group III (Shewan, Hobbs & Hodgkiss, 1960) NCMB 224
Pseudomonas group IV (Shewan *et al.* 1960) NCMB 114
Pseudomonas group IV (Shewan *et al.* 1960) NCMB 130
Pseudomonas fluorescent group A (Holding, 1960); supplied by Dr A. J. Holding, no. PT 2
Agrobacterium; supplied by Dr A. J. Holding, no. LK 11
Agrobacterium; supplied by Dr A. J. Holding, no. ZM 1
Pseudomonad producing gas from nitrate. Isolated by Miss Caroline Scarlett; Park no. 284
Vibrio alcaligenes NCTC 7048
Melanin-producing vibrio; supplied by Dr D. G. Smith

Twenty-five strains (see Table 2) gave indefinite results in O/F tests. Some strains did not change the pH value of O/F media during growth in the presence of glucose. Other strains produced a very weak acid reaction at the surface of O/F media in the presence of glucose, the medium becoming lighter green or yellow-green. A few strains gave an alkaline reaction in O/F media containing glucose. All these strains gave an alkaline reaction in O/F media without glucose so that, in most cases, there was a difference in pH value at the surface of media, with and without glucose. However, these differences were slight and the results of the tests difficult to interpret. It was in these cases that the enzyme method for detecting glucose disappearance was useful. All 25 of these strains (Table 2) removed glucose from +G MHL medium and from +G nutrient broth. Not all strains grew in +G MBH medium but those that did do so removed glucose.

DISCUSSION

An enzyme method for detecting the disappearance of glucose from liquid media showed that 25 strains of Gram-negative bacteria which gave indefinite results in the Hugh & Leifson (1953) O/F test were capable of metabolizing the sugar. The enzyme method is simple to use and has the advantage that it does not depend on the ability of an organism to use glucose as a sole carbon source or to decrease the pH value of a glucose-containing medium. It can be performed in a liquid medium like that described by Hugh & Leifson (1953) but lacking agar and bromothymol blue (MHL medium).

It is necessary to incubate the culture in 25+150 mm. tubes in the sloped position because strains which oxidize glucose leave sufficient sugar to give a reaction in the enzyme test when the medium is in 15+150 mm. tubes incubated vertically. A nutrient broth base, though generally satisfactory for the enzyme test, is not recommended because two strains (both achromobacters) which produced acid in O/F glucose tests and removed glucose from +G MHL medium without indicator did not always remove glucose from +G nutrient broth. This was presumably because components of the peptone and Lab Lemco were used in preference to glucose. A similar diauxic effect, in which citrate was used by *Pseudomonas aeruginosa* in preference to glucose, was reported by Hamilton & Dawes (1959, 1961).

The enzyme test showed that *Pseudomonas maltophilia* strains attacked glucose. Hugh & Ryschenkow (1961) reported that *P. maltophilia* did not produce acid from glucose except some old laboratory strains which produced a very weak acid reaction after prolonged incubation. The three strains examined by me (see Table 1) produced little or no acid at 7 days in O/F media which contained glucose but were shown by the enzyme method to attack glucose. Stanier, Palleroni & Doudoroff (1966) found that *P. maltophilia* utilized glucose as carbon+energy source when methionine, required as a growth factor, was present. Representatives of *Pseudomonas* group III and group IV of Shewan *et al.* (1960), which by definition do not produce acid in O/F glucose media, were found to attack glucose when tested by the enzyme method (Table 1).

It was confirmed by the enzyme method that representatives of strains which were classified as *Comamonas* by Davis & Park (1962) and Park (1962) did not attack glucose. These strains (Park nos. 11, 13, 23, 100; *Pseudomonas putrefaciens* NCIB 8615; *Comamonas percolans* NCTC 1937; *Vibrio cyclosites*, NCIB 2581; *V. neocystes*, NCIB 2582; *V. alcaligenes*, NCTC 9239) previously had been shown not to produce acid in O/F glucose media or to utilize glucose as a sole carbon+energy source. Strains of *P. alcaligenes* from Dr R. Hugh, (R. Hugh nos. 1155, 1278, 1288) and of *Comamonas terrigena* (R. Hugh nos. 31, 32, 37, 131, 1884) and the type strain of *Pseudomonas alcaligenes* NCTC 10367 were also shown by the enzyme method not to attack glucose. The taxonomic position of these strains with respect to the classification of Stanier *et al.* (1966) is not clear because, with the exception of the type strain of *Pseudomonas alcaligenes*, they do not appear to have been examined for nutritional pattern. Some are likely to be members of the acidovorans group or the alcaligenes group, but not all strains previously classified as *Comamonas* can be integrated into the scheme at present because the type strain of the genus *C. terrigena* (*C. percolans* NCTC 1937, ATCC 8461) has not yet been grown on a chemically defined medium (Stanier *et al.* 1966).

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Culture and Nutrition of Some Apochlorotic Diatoms of the Genus *Nitzschia*

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SUMMARY

Three distinct species of the genus *Nitzschia*, lacking chloroplasts and therefore obligately heterotrophic, were isolated in pure culture from various marine shores. The organisms reproduced rapidly in a defined medium based on an artificial sea water supplemented with mineral nutrients, thiamine and cobalamin. Lactate or succinate served as sole organic carbon source for all three species; two of the species also used glucose or glutamate. Two of the species were identified as *N. putrida* Benecke and *N. leucosigma* Benecke, respectively. The third organism appears to be a new species, here named *N. alba*.

INTRODUCTION

Almost all diatoms are capable of photosynthesis, but of these some can also grow heterotrophically by utilizing certain organic carbon substrates (Lewin & Lewin, 1960). A very few species, however, lack photosynthetic pigments and are therefore obligately heterotrophic. As pointed out by Benecke (1900) and Pringsheim (1951, 1956, 1963), 'colourless' (i.e. apochlorotic) diatoms are not uncommon in marine littoral habitats, especially on decaying seaweeds; none has been reported from a freshwater habitat. Two species were described by Benecke: *Nitzschia putrida* (with which he identified *Synedra hyalina* Provazek) and *N. leucosigma*. We have isolated several clones of colourless diatoms, clearly assignable to three distinct species, one hitherto undescribed. We present some information on the nutrition of these organisms, and a taxonomic description of the new species.

METHODS

Cultures of colourless diatoms were isolated from various habitats on marine shores (see Table 1). Fragments of source material were distributed on the surface of a 1% agar medium in Petri dishes. The medium, prepared with natural sea water, was supplemented with KNO_3, K_2HPO_4 and Tryptone (Difco), 0·2 g./l each. The addition of a mixture of pencillin (10^6 i.u./l.) and streptomycin sulphate (100 mg./l) was helpful in suppressing the growth of bacteria, but was not essential, since the diatoms tended to free themselves of contaminant organisms by their own movement over the

agar surface. Like Richter (1909), Wagner (1934) and Pringsheim (1951), we encountered little difficulty in establishing pure bacteria-free clones by picking individual diatoms and transferring them successively to fresh sterile media.

A basal medium of the following composition was used to replace sea water by a defined mixture of nutrient salts (g.): NaCl, 20·0; $MgSO_4.7H_2O$, 5·0; $CaCl_2.2H_2O$, 1·0; KCl, 1·0; sodium glycerophosphate, 0·1; $Na_2SiO_3.9H_2O$, 0·1; B, Fe, Mn, 0·5 mg. (of each element); Co, Cu, Mo and Zn, 0·01 mg. (of each element); distilled water, 1·0 l. The addition of tris buffer (Sigma; 1 g./l.) served to stabilize the pH value.

Monosodium glutamate and sodium lactate (60%, as syrup) were purchased from Fisher Scientific Co., as were most other salts used.

Media were sterilized by autoclaving at 120° for 15 min.; glucose was autoclaved separately and added aseptically.

Erlenmeyer flasks (125 ml.) containing 50 ml. medium were used for the nutritional studies. Growth (at 30°) was accelerated by shaking the cultures.

Table 1. *Sources of strains of apochlorotic marine diatoms (Nitzschia spp.)*

Species	Strain	Date of isolation	Locality	Substrate
Nitzschia alba	(Watson)	1958	Woods Hole, Mass., U.S.A.	Sargassum
	81-M	1958	Woods Hole, Mass., U.S.A.	Zostera
		1962	Ensenada, B.C., Mexico	Enteromorpha
	177-M	1964	La Jolla, California, U.S.A.	Aquarium outflow
	178-M	1964	La Jolla, California, U.S.A.	Pelagophycus
	179-M	1964	Split, Jugoslavia	Silt in harbour
N. putrida	176-M	1964	La Jolla, California, U.S.A.	Pelagophycus
N. leucosigma	180-M	1965	Chinook, Washington, U.S.A.	Fucus in marsh

RESULTS

Replacement of Tryptone by defined nutrients

A strain of *Nitzschia alba* from Ensenada was used for preliminary studies; unfortunately it died out before a suitable medium was evolved. Subsequent studies were carried out on strain 177-M, from La Jolla. In the enriched sea-water medium used to isolate these diatoms, Tryptone (Difco) served as the source of organic carbon, although the 0·2 g./l. used permitted only limited growth. Since lactate can be assimilated by many pennate diatoms for growth in darkness (Lewin & Lewin, 1960), various concentrations of sodium lactate were tested in liquid sea-water medium with KNO_3, K_2HPO_4 and Tryptone, each only 0·2 g./l. Na lactate (1·0 g./l.) appreciably stimulated growth. In media containing lactate, the Tryptone could be replaced by a vitamin mixture. It was thus clear that the organisms could use nitrate as source of nitrogen for growth.

It was then possible to determine whether any growth factors were required, by replacing the sea water (used in all media described above) by a suitable solution of mineral salts. This was effective with the defined mineral basal medium described in Methods.

In this basal medium, supplemented with suitable concentrations of sodium lactate (10 g./l.) and KNO_3 (1·0 g./l.), the vitamin mixture could be replaced by cobalamin (1 μg./l.) + thiamine (1 mg./l.).

Nutrition of various strains of Nitzschia

Defined media based on the foregoing studies were used to determine which vitamins were required and which organic substrates could be utilized for growth by various strains of apochlorotic Nitzschia species. The results are summarized in Table 2.

TAXONOMY

The classification of species within the vast genus *Nitzschia* is poorly established. Cleve-Euler (1952), following Grunow, distinguished 21 sub-genera, of which we are here concerned with the following six:

Sigmoideae; sigmoid in girdle view, more or less straight in valve view; keel median
Sigmata; as above, but keel marginal
Lineares; straight, linear, keel somewhat displaced from median axis
Dissipatae; straight, lanceolate, keel somewhat displaced from median axis
Spathulatae; straight, a line on each side of the keel
Lanceolatae; straight, lanceolate, keel close to one edge of the valve.

There seems little doubt that our sigmoid form, 180-M, is identifiable as *Nitzschia leucosigma* Benecke. The valves, though straight in surface view, are sigmoid in girdle view, which is the usual aspect one sees when looking down on an agar surface on which these organisms are crawling (Pl. 1, fig. 3–5).

The other strains, divisible into two main types, are less easily assigned to species. None agrees exactly with the description by Benecke (1900), according to whom *Nitzschia putrida* belongs to the Dissipatae or, possibly, to the Spathulatae (although he did not see or illustrate paracarinal lines characteristic of the latter subgenus). Pringsheim (1951) seems to have erred in referring *N. putrida* to the Sigmata, since he makes no reference to sigmoid form in his material. Benecke's illustrations of *N. putrida* clearly show the raphe only slightly displaced from the median axis. In most respects, our strain 176-M from La Jolla (Pl. 1, fig. 2) agrees with Benecke's description, though the organisms are considerably shorter (see Table 3). In view of the linear form of the valves, it would also be reasonable to assign this strain to the subgenus Lineares.

Our other isolates from La Jolla, however, like those from Woods Hole, Ensenada and Split, differ in the following features:

(*a*) The living organisms are more highly refractile by phase-contrast microscopy.

(*b*) In girdle view, the intact organisms are considerably thicker, about 5–6 μ; compare 2–4 μ for *N. putrida* 176-M.

(*c*) The cleaned valves are lanceolate, or, in the case of the shorter organisms, ovate, occasionally with a slight median constriction; compare our *N. putrida* 176-M, which has more or less straight sides.

(*d*) There is a clearly evident central nodule, where the two median puncta are spaced further apart then elsewhere (Pl. 1, fig. 3). Our *N. putrida* 176-M, like Benecke's illustrations of this species, lacks a central nodule.

(*e*) The position of the raphe is normally close to one margin of the valve. Our *N. putrida* 176-M, as in Benecke's illustration, has the raphe usually about one-third of the way across the valve.

This last character, in particular, seems to set apart our ovate-lanceolate forms in a

Table 2. *Nutrition of apochlorotic marine diatoms (Nitzschia species).*

		Substrates utilized as sole carbon sources				Utilized as nitrogen source		Vitamins required	
Species	Strain	Glucose (5 g./l.)	Na lactate (5 g./l.)	Na succinate (5 g./l.)	Na glutamate (10 g./l.)	K nitrate (1 g./l.)	Na glutamate (1 g./l.)	Thiamine (1 mg./l.)	Cobalamin (1 μg./l.)
Nitzschia alba	177-M	+	+	+	+	+	+	+	+
N. putrida	176-M	−	+	+	−	+	−	+	+
N. leucosigma	180-M	+	+	+	+	+	+	+	+

Table 3. *Diagnostic features of the silica shells of apochlorotic marine diatoms (Nitzschia species).*

Species	Strain	Valve length (μ)	Valve width (μ)	Striae/10μ	Keel puncta/10μ	Central nodule	Pores of striae
Nitzschia alba	81-M, 177-M, 178-M, Watson, Ensenada	(9–)22-28(–54)	4·5–6·5	40–48	15–20	Present	Distinct
N. putrida	Benecke (1900)	–70(–100)	4·4 (estd.)	.	10–13	Absent?	.
	Pringsheim (1951)	(10–)18–63	3·5–6·5	.	.	.	.
	176-M	10–55	2–3(–4)	55–60	15–20	Absent	Confluent
N. leucosigma	Benecke (1900)	–100(–130)	5(estd.)	.	10–13	Absent?	.
	180-M	27–65	4·5–5·0	30–35	10–12	Absent	Distinct

distinct subgenus the Lanceolatae. As far as we can determine, on the basis of the structure of their silica valves alone, our forms cannot be distinguished from *Nitzschia laevis* as described in the literature: form ± apiculate, length 12–25 μ, width 5–7 μ, puncta 10–14/10 μ, striae imperceptible by light microscopy (Hustedt, 1939). We are fortunate in having for direct comparison two clones of normally pigmented marine diatoms which we have previously identified as *N. laevis* (Lewin & Lewin, 1960). In the fine structure of the valves, our new apochlorotic species closely resembles *N. laevis* (our strain 72-M), though in the former the striae are somewhat coarser (44/10 μ; compare 52/10 μ for *N. laevis*). The question may be raised whether in 'cleaned' material, where the organic contents of the valves have been destroyed by heat or acid, it would be possible to distinguish between these two species. But we are inclined to agree with Benecke (1900) that loss of pigment must be regarded as constituting a specific character, if not indeed of generic importance (see Discussion).

A formal description in Latin follows.

Nitzschia alba spec.nov.

Subgenus: Lanceolatae. (Carina praeter aut prope marginem; valvae, lateraliter vistae, directae).

Cellulae sine chlorophyllo; mobiles; longae 9–54 μ, amplae 4·5–6·5 μ. Valvae lanceolatae sive aliquantum ellipsoides, aliquae in medio laeviter constrictae. Striae, manifestae solum a E-M, 44 in 10 μ, ordinum transversalium punctorum, diametro 120–200 nm compostae. Puncta carinae (inter claustra cognita a E-M) 13 in 10 μ, multa per axe elongata, 2–3 strias opponentes. Raphe (a E-M vista) in nodo centrale interrupta.

Hab. In algis lutoque litorum Adriaticae, Atlantici (Novae Angliae), Pacifici (Californiae), etc.

DISCUSSION

It seems clear that at least three distinct colourless species of Nitzschia occur in association with decaying seaweeds and other organic debris on temperate shores. (No apochlorotic diatom has been reported from a freshwater habitat, to the best of our knowledge.) The three species are distinguishable from one another not only by structural criteria, in the conformation of the siliceous shells, but also by physiological characters, notably in their different abilities to utilize exogenous sources of organic carbon for heterotrophic growth. Like many other heterotrophic microbes and like some photosynthetic diatoms (Lewin & Lewin, 1960), they require exogenous sources of thiamine and cobalamin.

We may speculate on the phylogenetic origin of these apochlorotic diatoms. It is reasonable to suppose that they originated as non-photosynthetic mutants of photosynthetic, normally pigmented strains, capable of rapid growth in the presence of organic substrates. They would presumably have to reproduce rapidly, in order to succeed in such environments in competition with other heterotrophic organisms such as bacteria and fungi. It is noteworthy that all of these apochlorotic diatoms grow considerably more rapidly than do any of the normally pigmented diatoms so far studied under heterotrophic conditions. *Nitzschia alba* may have arisen from a pigmented ancestor such as *N. laevis*, which it closely resembles in shell detail, and which was likewise shown to be capable of heterotrophic growth in media containing either

glucose or lactate (Lewin & Lewin, 1960). However, neither of our *N. laevis* strains required an exogenous source of vitamins, as do the apochlorotic species studied here. *N. putrida* resembles *N. angularis* in details of its silica valves; however, *N. angularis* can utilize glucose but not lactate (Lewin & Lewin, 1960), whereas *N. putrida* utilizes lactate but not glucose. As pointed out by Benecke (1900), *N. leucosigma* has many structural features in common with the pigmented species *N. sigma*; however, we have no information on the heterotrophic faculties of this last species, pure cultures of which have not yet been isolated.

We have considered, and rejected, the advisability of creating a special subgenus, with a name such as 'Hyalonitzschia', analogous to apochlorotic homologues of the green algae Raphidium (Hyaloraphidium), Chlorogonium (Hyalogonium), etc. In the case of the diatoms, where so much taxonomic emphasis is placed on the structure of the siliceous valves, it would probably be impractical at present to introduce other kinds of criteria. Furthermore, such a subgeneric distinction would cut across already existing subgeneric lines, based on structural features of the shells, which currently separate *N. alba*, *N. putrida* and *N. leucosigma*, respectively, in the subgenera Lanceolatae, Dissipatae and Sigmata.

We thank Dr S. M. Watson, Oceanographic Institution, Woods Hole, Mass., U.S.A., for first drawing our attention to an apochlorotic Nitzschia of doubtful identity; to Dr B. E. Reimann and Mrs Annika Sanfilippo, of the Scripps Institution of Oceanography, La Jolla, California, for the micrographs; and to the U.S. National Science Foundation, for financial support (to J.L.) under grant no. GB-4462.

This is a Contribution from the Scripps Institution, University of California, and from the Department of Oceanography, University of Washington (Contribution no. 413).

Permanent slide mounts and dried specimens of the cleaned silica walls of each of the three species discussed in this paper are being deposited in the California Academy of Sciences, San Francisco, and at the Smithsonian Institution, Washington, D.C., U.S.A.

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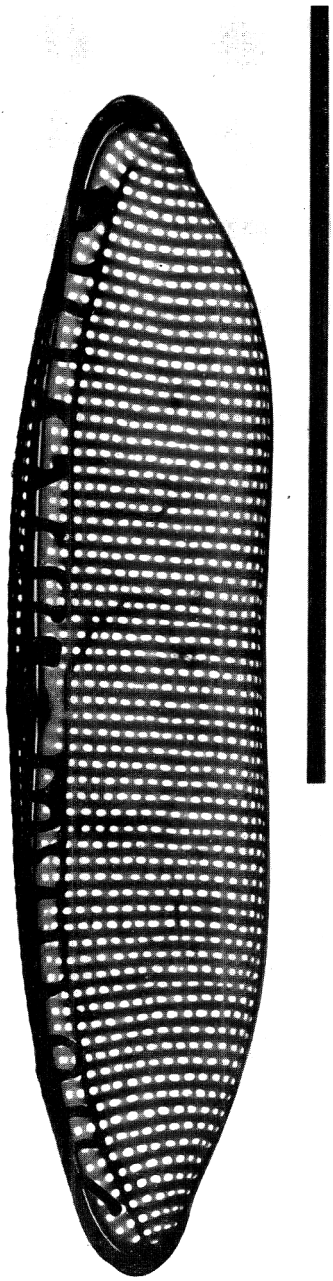

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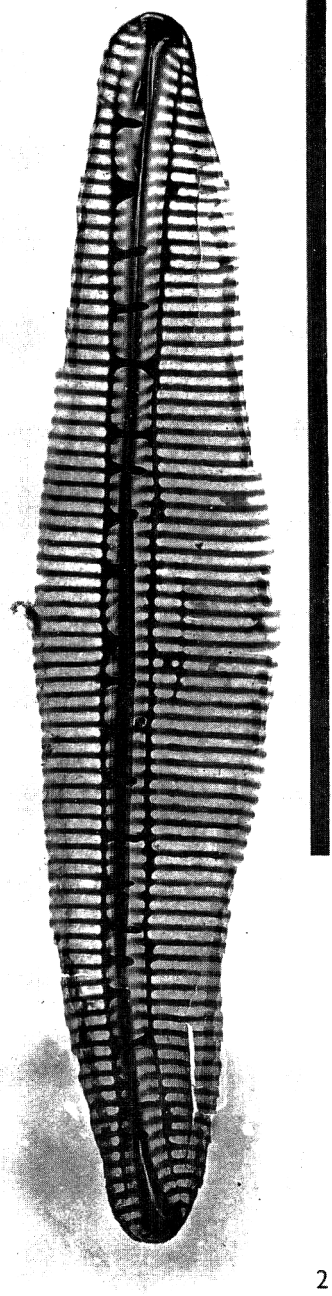

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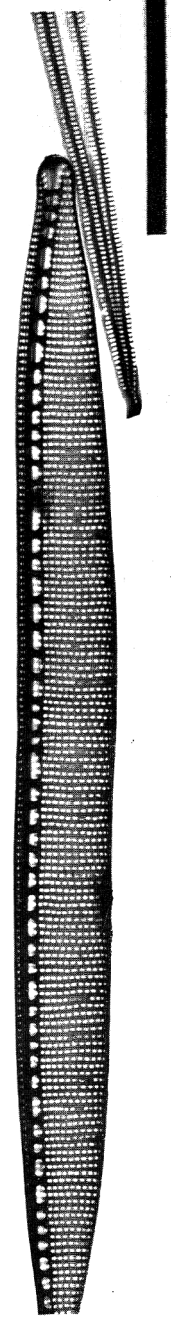

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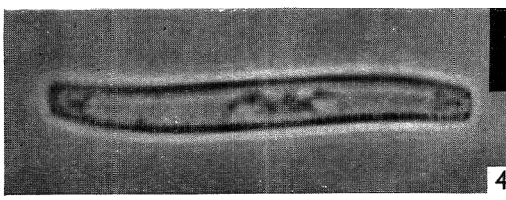

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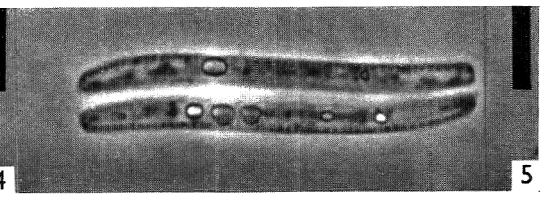

5

EXPLANATION OF PLATE

Scale bar = 10 μ in each photograph.

Fig. 1. *Nitzschia alba* strain 178-M.

Fig. 2. *Nitzschia putrida* strain 176-M.

Fig. 3. *Nitzschia leucosigma* strain 180-M. Acid-cleaned valves; electron micrographs by Dr B. E. Reimann.

Fig. 4. *Nitzschia leucosigma* strain 180-M. Single organism.

Fig. 5. *Nitzschia leucosigma* strain 180-M. Pair of sister organisms. Living material; phase-contrast micrographs by Mrs Annika Sanfilippo.

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DNA Base Composition and Taxonomy of Some Micrococci

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SUMMARY

The present report gives the results of studies on the % guanine+cytosine (GC) content of the DNA of 29 strains designated as *Micrococcus*, by using the methods of Marmur & Doty (1962) and Frédéricq, Oth & Fontaine (1961). In 17 strains agreement was found between their taxonomic position and their % GC content. Five micrococci formed a very tight cluster around a mean of 50% GC. It is recommended to classify them in the genus *Planococcus* Migula. From the DNA base compositions these cocci form a group hitherto not described, intermediate between the genera *Staphylococcus* and *Micrococcus*. *Micrococcus cryophilus* McLean, Sulzbacher & Mudd, and *Micrococcus cerolyticus* Friedman & Kern differ in their % GC values from those of the genus *Micrococcus* and should be discarded from the genus. It is confirmed that % GC content in the DNA in the genus *Micrococcus* lies within the range of 65–75%.

INTRODUCTION

The usefulness of the study of DNA base compositions for the taxonomy of bacteria has been emphasized by several authors. This approach has given important results in the problem of the classification of aerobic Gram-positive cocci. It was pointed out by Silvestri & Hill (1965) that aerobic Gram-positive cocci can be divided into two groups on the % guanine+cytosine (GC) content of their DNAs. The first group, with a low % GC in DNA, corresponds to the genus *Staphylococcus* and the second group, with a high % GC, corresponds to the genus *Micrococcus*. These conclusions were confirmed by the present authors in a previous paper (Boháček, Kocur & Martinec, 1965).

Equally significant is the application of this method in solving problems of intrageneric classification. As regards the species within the genus *Micrococcus* and their DNA base compositions, data on only some of the species can be found in the literature (Belozersky & Spirin, 1960; Marmur, Falkow & Mandel, 1963). With the exception of reports by Silvestri & Hill (1965), Auletta & Kennedy (1966) and Rosypalová, Boháček & Rosypal (1966), the % GC contents of DNA have not directly been used in the classification of the genus *Micrococcus*. The object of the present paper was to analyse the DNA base composition in species of the genus *Micrococcus* and to find whether the % GC content was in agreement with their classification by other characters.

METHODS

In the present study 29 strains of micrococci were investigated; they are listed in Table 1.

Most of the strains were grown on yeast-extract glucose agar (g./l.): yeast-extract, 5; peptone, 5; glucose, 10; agar, 20; pH 7·2. *Micrococcus halodenitrificans* was grown on the following medium: (g./l.): beef-extract, 5; peptone, 5; NaCl, 80; agar, 20; pH 7·2. *M. litoralis* was grown on the following medium (g./l.): yeast extract, 5; peptone, 5; glucose, 10; KCl, 2; $MgSO_4.7H_2O$, 25; NaCl, 150; agar, 25; pH 7·8. The marine micrococci were cultivated in the following medium (g./l.): beef extract, 10; peptone, 10; agar, 20; in sea water, 750 ml., tap water, 250 ml.

Table 1. *List of organisms used*

Name	CCM no.*	Other names or numbers
Micrococcus luteus	132	
	149	
	169	*M. lysodeikticus* ATCC 4698; NCTC 2665
	337	*Sarcina lutea* ATCC 382
	409	
	410	*Sarcina lutea* ATCC 272
	840	*M. ureae* ATCC 408
	1674	
Micrococcus roseus	679	*M. rubens* ATCC 186
	837	ATCC 416
	385	ATCC 185
	560	ATCC 179
	633	ATCC 412
	168	
	1405	*Staphylococcus roseus*
Micrococcus litoralis	2226	*Sarcina morrhuae*
Micrococcus sp.	740	*M. conglomeratus*
	825	*M. conglomeratus*
	836	
	2087	ATCC 401
Micrococcus cerolyticus	901	ATCC 12559
Micrococcus denitrificans	982	NCIB 8944; ATCC 13543
	1396	
Micrococcus halodenitrificans	286	ATCC 13511
Micrococcus radiodurans	1700	ATCC 13939
	1701	UI
Micrococcus cryophilus	900	ATCC 12226
Micrococcus aquivivus	316	ATCC 14404
Micrococcus eucinetus	2388	XQ 58
	2389	XQ 40

* CCM = Czechoslovak Collection of Microorganisms, University J. E. Purkyně, Brno.

The organisms were incubated at 30° for 24 hr with the exception of *Micrococcus litoralis*, which was incubated for 6 days, and *M. cryophilus* which was incubated at 10° for 2 days.

For the isolation of DNA a method combining chloroform deproteinization with phenol deproteinization was used, since when following the procedure of Marmur

(1961) it was found difficult with some DNA samples to decrease the protein content below 1% even after performing chloroform deproteinizations 8 times. Strains sensitive to lysozyme were incubated with 0·5 mg. lysozyme/ml. bacterial suspension in a NaCl + EDTA solution at 37° for 10–60 min. A 25% (w/v) solution of sodium dodecyl sulphate was then added to make the final concentration 2%; lysis was then complete at 60° in 10 min. After the mixture had been cooled to room temperature, sodium perchlorate was added to M concentration and one volume of a mixture of chloroform + isoamyl alcohol (24 + 1, v/v). After 30 min. shaking and 20 min. centrifugation at 5000 rev./min. the upper layer was removed and precipitated with 1·5 volume ethanol. The resulting fibrous DNA precipitate was dissolved in 10-fold dilution of standard saline citrate buffer pH 7 (0·15 M-NaCl + 0·015 M-sodium citrate, pH 7; hereafter SSC) and after its solution the saline citrate buffer concentration was adjusted to SSC with 10 × SSC and ribonuclease (5 × cryst. Reanal, Hungary) added 50 μg./ml. After incubation for 30 min. at 37°, NaCl was added to the solution to M concentration and a further equal volume of re-distilled phenol saturated with M-NaCl + 0·1 M-tris (pH 9). After shaking for 20 min. at 4°, the mixture was centrifuged at 75,000*g* for 30 min. and the clear supernatant layer precipitated with one volume of ethanol.

Deproteinization with a mixture of chloroform + isoamyl alcohol (24 + 1) was done twice and as a rule, after the second deproteinization, no interlayer of protein was formed after centrifugation. The DNA sample was precipitated with one volume of ethanol again. By this procedure, the amount of phenol in the sample decreased below 0·5%; this was detected spectrophotometrically by measuring the ratio $E_{260}:E_{235}$ in a medium of 0·1 M-NaOH (Boháček, 1966). The ethanol-precipitated DNA was dissolved in a 10-fold diluted SSC and 1/10 volume of 3 M-sodium acetate in 10^{-3} M-EDTA added. The DNA was precipitated by adding 0·55 to 0·7 volume of re-distilled 2-propanol with constant stirring. After twice washing in 75% (w/v) ethanol in water the DNA was dissolved in phosphate + EDTA (0·01 M-sodium phosphate + 0·001 M-EDTA, pH 7) buffer (PE).

For the measurement of the DNA melting curves a spectrophotometer, type Beckman DU, was used. The cell holder compartment was thermostated at both sides by two thermospacers for circulating hot water from a U-10 ultra thermostat. The temperature was measured with a rod thermometer directly in one of the cuvettes. The melting curves were not corrected for thermal expansion of the solution. The solvent used was 10^{-2} M-sodium phosphate buffer (pH 7) + 10^{-3} M-EDTA which contained the following substances per liter of solution: 0·608 g. $NaH_2PO_4.2H_2O$, 2·18 g. $Na_2HPO_4.12H_2O$, 0·372 g. disodium salt of EDTA, 1 ml. N-NaOH. An example of a melting curve and its evaluation is given in Fig. 1.

The % GC content was calculated according to the equation $T_m = 51 + 0{\cdot}45\,(GC)$ derived from the dependence of T_m on the GC content determined in DNA by paper chromatography (unpublished results) and according to the results from the method of Frédéricq *et al.* (1961). The width of the transition interval (ΔT) was established as the difference between temperatures from 17 to 83% of the over-all increase in E_{260} and the value $2\sigma = (\Delta T - 3).2{\cdot}5$ was calculated from it. The values 2σ and 3σ served then to express graphically the heterogeneity of the sample according to De Ley & van Muylem (1963).

As a second control method for determining the % GC content in isolated samples

of DNA, the method of Frédéricq *et al.* (1961) was used. This method is based on the fact that the extinction ratio of 260:280 mμ (E_{260}:E_{280}) measured in a medium of 0·1 M-acetic acid (pH 3) is dependent on the % GC content in DNA. The samples of DNA were dissolved in a PE medium in a concentration of approximately 2 mg./ml. Before measurement, 0·1 M-acetic acid was added to the final concentration of DNA, 20–30 μg/ml. The measurements of O.D. at 260 and 280 mμ respectively were made with a type Zeiss VSU-1 spectrophotometer. For pH measurements a pH-meter type PYE-Master was used.

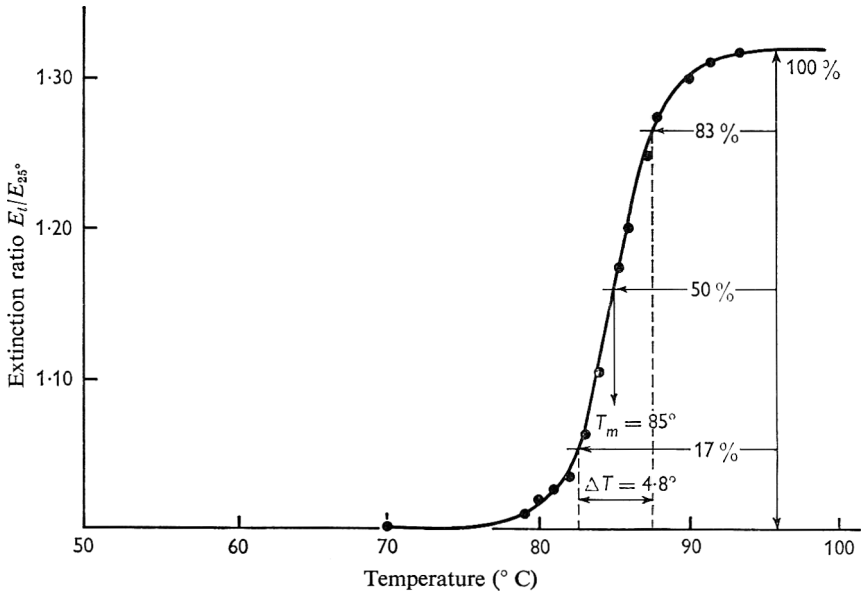

Fig. 1. Example of the evaluation of the melting curve of DNA of *Micrococcus luteus*, no. 409. Ordinate extinction ratio E_{260} at the given temperature (t) to E_{260} at the basal temperature 25°. The over-all increase in extinction ratio is designated as 100 %, T_m corresponds to 50 % of the over-all increase in E_{260}. From the course of the melting curve within the temperature range of 17–83 % E_{260}, the interval ΔT ($\Delta\frac{2}{3}$) is subtracted, from which 2σ = ($\Delta T - 3$) 2·5 is calculated.

RESULTS AND DISCUSSION

For determining the % GC content in the DNA of the micrococci examined, two different methods were used; besides the method of determining the % GC content from the T_m value (Marmur & Doty, 1962) we also used the method of determining % GC according to the ratio E_{260}:E_{280} at pH 3 (Frédéricq *et al.* 1961). The difference between the values obtained by the two methods were within the range of 1–3 % GC (see Table 2). Greater differences between the results given by the two methods would indicate the presence of impurities in the DNA preparations. In our opinion, the method recommended by Frédéricq *et al.* (1961) is useful for a rapid and orientating determination of % GC. However, since this method does not furnish data on the heterogeneity of DNA molecules it is advisable to use in parallel the method of T_m determination to obtain more detailed information.

The results (Table 2) show that the strains examined can be divided into three groups. The first, most numerous, group (Fig. 2) includes 17 strains (8 *Micrococcus luteus*,

5 *M. roseus*, 3 *Micrococcus* spp., 1 *M. litoralis*) whose % GC in DNA was in agreement with their classification as given by Kocur & Martinec (1962). Our results also confirm the opinion of these authors that aerobic Gram-positive yellow-pigmented cocci which do not attack glucose should be classified as *M. luteus*. The % GC values found in strains of *M. luteus* and *M. roseus* are in agreement with the data given by Belozersky & Spirin (1960) and by Silvestri & Hill (1965). In contrast, Rosypalová *et al.* (1966) found only 66·3% GC in the DNA of the type culture of *M. luteus* ATCC 398. Only one strain from our series (*M. luteus* CCM 169, NCTC 2665) was also studied by Silvestri & Hill (1965). Their result 72·8% GC agrees with the value of 73·3% GC found by us.

Table 2. *DNA base composition of some micrococci*

Name	CCM no.	T_m (°C)	% GC	2σ	$E_{260/280}$ pH = 3	% GC
Micrococcus luteus	132	83·0	71·0	2·5	1·130	67·0
	840	83·0	71·0	2·5	1·050	72·0
	1674	83·2	71·5	0	1·090	71·0
	337	83·6	72·3	4·5	1·100	70·0
	169	84·0	73·3	2·0	—	—
	410	84·2	73·7	2·5	1·100	70·0
	149	84·6	74·6	3·0	1·070	73·0
	409	85·0	75·5	3·30	1·080	72·0
Micrococcus roseus	679	82·0	69·0	3·0	1·120	68·0
	837	82·4	69·7	3·0	1·100	70·0
	385	83·0	71·0	4·5	1·120	68·0
	560	83·0	71·0	2·5	1·100	70·0
	633	83·8	72·8	0·75	1·100	69·0
	168	73·2	49·3	0·5	1·400	45·0
	1405	73·2	49·3	4·5	1·330	50·5
Micrococcus litoralis	2226	80·5	65·0	4·0	1·170	63·5
Micrococcus spp.	740	77·4	58·6	8·5	1·250	53·5
	836	81·7	68·3	1·5	1·165	64·0
	2087	81·7	68·3	0	1·165	64·0
	825	82·2	69·5	0	—	—
Micrococcus cerolyticus	901	67·0	35·6	5·0	14·90	39·0
Micrococcus denitrificans	982	79·8	64·0	4·5	1·160	64·0
	1396	80·6	66·3	1·5	1·130	67·0
Micrococcus halodenitrificans	286	80·2	65·0	0·5	1·140	66·0
Micrococcus radiodurans	1700	82·0	69·0	3·25	1·140	66·0
	1701	80·4	65·3	4·3	1·145	65·5
Micrococcus cryophilus	900	69·9	41·3	3·5	1·410	44·5
Micrococcus aquivivus	316	74·0	51·2	3·5	1·375	47·5
Micrococcus eucinetus	2388	72·6	48·0	3·25	1·370	47·5
	2389	73·6	50·3	4·0	1·350	49·0

The second heterogeneous group of strains (Fig. 2) consisted of those which would appear to need reclassification since they substantially differed in their % GC content from the value for the genus *Micrococcus*. This group contains *Micrococcus* sp. 740, *M. cerolyticus* 901, and *M. cryophilus* 900. With the exception of strain 740, these strains also differed in their cultural and biochemical properties from the species within the genus *Micrococcus*. *M. cerolyticus* had already been found by us to be identical on biochemical properties with *Staphylococcus epidermidis*. The finding of a low % GC

value (35·6–39·0) is compatible with the suggestion that this is a strain of *S. epidermidis*. As Marmur *et al.* (1963) reported, we also found a low % GC (41·3%) in the DNA of *M. cryophilus*. These results support the opinion of Mazanec, Kocur & Martinec (1966) that *M. cryophilus* should be discarded from the genus *Micrococcus*. The % GC values of *M. denitrificans*, *M. halodenitrificans* and *M. radiodurans* are lower than in most strains within the genus *Micrococcus*; these results agree with those of Marmur *et al.* (1963) and Moseley & Schein (1964). The above species were proposed by Baird-Parker (1965) for transfer to Gram-negative genera.

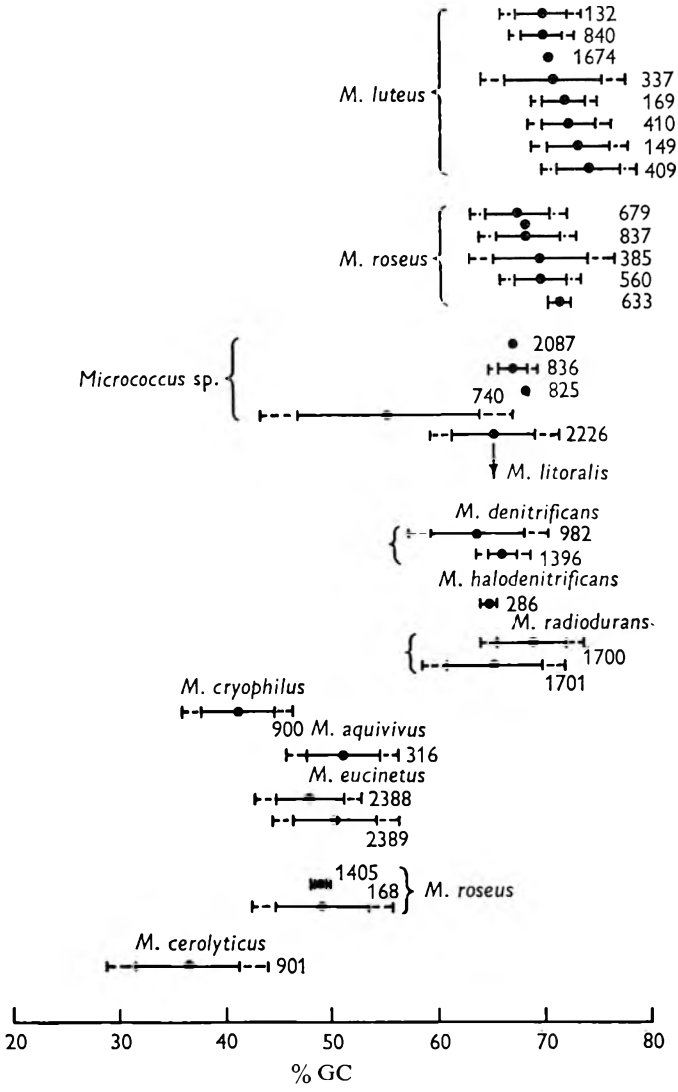

Fig. 2. Survey of the mean base composition and the exponential distribution of DNA molecules of various micrococci. The strains are designated by numbers (see Table 1). The horizontal lines are the projections of the Gaussian curves on the plane of the paper, their maxima being average % GC. The full horizontal lines represent % GC $\pm 2\sigma$, and encompass about 95% of the DNA molecules. The total length of each full line + dashed line represents % GC $\pm 3\sigma$ and encompasses about 99·7% of the DNA molecules.

The third group, comprising five strains, is of special interest from the standpoint of the % GC content in DNAs and its variability. The group includes two strains of *Micrococcus eucinetus*, two of *M. roseus* (168 and 1405), and one of *M. aquivivus*. These strains are interesting not only because they occupy an intermediate position in their % GC content (48–51 %) between the values for the genera *Staphylococcus* and *Micrococcus*, but also because they form a tight cluster around a mean value of 50% GC. Also interesting is the fact that three of these strains are marine strains, and in the case of the other two strains (*M. roseus* CCM 168 and 1405) the possibility of an original marine habitat cannot be excluded. Our results support to some extent Belser's (1964) conception of an evolutionary affinity among marine bacteria. Of course, in contrast to Belser's (1964) finding of a very tight cluster (around 41 % GC in DNA) among Gram-negative marine bacteria, our Gram-positive marine micrococci form a very tight cluster around a mean of 50% GC. On the other hand, we found (unpublished data) that some marine micrococci. e.g. *M. maripuniceus* ATCC 14399 had 73% GC similar to that of species of *Micrococcus*, but *M. euryhalis* ATCC 14389 had 33% GC in DNA, similar to the species of the genus *Staphylococcus*. Consequently, before general conclusions about the phylogeny of marine bacteria can be drawn from the above results, many more strains of these bacteria must be investigated. Since the strains of marine cocci studied by us show marked differences in their % GC content from species of *Micrococcus* it is recommended that these cocci should be discarded from the genus *Micrococcus* and a more suitable taxonomic position found for them. Komagata (personal communication) recommends that *M. eucinetus* should be placed in the genus *Planococcus* Migula in view of its motility and % GC content in DNA. In our opinion, for the same reason the strains designated as *M. aquivivus* CCM 316 and *M. roseus* CCM 168 and 1405 should also be placed in the genus *Planococcus*.

The results obtained in the present work support the correctness of the methodological principles suggested by Silvestri & Hill (1965), and also confirm the correctness of Evans's (1965) proposal to recognize only two species within the genus *Micrococcus*: *M. luteus* and *M. roseus*.

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Studies on the Mode of Action of Tetrin A

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SUMMARY

Tetrin A, a polyene antifungal antibiotic, induced permeability changes in the cell membrane of *Saccharomyces cerevisiae*, resulting in the loss of cell constituents, e.g. amino acids, 260 mμ absorbing material and the leakage of labelled metabolites from the organism previously grown with uniformly labelled glucose. Also, the uptake of glucose, glycine, and uracil was prevented by the antibiotic. Like other polyenes, the addition of exogenous cholesterol partially prevented the action of tetrin A, probably by the formation of a weak complex between tetrin A and cholesterol and resulting in a lower effective concentration of the antibiotic. When the non-sterol-containing fungus *Pythium ultimum* was grown on a medium containing cholesterol, this fungus was sensitive to filipin. However, prior growth of the fungus with cholesterol did not confer sensitivity to the polyene antibiotics tetrin A, nystatin, or pimaricin. Contrary to most polyene antibiotics which cause rapid lysis of mammalian erythrocytes even at low concentrations, tetrin A only caused partial lysis of calf red cells after long exposure to high concentrations.

INTRODUCTION

The modes of action of several polyene antibiotics, including filipin, nystatin, amphotericin B and pimaricin have been studied by many investigators (see reviews, Lampen, 1962; Kinsky, 1964). It is generally believed that polyene antibiotics induce permeability changes in the cell membrane of sensitive organisms causing loss of cell constituents which results in the death of the organism. Polyene-sensitive organisms bind these antibiotics, whereas insensitive bacteria and blue green algae do not. The ability of sensitive organisms to bind polyene antibiotics is apparently dependent on the presence of sterols in the cell membrane (Demel, Kinsky & van Deenen, 1965). Tetrin, a polyene antifungal antibiotic, was discovered by Gottlieb & Pote (1960). Further purification of the antibiotic showed that this tetrin contained two closely related compounds with antifungal activity, tetrin A and tetrin B (Rinehart, German, Tucker & Gottlieb, 1963). The present report is concerned with the mode of action of tetrin A.

METHODS

Organisms. Most of the mycelial fungi used here in the sensitivity tests were obtained from the culture collection in the Department of Botany, University of Pavia, Italy.

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Pythium ultimum and *Aspergillus niger* were obtained from the Department of Plant Pathology, University of Illinois, U.S.A., and *Glomerella cingulata* no. 10529 from the American Type Culture Collection (Rockville, Md., U.S.A.). *Saccharomyces cerevisiae* was obtained as an isolate from commercial Fleischmann's baker's yeast. All the fungi were maintained as slope cultures on a medium consisting of: glucose, 10 g.; yeast extract (Difco), 2 g.; agar, 20 g.; 1000 ml. water; pH 6·6 (glucose yeast extract medium). *S. cerevisiae* was grown on a reciprocal shaker at 28° on a liquid medium consisting of: glucose, 10 g.; dehydrated yeast nitrogen base (Difco), 6·7 g.; 1000 ml. distilled water (yeast nitrogen base medium). A 24-hr liquid culture of the yeast served as inoculum.

Testing for growth inhibition. The concentrations of tetrins A and B necessary for the complete inhibition of growth of mycelial fungi were determined by inoculating the fungi on the chemically defined agar medium of Davis & Mingioli (1950) to which various concentrations of the antibiotics had been added. The effect of various concentrations of tetrins A and B on the growth of *Saccharomyces cerevisiae* was determined by extinction measurements at 540 mμ of liquid cultures at given time intervals.

In all other studies with *Saccharomyces cerevisiae*, 12-hr cultures (log phase organisms) were used. The organisms were harvested by centrifugation, washed three times with equal volumes of distilled water, and resuspended in an equal volume of buffer or fresh medium to a final concentration equivalent to 1 mg. dry yeast/ml. suspending agent.

Measurement of leakage. The leakage of amino acids and 260 mμ absorbing materials from *Saccharomyces cerevisiae* treated with tetrin A was determined in 0·1 M-citrate buffer (pH 4 or 6) or in 0·1 M-potassium phosphate buffer (pH 8). After the addition of the antibiotic to the suspension of yeasts, samples were taken at various times, centrifuged, and the supernatant fluid analysed for amino acids as ninhydrin-positive materials (Spies, 1957), and 260-mμ absorbing materials. The leakage of 260 mμ absorbing materials was determined on a Beckman D.U. spectrophotometer and corrected for the absorbancy at 260 mμ due to the antibiotic.

Radioactive labelling. Yeast cells labelled with radioactive metabolites were obtained by growing the yeasts in the normal medium plus [uniformly ^{14}C]-labelled glucose at a concentration of 1 μC./ml. After harvesting, the yeast was washed with distilled water and resuspended in fresh yeast nitrogen base medium containing the antibiotic. At various times thereafter samples were collected, centrifuged, and the supernatant fluids plated on to metal planchets to determine the residual radioactivities in them.

Measurement of tetrin A effect on metabolite uptake. The effect of tetrin A on the uptake of glucose, glycine, and uracil was determined by suspending yeast cells in glucose nitrogen base medium containing 1 μC./ml. of [uniformly ^{14}C]-labelled glucose (specific activity, 3·9 μC./μmole), [uniformly ^{14}C]-labelled glycine (specific activity 67 μC./μmole) or [2-^{14}C]-labelled uracil (specific activity 30·0 μC./μmole). At appropriate times, samples were removed, centrifuged, and the residual radioactivity in the supernatant fluids determined. Radioactivity was determined on a S.E.L.O. gas flow counter (counting efficiency 10·2 %). Counts were made under conditions of negligible self-absorption. The radioactivity is expressed as counts/minute (c.p.m.)/ml.

Effect of tetrin A on Pythium ultimum. Methods similar to those of Schlösser & Gottlieb (1966) were used to determine the effect of tetrin A on *Pythium ultimum* after the fungus had been grown in the presence or in the absence of cholesterol. *Pythium*

ultimum was grown on the chemically-defined medium (MM-40) of Magni & Von Borstel (1962) and cholesterol (Applied Science Laboratories, Inc., State College, Pa., U.S.A.), 0·02 % (w/v), incorporated into the agar medium when appropriate. Following the growth of the fungus, discs were made from the advancing edge of the fungal colony with a cork borer. These discs were incubated on a reciprocal shaker for 4, 8 and 12 hr. in 0·1 M-citrate buffer (pH 6·0) which contained various concentrations of the antibiotics (1 disc/ml. buffer). After incubation, the discs were washed in distilled water, placed on glucose yeast extract agar plates, and their growth observed by measuring the colony diameter at selected time intervals.

Calf red cells. Calf red cells were obtained by centrifuging fresh blood at 2000*g* for 20 min. The red cells were washed twice with an equal quantity of 0·9 % sodium chloride. After the second washing, 2 ml. of the cells were resuspended in 1000 ml. 0·9 % sodium chloride and samples incubated at 28° with the antibiotics. At various times samples were collected, centrifuged and the extent of lysis determined by measuring at 550 mμ the haemoglobin present in the supernatant solution. Total haemolysis, as determined with 20 μg. filipin/ml. (Kinsky, 1963), resulted in an extinction of about 0·350 units.

In all studies the antibiotics as well as cholesterol were dissolved in dimethylformamide. The final concentration of dimethylformamide in the suspending solution was always 0·5 % (v/v) or less. Though at these concentrations dimethylformamide had no effect on the organisms, control tubes or plates containing equal amounts of organic solvent were always used.

Polyene sources. Tetrins A and B were isolated and purified by K. Rinehart and his colleagues at the University of Illinois. Pimaricin was obtained from B. L. Hutchings, Lederle Laboratories, Pearl River, New York, U.S.A.; filipin from Upjohn Company, Kalamazoo, Michigan, U.S.A., nystatin from Lepetit S.p.A., Milan, Italy.

RESULTS

Activity of the tetrins. The concentrations of tetrins A and B necessary to prevent the growth of nine mycelial fungi are given in Table 1. Generally, tetrin A was 2 to 8 times more active than tetrin B. However, *Pythium ultimum* was insensitive to both tetrins, even at tetrin A 100 μg./ml. and tetrin B 200 μg./ml. Tetrin A also inhibited the growth of *Saccharomyces cerevisiae* at much lower concentrations that did tetrin B (Fig. 1). Because tetrin A was more active than tetrin B, further studies were made on tetrin A only. The death of a population of viable *Saccharomyces cerevisiae* exposed to several concentrations of tetrin A is shown in Table 2. More than 99·5 % of yeasts were killed after 10 hr exposure to tetrin A 100 μg./ml.

Effect of tetrin A on metabolite leakage and uptake. The ability of tetrin A to induce leakage of ninhydrin-positive material (mostly amino acids) and 260 mμ absorbing material (nucleotides, nucleic acids) from yeasts was investigated. Tetrin A induced the leakage of amino acids and 260 mμ absorbing materials at concentrations in the same range as those lethal to the yeasts (Fig. 2). However, in general, the loss of amino acids occurred faster and at concentrations of tetrin A lower than those necessary for the loss of 260 mμ absorbing materials. Concentrations of tetrin A, as low as 12·5 μg./ml., resulted in a fairly rapid loss of amino acids (Fig. 2B) while only a slight loss of 260 mμ absorbing materials occurred at concentrations below 50 μg./ml. (Fig. 2A).

Nystatin at 50 μg./ml. induced the loss of amino acids and 260 mμ absorbing materials in a manner similar to that caused by tetrin A 100 μg./ml., while filipin 50 μg./ml. induced a more rapid leakage of these components. The optimum pH value of the leakage of 260 mμ absorbing materials caused by tetrin A was pH 6 (Fig. 3). A similar optimum pH of 6 was obtained for the leakage of amino acids.

Table 1. *Minimum inhibitory concentrations of tetrin A and tetrin B*

Fungus	Growth* period (hr)	Tetrin A	Tetrin B
		Minimal inhibitory concentration (μg./ml.)	
Alternaria solani	84	50	200
Aspergillus niger	48	12·5	100
Cephalosporium longiesporium	84	25	100
Giberella fujikuroi	84	50	100
Glomerella cingulata	84	25	100
Penicillium citrinum	48	100	> 200
Pythium ultimumi	48	> 100	> 200
Rhizoctonia solan	84	25	100
Verticillium albo-atrum	84	25	100

* Results determined after 48 or 84 hr growth of fungi.

Table 2. *Effect of tetrin A on the viability of Saccharomyces cerevisiae*

Initially $3{\cdot}7 \times 10^6$ viable yeasts/ml. 0·1 M-citrate buffer (pH 6·0); the suspensions were incubated with shaking at 28°. Viable counts were made on yeast nitrogen base agar medium and colonies scored after 48 hr incubation.

Treatment period (hr)	Concentration of tetrin A (μg./ml.)			
	0	25	50	100
	Yeasts killed (%)			
0	0	0	0	0
10	0	30	35	99·57
24	0	57	78	99·88
48	0	82	92	99·98

To determine whether tetrin A could cause leakage under normal growing conditions, yeasts previously grown on labelled glucose were used. The release of radioactive materials into the medium at various times in the presence of several concentrations of tetrin A and 50 μg. filipin is shown in Fig. 4. Tetrin A induced a loss of labelled materials from the yeasts, but not to the same extent as did filipin.

Tetrin A inhibited the uptake of labelled glucose by yeasts in a normal growth medium (Fig. 5). In addition, glycine and uracil uptake were inhibited to the same extent by the same concentrations of tetrin A as is shown for glucose. The uptake of these three compounds were also completely prevented by nystatin or filipin 50 μg./ml.

Effect of cholesterol on tetrin A activity. Because many reports (Gottlieb, Carter, Sloneker & Amman, 1958; Gottlieb, Carter, Wu & Sloneker, 1960; Gottlieb, Carter, Sloneker, Wu & Gaudy, 1961; Caltrider & Gottlieb, 1961) indicated at least partial prevention of the action of polyene antibiotics by the presence of sterols, the effect of various concentrations of cholesterol on leakage in *Saccharomyces cerevisiae* induced by tetrin A

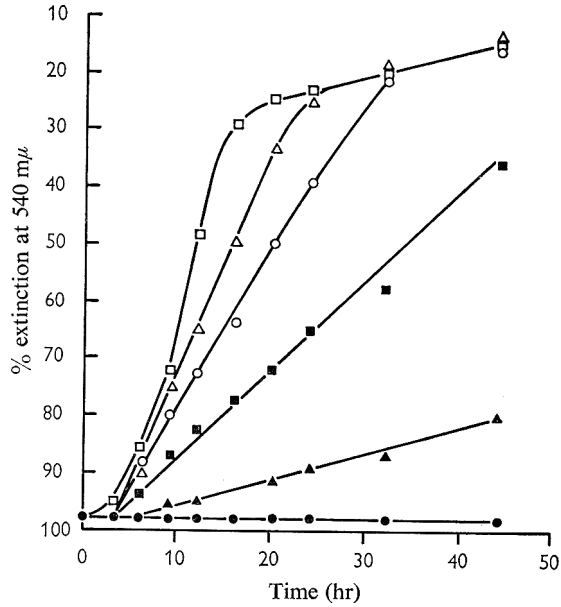

Fig. 1. The effect of various concentrations of tetrins A and B on the growth of *Saccharomyces cerevisiae*. (□), control or tetrin B 10 μg./ml.; (△), tetrin B 20 μg./ml.; (○), tetrin A 1 μg./ml.; (■), tetrin A 5 μg./ml.; (▲), tetrin B 50 μg./ml.; (●), tetrin A 10 μg./ml. or tetrin B 100 μg./ml.

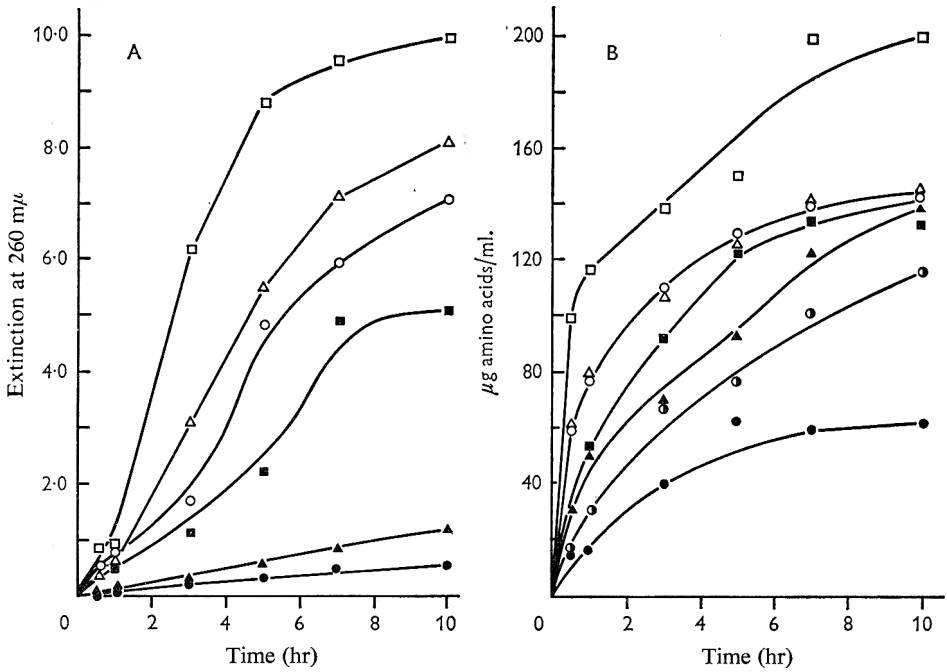

Fig. 2. Tetrin A, filipin and nystatin induced leakage of 260 mμ absorbing materials (2 A) and amino acids (2 B) from *Saccharomyces cerevisiae* suspended in 0·1 M-citrate buffer (pH 6·0). (●), control; (◑), tetrin A 12·5 μg./ml.; (▲), tetrin A 25 μg./ml.; (■), tetrin A 50 μg./ml.; (○), tetrin A 100 μg./ml.; (△), nystatin 50 μg./ml.; (□), filipin 50 μg./ml.

was examined. Cholesterol added at the same time as tetrin A, at a ratio of 1:1 (w/w), caused a slight decrease in the loss of amino acids and 260 mμ absorbing materials, while higher ratios resulted in a significant decrease (Fig. 6). Lampen, Arnow & Safferman (1960) suggested that the inhibition of polyene action by sterols is due to the formation of a sterol-polyene complex resulting in a lower effective concentration of the antibiotic.

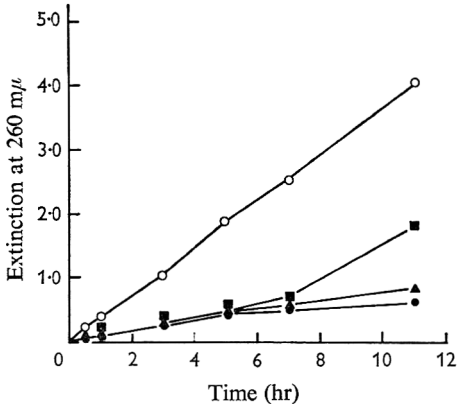

Fig. 3

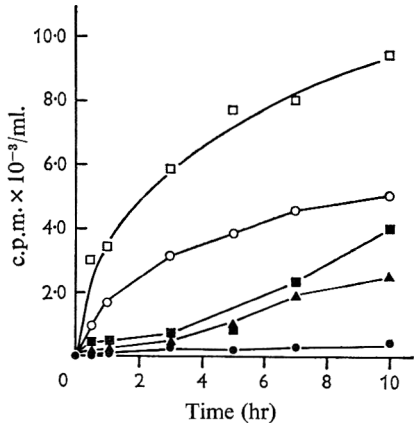

Fig. 4

Fig. 3. The effect of the pH value of the suspending buffer on the leakage of 260 mμ absorbing materials from *Saccharomyces cerevisiae* exposed to tetrin A 50 μg./ml. (●), control; (▲), pH 4·0; (○), pH 6·0; (■), pH 8·0.

Fig. 4. Leakage of [^{14}C]-labelled materials from *Saccharomyces cerevisiae* in normal growth medium exposed to filipin or tetrin A. (●), control; (▲), tetrin A 25 μg./ml.; (■), tetrin A 50 μg./ml.; (○), tetrin A 100 μg./ml.; (□), filipin 50 μg./ml.

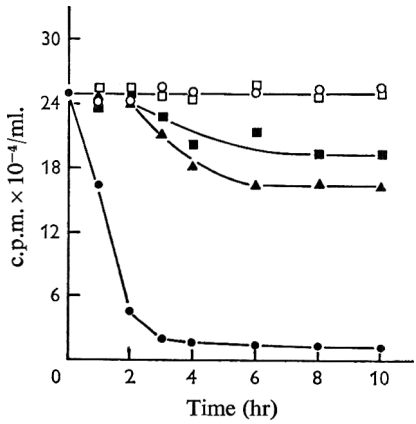

Fig. 5. Effect of tetrin A on the uptake of [uniformly ^{14}C]-labelled glucose by *Saccharomyces cerevisiae* in a normal growth medium. (●), control; (▲), tetrin A 25 μg./ml.; (■), tetrin A 50 μg./ml.; (○), tetrin A 100 μg./ml.; (□), filipin 50 μg./ml.

This might be one explanation for the observed effect of cholesterol on the leakage induced by tetrin A. For example, a ratio of cholesterol to tetrin A of 1:1 (w/w) resulted in a lower extinction of tetrin A (Fig. 7). Higher ratios of cholesterol to tetrin A resulted in a further decrease in the extinction. As with other polyenes, the complex of

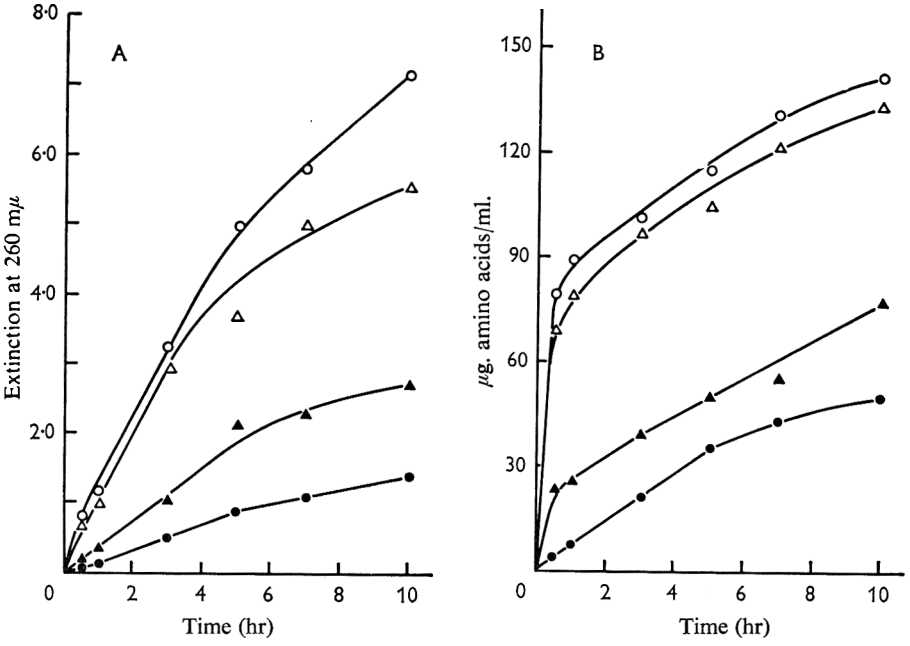

Fig. 6. The effect of exogenous cholesterol on the tetrin A (50 μg./ml.)-induced leakage of 260 mμ absorbing materials (6 A) and amino acids (6 B) from *Saccharomyces cerevisiae* suspended in citrate buffer (pH 6·0). (●), cholesterol 200 μg./ml.; (▲), tetrin A 50 μg./ml. +cholesterol 200 μg./ml.; (△), tetrin A 50 μg./ml.+cholesterol 50 μg./ml.; (○), tetrin A 50 μg./ml.

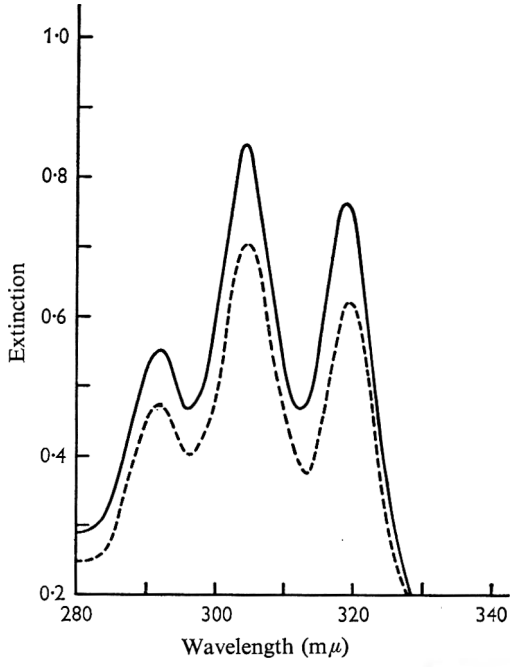

Fig. 7. Absorption spectrum of tetrin A (10 μg./ml.) in 0·05 M-potassium phosphate buffer (pH 6·0) in the presence (------) and absence (——) of cholesterol (10 μg./ml.)

tetrin A with cholesterol is apparently very weak since the decrease in extinction of tetrin A caused by cholesterol was annulled by the addition of an equal volume of dimethylformamide.

Further evidence that the addition of exogenous cholesterol might be causing a lower effective concentration of tetrin A is provided in Table 3. When tetrin A 50 μg./ml. was added to a population of viable *Saccharomyces cerevisiae* suspended in citrate buffer, 91% of the yeasts were killed after 48 hr. However, when cholesterol (200 μg./ml.) was added at the same time as the tetrin A, only 56% of the yeasts were killed. The addition of cholesterol to the yeast suspension 24 hr after the addition of tetrin A had no effect in restoring viability.

Table 3. *Effect of tetrin A on the viability of Saccharomyces cerevisiae in the presence of added cholesterol*

The initial yeast suspension contained $3{\cdot}3 \times 10^6$ viable yeasts/ml. buffer. Colonies were scored after incubation for 48 hr and rechecked after 96 hr; no differences were found. The incubation was at 28° with shaking. Cholesterol was added to 200 μg./ml. and tetrin A to 50 μg./ml.

	Treatment			
Time (hr)	Cholesterol only	Tetrin A only	Cholesterol and tetrin A added together at zero time	Cholesterol added 24 hr after the addition of tetrin A
	Yeasts killed (%)			
0	0	0	0	0
10	0	56	37	56
24	0	86	59	86
48	0	91	56	95

Effect of tetrin A on Pythium ultimum. Several investigators have indicated that the presence of sterols in an organism is a primary requirement for sensitivity to polyene antibiotics (see Demel, Kinsky & van Deenen, 1965, for a summary of evidence). Therefore, the absence of sterols from fungi of the genus *Pythium* might explain their resistance to polyene antibiotics (Schlösser & Gottlieb, 1966). However, Schlösser & Gottlieb (1966) note that when these fungi were grown on a sterol (cholesterol)-containing medium, they became sensitive to filipin. By using similar techniques to those of Schlösser & Gottlieb, we found that *Pythium ultimum* was sensitive to filipin after prior growth on a cholesterol-containing medium. Indeed all *P. ultimum* organisms exposed to filipin 250 μg./ml. for 4 hr were killed, but when *P. ultimum* was grown in the absence of cholesterol it was insensitive to filipin. However, prior growth of *P. ultimum* on cholesterol-containing medium followed by exposure to tetrin A, nystatin, or pimaricin 250 or 500 μg./ml. for periods up to 12 hr, had no effect on the resultant growth of the fungus when it was placed on fresh growth medium. Control discs of the sensitive fungus, *Glomerella cingulata*, were killed in less than 8 hr in the presence of nystatin, tetrin A, or pimaricin 500 μg./ml.

Effect of tetrin A on calf red cells. Because a previous report (Kinsky, 1963) indicated that many polyene antibiotics at low concentrations caused rapid and complete lysis of certain mammalian red cells, the effect of tetrin A on the lysis of calf red cells was examined. As shown in Table 4, tetrin A, even at 200 μg./ml. and after 6 hr in-

cubation, had little effect on the red cells, whereas, filipin at 2 μg./ml. caused complete lysis after a few min. Pimaricin and nystatin required higher concentrations and longer times than did filipin to cause complete lysis of the cells.

Table 4. *Lysis of calf red cells by tetrin A, nystatin, pimaricin and filipin*

	Treatment									
	Tetrin A (μg./ml.)			Nystatin (μg./ml.)			Pimaricin (μg./ml.)		Filipin (μg./ml.)	Control
Time (min.)	50	100	200	25	50	200	25	50	2	0
	Lysis (%)									
0	0	0	0	0	0	0	0	0	0	0
15	0	0	11	6	6	28	8	100	100	0
30	0	0	—	8	10	44	9	100	100	0
60	0	4	18	14	20	69	10	100	100	0
180	0	5	15	30	46	100	20	100	100	0
360	0	6	20	41	47	100	23	100	100	0

DISCUSSION

Tetrin A is apparently one of the least active polyene antifungal antibiotics because higher concentrations of the antibiotic are required for complete inhibition of fungal growth and for the killing of *Saccharomyces cerevisiae* than those reported for other polyenes (e.g. Bradley, Farber & Jones, 1961; Gottlieb *et al.* 1960; Kinsky, 1961). Nevertheless, tetrin A appears to act in a manner similar to that of other polyene antibiotics, namely by disrupting the permeability properties of the cells; most likely by acting on the ergosterol in the cell membranes. The disruption of cellular permeability causes a general leakage of cellular components and prevents the uptake of essential metabolites. Various compounds have been shown to leak out of fungal cells exposed to other polyene antibiotics, including amino acids and inorganic phosphate (Caltrider & Gottlieb, 1961), 260 mμ absorbing materials (Gottlieb *et al.* 1961; Kinsky, 1961), carboxylic acids (Scholz *et al.* 1959), and potassium (Marini, Arnow & Lampen, 1961).

Since tetrin A induced a leakage of metabolites, it is probably not surprising that the active uptake of essential compounds such as glucose, glycine and uracil was also inhibited. Stachiewicz & Quastel (1963) reported that glycine uptake was inhibited by nystatin.

Schlösser & Gottlieb (1966) reported that if pythium species were grown on a medium containing cholesterol the fungus became susceptible to the polyene filipin; we have confirmed such results with filipin. However, the polyenes, nystatin, pimaricin or tetrin A, even after 12 hr incubation at higher concentrations, had no effect on the growth of *Pythium ultimum* when it had previously been grown on cholesterol. The inability of these three polyenes to inhibit *P. ultimum* when grown in the presence of cholesterol cannot be entirely explained on the basis of a slower rate of lysis (Kinsky, 1962; Cirillo, Harsch & Lampen, 1964) since another fungus, *Glomerella cingulata*, was killed by these three antibiotics after 8 hr incubation. Moreover, if some or most of the *P. ultimum*-cells exposed to these antibiotics had been killed, a longer time period would have been necessary for the cell colony to grow. This did not occur.

A possible explanation for the ineffectiveness of these three polyene antibiotics may be that when the cholesterol is incorporated by the fungus, presumably into the cell membrane, it is oriented in such a manner that is accessible to the action of filipin, but not to nystatin, pimaricin, or tetrin A. Indeed, Lampen, Gill, Arnow & Magana-Plaza, 1963 reported that a sterol requiring pleuropneumonia-like organism, *Mycoplasma gallisepticum*, was sensitive to low concentrations of filipin but almost insensitive to nystatin at much higher concentrations, even though both antibiotics were bound by the organism. In addition, since filipin apparently caused a more extensive reorientation of an artificial membrane than did nystatin (Demel *et al.* 1965), it would not be surprising if the action of filipin were slightly different. While the presence of sterol in an organism is apparently a primary requirement for sensitivity to polyene antibiotics, in itself, this is not always sufficient for sensitivity. For example, *Mycoplasma laidlawii*, is resistant to the polyene antibiotics filipin and amphotericin B under certain temperature conditions, even though the organism contains sterol (Weber & Kinsky, 1965; Feingold, 1965).

Additional evidence that the alteration of the cell membrane brought about by tetrin A, nystatin and pimaricin is slightly different from that caused by filipin, and possibly other polyenes, is provided by the weaker ability of these antibiotics to induce lysis in mammalian red cells. Many polyene antibiotics, including filipin, cause rapid lysis of mammalian red cells even at low concentrations (Kinsky, 1963). Of the polyene antibiotics studied by Kinsky, nystatin and pimaricin were exceptions since higher concentrations were required for complete lysis of the red cells. Tetrin A resembled nystatin and pimaricin in this respect except that even higher concentrations of tetrin A were necessary to produce the lytic effect.

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Mutant Strains of *Aerobacter aerogenes* which Require both Methionine and Lysine for Aerobic Growth

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SUMMARY

Two types of mutants of *Aerobacter aerogenes* were obtained which grow anaerobically in a minimal medium with glucose as sole carbon source, but which require either succinate or methionine + lysine for aerobic growth in this medium. Only one type of mutant grew in minimal medium with succinate as sole carbon source. This type accumulated pyruvate and α-oxoglutarate during growth in minimal medium with glucose and succinate. It is apparently blocked in the oxidation of α-oxoacids. The other type of mutants accumulated only α-oxoglutarate during growth in minimal medium with glucose and succinate. Cell-free extracts of this type of mutant did not contain α-oxoglutarate dehydrogenase and washed organisms were unable to oxidize acetate. The aerobic molar growth yield of the mutant in minimal medium with glucose, methionine and lysine was much smaller than that of the wild type. Anaerobic growth of these mutants was strongly inhibited by nitrate; this inhibition was prevented by the addition of succinate.

INTRODUCTION

During the isolation of auxotrophic mutants of *Aerobacter aerogenes* a number of mutants were observed, which did not grow with glucose as sole carbon source in a minimal medium under aerobic conditions. Upon anaerobic incubation, however, these mutants were able to grow in the same medium. Aerobic growth occurred when the medium was supplemented with both methionine and lysine. One group of mutants lacked α-oxoglutarate dehydrogenase, the other group is blocked in the oxidation of α-oxoacids in general. These mutants were used to study the influence of the citric acid cycle on energy production by growing cells and on the mode of formation of succinyl-CoA during nitrate respiration.

METHODS

Bacteriological methods. The strain of *Aerobacter aerogenes* was that used in previous studies (Hadjipetrou, Gerrits, Teulings & Stouthamer, 1964 and Hadjipetrou & Stouthamer, 1965). The minimal medium and the growth conditions were as described previously (Hadjipetrou *et al.* 1964). Mutations were induced by treating the bacteria with *N*-methyl-*N*-nitroso-N^1-nitroguanidine by the method of Stouthamer, de Haan & Nijkamp (1965). Auxotropic mutants were enriched by the penicillin selection technique using the modification of de Haan, Stouthamer, Felix & Mol (1963) and were screened in the normal way for their specific requirements.

Enzyme assays. Extracts were prepared by submitting suspensions (approx. 2 g. wet weight/10 ml. in 0·01 M-phosphate buffer pH 6·8) to ultrasonic oscillation in a Mullard ultrasonic disintegrator (60 W., 20 kcyc./sec.) for 5 min. The resultant suspension was centrifuged at 12,500*g* for 30 min. in an MSE 18 centrifuge at 4°. α-Oxoglutarate dehydrogenase was assayed in the supernatant using the method described by Sanadi, Littlefield & Bock (1952) with ferricyanide as oxidant.

Manometric experiments. Oxidations with resting cells were measured by conventional Warburg techniques with about 15 mg. dry weight cells per vessel.

Analytical methods. Glucose was estimated with a Biochemica Test Combination, containing glucose oxidase and peroxidase (C. F. Boehringer and Soehne GMBH). Pyruvate and α-oxoglutarate were estimated with 2,4-dinitrophenylhydrazine according to the method of Reekers (1964). Protein was estimated with the Folin-Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as a standard.

RESULTS

Growth experiments. The growth of wild-type *Aerobacter aerogenes* and the mutants 19 and 42 on different media with fairly large inocula is shown in Table 1. It is evident that, for aerobic growth, mutants 19 and 42 are dependent on succinate. Other citric acid cycle intermediates or glutamate cannot replace succinate. Succinate can be replaced, however, by both methionine and lysine or methionine and diaminopimelic acid. It is known that succinyl-CoA is required for the biosynthesis of these amino acids and succinylated intermediates occur in these biosynthetic pathways (Rowbury & Woods, 1964; Gilvarg, 1957). From the results in Table 1 it seems likely that the

Table 1. *Growth of wild-type Aerobacter aerogenes and the mutants 19 and 42 under different growth conditions*

Aerobic incubation was in flasks with 8 ml. of medium; anaerobic incubation was in 12 ml. of medium. In both cases 0·2 ml. of washed cell suspension was used as inoculum.

Additions to minimal medium	Gas phase	Wild type	Mutant 19	Mutant 42
Glucose	Air	+	−	−
Glucose	95% N_2 + 5% CO_2	+	+	+
Glucose + succinate	Air	+	+	+
Succinate	Air	+	+	−
Citrate	Air	+	−	−
Glutamate	Air	+	−	−
Glucose + citrate	Air	+	−	−
Glucose + glutamate	Air	+	−	−
Fumarate	Air	+	−	−
Glucose + fumarate	Air	+	−	−
Glucose + methionine + lysine	Air	+	+	+

other citric acid cycle intermediates cannot be transformed into succinyl-CoA under aerobic conditions. Mutants 19 and 42 differ only in their growth response to succinate, only mutant 19 being able to use this compound as sole carbon source. Our mutants resemble mutant N309-1 of *Escherichia coli* described by Davis, Kornberg, Nagler, Miller & Mingioli (1959). The only difference is that this mutant requires

threonine in addition to methionine and lysine for aerobic growth. Threonine was not necessary for the growth of our mutants. Back & Westaway (1962) also studied a mutant of *E. coli*, which requires both methionine and lysine or methionine and diaminopimelic acid for aerobic growth. They found that methionine and lysine only permitted growth when fairly large inocula of washed organisms were used. Similar results were obtained for mutant 42. With small inocula in liquid medium a long lag phase was found. An inoculum of 0·003 ml. of washed suspension in 8 ml. growth medium had a lag phase of 6·5 hr before growth started. With even smaller inocula longer lag phases resulted and with inocula of less than 0·0001 ml. of washed suspension sometimes no growth was observed after 24 hr. Methionine and diaminopimelic acid had the same effects on the lag phase, when small inocula were used, as did methionine and lysine. On solid medium with methionine and lysine, mutants 19 and 42 produced very small colonies after 24 hr, whereas the mutants of Back & Westaway (1962) do not produce visible colonies on this medium at all. These results show that methionine and lysine (or diaminopimelic acid) are not sufficient to overcome completely the metabolic block in mutants 19 and 42 and in the *E. coli* mutant of Back & Westaway (1962).

Table 2. *Oxidation of a number of substrates by resting cells of wild-type Aerobacter aerogenes and the mutant 19 and 42 grown on different media*

The amount of substrate in the Warburg vessels was 10 μmoles. The figures indicate the oxygen uptake in moles/mole substrate. The sign > indicates that the reaction was not finished when the experiment was stopped after 180 min. All the figures have been corrected for endogenous O_2 uptake. The amount of cells in the vessels was approximately 15 mg. dry weight. Growth media: (1) nutrient broth + glucose, 0·2 % (2) nutrient broth (3) nutrient broth + citrate, 0·2 % (4) minimal medium + glucose, 0·2 % + methionine, 20 μg./ml. + lysine, 20 μg./ml. (5) minimal medium + glucose, 0·2 % + succinate, 0·24 %.

	Growth medium				
	1 or 2	1 or 2	1, 4 or 5	2	3
Substrate	Wild type	Mutant 42	Mutant 19		
Glucose	2·70	1·06	1·27	2·50	1·21
Acetate	1·10	0	0	0·72	1·12
Citrate	> 2·10	> 0·07	> 0·14	0·65	> 1·65
Succinate	> 1·89	0·55	1·47	1·95	1·09
Pyruvate	0·84	0·31	0·39	> 1·17	—
L-malate	—	0·57	0·55	1·13	—
α-Oxoglutarate	—	0	0	—	> 0·37

The results in Table 1 suggest that mutants 19 and 42 are blocked in the aerobic formation of succinyl-CoA. It is known that succinyl-CoA is not only involved in the biosynthesis of methionine and lysine, but also in the biosynthesis of porphyrins (for review, see Lascelles, 1962). Therefore the effect of adding δ-aminolaevulinic acid (5 μg./ml.) or haemin (1 μg./ml.) to the medium with methionine and lysine on the growth rate of cultures with a large inoculum or on the length of the lag with a small inoculum was tested. However, these additions were without effect.

Oxidation by resting cells. The final oxygen uptake by resting cells of wild-type *Aerobacter aerogenes* and mutants 19 and 42 grown under different conditions for a number of substrates are given in Table 2. Cells of mutant 42 never oxidize acetate. This is the result we expect when the formation of succinyl-CoA from other citric acid

cycle intermediates is blocked. The oxidation of acetate by resting cells of mutant 19 depends on the medium on which the cells had been grown. The oxidation of some substrates by glucose nutrient broth-grown cells of mutant 19 is shown in Fig. 1. These cells do not oxidize acetate and the other substrates are oxidized with very low oxygen uptakes. The oxidation of the same substrates by nutrient broth-grown cells is shown in Fig. 2. These cells oxidize acetate and the oxygen uptakes for the other substrates are much higher than in the experiment with glucose nutrient broth-grown cells. Thus there is a large difference in the oxidative capacities of cells grown in different media. Such differences were not found for mutant 42; cells grown in all the media had the same oxidative capacities.

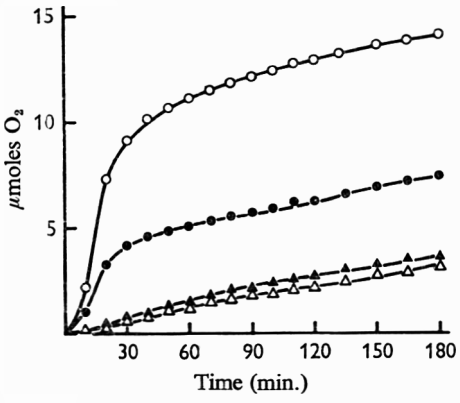

Fig. 1

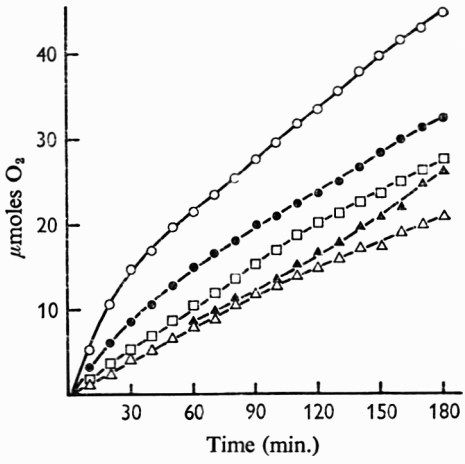

Fig. 2

Fig. 1. Oxidation of a number of substrates by glucose-nutrient broth grown cells of mutant 19 of *Aerobacter aerogenes*. The Warburg vessels contained 10 μmoles substrate and 15 mg. dry weight cells. The curves have not been corrected for endogenous respiration. ○—○ glucose; ●—● pyruvate; ▲—▲ citrate; △—△ endogenous and acetate.

Fig. 2. Oxidation of a number of substrates by nutrient broth grown cells of mutant 19 of *Aerobacter aerogenes*. Experimental conditions as in Fig. 1. □—□ acetate; △—△ endogenous; ○—○ glucose; ●—● pyruvate; ▲—▲ citrate.

Accumulation of α-oxoacids. The previous results suggest that mutants 19 and 42 are blocked in the formation of succinyl-CoA. Davis *et al.* (1959) showed that mutant N309-1 of *Escherichia coli*, had similar properties to our mutants and did not contain α-oxoglutaratedehydrogenase, therefore it seemed worthwhile to investigate the presence of this enzyme in our mutants. We can expect that when this enzyme is absent α-oxoglutarate will accumulate during the oxidation of a suitable substrate. Therefore the formation of α-oxoglutarate from a mixture of succinate and acetate and from citrate was tested. The results are shown in Table 3. During the experiments with mutant 19 using succinate and acetate we observed that there was also an accumulation of pyruvate. Therefore the formation of pyruvate and α-ketoglutarate during growth in minimal medium with glucose and succinate was tested. The result shown in Fig. 3 indicates that during growth large amounts of pyruvate and α-oxoglutarate are formed, which disappear again when the glucose has been fully utilized. Similar results were obtained when a large amount of washed suspension was inoculated into minimal

medium with glucose. In some experiments we have also measured the amount of acetate formed in these experiments. The results show that acetate is accumulated, but also disappears after growth has stopped. Thus there is a difference between the behaviour of resting cells after growth on these media (Table 1) and the behaviour of growing cells in these media. No accumulation of pyruvate by growing or resting cells of wild type *A. aerogenes* or of mutant 42 could be detected. We must conclude therefore, that mutant 19 is blocked in the oxidation of α-oxoacids in general and mutant 42 only in the oxidation of α-oxoglutarate.

Table 3. *Accumulation of α-oxoglutarate by resting cells of the mutants 19 and 42 of Aerobacter aerogenes during oxidation of mixtures of succinate and acetate or of citrate*

The bacteria had been grown on nutrient broth + citrate, 0·2%. The amount of substrate was 10 μmoles and the amount of bacteria about 15 mg. dry weight.

Mutant	Substrate	α-Oxoglutarate accumulation (μmolcs/ml.)
19	Acetate + succinate	1·92
	Citrate	5·80
42	Acetate + succinate	1·18
	Citrate	0·68

Demonstration of α-oxoglutarate dehydrogenase in cell-free extracts. It has been shown for *Escherichia coli* that the oxidation of α-oxoglutarate to succinyl-CoA is a very complex reaction (Hager & Kornberg, 1961), in which the first step is:

$$\alpha\text{-oxoglutarate} + \text{TPP} \rightleftarrows (\text{succinic semialdehyde})\text{-TPP} + CO_2.$$

Only this reaction has been measured. The oxidation of α-oxoglutarate by cell-free extracts of mutants 19 and 42 is illustrated in Fig. 4. In extracts of mutant 42 the enzyme is absent. Thus this mutant has the same metabolic defect as mutant N309-1 of *E. coli* (Davis *et al.* 1959; Hager & Kornberg, 1961). The enzyme was detected in extracts of mutant 19, grown on nutrient broth, nutrient broth + glucose and nutrient broth + citrate. No correlation was found between α-oxoglutarate dehydrogenase activity and acetate oxidation, since resting cells grown on nutrient broth + glucose did not oxidize acetate and still contained the enzyme.

Growth yields of mutant 19 and 42 in minimal medium with glucose. Previously, Hadjipetrou *et al.* (1964) found that during growth with glucose, acetate accumulated and the acetate was oxidized after glucose had been completely consumed. Acetate oxidation after glucose consumption does not contribute to the dry weight of bacteria. We found some evidence that acetate oxidation in the presence of glucose did contribute to the dry weight of bacteria, but to what extent was not known. Therefore the molar growth yields in minimal medium with glucose and methionine and lysine, with glucose alone and with succinate plus glucose were determined for wild-type *Aerobacter aerogenes* and mutants 19 and 42. The results shown in Table 4 indicate that in the absence of acetate oxidation the molar growth yield for glucose is decreased from 76·1 g. (wild type) to 45·3 g. (mutant 42). Thus the energy of acetate oxidation in the presence of glucose contributes significantly to the molar growth yield. The molar growth yields for mutant 19 are only somewhat smaller than for the wild type. This

confirms the results of the experiments in which the accumulation of α-oxoacids was tested. These experiments had shown that mutant 19 is able to oxidize glucose completely in a growth medium.

Nitrate respiration and nitrate assimilation by mutant 42. It has been shown that mutants 19 and 42 can grow anaerobically in a minimal medium with glucose as sole

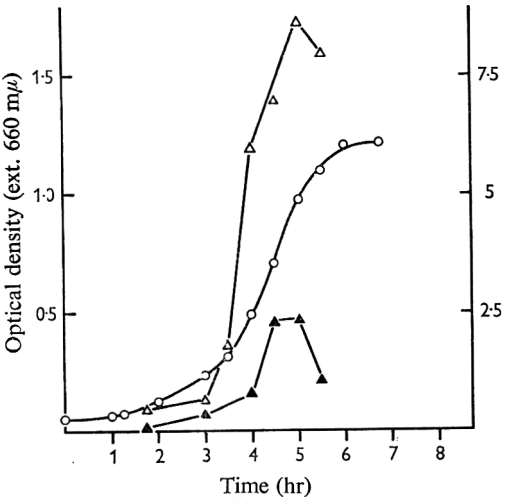

Fig. 3

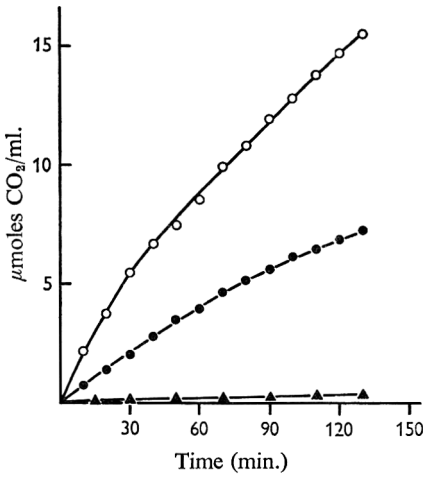

Fig. 4

Fig. 3. Accumulation of pyruvate and α-oxoglutarate by mutant 19 of *Aerobacter aerogenes*, growing aerobically in minimal medium with glucose, 11·1 μmoles/ml. + succinate, 20 μmoles/ml.. After 5·5 hr the glucose had been completely used. ○—○ optical density of culture; △—△ pyruvate (μmoles/ml.); ▲—▲ α-oxoglutarate (μmoles/ml.).

Fig. 4. Oxidation of α-oxoglutarate by cell-free extracts of mutants 19 and 42 of *Aerobacter aerogenes* with ferricyanide as electron acceptor. The cells had been grown on nutrient broth + citrate, 0·2%. The complete system in a Warburg vessel: α-oxoglutarate, 50 μmoles; $NaHCO_3$, 400 μmoles; thiamin-pyrophosphate, 200 μg; $MgCl_2$, 20 μmoles; bovine serum albumin, 30 mg. and cell-free extract in a total volume of 1·9 ml. The side-bulb contained 0·1 ml of 0·5 M-K_3 $Fe(CN)_6$. The gas phase was 100% CO_2. The CO_2 evolution was measured after tipping in the potassium ferricyanide. The incubation temperature was 37°. ○—○ cell-free, extract of mutant 19 (1·9 mg. of protein); ●—● cell-free extract of mutant 19 (0·95 mg. of protein); ▲—▲ cell-free extract of mutant 42 (2·4 mg. of protein).

Table 4. *Molar growth yields of wild-type Aerobacter aerogenes and mutants 19 and 42*

The glucose concentration was 1·5 μmoles/ml.; succinate concentration 5 μmoles/ml. The inoculum was 4 ml. of washed bacteria in 200 ml. of the indicated growth medium. 5 ml. samples were taken at intervals and the extinction at 660 mμ was measured. The molar growth yields were calculated from the dry weights at the moment of maximal extinction. Dry weight was calculated from the previously established relationship: dry weight of bacteria = 380 × extinction at 660 mμ (Hadjipetrou *et al.* 1964).

Medium	Wild type	Mutant 19	Mutant 42
Min. medium + glucose	72·1*	0	0
Min. medium + glucose + succinate	76·1	67·5	45·3
Min. medium + glucose + methionine + lysine	77·0	76·6	44·4

* From Hadjipetrou *et al.* (1964).

carbon source. In this case succinyl-CoA is formed by reduction of oxaloacetate. We were interested to know how the cells satisfied their need for succinyl-CoA under anaerobic conditions in the presence of nitrate. It was found that mutant 42 gave very slow growth in a medium with nitrate as sole nitrogen source, in sharp contrast to the

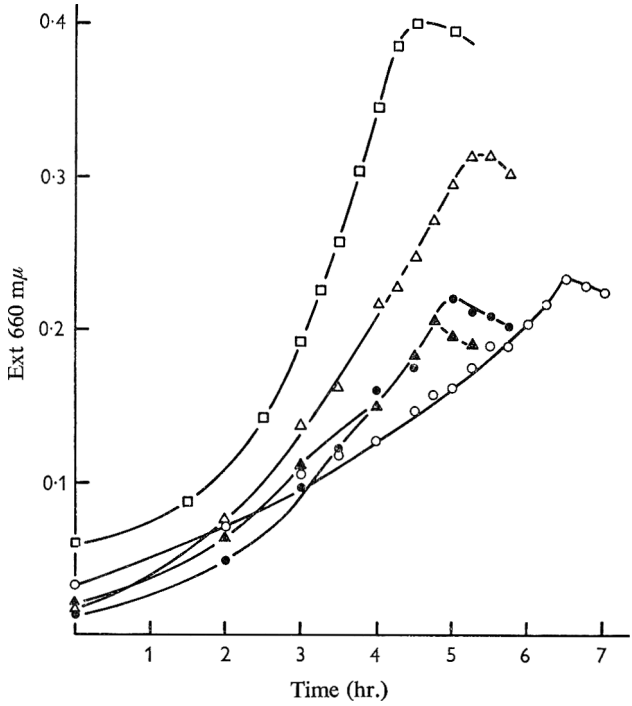

Fig. 5. Growth of cultures of wild-type *Aerobacter aerogenes* and of mutant 42, under anaerobic conditions with 3 μmoles glucose/ml. in minimal medium, with different nitrogen sources. The gas phase was 95 % N_2 + 5 % CO_2. The inoculum was 20 ml. of washed bacteria in 200 ml. of the indicated growth medium. 5 ml. samples were taken at intervals and the extinction at 660 mμ was measured. ●—● mutant 42, NH_4Cl (0·2 %); ▲—▲ mutant 42, NH_4Cl + succinate (5 μmoles/ml.); △—△ mutant 42, KNO_3 + succinate; ○—○ mutant 42, KNO_3 (0·35 %); □—□ wild type, KNO_3.

Table 5. *Molar growth yields of wild-type Aerobacter aerogenes and mutant 42 grown anaerobically with glucose under different conditions*

The glucose concentration was 3 μmoles/ml., the succinate concentration 5 μmoles/ml. The inoculum was 20 ml. of washed bacteria in 200 ml. of the indicated medium. The molar growth yield was determined in Table 4.

Composition of minimal medium		Wild	Mutant
C-source	N-source	type	42
Glucose	NH_4Cl	26·1*	26·2
Glucose	KNO_3	43·0*	26·6
Glucose	$KNO_3 + NH_4Cl$	45·5*	29·9
Glucose + succinate	NH_4Cl	28·7	23·3
Glucose + succinate	KNO_3	Not done	37·0
Glucose + succinate	$KNO_3 + NH_4Cl$	48·5	38·1

* From Hadjipetrou & Stouthamer (1965).

very good growth which the wild type shows under these conditions (Fig. 5). In this experiment a large inoculum of washed organisms was used. With smaller inocula the difference between the growth curves of the culture with ammonia and that with nitrate was much larger. In a separate experiment it was found that anaerobic growth in a medium with both nitrate and ammonia is slower than in a medium with only ammonia. This indicates that the slow growth with nitrate as sole nitrogen source is not due to slow assimilation of nitrate but to the fact that in its presence nitrate respiration occurs. Normal growth of mutant 42 in the presence of nitrate can be obtained when succinate is added (Fig. 5). These effects are not found with wild type *Aerobacter aerogenes*. The molar growth yields of wild type *A. aerogenes* and mutant 42 under these conditions are given in Table 5. The molar growth yields of mutant 42 during anaerobic growth in the presence of nitrate are hardly larger than in its absence, in sharp contrast to the results with the wild type. The metabolic block in mutant 42 prevents the efficient use of nitrate respirations.

DISCUSSION

Experiments have shown that *Aerobacter aerogenes* mutant 42 is blocked in the decarboxylation of α-oxoglutarate. The metabolic block in mutant 19 is unknown. Because the oxidation of both pyruvate and α-oxoglutarate is impaired, we must look for a reaction which is involved in the oxidation of both compounds. The metabolic block in mutant 19 is not complete because in a growth medium glucose is completely oxidized (Table 4), whereas resting cells in some media do not oxidize glucose completely (Table 2). The reason for this difference in behaviour between growing and resting cells is not known. Experiments are in progress to define more clearly the metabolic block in mutant 19. When this is known the differences between growing and resting cells may become clear. Because the oxidation of pyruvate is retarded the flow of metabolites to the citric cycle is diminished, and because in addition the oxidation of α-oxoglutarate is retarded the observed shortage of succinyl-CoA for biosynthetic purposes can be explained. A combination of methionine and lysine could not completely replace the requirement for succinate. Good growth in this medium was not obtained for the methionine- and lysine-requiring mutants of *Escherichia coli*, which were described by Back & Westaway (1962). Haemin, which is derived from succinyl-CoA, did not improve growth in the presence of methionine and lysine. Haemin biosynthesis does not seem to be impaired in these mutants because cells grown on minimal medium with glucose, methionine and lysine (medium 4, Table 2) respire normally and preliminary experiments indicate that they contain normal levels of NADH oxidase. Two explanations may be offered for these observations: (1) Succinyl-CoA is not an intermediate in the biosynthesis of haemin in *A. aerogenes*, or (2) more likely the metabolic block in both mutants is leaky, permitting the synthesis of a small amount of succinyl-CoA, which is used preferentially for the biosynthesis of haemin. The results show, that poor growth in minimal medium with glucose, methionine and lysine is not due to a shortage of haemin. Two explanations for the poor growth in this medium may be given. (1) Succinyl-CoA is required for another unknown reaction, or (2) the combination of methionine and lysine produces repression of the aspartokinases, and perhaps of some other enzymes leading to a shortage of the aspartic acid family of amino acids (methionine, lysine, diaminopimelic acid, threo-

nine and isoleucine). Repression of the enzymes involved in the biosynthesis of these amino acids in *E. coli* is very complex (Cohen & Patte, 1963). This phenomenon may also explain the requirement for threonine by mutant N319-1 of *E. coli* (Davis *et al.* 1959). The experimental results are insufficient at this moment to permit a choice to be made between these possibilities.

The metabolic block in mutant 42 gives rise to a very sharp decrease in the molar growth yield with glucose, indicating that the energy of acetate oxidation contributes to the dry weight of bacteria, a fact which confirms a previous suggestion by Hadjipetrou *et al.* (1964). The molar growth yield of mutant 42 is about the same after aerobic growth as the yield obtained from wild type after anaerobic growth in the presence of nitrate (compare Tables 4 and 5). In *Aerobacter aerogenes* the citric acid cycle does not function anaerobically in the presence of nitrate (Pichinoty, Mottet, Bigliardi-Rouvier & Forget, 1963: Hadjipetrou & Stouthamer, 1965). The absence of α-oxoglutaratedehydrogenase in mutant 42 also leads to a failure of the citric acid cycle. The fact that the molar growth yields of mutant 42 grown aerobically and of wild type grown anaerobically in the presence of nitrate, gives further support to our previous conclusion that nitrate respiration is as efficient as normal respiration (Hadjipetrou & Stouthamer, 1965).

Mutants 19 and 42 can grow anaerobically in a minimal medium with glucose as sole carbon source. Succinate, which is formed by reduction of oxaloacetate, is a normal end product of glucose fermentation in this strain of *Aerobacter aerogenes* (Hadjipetrou, 1965). Succinate can be converted into succinyl-CoA and in this way the cells satisfy their need for succinyl-CoA. Anaerobic growth of mutant 42 is strongly inhibited by the presence of nitrate and this inhibition is prevented by the addition of succinate. In the presence of nitrate the hydrogen donor, necessary for the reduction of oxaloacetate to succinate, is reoxidized by nitrate. This explains the observation that addition of nitrate to an anaerobically growing culture causes a shortage of succinyl-CoA. These results indicate that the wild type grown anaerobically in the presence of nitrate forms succinyl-CoA by the oxidation of α-oxoglutarate with nitrate as hydrogen acceptor. Thus although the citric acid cycle does not function to complete the oxidation of acetate under anaerobic conditions in the presence of nitrate, the cycle is utilized more extensively for biosynthesis than in the absence of nitrate. The reasons for the absence of anaerobic acetate oxidation in the presence of nitrate are being studied at this moment.

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The Urease Activity of *Acinetobacter lwoffii* and *A. anitratus*

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SUMMARY

The production of urease by *Acinetobacter lwoffii* and *A. anitratus* was studied in 20 strains. It is concluded that both species may produce urease under appropriate conditions and that this property is of no value in differentiating between them. This conclusion was tested in 40 other strains of which 37 produced urease independently of saccharolytic activity.

INTRODUCTION

Many strains of *Acinetobacter lwoffii* and *A. anitratus* have now been studied by numerous workers; the reports reveal diversity of opinion about the presence of urease activity. A list of the reports and techniques used is given in Table 1. The present study was undertaken on three grounds: (1) The diversity of results recorded in Table 1. (2) A strain of *A. anitratus* (reported by Lemoigne, Girard & Jacobelli, 1952, as *Neisseria winogradskyi* and stated by these workers to be vigorous urease producers) did not give the urease reaction on Christensen medium (Christensen, 1946) when tested by the present author. (3) Jeffries (1964) suggested that certain strains of *A. anitratus* might fail to show urease activity when grown on ordinary test media, but that these strains might contain intracellular urease revealed only after ultrasonic disruption of the organism.

Table 1. *Urease activity of Acinetobacter lwoffii and A. antitratus according to various investigators*

Investigators	Date	No. of strains tested	No. of strains found to produce urease	Technique
Schaub & Hauber	1948	15	15	Christensen (1946)
Stuart, Formal & McGann	1949	55	0	Rustigian & Stuart (1941)
Ferguson & Roberts	1950	109	0	Rustigian & Stuart (1941)
Brooke	1951	86	29	Not stated
Lemoigne, Girard & Jacobelli	1952	18	18	Modification of Grelet (1946)
Henderson	1965	60	10	Christensen (1946)

The present investigation falls into four parts: (1) The testing of strains of *Acinetobacter anitratus* and *A. lwoffii* on a variety of media containing urea. (2) The estimation of urea consumption when the bacteria were grown in urea + peptone

water. (3) The estimation of urea consumption when the supernatant fluid of centrifuged disrupted bacteria was suspended in an aqueous solution of urea. (4) The estimation of ammonia produced during the growth of the bacteria in aqueous solutions of urea.

METHODS

Organisms. Most strains were obtained from culture collections with the addition of a few wild strains (see Table 2).

Identity of organisms

The organisms were required to conform to the description of Henderson (1965), namely: coccobacilli; catalase produced; indole and acetoin not produced; no acid from carbohydrates, when tested against 1% (w/v) solutions of carbohydrate in peptone water, or, only from pentoses and hexoses, never dissacharides, trisaccharides, polysaccharides or alcohols, and the acid should be produced oxidatively and never fermentatively; ammonia always produced from peptone water; presence or absence

Table 2. *Cultures of Acinetobacter*

A. lwoffii (*Mima polymorpha*)	*ACTC 9957
A. lwoffii (*Moraxella lwoffii*)	Inst. Pasteur, 5382
A. lwoffii (*Moraxella lwoffii*)	Inst. Pasteur, 53116
A. lwoffii	Wild strain A
A. lwoffii	Wild strain B
A. lwoffii var. *oxydans*	Wild strain C
A. lwoffii var. *oxydans*	Wild strain D
A. lwoffii var. *oxydans*	Wild strain E
A. anitratus (B5W)	†NCTC 10292
A. anitratus	NCTC 7250
A. anitratus	NCTC 7412
A. anitratus	NCTC 7461
A. anitratus	NCTC 7844
A. anitratus	NCTC 9427
A. anitratus (*Moraxella glucidolytica*)	Inst. Pasteur, 5497
A. anitratus (*Neisseria winogradskyi*)	
A. anitratus	Wild strain F
A. anitratus	Wild strain G
A. anitratus	Wild strain H
A. anitratus	Wild strain I

* ACTC, American Collection of Type Cultures.
† NCTC, National Collection of Type Cultures, Colindale, London, England.

of oxidase unimportant; nitrate not reduced to nitrite in broth. Jyssum & Joner (1965*a*, *b*) have shown that these bacteria in fact utilize nitrite or nitrate as sole source of nitrogen and that repression of the nitrate-reducing system is caused by aspartic acid, alanine and amino acids biosynthetically related to them, though not by the ammonium ion. On the other hand, the activity of the nitrate-reducing system is inhibited by the ammonium ion, and ammonia is produced from the peptone which is the usual base of nitrate broths. The organisms are also known to convert nitrate to nitrite when grown in a succinate + nitrate medium but not in the presence of ammonia (personal communication from Dr C. A. Fewson, Department of Biochemistry, University of Glasgow).

PHYSIOLOGICAL TESTS

All tests were made after incubation for 7 days at 37°.

Indole production was tested in peptone water with the reagent of Ehrlich (Cowan & Steel, 1965).

Catalase was detected by effervescence on adding '10 vol.' hydrogen peroxide to peptone water cultures.

Acetoin was tested for by the method of Barrit (1936) in the simple medium recommended by the Ministry of Health (1934).

Production of acid from carbohydrates was examined by using 1 % (w/v) carbohydrate in peptone water with 1 % acid fuchsin in tubes.

Oxidase production was tested by pouring on colonies on nutrient agar a freshly prepared 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine dihydrochloride containing 1 % (w/v) ascorbic acid to delay autoxidation of the reagent (Steel, 1962).

Estimation of urea was by the diacetyl method of Natelson, Scott & Beffa (1951), but for the preliminary precipitation of protein the barium hydroxide and zinc sulphate method recommended by Dickenman, Crafts & Zak (1954) was used.

Ultrasonic disruption of bacteria was done in a Dawe's Soniprobe (Dawe Instruments Ltd., Western Avenue, Acton, London, W. 3) with subsequent centrifugation and sterilization of the supernatant fluid through membrane filters (Oxoid brand; size 0·5–1μ in diameter).

Estimations of cytoplasmic protein concentration were done by the Kjeldahl Nesslerization method (after Varley, 1960).

Test media for urease production. The following media were used: Rustigian & Stuart (1941); Christensen (1946); the modification of the latter marketed by Messrs Oxoid Ltd; the medium of Grelet (1946) modified by Lemoigne *et al.* (1952); Schneider & Gunderson (1946); Elek (1946).

RESULTS

Detection of urease by cultural methods

For most of the substrates used, the instructions of the various originators were followed closely. In the case of the reagent used by Lemoigne *et al.* (1952), the authors simply stated that urea was used in Grelet's base; accordingly urea was added to this in an arbitrary concentration of 2 %. Incubation in all cases was for 1 week at 37° except in the case of Elek's method where the tests were made after incubation for 3 hr in a water bath at 37°.

The results are summarized in Table 3.

Lemoigne *et al.* (1952), when using urea dissolved in Grelet's base, found that their strains of *Neisseria winogradskyi* (*Acinetobacter anitratus*) all yielded urease as revealed by ammonia production. However, the medium contains peptone and Henderson (1965) showed that all his strains produced ammonia from peptone, so the results obtained by the use of the medium that Lemoigne *et al.* used should be discounted. The varying results obtained from the use of the medium of Christensen (1946) and the Oxoid modification of it, and from the medium of Schneider & Gunderson (1946), may have been due to a similar cause, since all contain peptone or tryptone. The medium of Rustigian & Stuart (1941) supported growth badly; it is noteworthy that the two groups of workers who failed to find urease used this medium.

The results obtained by use of the technique of Elek (1946) are acceptable if it can be shown that negative results are not due to utilization of the ammonia produced from the urea.

Table 3. *Production of urease on various test media*

	Media				
	Elek	Christensen	Oxoid	Schneider & Gunderson	Grelet
Acinetobacter anitratus					
NCTC 7250	+	0	2	0	1
NCTC 7412	+	0	0	0	1
NCTC 7461	+	0	1	0	1
NCTC 9427	+	0	1	0	1
Wild strain F	+	2*	1	4	1
Wild strain G	+	2	1	3	1
Wild strain H	+	2	1	4	1
Wild strain I	+	2	1	3	1
NCTC 10292	+	0	0	0	1
NCTC 7844	0	0	5	0	1
Inst. Pasteur, 5497	0	0	0	0	1
Acinetobacter lwoffii					
Wild strain A	+	5	2	5	1
Wild strain C	+	0	0	0	1
ACTC 9957	0	0	0	0	1
Inst. Pasteur, 53116	0	0	0	0	1
Inst. Pasteur, 5382	0	0	0	0	1
Wild strain B	0	0	0	0	1
Wild strain D	0	0	0	0	1
Wild strain E	0	0	0	0	1

+ = Positive reaction. 0 = Negative reaction. 1, 2, etc. = no. of days incubation required for positive reaction.

Detection of urease by estimation of bacterial consumption of urea

When these bacteria grow in peptone water ammonia is produced. Accordingly, it was not possible to grow the bacteria in a solution of urea in peptone water and estimate residual urea after conversion to ammonia. The reagents available in such a contingency are: diacetyl monoxine (Barker, 1944), α-isonitrosopropriophenone (Archibald *et al.* 1945); diacetyl (Natelson, Scott & Beffa, 1951; Dickenman, Crafts & Zak, 1954); acetylbenzoyl (Dickenman *et al.* 1954). It was decided to adopt a diacetyl method as utilized by Jeffries (1964).

Preliminary experiments showed that the method of Natelson *et al.* (1951) yielded results that were more linear between the limits 10 and 100 mg. urea/100 ml. in the present investigator's hands than did the technique of Dickenman *et al.* (1954). Natelson *et al.* recommended reading the results in a spectrophotometer at 480 mμ, and it was found that peptone increased the extinction but did not affect the wavelength of maximal absorption. However, even trivial differences in boiling time due, for example, to varying numbers of tubes cooling the water in the bath caused large differences in the final extinction, with the result that graphs from different runs were dissimilar and a set of tubes for a full calibration curve had always to be included.

Bacteria were grown on nutrient agar, washed, and added to peptone water containing urea 100 mg./100 ml., so that each 5 ml. sample of urea contained 0·005 g.

bacteria (wet weight). These urea solutions were incubated at 37° and each day the bacteria were removed by centrifugation from one sample, chloroform added, and the supernatant fluid kept at 4°. At the end of 6 days the residual urea was estimated in each sample.

No graphs of the results are given since no convincing instance of urea consumption was noted. It was surprising that not one instance of a decrease in urea concentration was encountered. The author is indebted to Dr C. A. Fewson (Department of Biochemistry, University of Glasgow) for his suggestion that the presence of nitrogenous substances such as ammonia in the culture medium may suppress urease formation.

Urea consumption by supernatant fluid of ultrasonically disrupted bacteria

Preliminary tests indicated that optimal results were obtained after ultrasonic treatment for 10–15 min., followed by incubation with urea for 10 days. The bacteria were grown on nutrient agar, washed, and disrupted ultrasonically for 12 min., the suspension centrifuged, the supernatant fluid sterilized by filtration through membrane filters and the protein estimated and diluted to 1 mg./ml. Samples of this filtrate were then added to an equal volume of an aqueous urea solution at 200 mg. and 1 mg./ml. respectively. Immediately on mixing, a small sample was withdrawn and stored at

Table 4. *Urea consumption by membrane-sterilized filtrates of supernatant fluids after centrifugation of ultrasonically disrupted washed bacteria*

Organism	Decrease in urea concentration (mg./ml.)
Acinetobacter lwoffii	
ACTC 9957	0
Inst. Pasteur, 53116	0
Wild strain A	0
Wild strain C	0
A. anitratus	
NCTC 7844	0
NCTC 7412	0
NCTC 9427	0
A. lwoffii	
Inst. Pasteur, 5382	0–25
Wild strain B	0–25
Wild strain D	0–25
Wild strain E	0–25
A. anitratus	
NCTC 7461	0–25
A. anitratus	
NCTC 7250	24–50
NCTC 10292	25–50
Wild strain F	25–50
Wild strain H	25–50
Wild strain I	25–50
A. anitratus	
Inst. Pasteur, 5497	75–100
Wild strain G	75–100

$-20°$ to act as a control. The mixtures were then incubated at 37° for 10 days, and the urea concentration remaining then estimated and compared with the controls.

The results are shown in Table 4.

The use of diacetyl as a reagent for the detection of urea was unsatisfactory in my hands as discussed above; very small variations in the boiling time caused large variations in the extinction; even the time required for the water to boil again after the cooling by immersion of the tubes caused great variation in the intensity of the colour produced. Similarly, the delay in transferring the tubes to the ice bath caused some tubes to have relatively longer heating. It was not found practicable to immerse large numbers of tubes in a thermostatically controlled, electrically heated bath, as the action of the paddle did not prevent unequal heating: the tubes nearer the heating element always attained a higher extinction value.

Table 5. *Production of ammonia in g. per 100 g. urea by Acinetobacter lwoffii and A. anitratus*

Acinetobacter anitratus	Ammonia production in g. from 100 g. urea	*Acinetobacter lwoffii*	Ammonia production in g. from 100 g. urea
NCTC 7250	0·5	ACTC 9957	0·8
NCTC 7412	0·5	Inst. Pasteur, 5382	1·3
NCTC 7461	0·5	Inst. Pasteur, 53116	1·5
NCTC 7844	0·8	Wild strain A	1·0
NCTC 9427	1·1	Wild strain B	0·8
NCTC 10292	1·3	Wild strain C	0·8
Inst. Pasteur, 5497	1·4	Wild strain D	0·5
Wild strain F	3·2	Wild strain E	0·5
Wild strain G	1·9		
Wild strain H	2·2		
Wild strain I	3·2		

Because of these difficulties and because the urea concentration tended to decrease on incubation, it was decided to accept decreases in urea concentration of 0–25 mg./100 ml. as not significant, 25–50 mg./100 ml. as slight urease production, and 50–100 mg./100 ml. as definite urease production.

All the strains of *Acinetobacter lwoffii* failed to produce urease, but 5 of 11 strains of *A. anitratus* gave similar negative results. Of the remaining strains of *A. anitratus*, 4 of 11 gave weak positive results and 2 of 11 definite positive results.

Estimation of ammonia produced during growth in urea

In each instance, one loopful of an overnight growth of the organism on nutrient agar was placed in 1·5 ml. of Elek urea medium. After incubation for 3 hr in a water bath at 37° the solutions were cleared by centrifugation, 0·5 ml. of each was withdrawn and added to 12 ml. distilled water; similarly 0·5 ml. of a solution of ammonium sulphate (1 mg./ml.) was added to 12 ml. distilled water. From each of the urea solutions 2 ml. was withdrawn and added to 5 ml. water and 3 ml. Nessler reagent; from the ammonium solution 1 ml. was taken and added to 6 ml. water and 3 ml. Nessler reagent; then the ammonia concentrations were estimated in a photoelectric colorimeter.

The results are given in Table 5. It is seen that every organism produced ammonia from urea. The quantities are not comparable since the inocula were unequal.

Forty more strains were examined by Elek's method (20 from human sources, 20 freshly isolated from soil). These results are shown in Table 6.

Table 6. *Production of urease by 40 additional strains*

Source	Urease activity	Saccharolytic activity	
Human	19+	17+	2−
	1−	0+	1−
Earth	18+	12+	6−
	2−	1+	1−

DISCUSSION

All of the strains of bacteria examined, no matter whether regarded as *Acinetobacter lwoffi* or *A. anitratus*, produced urease when no peptone was present and when incubation was brief. Thus no distinction can be made between these two organisms on this basis. Confusion in the past appears to have arisen from three sources: the liberation of ammonia from peptone in the basal media; no allowance being made for the possibility that an organism might resynthesize urea from other nitrogenous precursors; the role of ammonia in suppressing urease formation. König, Kaltwasser & Schlegel (1966) have demonstrated similar suppression of urease formation by ammonia in a Hydrogenomonas strain.

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A Model for the Identification of Bacteria

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SUMMARY

Two methods are described for determining the shortest route to an identification. The methods are based upon the reduction to a minimum test set, as suggested by Gyllenberg (1963), which will result in the most efficient separation of the organisms. The first method is used when no test results for characters are considered variable; the second method is applicable when some characters are variable and others are not. To illustrate the methods two sets of data are reduced. One set includes the reduction to a minimum set of 6 tests from 32 tests for 36 organisms in the Enterobacteriaceae. The other set includes the reduction to a minimum set of 3 tests from 34 tests for 8 Pasteurella species. The methods described may be used manually or be programmed for a computer.

INTRODUCTION

For the efficient identification of large numbers of organisms, it would be desirable to know which set of tests would be most effective in separating a group of organisms into progressively smaller subgroups with equal, or nearly equal, numbers of organisms. The number of subgroups ideally would equal the original number of organisms. If this situation could be attained, there would be complete separation of the organisms in a group. If only one organism were separated into a subgroup, this would constitute an identification. In addition, a minimal number of tests would have been done in order to effect separation, that is, the shortest route to an identification would have been used. Gyllenberg (1963, 1964) has given consideration to a general method of determining the 'minimum set' of tests required for separation of groups of organisms. His methods, with some modification, have been applied to representative species in the Enterobacteriaceae to develop a generalized programme for the Burroughs 5500 Computer.

METHODS

An idealized example. The number of tests required for the minimum set is determined by the number of organisms to be separated. Considering only two-state tests, that is, tests for characters that may be recorded as present (+ or 1) or absent (− or 0), two organisms may be separated by one test, three to four organisms would require a minimum of two tests, five to eight organisms three tests, nine to sixteen organisms four tests, etc. These are idealized circumstances. According to Gyllenberg (1963), the minimum set of tests is $T_{\min.} = \log_2 G$, or in exponential notation, $2^T = G$, where

T is the number of tests in the minimum set and G the number of organisms in the group.

The idealized situation is illustrated in Table 1. In the three examples in this table each test has an equal number of 1 and 0 results although the alternating sequence of 1's and 0's varies. Thus, for the most efficient separation of organisms each test in the minimum set would have an equal number of alternating sequences of 1 and 0 test results. The proper number of alternating sequences of 1 and 0 test results, where N the number of organisms is even, would be $N/2$, $N/4$,..., N/N. For example, with 8 organisms and 3 tests, $8/2 = 4$, $8/4 = 2$, and $8/8 = 1$. In the third example in Table 1, test 1 (four 1's and four 0's) separates the organisms into 2 subgroups each with 4 organisms. Test 2 (alternating two 1's and two 0's) separates the 2 subgroups into 4 subgroups each with 2 organisms. Test 3 (alternating 1's and 0's) separates the 4 subgroups into 8 subgroups each with 1 organism.

Table 1. *Idealized separation of organisms into subgroups*

Organisms	Tests and results		
	One test separates 2 organisms*		
	Test 1		
A	1 (or+)		
B	0 (or−)		
	Two tests separate 3 to 4 organisms*		
	Test 1	Test 2	
A	1	1	
B	1	0	
C	0	1	
D	0	0	
	Three tests separate 5 to 8 organisms*		
	Test 1	Test 2	Test 3
A	1	1	1
B	1	1	0
C	1	0	1
D	1	0	0
E	0	1	1
F	0	1	0
G	0	0	1
H	0	0	0
$N = 8$	$8/2 = 4$	$8/4 = 2$	$8/8 = 1$

* One two-state test has 2 possible results, 1 (or+) and 0 (or−). Two two-state tests have 4 possible results, 11 (or++), 10 (or+−), 01 (or −+), and 00 (or−−), or in general the number of possible unique combinations for two-state tests is 2^T where T is the number of tests.

It will be noticed in the last example in Table 1 that each of the 3 tests results in the same number of separations. Test 1 for organism A is 1, and this separates A from organisms E, F, G, and H which are 0 for this test. Hence, there are 4 separations. The same applies to organisms B, C and D which are 1 for the first test and organisms E, F, G, and H which are 0. There are a total of 16 separations effected by test 1. This procedure is applicable in a similar manner for tests 2 and 3 each of which results in 16 separations.

The same result is obtained if the number of 1's (n_1) and the number of 0's (n_0) for

each test are multiplied together, that is, $n_1 n_0$. This is the 'separation figure (S)' of Gyllenberg (1963), $S = UV$ or $S = n_1 n_0$. If the tests for the organisms in a group are analysed by this method, that test with the highest S value will be the first best test. It usually will separate the organisms into two subgroups with an equal, or nearly equal, number of organisms. The application of Gyllenberg's formula is not limited necessarily to one test, that is, it may be used to calculate the S value for two or more tests. A generalized equation, applicable when no test results are variable, has been derived by I. G. Bowen. The term 'combination' as used in this derivation refers to the possible unique combinations of 1's and of 0's when all of the tests (T) in the minimum set are considered variable (v). Thus, if there were 5 tests there would be $2^{T_v} = 2^5 = 32$ possible unique combinations. Examples of these combinations are given in Table 1 and at the bottom of Tables 3, 4, 9, 12, and 14. Let

N = total number of organisms,
n_a = total number of organisms with combination a,
n_b = total number of organisms with combination b,
n_i = total number of organisms with combination i.

Then, $(N-n_a)$ is the number of organisms not having combination 'a' and consequently is the number of separations for each 'a' organism. For n_a organisms, the separations would be $n_a(N-n_a)$. Similar expressions can be written for organisms with combinations 'b'...'i'; however, in arriving at the total number of separations it should be remembered that each separation has been counted twice. Thus, the total separations, S, is given by

$$S = \tfrac{1}{2}[n_a(N-n_a)+n_b(N-n_b)+\ldots+n_i(N-n_i)]$$

which reduces to

$$S = \tfrac{1}{2}[N^2-(n_a^2+n_b^2+\ldots+n_i^2)]. \quad (1)$$

The application of this equation is illustrated next.

When no characters are considered variable. If one is dealing with a large number of organisms to be identified, it usually is impossible to find a sufficient number of characters for the determinative scheme that, as far as the available data indicate, are not variable. That is, few characters are definitely always present or always absent. A character that is variable is useful for a determinative scheme if the frequency it is present or absent is known. Cowan & Steel (1965), in their diagnostic tables, state that a positive or negative test result for a character means 80–100 % positive or negative and 0–20 % negative or positive, respectively. The procedures to be discussed are applicable to finding the minimum set of tests for separation of a group of organisms when no characters are variable or when confidence levels for the relative frequency that variable characters are positive or negative have been established. Gyllenberg (1963) has given consideration to these procedures.

Hypothetical data are given in Table 2 for 8 organisms and 5 tests. The left part of the table gives the data for the test results of the organisms and the S values for the tests. The right side of the table shows the tests arranged in descending order of their S values. Ideally 3 two-state tests ($2^3 = 8$) would be required to separate the 8 organisms as illustrated in Table 1. The problem is to find the combination of 3 of the 5 tests that will result in the most efficient separation of the 8 organisms. For illustrative purposes we shall find the first 2 tests and then the first 3. It should be emphasized, however, that we could directly find the first 3 tests.

One two-state test could separate 2 or more organisms into 2 subgroups, those organisms that were 1 for the test and those organisms that were 0. As indicated in Table 2, test 4 gives the largest number of separations for one test. Two two-state tests could separate the organisms into subgroups with the following possible unique combinations of test results, 11, 10, 01, 00 (or $++$, $+-$, $-+$, $--$). First it is desired to

Table 2. *Hypothetical data for organisms and their test results. The S values are calculated for each test and the tests are rearranged in descending order of their S values in the right part of the table*

	Tests						Tests				
Organisms	1	2	3	4	5	Organisms	4	2	1	3	5
1	0	1	0	1	1	1	1	1	0	0	1
2	0	0	0	1	1	2	1	0	0	0	1
3	0	1	1	1	1	3	1	1	0	1	1
4	0	0	0	0	1	4	0	0	0	0	1
5	1	1	0	0	0	5	0	1	1	0	0
6	0	1	0	0	1	6	0	1	0	0	1
7	1	1	0	0	1	7	0	1	1	0	1
8	0	0	1	1	0	8	1	0	0	1	0
n_1	2	5	2	4	6	Tests arranged in decending order of					
n_0	6	3	6	4	2	S values					
S^* =	12	15	12	16	12		16	15	12	12	12

* $S = \frac{1}{2}[N^2-(n_1^2+n_0^2)] = n_1 n_0$.

find that combination of 2 tests which most closely would approach the ideal condition of separating 8 organisms into 4 subgroups each with 2 organisms. To determine this it only is necessary to find the number of combinations (C) of 5 tests taken 2 at a time, or

$$C_2^5 = \frac{5(5-1)}{2!} = \frac{5 \cdot 4}{1 \cdot 2} = 10.$$

Next a count is made of the number of organisms which are separated into the 4 possible unique combinations of results for 2 two-state tests. This is illustrated in Table 3. Of the 10 possible combinations of 5 tests taken 2 at a time, tests 4 and 2 appear to approach most closely the ideal separation.

This same result could be obtained by using equation (1) in the form

$$S = \tfrac{1}{2}[N^2-(n_{11}^2+n_{10}^2+n_{01}^2+n_{00}^2)],$$

where again N is the number of organisms in the group and n_{11}, n_{10}, n_{01}, and n_{00} are the number of organisms with the 4 possible unique combinations of 2 two-state tests. S will approach a maximum value when n_{11}, n_{10}, n_{01}, and n_{00} each approach $N/4$ as a limit. That is, when $N/4 = 8/4 = 2 = n_{11} = n_{10} = n_{01} = n_{00}$, the maximum value for S is 24. The S value for the 10 combinations of 2 two-state tests are given at the bottom of Table 3. The test combination 4–2 most closely approaches the ideal separation ($S = 24$) with a value of 23 and these 2 tests are selected for the first 2 best tests.

This process is repeated again to find the 3 first best tests. In this case, however, equation (1) becomes

$$S = \tfrac{1}{2}[N^2-(n_{111}^2+n_{110}^2+n_{101}^2+n_{100}^2+n_{011}^2+n_{01C}^2+n_{001}^2+n_{000}^2)]$$

and the maximum value for S will be obtained when each n approaches $N/8$ as a limit. This value is 28. The possible combinations of 5 tests taken 3 at a time are 10.

$$C_3^5 = \frac{5 \cdot 4 \cdot 3}{3!} = 10.$$

Table 3. *Possible combinations of 5 tests taken 2 at a time (from Table 2) and the number of organisms that are separated into each unique combination of 2 two-state tests*

	Combination of tests																			
Organism	4	2	4	1	4	3	4	5	2	1	2	3	2	5	1	3	1	5	3	5
1	1	1	1	0	1	0	1	1	1	0	1	0	1	1	0	0	0	1	0	1
2	1	0	1	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	0	1
3	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	1
4	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1
5	0	1	0	1	0	0	0	0	1	1	1	0	1	0	1	0	1	0	0	0
6	0	1	0	0	0	0	0	1	1	0	1	0	1	1	0	0	0	1	0	1
7	0	1	0	1	0	0	0	1	1	1	1	0	1	1	1	0	1	1	0	1
8	1	0	1	0	1	1	1	0	0	0	0	1	0	0	0	1	0	0	1	0

Number of organisms in each unique combination of 2 two-state tests

Combination	n	n	n	n	n	n	n	n	n	n
11	2	0	2	3	2	1	4	0	1	1
10	2	4	2	1	3	4	1	2	1	1
01	3	2	0	3	0	1	2	2	5	5
00	1	2	4	1	3	2	1	4	1	1
S*	23	20	20	22	21	21	21	20	18	18

* $S = \frac{1}{2}[N^2 - (n_{11}^2 + n_{10}^2 + n_{01}^2 + n_{00}^2)]$.

Table 4. *Possible combinations of 5 tests taken 3 at a time (from Table 2) and the number of organisms that are separated into each unique combination of 2 two-state tests*

	Combination of tests																											
Organism	4	2	1	4	2	3	4	2	5	4	1	3	4	1	5	4	3	5	2	1	3	2	1	5	2	3	5	1	3	5
1	1	1	0	1	1	0	1	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	0	1	1	0	1	0	0	1
2	1	0	0	1	0	0	1	0	1	1	0	0	1	0	1	1	0	1	0	0	0	0	0	1	0	0	1	0	0	1
3	1	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1	0	1	1	1	1	0	1	1
4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1
5	0	1	1	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	1	1	0	1	1	0	1	0	0	1	0	0
6	0	1	0	0	1	0	0	1	1	0	0	0	0	0	1	0	0	1	1	0	0	1	0	1	1	0	1	0	0	1
7	0	1	1	0	1	0	0	1	1	0	1	0	0	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1	0	1
8	1	0	0	1	0	1	1	0	0	1	0	1	1	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	1	0

Number of organisms in each unique combination of 3 two-state tests

Combination	n	n	n	n	n	n	n	n	n	n
111	0	1	2	0	0	1	0	1	1	0
110	2	1	0	0	0	1	2	1	0	0
101	0	1	1	2	3	2	1	3	3	1
100	2	1	1	2	1	0	2	0	1	1
011	2	0	2	0	1	0	0	0	0	1
010	1	3	1	2	1	0	0	0	1	1
001	0	0	1	0	2	3	1	2	2	4
000	1	1	0	2	0	1	2	1	0	0
S* =	25	25	26	24	24	24	25	24	24	22

* $S = \frac{1}{2}[N^2 - (n_{111}^2 + n_{110}^2 + n_{101}^2 + n_{100}^2 + n_{011}^2 + n_{010}^2 + n_{001}^2 + n_{000}^2)]$.

The reduction of data for finding the 3 first best tests is presented in Table 4. The highest S value is 26 for the combination of tests 4, 2 and 5, and these tests are selected for the first 3 which effect maximum separation.

Gyllenberg (1964) used organism versus organism matrices to determine the completeness of the test set. A test that separates 2 organisms is entered into the proper space of the matrix (a 'point of separation') and when all parts of the matrix are filled, the test set is complete and all organisms may be separated. The number of spaces to be filled in the matrix is $t(t-1)/2$ (t = number of organisms) or these are the number of spaces to be filled below the diagonal line in Table 5. The top right-hand side of the table is not filled-in because it is a mirror image of the bottom left-hand side of the table. There are 28 spaces ($t(t-1)/2 = 8(8-1)/2 = 28$) to be filled-in. This is the same answer given by equation (1) for the ideal conditions that 3 tests would separate completely 8 organisms. Gyllenberg's (1964) method, $t(t-1)/2$, is less cumbersome.

Table 5. *An organism versus organism matrix in which the test number* that results in a separation of 2 organisms is recorded in the appropriate square*

	Organism 1	2	3	4	5	6	7	8
1								
2	2							
3		2						
4	2, 4	4	2, 4					
5	4, 5	2, 4, 5	4, 5	2, 5				
6	4	2, 4	4	2	5			
7	4	2, 4	4	2	5			
8	2, 5	5	2, 5	4, 5	4, 2	2, 4, 5	2, 4, 5	

* Test numbers refer to test combination 4, 2, and 5 which has an S value of 26. This value agrees with the number of spaces filled in the matrix above. See Table 4.

However, equation (1) can be used directly to determine the number of spaces that have been filled by the selection of the first, second, third,..., etc., tests—and this obviates the use of matrix analysis. By counting the spaces in Table 5 occupied by tests 2, 4, and 5, or any combination of these tests, it can be determined that the number of spaces occupied in the matrix agrees with the answers obtained by using equation (1). When two or three of the tests occur together in the same space in the matrix they are counted only once.

Equation (1) also may be used if test results are variable but in this case the entire table of organisms and their test results must be expanded first to indicate the possible

unique combinations. For example, if organism *A* is variable (v or 2) for two of five tests there are 4 possible combinations of results for 2 two-state tests. The expansion of the results for organism *A* would be as follows:

	Tests				
Organism	1	2	3	4	5
A	1	2	0	1	2
A_1	1	1	0	1	1
A_2	1	1	0	1	0
A_3	1	0	0	1	1
A_4	1	0	0	1	0

Tests 2 and 5 are variable and there are 4 possible combinations ($2^{T_v} = 2^2 = 4$) for 2 variable tests, 11, 10, 01, and 00. Test 2, for the 4 possible combinations is written in the sequence: 1, 1, 0, 0, and test 5: 1, 0, 1, 0. This gives 4 unique combinations of results for organism *A*.

If equation (1) is used to determine the first test by this method the most variable test, in general, will be selected because the number of 1 and of 0 reactions would be about equal after expansion of the table. In addition, the method is not efficient when a large number of variable characters occur. If 30 characters for an organism were being considered, and 10 of the characters were variable, the expansion of the table for this organism would be $2^{T_v} = 2^{10} = 1024$. In actual practice this situation frequently arises because of lack of information. Characters for which no data are available are considered variable on the assumption that the character must be either present or absent.

When some tests are considered variable. In the diagnostic laboratory or when bacterial flora studies are undertaken and it is not known on the basis of previous experience what organisms may be identified, the study will only be as comprehensive as the primary environmental conditions used for isolation and the inclusiveness of the keys to be used for identification. This situation is complicated further because classifications into which organisms are to be identified are dynamic and not static. That is, as more information becomes available justification can be found for reclassification. This, in a sense, also reveals the dynamic state of bacterial populations.

Ideally, in either case, that of the diagnostic laboratory or larger bacterial flora studies, the keys constructed for identification should be as inclusive as possible. If this is done the concept of the minimum test set of Gyllenberg (1963) becomes important particularly in regard to the volume of work and the time required for inoculation of media and reading of tests. That is, unless a laboratory has unlimited resources of time and personnel the shortest route to an identification must be taken.

A separate table is used to collect data for each organism to be considered. The data for all organisms then are compiled into one large table and from this the minimum set of tests required for maximum separation of the organisms is determined. In constructing tables of organisms and their test results it is necessary to decide what constitutes a positive reaction, a negative reaction, and a variable reaction. This may vary depending upon the findings of different investigators. A very rough approximation was made by analysing the results of each test for a species as designated by various investigators, and the test results were divided into 4 approximate confidence levels, 99, 95, 90, and 80%. This is illustrated in Table 6 for *Escherichia coli.*

All references agree mannitol fermentation is positive 99% or more of the time and this is recorded positive at all 4 confidence levels. Lactose fermentation is considered positive by 4 references, 90·5% positive and 5·5% delayed positive by Edwards & Ewing (1964), and 92% positive by Møller (1954). Lactose fermentation is summarized variable at the 99 and 95% confidence levels and positive at 90 and 80%. In general, delayed positive reactions are considered negative and for this reason citrate utilization is summarized as negative at all four confidence levels. Qualitative data for variable reactions such as that found in *Bergey's Manual* (1957) arbitrarily were given precedence over quantitative data and the summary for the reaction would be considered variable. This approach is biased and allows for more variation and fewer separations. However, on the basis of the minimum test set it is desired to include, and not exclude, as many organisms as possible. Quantitative data using the same standardized test procedures will be required before significant results can be obtained for variable reactions.

Table 6. *Approximating confidence levels of test results for Escherichia coli*

	Tests		
Source*	Mannitol	Lactose	Citrate
a	+	+	−
b	+ > 99%	+90·5% (5·5%)†	+0·3% (0·8%)†
c	+	+	−
d	+	+	−
e	+	+92%	−9*x*‡
f	+	+	−
	Estimate of confidence level		
99%	+	*v*	−
95%	+	*v*	−
90%	+	+	−
80%§	+	+	−

* Data from: *a*, *Bergey's Manual* (1957); *b*, Edwards & Ewing (1964); *c*, Lysenko (1959); *d*, Cowan (1956); *e*, Møller (1954); *f*, Kauffmann (1956).

† Figures in parentheses represent percentage delayed positive reactions which arbitrarily are considered negative.

‡ 91% of strains negative, 9% delayed or weakly positive.

§ If data from Cowan & Steel (1961, 1965) are applicable the confidence level is 80% because *a*+ or − means 80–100%.

In Table 7 will be found data for ten organisms in the Enterobacteriaceae. The confidence level is approximately 80%. Two different procedures may be used to determine if sufficient tests are included to give complete separation of the organisms in the group. The test results for each organism may be compared with those of each other organism. The results for organism 1 are compared with those of organisms 2, 3, 4, etc., and whenever a 1–0 or 0–1 combination occurs there is separation. If no 1–0 or 0–1 combination occurs the organisms cannot be separated. Next, results for organism 2 are compared against organisms 3, 4, 5, etc., and this process is continued until all comparisons are made. When this is completed it is found that organisms 1, 2, 4, 8, and 10 are separated, and that the following organisms are not separated, 3 from 7, 5 from 7 and 9, 6 from 7, and 7 from 9. The data in Table 7 also may be used

as input for the computer and the output is a listing of the organisms separated and the tests required for separation. The organisms not separated are not listed.

From this type of analysis, either manual or automatic, it is known that the test set is incomplete and not all the organisms are separated as single answers. To keep the illustration within bounds, however, these data will be used to determine which minimum set of tests will result in the most efficient separation of the organisms.

Table 7. *Test results* for 10 organisms in the enterobacteriaceae*

		Tests							
No.	Organisms	1 LD	2 KCN	3 H_2S	4 LAC	5 OD	6 ARAB	7 INOS	8 CIT
1	*Escherichia coli*	2	0	0	1	2	1	0	0
2	*E. freundii*	0	1	1	1	2	1	2	2
3	*Aerobacter aerogenes*	1	1	0	1	1	1	1	2
4	*A. cloacae*	0	1	0	1	2	1	0	2
5	*Klebsiella pneumoniae*	1	2	0	2	0	1	2	1
6	Arizona group	1	0	1	2	1	1	0	2
7	Serratia	1	2	2	2	2	2	2	1
8	*Proteus vulgaris*	0	1	1	0	0	0	0	2
9	*Salmonella typhi*	1	0	2	0	0	2	2	2
10	*Shigella dysenteriae*	0	0	0	0	0	2	0	0
	n_1	5	4	3	4	2	6	1	2
	n_0	4	4	5	3	4	1	5	2
	S† =	20	16	15	12	8	6	5	4

* A positive reaction (1) or negative reaction (0) means positive or negative approximately 80% of the time. Variable reactions are designated 2. LD = lysine decarboxylase, LAC = lactose, OD = ornithine decarboxylase, ARAB = arabinose, INOS = inositol, CIT = citrate.

† $S = \frac{1}{2}[N^2-(n_1^2+n_0^2)] = n_1 n_0$. Variable test results, designated 2, are not used in the calculation.

The first best test is based upon the highest S value. In Table 7 the tests are arranged in descending order of their S values as indicated at the bottom of the table. In calculating S values, reactions which are variable, designated by 2, are omitted and only the 1 and 0 reactions are considered. It will be noted in Table 7, in general, that the S values decrease as the number of variables increases. An inversion occurs with tests 5 and 6, i.e. test 5 has 4 variables and test 6 has 3 variables and their S values are 8 and 6 respectively.

To find the first 2 best tests, there are 28 possible combinations of 8 tests taken 2 at a time. However, in the automatic selection of the first 2 best tests, the computer does not make every possible combination. After the selection of the first best test, each remaining test is used with the first test and the combination that effects the largest number of separations is selected for the first 2 best tests. In this case 7 combinations of 2 tests were made, i.e. 1–2, 1–3, 1–4, 1–5, 1–6, 1–7, and 1–8. The combination of tests 1–2 and 1–3 each effect 29 separations which is the maximum separation for any 2 tests. This is illustrated in an organism versus organism matrix in Table 8. Note there are 45 spaces in the matrix to be occupied under the diagonal line. The separation spaces (SS) for any combination of 2 tests is: SS_{test_1} + (number of times test being compared with 1 occurs independent of 1). The computer selects both combinations 1–2 and 1–3 for the first 2 best tests and compares each remaining test with these 2 combinations to find that combination of 3 tests that effect maximum separation of 10 organisms. Test combinations 1–2–3 and 1–2–4 effect 33 separations each, but com-

bination 1–3–4 effects 34 separations and this is selected for the best combination of 3 tests.

By the first analysis it was found the test set was incomplete and organisms could not be separated: 3 from 7, 5 from 7 and 9, 6 from 7, and 7 from 9. This means there are 5 separation spaces that cannot be occupied. The total number of spaces is 45, therefore, the maximum number of spaces that can be filled is 40.

Table 8. *An organism versus organism matrix in which the test number* that results in a separation of 2 organisms is recorded in the appropriate square*

Organism	1	2	3	4	5	6	7	8	9	10
1										
2	2, 3									
3	2	1, 3								
4	2	3	1							
5		1, 3		1						
6	3	1, 2	2, 3	1, 2, 3	3					
7		1		1						
8	2, 3		1, 3	3	1, 3	1, 2	1			
9		1, 2	2	1, 2				1, 2		
10		2, 3	1, 2	2	1	1, 3	1	2, 3	1	

* Test numbers refer to test combinations 1 and 2 or 1 and 3, each combination effecting 29 separations. To determine the number of separations for each combination, the separation spaces (*SS*) are found by: SS_{test_1} + (number of times test being compared with 1 occurs independent of 1).

Ten organisms ideally could be separated by a minimum test set of 4. The number of combinations of 8 tests taken 4 at a time is 70,

$$C_4^8 = \frac{8 \cdot 7 \cdot 6 \cdot 5}{4!} = 70.$$

The computer, however, only makes 5 of these: 1–3–4–5, 1–3–4–6, 1–3–4–7, 1–3–4–8, and 1–3–4–2 which effect the following number of separations, 36, 34, 35, 36, and 36 respectively. Three combinations of 4 tests occupy 36 separation spaces which is the maximum for these tests. Any one of these combinations could be selected as the minimum set. Maximum separation occurs if 40 spaces are occupied; the minimum set occupies 36, and therefore separation is 90 % completed. Four two-state tests have 16 unique combinations ($2^4 = 16$). The number of organisms separated into these 16

possible unique combinations by test combinations 1–3–4–5, 1–3–4–8, and 1–3–4–2 are illustrated in Table 9. Manual analysis of these data indicates that another combination 1–2–4–5 also gives 36 'points of separation' and that the computer did not select this combination.

The largest subgroup for any combination of 4 tests contains 3 organisms. The organisms and the remaining tests in any subgroup that contain 2 or 3 organisms are used as input again and the output is the test or tests that effect separation. This process is continued until separation is complete or until no further separations can be made.

Table 9. *Separation of 10 organisms by 3 minimum sets of 4 tests*

	Minimum tests											
	a				*b*				*c*			
Organisms	1	3	4	2	1	3	4	5	1	3	4	8
1	2	0	1	0	2	0	1	2	2	0	1	0
2	0	1	1	1	0	1	1	2	0	1	1	2
3	1	0	1	1	1	0	1	1	1	0	1	2
4	0	0	1	1	0	0	1	2	0	0	1	2
5	1	0	2	2	1	0	2	0	1	0	2	1
6	1	1	2	0	1	1	2	1	1	1	2	2
7	1	2	2	2	1	2	2	2	1	2	2	1
8	0	1	0	1	0	1	0	0	0	1	0	2
9	1	2	0	0	1	2	0	0	1	2	0	2
10	0	0	0	0	0	0	0	0	0	0	0	0

Organisms separated into the unique combinations of 4 two-state tests

Combination	Organisms *a*	Organisms *b*	Organisms *c*
1111	7	6, 7	6, 7
1110	6, 7	7	6
1101	7	6, 7	6, 7, 9
1100	6, 7, 9	7, 9	6, 9
1011	3, 5, 7	1, 3, 7	3, 5, 7
1010	1, 5, 7	1, 5, 7	1, 3
1001	5, 7	7	5, 7, 9
1000	5, 7, 9	5, 7, 9	9
0111	2	2	2
0110	None	2	2
0101	8	None	8
0100	None	8	8
0011	4	1, 4	4
0010	1	1, 4	1, 4
0001	None	None	None
0000	10	10	10

The programme for the automatic selection of a minimum set of tests agrees, in general, with the manual analysis. Inversions, however, may occur occasionally, but a programme can be devised that will analyse every possible combination of tests.

RESULTS

Different laboratories may identify the same organism by use of various determinative schemes, that is, different routes to an identification may be used. An attempt has been made, using modified procedures of Gyllenberg (1963, 1964) to illustrate a

generalized method of reducing data of organisms such that the shortest route to an identification will be taken. This entails determining which tests, on the basis of available information, can give maximum separation of organisms in a group. Two examples are used to illustrate these methods.

Selection of 6 tests from 32 tests to separate 36 organisms in the Enterobacteriaceae

In the first example 36 organisms in the Enterobacteriaceae are used. The data for 32 test results for the organisms are reduced to determine which 6 tests would effect maximum separation of the organisms into 64 possible unique combinations ($2^6 = 64$). Data are not available for all organisms and all tests. When this situation is encountered the reaction is considered variable (*v* or 2) on the assumption that the test must be either 1 or 0. The confidence level is approximately 80 % but it is emphasized this is biased in favour of indicating more reactions variable.

In general, the classification and nomenclature of *Bergey's Manual* (1957) or Cowan & Steel (1961, 1965) were used. For this reason, delayed positive reactions are considered negative for the first 24–48 hr in order to differentiate *Paracolobactrum* species from *Escherichia coli*, *E. freundii*, *E. intermedium*, *Aerobacter aerogenes*, and *A. cloacae*. The latter organisms, although lactose negative some of the time, are all considered lactose positive. This is illustrated in Table 10, the input for the computer, which is a summary of test results for the 36 organisms. The following references: *Bergey's Manual* (1957), Cowan (1956), Cowan & Steel (1961, 1965), Davis, Ewing & Reaves (1957), Edwards & Ewing (1964), Kauffmann (1956), Manclark & Pickett (1961), and Møller (1954), were used to prepare summaries similar to that illustrated in Table 6.

With 36 organisms there are a total of 630 separation spaces ($t(t-1)/2 = 36{\cdot}35/2 = 630$) to be occupied. The 6 first tests selected to effect maximum separation and the number of separations are indicated in Table 11. Separation is 75·7 % completed by use of these 6 tests ($477/630 \times 100 = 75{\cdot}7\,\%$). Table 12 indicates the subgroups of organisms and which one of the unique combinations each subgroup occupies after use of the minimum test set. Seventeen of the 64 combinations are not occupied, 11 contain 1 organism, 9 contain 2 organisms, 4, 3 organisms, 13, 4 organisms, 3, 5 organisms, 1, 6 organisms, and 6, 7 organisms.

Each of the subgroups with more than 1 organism are separated further using the organisms in the subgroup and the remaining 26 tests as input with an output of the best tests required to effect maximum separation. A minimum set, dependent upon the number of organisms in the subgroup to be separated, is not determined necessarily in these cases. Tests for separation may be selected at this point until all organisms are separated or until further separation is impossible.

Selection of 3 tests from 34 tests to separate 8 species of Pasteurella

In the second example, the data of Smith & Thal (1965), used in a numerical taxonomic study of 8 species of the genus *Pasteurella*, was used for input (Table 13). The output was the minimum test set and the organisms separated into each of the possible combinations of 3 two-state tests (Table 14). The number of separation spaces to be occupied is 28.

Table 10. *Data used to determine the minimum set of 6 tests that result in maximum separation of 36 organisms*

	KCN	Mannitol	Lactose	Glucose (gas)	Lysine	Sorbitol	Arabinose	Voges-Pros.	Salicin	Indole	Malonate	Citrate	Phenylalanine	Urease	Inositol (acid)	Maltose	Ornithine	Rhamnose	Gelatin	Adonitol	Motility	Sucrose	H_2S	Xylose	Methyl red	Dulcitol	Glutamic acid	Raffinose	Arginine	Glucose (acid)	Inositol (gas)	Nitrate red
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1 *Escherichia coli*	0	1	1	1	2	2	1	0	2	2	0	0	0	0	0	2	2	2	0	0	2	2	0	2	1	2	1	2	2	1	2	1
2 *E. aurescens*	2	1	1	1	2	2	2	0	1	1	2	0	2	2	2	2	2	2	0	0	1	2	0	2	1	0	2	0	2	1	2	2
3 *E. freundii*	1	1	1	1	0	1	2	0	1	2	2	0	0	2	2	1	2	1	0	0	2	2	0	1	1	2	2	0	2	1	2	1
4 Bethesda–Ballerup group	1	1	0	1	0	1	1	0	2	2	0	2	0	2	0	1	2	1	0	0	1	2	0	1	1	2	2	0	2	1	2	2
5 *Escherchia intermedia*	2	1	1	1	2	2	1	0	2	2	0	1	2	2	2	1	2	1	0	0	1	2	0	1	1	2	2	0	2	1	2	2
6 *Aerobacter aerogenes*	1	2	1	1	1	1	1	1	2	2	2	2	0	2	1	1	1	2	2	2	2	2	1	2	0	2	2	2	2	1	2	2
7 *A. cloacae*	1	1	1	1	2	1	1	1	2	2	0	2	0	2	1	2	2	2	2	1	2	2	0	2	1	2	2	2	2	1	2	2
8 *A. liquefaciens*	1	1	2	1	1	1	1	2	1	0	0	2	0	2	1	2	1	2	1	0	2	2	0	2	1	2	2	0	2	1	2	2
9 *Klebsiella pneumoniae*	2	1	2	1	1	1	1	2	1	2	1	2	0	2	2	1	0	2	0	1	0	2	0	1	1	2	2	0	2	1	2	2
10 *K. ozaenae*	2	1	2	2	0	1	2	1	1	0	1	0	0	2	1	1	0	2	0	1	0	2	0	1	1	2	2	0	2	1	2	2
11 *K. rhinoscleromatis*	2	1	2	1	1	2	1	0	1	1	1	1	0	2	2	1	0	2	0	2	0	2	0	2	1	2	2	0	2	1	2	2
12 *K. aerogenes*	1	1	1	1	1	2	1	1	2	0	2	1	0	2	2	2	1	2	0	2	1	2	0	2	0	2	2	0	2	1	2	2
13 *K. oxytoca*	1	1	1	1	1	2	1	1	2	1	2	1	0	2	2	2	1	2	0	2	1	2	0	2	1	2	2	0	2	1	2	2
14 *K. edwardsii* var. *edwardsii*	1	1	0	0	1	2	2	1	2	0	2	2	0	2	2	2	1	2	0	2	2	2	0	2	1	2	2	0	2	1	2	2
15 *K. edwardsii* var. *atlantae*	1	1	1	0	1	2	2	2	2	0	2	1	0	2	2	1	2	2	0	2	2	2	0	2	1	2	2	0	2	1	2	2
16 Hafnia group	1	1	2	2	1	1	2	1	2	2	0	2	0	2	2	1	2	2	0	2	2	2	0	2	1	2	2	0	2	1	2	2
17 *Paracol. aerogenoides*	1	2	0	1	2	1	2	0	2	2	2	2	0	2	2	1	1	2	0	2	2	2	0	2	1	2	2	0	2	1	2	2
18 *P. intermedium*	1	1	0	1	1	2	1	1	2	2	2	2	0	2	2	1	2	1	0	2	2	2	0	2	1	2	2	0	2	1	2	2
19 *P. arizonae*	0	1	2	1	1	1	1	0	2	2	1	2	0	2	0	2	2	2	0	2	1	2	0	2	1	2	2	0	2	1	2	2
20 *P. coliforme*	0	1	0	1	2	1	2	0	2	2	0	2	0	2	0	2	2	2	0	2	2	2	0	2	1	2	2	0	2	1	2	2
21 Alginobacter	2	1	1	1	2	2	1	2	1	2	0	2	0	2	0	2	2	2	0	2	1	2	0	2	1	2	2	0	2	1	2	2
22 Erwinia	1	2	2	1	2	1	0	2	1	2	2	1	0	2	2	2	2	2	0	2	1	2	1	2	1	2	2	0	2	1	2	2
23 Serratia group	2	1	2	2	1	2	2	0	2	1	2	1	0	2	2	1	2	2	1	2	1	2	1	2	1	2	2	0	2	1	2	2
24 *Proteus vulgaris*	1	0	0	2	0	0	2	0	2	1	0	2	1	1	0	1	0	2	1	0	1	2	1	1	1	0	2	0	2	1	2	2
25 *P. mirabilis*	1	0	0	2	0	0	2	0	2	0	0	2	1	1	0	1	0	2	1	0	1	2	1	1	1	0	2	0	2	1	2	2
26 *P. morganii*	1	0	0	2	0	0	2	0	2	1	0	2	1	1	0	1	0	2	0	1	0	2	0	1	1	0	2	0	2	1	2	2
27 *P. rettgeri*	1	2	0	0	2	0	2	0	2	1	0	2	1	1	2	1	0	2	0	1	0	2	0	2	1	2	2	0	2	1	2	2
28 *P. inconstans*	1	2	0	0	2	0	2	0	2	1	0	2	1	1	2	1	0	2	0	1	0	2	0	2	1	2	2	0	2	1	2	2
29 *Salmonella typhi*	0	1	0	0	2	1	2	0	2	0	0	2	0	2	2	1	0	2	0	2	0	2	0	2	1	2	2	0	2	1	2	2
30 Other salmonellas	0	1	0	0	2	1	2	0	2	0	0	2	0	2	2	1	0	2	0	2	0	2	0	2	1	2	2	0	2	1	2	2
31 Other shigellas	2	0	0	0	2	0	2	0	2	0	0	2	0	2	2	1	0	2	0	2	0	2	0	2	1	2	2	0	2	1	2	2
32 *Shigella dysenteriae*	0	0	0	0	2	0	2	0	2	0	0	2	0	2	2	1	0	2	0	2	0	2	0	2	1	2	2	0	2	1	2	2
33 *S. boydii*	0	0	0	0	2	0	2	0	2	0	0	2	0	2	2	1	0	2	0	2	0	2	0	2	1	2	2	0	2	1	2	2
34 *S. flexneri*	0	0	2	0	2	0	2	0	2	0	0	2	0	2	2	1	0	2	0	2	0	2	0	2	1	2	2	0	2	1	2	2
35 *S. sonnei*	0	1	2	0	2	0	1	0	2	0	0	2	0	2	2	1	0	2	0	2	0	2	0	2	1	2	2	0	2	1	2	2
36 Alkalescens–Dispar group	0	1	2	0	2	1	2	0	2	2	0	0	0	0	0	1	2	1	0	0	0	2	0	1	1	2	1	2	2	1	2	2

When the S values for each test are calculated, 3 of the 34 tests, 6, 7, and 17, have a maximum S value of 16. This meets the first part of the idealization previously illustrated (Table 1) that the first 3 tests should have an equal number of 1 and of 0 test results. However, the second part of the idealization is not fulfilled because the alternating sequences of 1's and 0's are not 11110000, 11001100, and 10101010. The S value for these 3 tests is 26 which is not the highest possible value for a combination of 3 of the tests.

Table 11. *The first 6 tests and the number of separations effected by successive use of these tests*

Tests						'Points of separation'
1	2	3	4	5	6	
KCN	—	—	—	—	—	180
KCN	LAC*	—	—	—	—	293
KCN	LAC	LD*	—	—	—	367
KCN	LAC	LD	ADON*	—	—	419
KCN	LAC	LD	ADON	H_2S	—	452
KCN	LAC	LD	ADON	H_2S	MALT*	477

* LAC = lactose, LD = lysine decarboxylase, ADON = adonitol, MALT = maltose.

Table 12. *Organisms separated into the 64 possible combinations for 6 two-state tests by the minimum set of 6 tests*

	Combination	Organisms		Combination	Organisms
1	111111	21, 23	33	011111	21, 23
2	111110	None	34	011110	None
3	111101	5, 6, 9, 10, 12, 13, 23	35	011101	5, 9, 10, 23
4	111100	12	36	011100	None
5	111011	16, 21, 23	37	011011	19, 21, 23
6	111010	22	38	011010	None
7	111001	2, 5, 6, 8, 12, 16, 23	39	011001	1, 2, 5, 23, 36
8	111000	2, 8, 12, 22	40	011000	1, 2
9	110111	21	41	010111	21
10	110110	None	42	010110	None
11	110101	5, 7, 10, 11	43	010101	5, 10, 11, 35
12	110100	None	44	010100	None
13	110011	3, 21	45	010011	21
14	110010	22	46	010010	None
15	110001	2, 5, 7, 8	47	010001	1, 2, 5, 35, 36
16	110000	2, 8, 22	48	010000	1, 2
17	101111	23	49	001111	23
18	101110	None	50	001110	None
19	101101	9, 10, 14, 15, 17, 23, 31	51	001101	9, 10, 23, 31
20	101100	14, 15	52	001100	None
21	101011	16, 23	53	001011	19, 23, 29, 30
22	101010	22	54	001010	30
23	101001	8, 14, 15, 16, 17, 23, 31	55	001001	20, 23, 29, 30, 31, 33, 36
24	101000	8, 14, 15, 22	56	001000	20, 30, 33
25	100111	18, 28	57	000111	None
26	100110	28	58	000110	None
27	100101	10, 11, 17, 18, 28, 31	59	000101	10, 11, 31, 35
28	100100	27, 28	60	000100	None
29	100011	4, 18, 24, 28	61	000011	None
30	100010	22, 25, 26, 28	62	000010	None
31	100001	8, 17, 18, 28, 31	63	000001	20, 31, 32, 33, 34, 35, 36
32	100000	8, 22, 26, 28	64	000000	20, 32, 33, 34

Table 13. *Data from Smith & Thal (1965) used to determine the minimum set of 3 tests that results in maximum separation of 8 pasteurellas*

Organisms	*	1	2	3	4	5	6	7	8
Motility, 22°	1	0	0	0	0	0	1	0	0
Haemolysis	2	0	0	1	1	1	0	0	0
MacConkey	3	0	1	0	1	1	0	0	0
Indole	4	1	0	1	0	0	1	0	0
Voges–Proskauer	5	0	0	0	0	0	0	1	0
Methyl red	6	0	1	0	0	0	1	0	0
Urease	7	0	1	0	0	0	1	0	0
KCN	8	0	2	0	0	0	2	0	0
Oxidase	9	0	1	0	1	1	0	1	0
Litmus	10	0	0	1	0	0	1	0	0
Lysine decarboxylase	11	0	0	0	0	0	0	0	0
Arginine decarboxylase	12	0	0	0	0	0	0	0	0
Ornithine decarboxylase	13	0	0	0	1	0	0	0	0
Aesculin	14	0	0	0	0	0	0	0	0
Adonitol	15	0	0	0	0	0	0	0	0
Amygdalin	16	0	2	0	0	1	0	0	0
Arabinose	17	0	0	0	0	0	1	0	0
Cellobiose	18	0	0	0	0	1	0	0	0
Dextrine	19	1	1	0	0	0	1	0	0
Galactose	20	1	1	0	0	0	1	0	0
Glycerol	21	2	0	0	0	1	0	0	0
Glycogen	22	0	0	0	0	0	0	0	0
Inositol	23	0	0	0	0	0	0	0	0
Maltose	24	1	1	0	0	0	1	0	0
Mannitol	25	0	1	0	0	0	1	0	0
Mannose	26	1	1	0	0	0	1	0	0
Raffinose	27	2	0	0	0	0	0	0	0
Rhamnose	28	0	0	0	0	0	0	0	0
Salicin	29	0	0	0	0	0	1	0	0
Sorbitol	30	0	1	0	0	0	1	0	0
Starch	31	1	0	0	0	0	1	0	0
Sucrose	32	1	1	0	0	0	1	0	0
Trehalose	33	1	1	0	0	0	1	0	0
Xylose	34	0	0	0	0	0	1	0	0
n_1		2	3	4	3	1	4	4	0
n_0		6	5	3	5	7	4	4	5
S'		12	15	12	15	7	16	16	0

* 1, *Pasteurella pneumotropica*; 2, *P. hemolytica* var. *ureae*; 3, *P. multocida*; 4, *P. hemolytica* type A; 5, *P. hemolytica*, type T; 6, *Pasteurella* X; 7, *P. pestis*; 8, *P. pseudotuberculosis*.

For the first 2 tests 6–4, 6–2, and 6–29 each have an S value of 23. However, for the first 3 tests 2, 29, and 7 are selected. This combination results in 27 separations of the required 28 (Table 14). A fourth test, for example, methyl red, used with the first 3, will separate all the organisms. This type of analysis suggests that input data used in numerical taxonomic studies may be used for constructing identification schemes.

Table 14. *Separation of 8 species of Pasteurella into 8 possible combinations of 3 two-state tests*

	Tests		
	2	29	7
Organisms	Haemolysis	Salicin	Urease
1. *P. pneumotropica*	0	0	1
2. *P. hemolytica* var. *ureae*	1	0	1
3. *P. multocida*	0	0	0
4. *P. hemolytica*, type A	1	0	0
5. *P. hemolytica*, type T	1	1	0
6. *Pasteurella* X	0	0	1
7. *P. pestis*	0	1	0
8. *P. pseudotuberculosis*	0	1	1

Separation of organisms into 8 possible combinations of 3 two-state tests

Combination	Number (n)	Organisms
111	0	None
110	1	*P. hemolytica*, type T
101	1	*P. hemolytica* var. *ureae*
100	1	*P. hemolytica*, type A
011	1	*P. pseudotuberculosis*
010	1	*P. pestis*
001	2	*P. pneumotropica Pasteurella* X
000	1	*P. multocida*

DISCUSSION

With increasing interest in methods of classification in the biological sciences it is important that accurate, rapid, and practical methods be devised for identification of organisms that can keep pace with increasing information content. The method described here involves establishing the number of organisms to be separated. This determines the number of tests required for the minimum set as calculated by Gyllenberg's (1963) $T_{min} = \log_2 G$. If each of the tests in the minimum set is considered variable, the number of possible combinations of sequential positive (+ or 1) and negative (− or 0) test results is 2^{T_v} or 2 raised to the power of the number of tests (T) in the minimum set all of which are considered the variable (v). When all possible combinations are constructed this represents the 'logically possible patterns of traits' as described by Birnbaum & Maxwell (1960). These patterns cover all possibilities of test results for the minimum set for all organisms in the group and do not exclude the use of tests for characters that may be variable for some organisms. This makes it possible to account for known variation in the organisms—or in the sensitivities of different test procedures that may be used to determine the presence or absence of the same trait or character. Once the minimum set is determined on the basis of having the highest number of separations of any combination of tests which the computer is

programmed to check, a table is constructed of the output which lists all the possible combinations of results for the minimum set and which organisms are placed in each unique combination. This is similar to Table 12 and the bottom of Table 14 which list the possible patterns of combinations for the organisms.

Data for the minimum set of tests for an organism to be separated can be scanned readily on this type of table. An organism may be identified by the minimum set if there is only one organism separated into a unique combination or the organism may be separated into a combination containing more than one organism. In the latter case a second table is scanned which contains another minimum set or the tests selected from those remaining tests required to effect identification of the organisms in the subgroup. If some organisms in a subgroup cannot be separated, a programme has been prepared (R.B.) based upon the relative frequencies of positive and negative results for variable tests which give the most likely answers. In most cases, at present, quantitative data are wanting concerning variable reactions. Notable exceptions are Edwards & Ewing (1964) and Møller (1954). This part of the programme is analogous to that described by Payne (1963*a*, *b*) for use in the automatic library facility, and Möller (1962) has given consideration to probablistic identification keys. Not all laboratories have access to automatic facilities and laboratories in remote areas may not have communication with such facilities. For this reason up-dated print-out tables should be made available for manual use. The use of these tables is similar to the 'Determinator' described by Cowan & Steel (1960). In addition, data concerning variable characters may vary geographically and temporarily and the Bayesian approach is suggested (Ledley & Lusted, 1959).

A limitation of the automatic programme is that all possible combinations of tests are not made. That is, some combination of tests are not made and occasionally these could have higher separation values than those combinations that are selected. However, in Table 10, with 36 organisms and 32 tests, a total of 906, 192 combinations of tests would have to be made to find the 32 tests taken 6 at a time that would give the highest number of separations. For this reason a first best test is determined by the separation figure of Gyllenberg (1963) and the tests are arranged in descending order of the S values. Then, each of the remaining tests are used with the first test to determine that combination of 2 tests which give the highest number of separations. On the basis of the highest number of organisms separated by some combination of 2 tests, the tests are rearranged again in descending order of the number of separations. The remaining tests are used with the first 2 to determine the first 3 tests, and this process is continued until the required minimum set is complete.

In a report of a conference on microbial classification, Skerman (1964) introduced the discussion on biochemical tests, and comment was made that standardization of tests may be required for each group of organisms. Discrimination was not made concerning which tests were to be considered for use in identification. In the summary of tests for characters in Table 10 it was not possible always to know if all tests were done by the same procedures. This means there may be an additional bias in this table. For diagnostic purposes some of the tests no doubt could be considered unsatisfactory because of the time required before the test could be read positive or negative.

With the limitation in mind, for example, that the method for determining glucose utilization by *Neisseria gonorrhoeae* is different than that for *E. coli* it still is possible to reduce data for a large number of organisms when it is known that the same test

procedure may be used to reveal the presence or absence of a character. A consideration of the environmental conditions necessary for the expression of a character also is useful in identification procedures.

One thing that is needed is more data concerning the characteristics of the bacteria and to reduce this data to determine which tests give the most efficient separations. The diagnostic tables of Cowan & Steel (1961, 1965) help fill this data gap, and Williams (1966) has summarized some of the difficulties of instituting automatic data processing procedures.

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Pectolytic Activity of Phytopathogenic Xanthomonads

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SUMMARY

Cultures of 10 of 19 nomenspecies of the genus *Xanthomonas* liquefied a nutrient pectate gel. None of the 19 nomenspecies produced either hydrolytic polygalacturonase or pectin *trans*-eliminase. Seventeen of the nomenspecies showed a detectable, at times weak, pectinesterase activity. Seven of the 10 pectate-liquefying nomenspecies excreted polygalacturonic acid *trans*-eliminase in culture fluids containing pectin as growth substrate. The enzymological basis, if any, is not at present known for the liquefaction of the nutrient pectate gel in the remaining three xanthomonads, which all lacked both the hydrolytic and the eliminative enzymes. Pectinesterase and polygalacturonic acid *trans*-eliminase were produced inducibly on pectin by most *X. campestris* cultures which were examined; some strains of this phytopathogen formed polygalacturonic acid *trans*-eliminase constitutively on glucose, but pectinesterase was never constitutive. The polygalacturonic acid *trans*-eliminase excreted by the tested xanthomonads degraded polygalacturonic acid in a random manner. The major end-products were unsaturated di- and tri-galacturonic acids, accompanied by lesser amounts of saturated mono-, di-, and tri-galacturonic acids.

INTRODUCTION

The symptoms caused in plants by phytopathogenic bacteria of the genus *Xanthomonas* are sufficiently unlike those induced by the pectolytic soft-rot Erwinia, Bacillus and Pseudomonas species to foster the impression that xanthomonads lack ability to degrade pectic substances. Nevertheless, several reports attest to the pectolytic activity of xanthomonads. For example, one of the present writers had observed two decades ago (Burkholder & Starr, 1948) that 38 of 77 xanthomonad cultures (14 of 25 *Xanthomonas* spp.) could liquefy a pectate gel, although more slowly and less vigorously than did soft-rot erwinias. Similar observations and crude enzymological surveys were subsequently presented by other workers (Sabet & Dowson, 1951; Smith, 1958*a*, *b*; Dye, 1960). By present standards, a certain confusion about the nomenclature of pectic substances and enzymes is evident in some of these writings. An assortment of methodological and interpretative procedures, ingenious or naive, were used; these make almost impossible any intelligent comparisons among the results obtained by individual workers. Moreover, following the discovery by Albersheim, Neukom & Deuel (1960) of fungal pectin *trans*-eliminase (PTE), there have been elucidated eliminative splits of pectic substances in soft-rot phytopathogenic bacteria such as *Erwinia carotovora* (Starr & Moran, 1962) and *Bacillus polymyxa* (Nagel & Vaughn, 1961*b*), in addition to the classical hydrolytic action (Kraght & Starr, 1953) which has recently been re-examined with purified enzyme in *Erwinia carotovora* (Nasuno &

Starr, 1966*a*). Except for our preliminary abstract (Starr & Nasuno, 1963), there has been no published work on the eliminative split of pectic substances by xanthomonads; indeed, not much is known about the enzymology of pectic substances by *Xanthomonas*, by comparison with the knowledge now available for other groups of microbes. A comprehensive study was undertaken with the hope of unravelling the kinds of pectolytic activity in various xanthomonads, and their distribution among the various so-called species (actually, nomenspecies; see Stolp, Starr & Baigent, 1965). The present report surveys the pectolytic capacities of a representative assortment of xanthomonads; the enzymological details with respect to a typical strain will be published separately (Nasuno & Starr, 1967).

METHODS

Chemicals. Pectin N.F. (68% esterified) and polygalacturonic acid (no. 491) were obtained from Sunkist Growers, Inc. (Corona, California); they were used without additional treatment. 0-(4-Deoxy-β-L-threo-hexopyranos-4-enyluronic acid)-(1 → 4)-D-galacturonic acid (unsaturated digalacturonic acid) was kindly supplied by Dr C. W. Nagel (Department of Horticulture, Washington State University, Pullman, Washington). Di-, tri-, and tetra-galacturonic acids were the generous gifts of Dr H. J. Phaff (Department of Food Science and Technology, University of California, Davis, California). Pure D-galacturonic acid was prepared from the commercial product by recrystallization from acetone.

Cultural methods. Stock cultures of 27 species or strains of the genus *Xanthomonas* were obtained from the International Collection of Phytopathogenic Bacteria (ICPB), maintained in this Department; they were grown on slopes of yeast extract, glucose and $CaCO_3$ (YDC) agar during the course of this programme.

A nutrient sodium pectate gel (Starr, 1947) was used for liquefaction tests. This medium consisted of Exchange Brand sodium ammonium pectate (Sunkist Growers, Inc., Corona, California), 3 g.; 10% (w/v) $CaCl_2.2H_2O$, 0·6 ml., N-NaOH, 0·9 ml.; yeast extract (Difco), 1 g.; distilled water, 100 ml.; pH 6·4 after autoclaving.

The basal medium consisted of two parts: (i) pectin N.F., 5 g./200 ml.; (ii) KH_2PO_4, 1·6 g.; Na_2HPO_4, 1·6 g.; $MgSO_4.7H_2O$, 0·2 g.; $CaCl_2.2H_2O$, 0·1 g.; yeast extract (Difco Laboratories, Inc., Detroit, Michigan), 5 g.; total volume, 800 ml. Both solutions were adjusted to pH 6·8 autoclaved separately, and mixed before use. For the calcium-deficient medium, the $CaCl_2$ was omitted.

Bacterial growth in liquid culture was determined as the dry weight of bacteria/ml. culture.

Enzyme preparations. The bacteria were grown at 28° in a basal medium, or in a calcium-deficient medium, containing pectin, on a rotary shaker (3 cm. amplitude and 100 rotations/min.) for 48 hr. When pectin was replaced with glucose, the bacteria were grown for 24 hr, at which time maximum growth had been attained. The culture fluid was centrifuged at 6000 *g* for 10 min., filtered through a 0·45 μ Millipore filter (Millipore Filter Corp., Bedford, Massachusetts), dialysed against distilled water for 24 hr at 4°, and tested for the presence of pectic enzymes.

Assays of pectic enzymes. Heated enzyme was used as a control in all enzyme assays. Pectinesterase (PE) activity was determined by a titration method. The reaction mixture contained 1% (w/v) pectin N.F., 0·1 M-NaCl., and 10% (v/v) enzyme solution in a total volume of 10 ml. The reaction mixtures were adjusted to pH 7·0 after

addition of enzyme, incubated at 30° for 1 hr, and titrated to pH 7·0 with 0·01 N-NaOH at the end of the incubation period. PE activity was expressed in terms of μmoles ester hydrolysed/hr/ml. reaction mixture, under the above conditions.

Polygalacturonic acid *trans*-eliminase (PATE) activity was determined, by spectrophotometry. Reaction mixtures contained 10% (v/v) enzyme solution, in 0·05 M-glycine-NaOH buffer (pH 9·5), in a total volume of 6 ml. Samples (0·5 ml.) were removed immediately after enzyme was added and again after 1 hr incubation at 30°, placed in 4·5 ml. or 9·5 ml. of 0·01 N-HCl to stop enzyme action, and the extinction measured in the Beckman model DU spectrophotometer. The relative activity of PATE is expressed as the increase in extinction at 235 mμ/hr, under the above conditions.

The same principle used in the PATE assay was used for the attempted detection of pectin *trans*-eliminase (PTE) activity. Reaction mixtures containing 10% (v/v) enzyme solution, 0·5% (w/v) pectin N.F., and 0·4 M-NaCl in 0·05 M-sodium acetate buffer (pH 5·2) were incubated at 30° for 1–24 hr, and the extinction measured at 235 mμ (Edstrom & Phaff, 1964 *a*).

The iodometric method of Yemm (1935) was used to detect polygalacturonase (PG) activity by following the release of reducing groups during hydrolysis of the substrate. Replicate flasks were set up containing 10% (v/v) enzyme solution, 0·5% (w/v) polygalacturonic acid, and 0·1 M-NaCl in 0·05 M-sodium acetate buffer (pH 5·2) in a total volume of 5 ml. After suitable periods of reaction (1 hr and 24 hr), the reaction was stopped by adding 0·35 ml. of M-Na_2CO_3 and then 2 ml. of the iodine solution were added; the initial values were obtained by adding the Na_2CO_3 before the enzyme. After 20 min., the reaction mixture was acidified with 0·8 ml. of 2N-H_2SO_4 and the liberated iodine was titrated with 0·01 N-$Na_2S_2O_3$ with starch as indicator.

Paper chromatography. After 24 hr of incubation, samples (1 ml.) were taken from each reaction mixture for paper chromatography. The tubes containing these samples were immersed in boiling water for 5 min., and the samples dried at 70° over $CaCl_2$ under vacuum after removing cations with Dowex 50 cation exchange resin in the hydrogen form. The residues were dissolved in 0·1 ml. distilled water, and 10 μl. of each sample was chromatographed on Whatman no. 4 paper. The solvent was a mixture of pyridine + ethyl acetate + acetic acid + water (5 + 5 + 1 + 3 by vol.) applied for 18 hr at room temperature. The marker spots were mono-, di-, tri-, and tetragalacturonic acids, and unsaturated di-galacturonic acid. The silver nitrate reagent (Block, Durrum & Zweig, 1955) was used to detect saturated and unsaturated oligogalacturonides and galacturonic acid. Under these conditions, 0·02 μmole galacturonic acid gives a definite black spot at room temperature. Unsaturated compounds were also located by spraying with a solution containing 0·01 % (w/v) quinine sulphate and 0·04 N-sulphuric acid in 95% (v/v) ethanol in water (Edstrom & Phaff, 1964 *b*). After drying at room temperature, the presence of the unsaturated compounds was detected under ultraviolet radiation as a dark spot on a light violet to white fluorescent field. Amounts of less than 0·2 μmole of unsaturated digalacturonic acid were readily located by this method.

RESULTS AND DISCUSSION

Pectic enzymes produced by Xanthomonas species

Tables 1 and 2 summarize the results of the pectate gel liquefaction tests and the assays for the various pectic enzymes in xanthomonad culture supernatant fluids. Even though every culture examined showed a good growth on the pectate gel medium (probably at the expense of the yeast extract in the medium), cultures of only 10 of 19 species were able to bring about prompt liquefaction of the gel; viz. *Xanthomonas badrii*, *X. campestris*, *X. carotae*, *X. geranii*, *X. lespedezae*, *X. manihotis*, *X. nakataeolitorii*, *X. papavericola*, *X. pelargonii*, *X. ricinicola*. These results are generally in accord with those presented earlier by Burkholder & Starr (1948) and Dye (1960).

Table 1. *Distribution of pectolytic enzymes in Xanthomonas species*

The strains are designated by their accession numbers in the International Collection of Phytopathogenic Bacteria (ICPB); many of the cultures are deposited also in the National Collection of Plant Pathogenic Bacteria, Harpenden. Liquefaction (*a*) of nutrient pectate gel (Starr, 1947) was scored after 24 hr at 28°. Growth (*b*) is expressed as mg. dried cells per ml. culture fluid. Pectinesterase (PE) activity (*c*) is reported in terms of the increase in μmoles of carboxyl groups per ml. reaction mixture per hour. Polygalacturonic acid *trans*-eliminase (PATE) activity (*d*) is recorded as change per hour in absorbancy at 235 mμ; reaction mixtures were diluted 20-fold with 0·01 N-HCl before measuring absorbancy.

Species	Strain ICPB	Liquefaction (*a*)	Growth (*b*)	Enzymic activity* PE (*c*)	Enzymic activity* PATE (*d*)
X. badrii	XB 103	+	0·76	1·10	1·63
X. beticola	XB 109	−	0·39	0·05	0
X. campestris	XC 135	+	0·98	0·09	0·69
X. carotae	XC 139	+	1·17	0·57	0·14
X. corylina	XC 12	−	0·46	0·05	0
X. desmodii-gangetici	XD 106	−	0·63	0	0
X. geranii	XG 10	+	1·05	0·02	0·06
X. holcicola	XH 3	−	0·83	0·06	0
X. hyacinthi	XH 110	−	0·83	0	0
X. juglandis	XJ 103	−	0·51	0·10	0
X. lespedezae	XL 2	+	0·48	0·10	0
X. manihotis	XM 12	+	1·06	0·02	0·55
X. nakatae-olitorii	XN 101	+	0·95	0·01	0
X. papavericola	XP 5	+	1·24	0·61	0·02
X. pelargonii	XP 8	+	0·84	0·04	0
X. phaseoli	XP 104	−	0·83	0·01	0
X. ricinicola	XR 4	+	0·33	0·67	0·83
X. taraxaci	XT 11	−	0·48	0·04	0·12
X. vesicatoria	XV 3	−	0·10	0·09	0

* Not even a trace of hydrolytic polygalacturonase or pectin *trans*-eliminase activity was detectable in any of the culture filtrates.

Some pectinesterase (PE) activity was found in all pectate-liquefying species and even in some cultures which did not liquefy the gel. The level of PE activity in *Xanthomonas badrii*, *X. campestris* (strains ICPB-XC 147 and ICPB-XC 149), *X. carotae*, *X. papavericola* and *X. ricinicola* is comparable to that reported for *Pseudomonas solanacearum* (Winstead & Walker, 1954), *Erwinia carotovora* (Goto & Okabe, 1962*a*) and *X. malvacearum* (Abo-El-Dahab, 1964), based on the same enzyme concentration and

the same reaction time. The lesser amounts of PE produced by other species or strains also are comparable on the same bases to that excreted by *Pseudomonas marginalis* (Ceponis & Friedman, 1959; Nasuno & Starr, 1966*b*).

Table 2. *Production of pectolytic enzymes by various strains of Xanthomonas campestris grown in the medium containing glucose or pectin as substrate**

Strain ICPB	Plant sources	Liquefaction (*a*)	Glucose-grown organisms: Growth (*b*)	Glucose-grown organisms: Enzymic activity PE (*c*)	Glucose-grown organisms: Enzymic activity PATE (*d*)	Pectin-grown organisms: Growth (*b*)	Pectin-grown organisms: Enzymic activity PE (*c*)	Pectin-grown organisms: Enzymic activity PATE (*d*)
xc 4	Horseradish	−	1·24	0	0	0·47	0·04	0
xc 7	Cabbage	+	0·99	0	0·04	0·52	0·12	0·12
xc 10	Turnip	+	1·42	0	0·12	0·42	0·06	0·95
xc 15	Cabbage	+	1·10	0	0·01	0·44	0·02	0·30
xc 117	Cauliflower	+	1·12	0	0·05	0·41	0·02	0·04
xc 132	Candytuft	+	1·26	0	0·10	0·71	0·02	0·45
xc 147	Cabbage	+	1·00	0	0·43	0·37	0·78	0·36
xc 149	Brussels sprout	−	1·62	0	0·04	0·51	0·47	0·07

* See the sublegend of Table 1 for notes; the only exception in the present instance is that the samples for determination of PATE activity (*d*) were diluted ten-fold with 0·01 N-HCl before measuring absorbancy.

The excretion of polygalacturonic acid *trans*-eliminase (PATE) was observed in all pectate-liquefying species except *Xanthomonas lespedezae*, *X. nakatae-olitorii* and *X. pelargonii*. Since these three cultures do not produce any polygalacturonase (PG), although they do form pectinesterase, the basis for liquefaction of the pectate gel deserves further study. Dye (1960), who observed a similar pattern, suggested that weak liquefaction of a pectate gel might well have been caused by other than enzymic factors. To further complicate the picture, it must be noted that PATE is shown in the present study to be produced by *X. taraxaci* which does not, however, detectably liquefy the pectate gel.

Under the assay-conditions used in the present study, neither polygalacturonase nor pectin *trans*-eliminase activity could be detected, even after 24 hr of reaction, in the culture supernatant fluids of any tested xanthomonads. According to Goto & Okabe (1962*b*), *Erwinia carotovora* can produce a pectic glycosidase active at pH 4·0 only when grown in a medium at very low calcium ion levels. However, polygalacturonase was not excreted by any xanthomonad cultures, even in a calcium-deficient medium. It might be noted that polygalacturonic acid *trans*-eliminase production in this calcium-deficient medium was either remarkably reduced (*Xanthomonas badrii*, *X. campestris*, *X. carotae*, *X. ricinicola*) or completely halted (*X. geranii*, *X. manihotis*, *X. papavericola*, *X. taraxaci*) in the case of xanthomonads which normally form PATE in the presence of calcium. Static culture conditions were ineffective in stimulating polygalacturonase excretion by xanthomonads, although this enzyme is produced by yeasts in static culture (Luh & Phaff, 1954 *a*, *b*).

The production of a 'pectin polygalacturonase' was reported by Dye (1960) to occur in many xanthomonads. It is more likely that the enzyme so designated by Dye is actually a combination of polygalacturonic acid *trans*-eliminase (PATE) and pectinesterase rather than the hydrolytic enzyme. He determined this enzyme activity

in growing cultures on a pectic agar media adjusted to pH 6·2–7·0 at which pH value the PATE of xanthomonads is still somewhat active (Nasuno & Starr, 1967). Moreover, a shift in pH value of the growth medium to the alkaline range favourable for PATE normally occurs when xanthomonads are grown on the medium used by Dye. The distribution of PATE+pectinesterase among the various xanthomonad species coincides well with Dye's (1960) report of what he terms 'protopectinase' ('PP') activity in xanthomonads (actually measured by Dye as maceration of potato tuber tissue). The good agreement between distribution of 'PP' and PATE+pectinesterase suggests that the so-called 'PP' of xanthomonads in most cases is probably a combination of polygalacturonic acid *trans*-eliminase (PATE) and pectinesterase.

Effects of substrate on pectic enzyme production

Various studies have shown that some pectic enzymes are inducible, whereas others seem to be constitutive; most pectic enzymes produced by phytopathogenic bacteria are inducible. Kraght & Starr (1953), Ozawa & Okamoto (1956) and Goto & Okabe (1962*b*) reported that pectin was superior to glucose as the carbon source for production of pectic glycosidase by Erwinia species. Similar results were found for *Bacillus polymyxa* by Nagel & Vaughn (1961*a*), and for *Xanthomonas malvacearum* by Abo-El-Dahab (1964). When eight strains of *X. campestris* were grown in a medium containing glucose but no pectic substances, there was no pectinesterase activity in the culture fluids of any strain; however, polygalacturonic acid *trans*-eliminase (PATE) was produced to some extent under these conditions by some strains (Table 2). On the other hand, in all these strains, pectinesterase was induced by the presence in the growth medium of pectin, which remarkably stimulated PATE production by some strains. Constitutive production of PATE is, thus, clear in several *X. campestris* strains. Somewhat similar results were recently found in *Pseudomonas marginalis* (Nasuno & Starr, 1966*b*), in which one of two strains excreted PATE regardless of the presence of a pectic substrate in the culture medium.

End-products from pectic substances formed by Xanthomonas species

Table 3 shows the results of paper chromatographic analysis of the breakdown products from polygalacturonic acid by polygalacturonic acid *trans*-eliminase (PATE) excreted by various Xanthomonas species or strains. The experimental conditions were 10% (v/v) enzyme solution, 0·5% (w/v) substrate, and 0·001 M-$CaCl_2$ in 0·05 M-glycine-NaOH buffer (pH 9·5), and a reaction time of 24 hr at 30°. With enzyme preparations showing high activity, the reaction products were unsaturated di- and trigalacturonic acids accompanied by saturated mono-, di-, tri- and tetra-galacturonic acids. With weakly active enzyme preparations, the mono- and di-galacturonic acid and sometimes even the unsaturated di-galacturonic acid were not detectable in the case of some xanthomonads

In earlier work it was believed that some 'polygalacturonase' preparations degrade the substrate completely to galacturonic acid, whereas others seemed to produce only oligalacturonides but not galacturonic acid. For example, in the reaction mixture with the pectic glycosidase from *Pseudomonas marginalis* and a pectic substrate, Ceponis & Friedman (1959) could not detect galacturonic acid which recently was found to be formed with concentrated enzyme under similar conditions (Nasuno &

Starr, 1966*b*). With tomato polygalacturonase, the reaction product was first thought to be pentagalacturonic acid (McColloch & Kertesz, 1949), then tri-, di-, and galacturonic acids (Roelofsen, 1953); eventually, mono- and di-galacturonic acids were confirmed as the real end-products (Luh, Leonard & Phaff, 1956).

Table 3. *Paper chromatographic analysis of the reaction products from polygalacturonic acid by polygalacturonic acid trans-eliminase produced by various Xanthomonas species*

Species	Strain ICPB	Galacturonides formed*						
		Saturated				Unsaturated		
		Mono-	Di-	Tri-	Tetra-	Di-	Tri-†	ΔE_{253}‡
X. badrii	XB 103	(+)	++	+	(+)	+++	++	0·500
X. campestris	XC 7	.	+	+	+	++	++	0·305
X. campestris	XC 10	(+)	+	+	+	++	++	0·480
X. campestris	XC 15	.	.	.	+	(+)	.	0·046
X. campestris	XC 117	.	.	.	+	(+)	.	0·035
X. campestris	XC 132	(+)	+	+	+	++	++	0·320
X. campestris	XC 135	(+)	+	+	(+)	++	++	0·470
X. campestris	XC 147	(+)	+	+	+	++	++	0·445
X. campestris	XC 149	.	+	+	+	+	+	0·110
X. carotae	XC 139	.	+	+	++	++	++	0·300
X. geranii	XG 10	.	.	+	+	+	.	0·135
X. lespedezae	XL 2	.	(+)	+	++	(+)	++	0·085
X. manihotis	XM 12	+	++	+	(+)	+++	++	0·545
X. papavericola	XP 5	.	.	.	+	(+)	.	0·035
X. ricinicola	XR 4	+	++	+	(+)	+++	++	0·504
X. taraxaci	XT 11	.	.	+	+	+	+	0·164

* (+) represents very weak spots; +++ intense spots.

† From their relative positions on the paper, these spots were only tentatively assumed to represent unsaturated trigalacturonic acid.

‡ Reaction mixtures were diluted 100-fold with 0·01 N-HCl for extinction measurement. Reaction period, 24 hr.

Although various patterns of breakdown products were transiently found in the reaction mixtures when culture supernatant fluids of xanthomonads were used as the enzyme preparations, the major reaction products from polygalacturonic acid acted upon by concentrated crude polygalacturonic acid *trans*-eliminase from all Xanthomonas cultures were unsaturated di- and tri-galacturonic acids with lesser amounts of saturated mono- and di-galacturonic acids. It can now be concluded that reports in the earlier literature to the effect that the monomer was not formed by some xanthomonads might stem from: (*a*) the use of samples too small to permit detection of the galacturonic acid, or (*b*) enzyme activity insufficient to produce monomer in a quantity detectable by the methods used.

The sequential appearance of the several products of pectic digestion by polygalacturonic acid *trans*-eliminase-forming Xanthomonas species was followed in culture fluids by paper chromatography of samples taken at 24 hr intervals of incubation. In the medium containing pectin, saturated and unsaturated oligogalacturonides first appeared after 24 hr and then monomer was detected after 48 hr. Saturated mono-, di-, and tri-galacturonic acids and unsaturated di- and tri-galacturonic acids were found as major end-products after 7 days of incubation. The sequence was the same,

but this process was shortened, when polygalacturonic acid replaced the pectin in the culture medium; this suggested that pectinesterase activity might be limiting. After incubation for 4 days in the polygalacturonic acid medium, the sole end-product was galacturonic acid which cannot be utilized further by any Xanthomonas culture examined. Therefore, it is plausible that this uronic acid is one of the real end-products of pectin digestion by Xanthomonas species. These results contrast markedly with the non-accumulation of galacturonic acid in the culture fluid of *Erwinia carotovora* (Kraght & Starr, 1953); but this organism can rapidly metabolize galacturonic acid through the pathway elucidated by Kilgore & Starr (1959).

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Fine Structure of Lymphogranuloma Venereum Agent and the Effects of Penicillin and 5-Fluorouracil

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SUMMARY

Growth of lymphogranuloma venereum (LGV) agent in HeLa cells has been studied by light and electron microscopy. Infective elementary bodies enter the host cell by phagocytosis, transform directly into the larger forms known as initial bodies, and multiply by fission within a vesicular cytoplasmic inclusion. Later in the growth cycle, elementary bodies are produced by a process involving internal condensation and a progressive reduction in size of the dividing initial bodies. An envelope with two layers, ribosome-like granules and filamentous 'nuclear' material are components common to all developmental forms of the LGV agent. Penicillin prevents multiplication of initial bodies, but not their growth; the very large and vacuolated penicillin forms are similar to bacterial spheroplasts. 5-Fluorouracil affects chiefly the later part of the LGV growth cycle; non-infective forms develop, and the maturation of elementary bodies is blocked. The findings endorse the view that the psittacosis–lymphogranuloma–trachoma group agents are not viruses, but have close affinities with rickettsiae and certain bacteria.

INTRODUCTION

The taxonomic position of the psittacosis–lymphogranuloma–trachoma (PLT) group of organisms, particularly their relation to viruses, is a subject of long-standing controversy (see Weiss, 1955; Bedson, 1959; Moulder, 1964; Andrewes, 1964). Current views on the essential attributes of viruses are more precise than formerly, but the position of the PLT group has remained uncertain, due largely to the equivocal nature of data on some basic properties including their mode of replication. The original studies using light microscopy (e.g. Bedson, Western & Levy–Simpson, 1930; Findlay, Mackenzie & MacCallum, 1938; Bedson & Gostling, 1954) showed these agents to be obligate intracellular parasites with a complex growth cycle. This involved the development of cytoplasmic inclusions containing relatively large particles (*c.* 1 μ) termed initial bodies, which appeared to multiply by binary fission and give rise to a population of smaller infective particles (*c.* 0·3 μ) termed elementary bodies. However, some crucial aspects of the growth cycle were not defined. The mode of entry into the host cell, the possibility of eclipse or breakdown of the invading particles, the origin of initial bodies and the mechanism of elementary body formation, all remained conjectural.

Electron microscopical studies on the development of PLT agents have been under-

taken in several laboratories (Gaylord, 1954; Mitsui *et al.* 1958; Higashi, 1959; Litwin, Officer, Brown & Moulder, 1961; Bernkopf, Mashiah & Becker, 1962), but these observations did not at once clarify the relationship of the different types of particles, either to the host cell or to each other. Examination of cell cultures at known intervals following infection suggested the occurrence of a virus-like matrix prior to the appearance of dividing particles (Tajima, Nomura & Kubota, 1957; Armstrong, Valentine & Fildes, 1963; Higashi, Tamura & Iwanaga, 1962; Mitsui, Fujimoto & Kajima, 1964). Re-investigation of this somewhat surprising observation led to recognition of a misleading technical artifact which affects the morphology of developing PLT organisms, even though the host cell may be well preserved (Armstrong & Reed, 1964). In HeLa cells infected with the agent of lymphogranuloma venereum (LGV) it was shown that elementary bodies from the inoculum entered cells as a result of phagocytosis; within 6 hr, and with no loss of structural integrity, engulfed particles transformed directly into discrete initial bodies. Previous descriptions of a viral matrix in PLT-infected cells, including our own, evidently referred to the products of accidental disruption of initial bodies caused by inadequate fixation. Similar findings have been reported recently by Higashi (1965) following use of improved techniques.

Susceptibility of the PLT group to antibiotics is well known. Weiss (1950) drew attention to the development of abnormally large particles in the presence of penicillin, and Moulder (1964) has pointed to an analogy with penicillin-induced bacterial spheroplasts; but so far there has been no satisfactory description of the penicillin forms in fine structural terms.

The present paper describes experiments aimed at further defining the morphological characteristics of the LGV agent, and its replicating mechanism in cell cultures. In addition, observations have been made on the effects of penicillin and of 5-fluorouracil (5-FU) on the structure and multiplication of this agent.

METHODS

Tissue culture and inoculation. HeLa cell monolayers were grown in Hanks balanced salt solution with 0·5% (w/v) lactalbumen hydrolysate, 10% (v/v) calf serum and 200 μg./ml. streptomycin. After inoculation the cultures were normally maintained on medium 199 with 5% (v/v) horse serum; but in experiments involving the use of 5-FU, Eagle's medium with 10% (v/v) horse serum was employed. 5-FU was added to the medium at a concentration of 4 μg./ml. and the cells were pretreated with this inhibitor for 18 hr prior to inoculation. The sodium salt of benzyl penicillin was used at a concentration of 0·1 unit/ml. An inoculum containing the JH strain of LGV agent was prepared by treatment of 42 hr-infected HeLa cells for 4 min. in the MSE ultrasonic disintegrator. Bottles to be inoculated were rinsed with Hanks solution, the cell sheet was covered with LGV suspension and incubated for 30 min. at 36°; the inoculum was then removed, the monolayers rinsed with Hanks solution and maintenance medium added. Cultures were incubated at 36°.

Infectivity titrations were made in HeLa cell cultures using a method of inclusion counting, based on that described by Furness, Graham & Reeve, (1960).

Light microscopy. Sample coverslips were removed after periods of 18, 24, 48 and 72 hr following inoculation. Some were observed in the living state by phase-contrast;

others were rinsed in Hanks solution, fixed in methanol or formol-saline, and stained by the Giemsa method.

Electron microscopy. Inoculated and control cultures, including those with added 5-FU, were prepared for thin-section electron microscopy at intervals from 15 to 72 hr from the time of inoculation. Penicillin-treated cultures were sampled at 24 and 48 hr stages only. Processing was as follows: after a brief rinse with Hanks solution the monolayers were fixed *in situ* for 5 min. with the solution of Kellenberger, Ryter & Séchaud (1958), i.e. 1 % (w/v) osmium tetroxide in veronal–acetate–saline buffer, with calcium ions, at pH 6·1. They were then pushed gently from the glass; the contents of several bottles were pooled, and fixation continued for 1 hr at 4°. This was followed by 1 hr in the 0·5 % (w/v) buffered uranyl acetate washing solution of Kellenberger *et al.* (1958), and embedding in Vestopal W or in pre-polymerized methacrylate. Contrast in the sections was enhanced by staining with uranyl acetate or with lead citrate as recommended by Reynolds (1963). Micrographs were obtained with a Philips EM 75b electron microscope.

RESULTS

Infectivity titrations

HeLa cells inoculated with LGV agent and incubated for 48 hr. were resuspended in a volume of fresh medium equal to that in the original bottles. After treatment for 4 min. in the ultrasonic disintegrator a preparation grown in medium containing neither penicillin nor 5-FU gave a titre of $1{\cdot}6 \times 10^6$ inclusion-forming units (i.f.u.) per ml. when titrated on fresh monolayers. The corresponding 48 hr preparation in the presence of penicillin or 5-FU possessed no detectable infectivity. There was again no sign of infectivity in specimens grown for 72 hr in the presence of 5-FU. After 72 hr in the presence of penicillin scattered inclusions were produced in the test monolayer, but infectivity of the material was well below the lower limit of accuracy of the titration method, i.e. approximately 10^3 i.f.u./ml.

Light microscopy

Phase-contrast observations. In unfixed monolayers typical cytoplasmic inclusions were visible in more than 75 % of the cells 18 hr after inoculation; each inclusion contained a cluster of discrete, rounded and optically dense initial bodies with a diameter of approximately 1 μ. After 24 hr inclusions were larger and the initial bodies seemed to be more numerous. If the monolayer was passed successively through the fixative and washing solutions of Kellenberger *et al.* (1958) the initial bodies became more clearly visible through enhanced contrast (Pl. 1, fig. 1); on the other hand, brief immersion in the standard fixative of Palade (1952) produced inclusions with ill-defined or diffuse contents. In 48 hr specimens the inclusions were massive and contained innumerable minute elementary bodies, measuring less than 0·5 μ. These were frequently in a state of vigorous Brownian movement.

In penicillin-treated monolayers abnormal cytoplasmic inclusions were detectable in a majority of cells 24 hr after inoculation; in general they were smaller than normal inclusions at the same stage, and instead of the usual initial bodies they contained a small number (usually only one or two) of oval or rounded bodies corresponding to the penicillin forms of other PLT agents as described by Weiss (1950). They were much larger than normal initial bodies, and commonly vacuolated (Pl. 3, fig. 7). Further

enlargement of the penicillin forms was evident after 48 hr and 72 hr, with no apparent increase in their number; many had assumed the form of vesicular structures with a dense crescentic margin. There was no sign of elementary bodies. Immersion of the monolayers in Palade's fixative for a few minutes caused many of the penicillin forms to rupture.

In the cultures treated with 5-FU cytoplasmic inclusions were readily seen in the living cells 24 hr after inoculation, and subsequently they enlarged in the normal way to occupy much of the cell. At first their contents were not unlike the normal initial bodies but from 48 hr onwards there was marked variation in the appearance of inclusions in different cells (Pl. 4, fig. 10). Some small inclusions contained only a few rather large particles; elsewhere the inclusions were larger, and these contained particles of various sizes at least some of which resembled elementary bodies.

Stained monolayers. A comparison of fixed and stained monolayers with the parallel cultures examined by phase-contrast suggested that normal initial bodies are deformed and sometimes ruptured as a result of methanol fixation. The larger penicillin forms and those developing in the presence of 5-FU showed even greater susceptibility to methanol damage. Formol-saline preserved the inclusions in a form nearer to that seen in the living cells.

Electron microscopy

Normal structure and fate of initial bodies. Cytoplasmic LGV inclusions are well-defined vesicular structures, sometimes loculated, situated in close relation to the Golgi complex of the cell. A single unit membrane limits the inclusion, separating its contents from the surrounding cytoplasm. Study of sequential stages following inoculation suggests that the typical juxtanuclear inclusion arises from coalescence of the phagocytic vesicles containing individual ingested elementary bodies; indirectly, therefore, the inclusion wall is derived from the host cell membrane. Sections of inclusions at the 15 hr stage show a collection of discrete initial bodies suspended freely in an electron-transparent medium (Pl. 1, fig. 2). Initial bodies are rounded or oval in shape, and measure between 1 and 1·5 μ in diameter. Each is enclosed in a limiting envelope which, in appropriate planes of section, is seen to be a bilaminar structure. Both layers are typical unit membranes, and approximately 7 mμ in thickness (Pl. 2, figs. 3, 4). A clear zone about 10 mμ wide normally separates the two dense layers, though in many places the outer is found to be lifted slightly with a consequent widening of the intervening clear zone. The inner layer of the envelope everywhere adheres closely to the contents of the initial body. Two internal components are recognizable in all sectioned initial bodies (Pl. 1, fig. 2; Pl. 2. figs. 3, 4). Dense granular material consisting largely of ribosome-like particles occupies the peripheral regions of the organism, and also extends in the form of irregular strands into the centre; here there are intervening areas of lower electron density containing a network of very fine filaments. There are obvious similarities in terms of fine structure, between LGV initial bodies and many bacterial and rickettsial cells. The two layers of the limiting envelope can be compared, respectively, to the cell wall and cytoplasmic membrane as found in Gram-negative bacteria; whilst the filamentous and granular elements are similar in character to bacterial nuclear and cytoplasmic regions.

Comparison of LGV-infected cells 15, 18 and 24 hr after inoculation reveals an increase both in the size of inclusions and in the number of contained initial bodies.

Moreover, initial bodies showing constricted or dumb-bell profiles are very common and are strong evidence for the view that the LGV agent multiplies by a process of fission (Pl. 1, fig. 2; Pl. 2, figs. 3, 5). It appears that division of an initial body is preceded by enlargement and slight elongation of the organism, that this is followed by the development of a circumferential constriction at or near to the mid-point, and that final separation of the two halves produces a pair of virtually identical daughter initial bodies. The two layers of the envelope remain distinct and in close apposition whilst growing centripetally at the point of constriction; and there is no sign of preliminary cross-wall formation. A closely comparable mode of cell divisions has been described in certain Gram-negative bacteria, and in rickettsiae (Conti & Gettner, 1962; Bladen & Waters, 1963; Ito & Vinson, 1965).

Some signs of a departure from this pattern of multiplication are detectable in the cultures examined 24 hr after inoculation, and are quite obvious in the 48 and 72 hr specimens. The initial bodies, though still readily identified, tend to be smaller than in the early part of the growth cycle. Measurement of dividing forms suggests that organisms of progressively smaller size are being formed. Where the diameter of the structure falls much below 1 μ there are additional signs of modified internal organization: the filamentous and granular elements tend to separate, with the 'nuclear' material becoming limited to a central mass of high density whilst the ribosome-like granules are confined to the periphery (Pl. 2, fig. 4). Most inclusions from 48 hr onwards thus contain a wide spectrum of particles, ranging from initial bodies down to small, dense-centred forms that are typical elementary bodies (Pl. 2, fig. 6). Profiles such as the comparatively small dividing form shown in Pl. 2, fig. 5, are of particular interest, as this would seem to represent the formation of a pair of elementary bodies. It is seen that the two layers of the limiting envelope are becoming widely separated, as the inner layer retracts in company with the condensed contents of the organism.

The prominent external membrane that is a familiar feature of all PLT-group elementary bodies evidently corresponds morphologically with only the outer layer of the bilaminar envelope possessed by initial bodies. In sections through mature elementary bodies (Pl. 2, fig. 6) it becomes difficult as a rule to identify more than a small part of the inner layer of the envelope, so intimate is its association with the dense core structure.

Effects of penicillin. 24 hr after inoculation, in the presence of the antibiotic, cytoplasmic inclusions containing abnormal developmental forms of LGV agent are readily identified (Pl. 3, fig. 9). The penicillin forms appear as large vacuolated organisms measuring about 10 μ, occasionally more, in diameter. There is a complete limiting envelope, consisting of two layers which in thickness and spacing appear to be much the same as in normal initial bodies. Inside are the usual granular and fibrillar components, but in contrast to normal initial bodies the relative development of these components is very variable. Particularly in the largest penicillin forms there is often a striking preponderance of the filamentous, or 'nuclear', material (Pl. 3, fig. 8). Vacuoles are usually multiple, and bounded by a single membrane; points of continuity between vacuoles and the clear zone between the layers of the limiting envelope can be found, suggesting that vacuolation may have developed through ballooning of the envelope. Vacuoles are larger and more numerous after 48 hr, resulting in correspondingly greater morphological distortion. This evidently accounts for the crescentic forms observed in living cultures by phase-contrast, and in thin sections gives rise

to bizarre profiles. Deep invaginations from the inner layer of the envelope are sometimes present, producing irregular internal subdivisions; such organisms, however invariably remain enclosed within a common outer membrane and there is no sign of true division.

Effects of 5-FU. Electron microscopy of the 5-FU-treated cultures suggests that, in contrast to penicillin, the concentration of 5-FU used in this experiment has little effect on structure and multiplication of LGV agent during the first 24 hr of growth in HeLa cells. Initial bodies, very similar to those seen normally in infected cell cultures, are present in the inclusions 15 and 24 hr after inoculation. In addition, profiles suggesting fission are also found. By 48 hr most inclusions are generally smaller than they would be normally, and contain a proportion of abnormal initial bodies which show vacuolation and segmentation due to ingrowths from the inner layer of the envelope. After 72 hr (Pl. 4, fig. 11) nearly all of the organisms are obviously affected and many are undergoing fragmentation into irregular debris. Mature elementary bodies are not found.

DISCUSSION

In recent papers (Armstrong & Reed, 1964; Higashi, 1964, 1965) evidence was presented that in cell cultures infected with LGV, trachoma or psittacosis agents, initial bodies originate during the first few hours of the growth cycle by enlargement and reorganization of phagocytosed elementary bodies. Factors that initiate and control the transformation remain uncertain, but the influence of the host cell enzymes is suggested by intimate association of the engulfed particles with lysosome-like vesicles in the cell cytoplasm.

Distinct morphological similarities exist between LGV initial bodies, rickettsiae and certain bacterial cells, notably in respect of the limiting envelope and the presence of what may be regarded as 'nuclear' and 'cytoplasmic' internal components. That this is true of the PLT group as a whole is evident from recently published micrographs showing the initial bodies of psittacosis, trachoma and meningopneumonitis agents (Erlandson & Allen, 1964; Kajima, Sharon & Pollard, 1964; Higashi, 1965; Anderson, Hopps, Barile & Bernheim, 1965). The essential form of initial bodies would have been recognized sooner but for their peculiar fragility: an isotonically balanced fixative is required for their adequate preservation, especially in the first 12 hr of intracellular growth. Multiplication of initial bodies during the first 24 hr of growth in HeLa cells is exclusively by fission, without preliminary cross-wall formation; and the daughter organisms retain typical initial body morphology. Division of initial bodies continues beyond the 24 hr stage, but with a gradual reduction of their size. Segregation and condensation of the filamentous and granular internal components then becomes apparent, and the layers of the limiting envelope become widely separated; in this way the particles assume the form of elementary bodies. The transformation is, in effect, the reverse of that observed during the first few hours of the growth cycle. By 72 hr particles of the elementary body type predominate, and forms that are obviously dividing are found less frequently. It seems likely that once the filamentous material, that is assumed to contain DNA, becomes condensed it does not replicate further until the particle enters a new host cell. At all developmental stages it is possible to identify the two layers of the limiting envelope, and the same two internal components;

however, the arrangement of these elements, relative to one another, differs appreciably in the particles seen at each stage of the growth cycle. The different fine-structural organization of initial and elementary bodies evidently reflects the markedly different biological properties of these particles.

As expected, growth and multiplication of LGV agent was considerably modified in the presence of penicillin. Adsorption and engulfment of elementary bodies, and their subsequent enlargement are clearly not prevented by the antibiotic. However, the initial bodies do not multiply, but develop into large, abnormal organisms which entirely fail to revert to elementary body form. The present findings are in full agreement with the conclusions of Weiss (1950) that the effect of penicillin on the PLT group is essentially an interference with fission. From the structure of the penicillin forms it can be deduced that the outer layer of the envelope fails to participate in attempts at division. Members of the PLT group have been shown to contain muramic acid, a characteristic component of bacterial cell-wall mucopeptide (Perkins & Allison, 1963); since penicillin is known to inhibit mucopeptide synthesis in bacteria it probably acts in the same way on the PLT group. Disproportionate development of 'nuclear' and 'cytoplasmic' components, as found in some of the penicillin forms, suggests that an additional effect may be disturbance of the normal balance of nucleoprotein synthesis. Spheroplasts induced by treatment of Gram-negative bacteria with penicillin are swollen and vacuolated structures, osmotically labile and dependent on a medium of high tonicity for stabilization (Lederberg & St Clair, 1958; McQuillen, 1960; Martin, 1963). At the fine structural level (Thorsson & Weibull, 1958; Hines, Freeman & Pearson, 1964; Weibull & Mohri, 1965) spheroplasts are markedly pleomorphic, but often bear a close resemblance to the penicillin forms seen in the present study.

5-FU is known to become incorporated into ribonucleic acid as a uracil analogue in mammalian cells, bacteria and viruses; replication of DNA is also impaired due to an inhibition of thymine synthesis (Cohen *et al.* 1958; Horowitz, Saukkonen & Chargaff, 1960). The compound evidently interferes also with synthesis of bacterial cell walls (Tomasz & Borek, 1960; Rogers & Perkins, 1960). In the present study the effect of 5-FU is seen mainly in the later stages of the LGV growth cycle. Formation of initial bodies and their multiplication proceeds normally for at least a few hours, and there is marked variation from cell to cell concerning the point in the growth cycle at which definite effects of 5-FU are seen. Eventually, however, atypical initial bodies develop; they are not unlike some of the penicillin forms, which suggests an effect on the cell wall, but they show a marked tendency to spontaneous fragmentation.

In cytochemical studies using the acridine orange fluorescence technique (Pollard, Starr, Tanami & Elliott, 1960; Tanami, Pollard & Starr, 1961; Pollard & Starr, 1962) it was observed that treatment of cell cultures with 5-FU 14 hr after inoculation with PLT agents resulted in highly abnormal patterns of diffuse RNA-containing material in the region of the cytoplasmic inclusions. This was interpreted as host-cell-contributed material. However, fine structural appearances after fixation by Kellenberger's method suggest that inclusions which develop in the presence of 5-FU contain discrete though abnormal forms of the infective agent; no diffuse matrix is demonstrable. Damage due to alcohol-containing fixatives could perhaps give rise to an appearance simulating a diffuse matrix when viewed by fluorescence microscopy.

Moulder (1964) has presented a detailed and lucid account of evidence bearing upon the possible relationship between PLT agents and bacteria. Additional biochemical

studies have shown that PLT agents possess at least some degree of autonomous energy metabolism (Weiss, Myers, Dressler & Chun-Hoon, 1964), though they depend upon ATP supplied by the host cell as an energy substrate (Weiss, 1965). The present findings lend further support to the concept of the PLT group as complex organisms, well adapted to a life of intracellular parasitism, and with close affinities to the rickettsiae. They have only a superficial likeness to viruses, as these are presently defined. In purely morphological terms, initial bodies of the PLT group and the rickettsiae both have much in common with the free-living Gram-negative bacteria.

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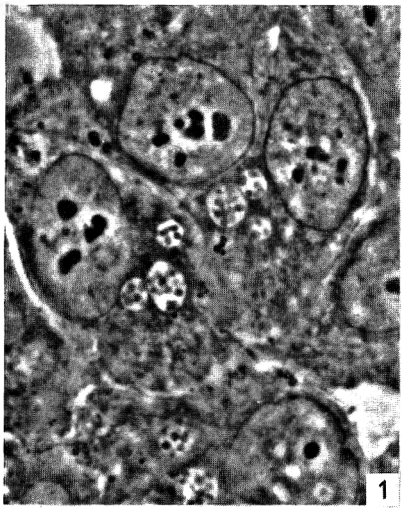

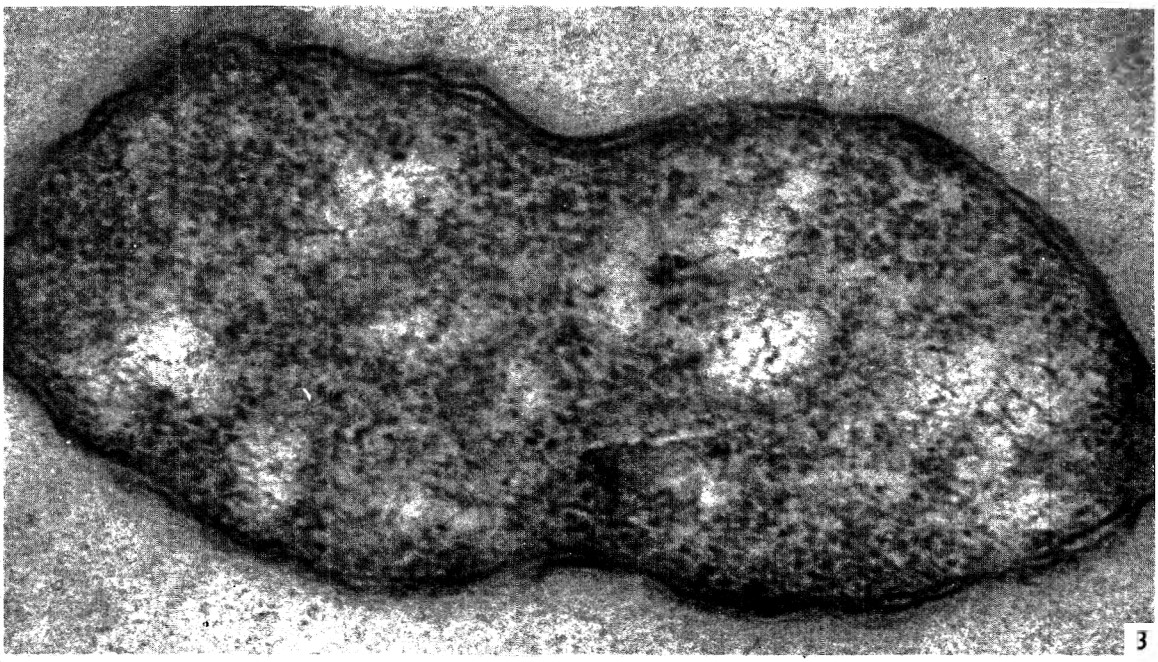
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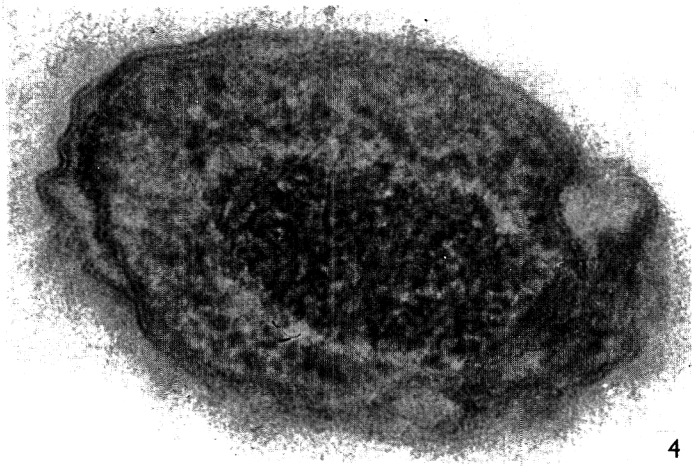
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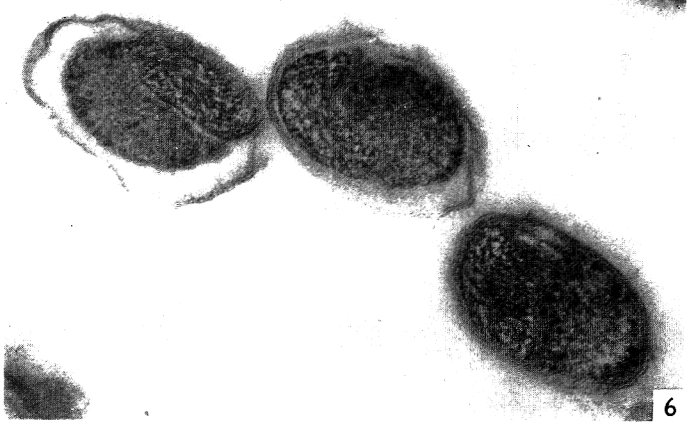
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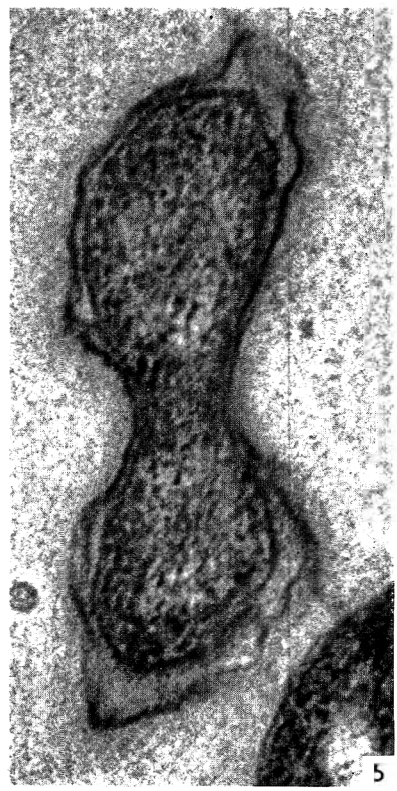
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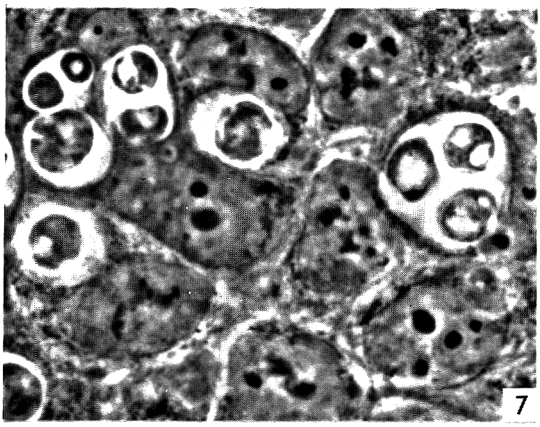
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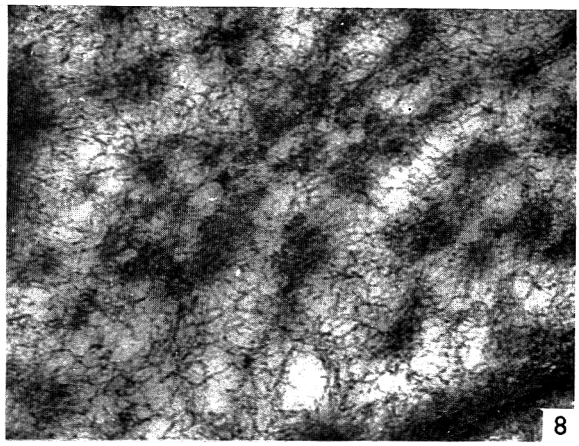
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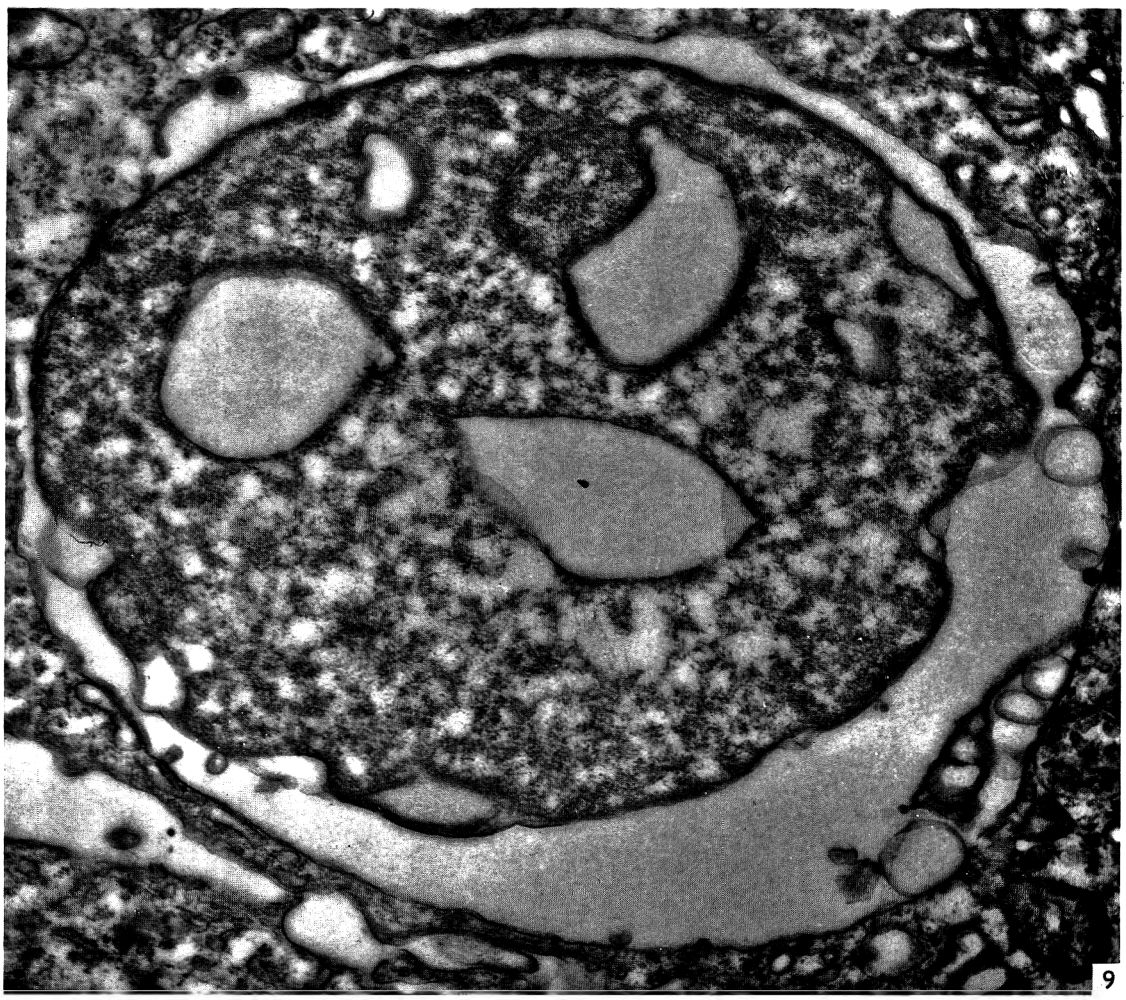
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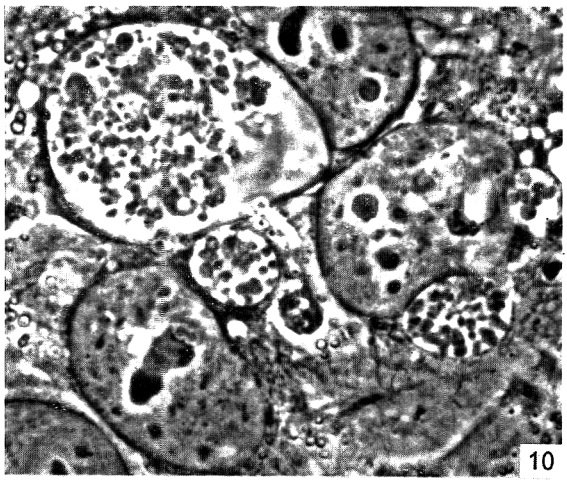

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. HeLa cells in a monolayer culture 18 hr after LGV inoculation. Discrete initial bodies are visible in the vesicular cytoplasmic inclusions. Phase-contrast, after Kellenberger fixation (× 1350).

Fig. 2. Electron microscopical appearance of initial bodies in a cytoplasmic inclusion 18 hr after inoculation. A dividing form is seen at the top, and possibly another at lower right (× 64,000).

PLATE 2. Electron micrographs

Fig. 3. Section through a dividing initial body, seen at higher magnification. Note the elongated shape and developing central constriction, also the bilaminar character of the limiting envelope (× 120,000).

Fig. 4. A particle of transitional type in a 48 hr inclusion, interpreted as intermediate between the typical initial body and elementary body forms. The filamentous 'nuclear' material is seen here as a central dense mass, and both layers of the envelope have unit-membrane structure (× 104,000).

Fig. 5. Thin section profile of a small dividing particle in a 48 hr inclusion. The layers of the envelope are more widely separated than in initial bodies, and the inner layer adheres to the particle contents (× 120,000).

Fig. 6. Section through three morphologically mature elementary bodies (× 100,000).

PLATE 3

Fig. 7. HeLa cell monolayer 24 hr after LGV inoculation, in the presence of penicillin. The inclusions contain large penicillin forms of the LGV agent. Phase-contrast after Kellenberger fixation (× 1300)

Fig. 8. Thin section illustrating the predominantly filamentous nature of the interior of a penicillin form, 48 hr after inoculation (× 64,000).

Fig. 9. Section of 24 hr LGV inclusion, in the presence of penicillin. It contains a single, vacuolated penicillin form of LGV agent (× 32,000).

PLATE 4

Fig. 10. HeLa cell monolayer 72 hr after LGV inoculation, in the presence of 5-fluorouracil. The inclusions vary in size, with irregular and poorly defined contents. Phase contrast after Kellenberger fixation (× 1300).

Fig. 11. Electron microscopical appearance 72 hr after inoculation in the presence of 5-fluorouracil. The inclusion is of about normal size, but the contained particles are pleomorphic. Some resemble misshapen initial bodies. There are numerous irregular membrane-bound fragments, but no mature elementary bodies (× 32,000).

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X-Ray Diffraction and Infrared Study of the 'Sulphur Granules' of *Actinomyces bovis*

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SUMMARY

Bovine actinomycosis exudate was studied to determine the chemical composition and mineral phase(s) of the so-called 'sulphur granules'. The major constituents, CaO and P_2O_5, accounted for 86–89% of the ashed granules. The granules also contained Na, K, Mg (as oxides) and Cl which added up to 10–14%. Although the X-ray diffraction patterns indicated only poorly formed apatite; the infrared spectrum could be interpreted as arising from poorly crystallized apatite and/or a partially dehydrated or hydrolysed octacalcium phosphate either or both mixed with anhydrous dicalcium phosphate. The formation of pyrophosphate (44·5%) in the ignited granules indicated the presence of considerable acid phosphate in the original material.

INTRODUCTION

Because of similarities in the morphology of the organisms and pathological descriptions of actinomycosis (lumpy jaw) in cattle and cervico-facial actinomycosis in man, it was considered for many years that the organisms causing these diseases were the same. Recently, morphological, physiological and biochemical studies (Erikson, 1940; Thompson, 1950; Pine, Howell & Watson, 1960) indicated that the animal disease is caused by *Actinomyces bovis* and the human infections are caused by *A. israelii*, *A. naeslundi* (Thompson & Lovestedt, 1951; Howell, Murphy, Paul & Stephan, 1959) and *Norcardia asteroides* (Burnett & Scherp, 1957).

Although small differences have been found between the animal and human strains, so-called 'sulphur granules' are invariably present in the clinical infection. They can be observed and easily isolated from the draining sinuses; their presence is regarded as a valuable aid in the diagnosis of the disease. The granule is generally a yellowish, hard, spherical body containing a peripheral arrangement of 'clubs' which can be seen upon microscopic examination (Pine *et al.* 1960).

There has been considerable speculation concerning the structure of the granule and the clubs surrounding it. Recent studies (Pine & Overman, 1963) indicated that the granule formed by *Actinomyces bovis* is a mycelial mass cemented together by a polysaccharide + protein complex probably excreted by the organism. The centre of the mass is calcium phosphate according to Pine & Overman (1963), but the mineral phase(s) have not been identified.

Viable *Actinomyces israelii*, *A. naeslundi* and other bacterial species become mineralized, i.e. form apatite when implanted in dialysis bags in the peritoneal cavity of rats (Rizzo, Martin, Scott & Mergenhagen, 1962). The mineral is found intra- and extra-

cellularly, but granules were not observed (Rizzo, Scott & Bladen, 1963). Formation of 'sulphur granules' seems to require the local conditions associated with the chronic infection, because they have been observed only once *in vitro* (Wright, 1905). Our study, therefore, was undertaken to determine the chemical composition and to characterize the mineral phase(s) of the granules.

METHODS

The 'sulphur granules' used for this study were obtained from bovine actinomycosis exudates. Sample 1 (from the National Animal Disease Laboratory, Ames, Iowa) consisted of a pooled exudate fixed in an aqueous solution of 10% (v/v) glycerol and 2% formalin. Sample 2 was removed directly from an infected steer. The identity of the micro-organisms from sample 1 was established by culture and microscopic examination. Sample 2 was considered to be an actinomycosis exudate on the basis of the clinical infection and the morphology of the granules.

Initially, both samples were washed with water at about 40° for 8 hr, centrifuged and dried at 40° in vacuum. To decrease the organic component of the exudate, a portion of sample 1 was ashed by the low-temperature incineration method for ashing micro-organisms described by Thomas (1964). Extraction with organic solvents or strong base was avoided because of the increased possibility of introducing changes in the inorganic phase(s). Portions of the washed and low-temperature incinerated granules from sample 1 were ignited at 400° (50 hr) 500° (24 hr) and 900° (12 hr). Sample 2 was ashed directly at 650° and 900°.

X-ray diffraction and infrared analyses were done on the original water-washed and ashed granules. Diffraction patterns were recorded in a Debye–Scherrer type powder camera (114·59 mm. diameter) using Ni-filtered Cu radiation. Exposure time at 45 kV and 35 mA was 6 hr. Infrared spectra of the samples in KBr pellets were obtained utilizing a double-beam spectrophotometer covering the range 4000–200 cm.$^{-1}$.

Portions of the water-washed granules were ashed (sample 1 at 500° and sample 2 at 650°) and subsequently analysed for Ca, P, Na, K, Mg, Cl and trace elements. In addition, samples of the low-temperature incinerated portion of sample 1, ignited at 400° and 500°, were analysed for orthophosphate and pyrophosphate. The CO_2 content was estimated from infrared spectra.

RESULTS

Chemical analyses

Chemical analyses of the ashed samples for selected constituents are given in Table 1. The major constituents Ca and P, calculated as the oxides, account for 89% of sample 1 and 86% of sample 2. Except for CO_3, infrared active anions other than ortho- and pyrophosphate were not evident from the infrared spectra of the ashed samples. The Na, K, Mg (as oxides) and Cl percentages totaled accounted for 10% of sample 1 and 14% of sample 2. Approximately 1% of ashed sample 1 was estimated to be carbonate. The Ca:P molar ratios for ashed samples 1 and 2 were 1·45 and 1·21 respectively. The Ca:P molar ratio for sample 1 compares favourably with the 1·40 ratio calculated from the data presented by Pine & Overman (1963). Their ash value was much higher (56·0% compared to 12·0%) apparently caused by the removal of

pus and organic debris by extracting the granules with strong base (Dr L. Pine, personal communication). The low-temperature incinerated granules ignited at 400° and 500° yielded 40·6 and 44·5 % of the phosphorus as pyrophosphate, respectively.

Table 1. *Analyses of ashed sulphur granules*

	Sample 1	Sample 2
Ash	12·0 %	4·0 %
Ca/P molar ratio	1·45	1·21
Pyrophosphate		
(A) 400°	40·6 %	—
(B) 500°	44·5 %	—
Constituent	%	%
CaO	47·50	42·00
P_2O_5	41·49	43·99
Na_2O	0·34	0·63
K_2O	0·24	0·65
MgO	7·29	9·62
Cl	2·2	3·1
Trace elements*	0·02	0·05
Oxygen equivalent of chlorine	−0·50	−0·70
	98·58	99·34
CO_2	~1·0	Nil

* Sr, Br, Si, As, Fe, Zn, Cu and Pt summed.

X-ray and infrared analyses of original and low-temperature incinerated granules

The X-ray diffraction patterns of the washed and non-washed granules were characteristic of poorly crystallized apatite. The diffraction lines were weaker and more diffuse than those observed for normal bone (*ASTM publication*, 1960; Carlström & Engström, 1956; Posner, Eanes, Harper & Zipkin, 1963; Frazier, Zipkin & Mills, 1967). The diffraction pattern of the low-temperature incinerated granules remained indistinguishable from poorly crystallized apatite; the primary difference in the diffraction pattern as compared with that of the original was a decrease in background (P. D. Frazier, F. J. Brown, L. S. Rose & B. O. Fowler, in preparation).

Interpretation of the infrared spectra of the original 'sulphur granules', with regard to the inorganic phase, was hindered by the organic component (~ 90 %), although poorly defined bands were observed in the proper frequency ranges for apatite.

The infrared spectrum of the inorganic component(s) of the low-temperature incinerated granules consisted of better resolved ν_3, ν_1, ν_4 and ν_2 bands of apatite. In addition, the OH stretching band of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) was observed at 3570 cm.$^{-1}$, but was very weak, and the OH librational mode at 631 cm.$^{-1}$ was barely detectable (Fowler, Moreno & Brown, 1966). A broad shoulder from about 930 to 850 cm.$^{-1}$ was present. This band could partially arise from anhydrous dicalcium phosphate ($CaHPO_4$) which has an absorption band at 892 cm.$^{-1}$ (Fowler *et al.* 1966). Also, octacalcium phosphate ($Ca_8H_2(PO_4)_6.5H_2O$) has weak absorption bands in this region at 910 and 865 cm.$^{-1}$ which after partial dehydration, either by heating to 150–200° (Fowler *et al.* 1966) or by hydrolysis (B. O. Fowler, unpublished data) results in a shoulder at about 875–865 cm.$^{-1}$. In general, the spectrum could be inter-

preted as arising from a poorly crystallized hydroxyapatite and/or a partially dehydrated or hydrolysed octacalcium phosphate, either or both mixed with anhydrous dicalcium phosphate.

X-ray and infrared analyses of ignited granules

The X-ray and infrared patterns of the low-temperature ashed and non-ashed granules ignited at 400° for 50 hr showed poorly crystallized apatite; in addition, the infrared spectrum contained pyrophosphate bands. After continued ignition of the original granules at 400° for 3 weeks, both apatite and γ-$Ca_2P_2O_7$ were detected from the X-ray and infrared patterns. Further ignition of the low-temperature incinerated portion of sample 1 at 500° for 24 hr resulted in the formation of β-$Ca_2P_2O_7$ along with the apatite.

Relative intensities indicated additional X-ray diffraction lines nearly superimposed on the 2·88 and 3·21 Å lines of β-$Ca_2P_2O_7$ and the 2·63 Å line of apatite. These lines could arise from β-$Ca_3(PO_4)_2$ or a magnesium substituted β-tricalcium phosphate (Mg content of this sample 4·4 %) which have been shown to form at temperatures as low as 100° in the presence of magnesium (Trautz, Fessenden & Newton, 1954). The portion of ashed sample 1 pre-ignited in the low-temperature incinerator gave an infrared pattern with better resolved pyrophosphate bands as compared with the portion of ashed sample 1 which was not pre-ignited in the low-temperature incinerator. The effects of prior removal of the organic fraction, by this technique, on recrystallization and/or pyrophosphate formation on ignition are at present under investigation.

Sample 2, which was ignited at 650° for 24 hr, formed α-$Ca_2P_2O_7$ and β-$Ca_3(PO_4)_2$; the α-$Ca_2P_2O_7$ converted to β-$Ca_2P_2O_7$ after ignition at 900° for 12 hr. The phase transition from β- to α-$Ca_2P_2O_7$ is reported as 1140° (Hill, Faust & Reynolds, 1944). Formation of α-$Ca_2P_2O_7$ below the reported phase transition temperature (1140°) in apatitic calcium phosphates (ignited at 600°) has been reported by Rowles (1964). Sample 1 formed, as expected, β-$Ca_2P_2O_7$ and β-$Ca_3(PO_4)_2$ on ignition at 900°.

DISCUSSION

Several investigators have used the formation of pyrophosphate in pyrolysed synthetic calcium phosphates as an index of the presence of acid phosphate in the original material (Gee & Deitz, 1955; Winand, 1961; Herman, Francois & Fabry, 1961; Kühl & Nebergall, 1963; Fowler *et al.* 1966). The pyrophosphate formation in mineralized tissues, upon ignition, has also served as an indication of the presence of acid phosphate in the original material (Herman & Dallemagne, 1961; Francois & Herman, 1961; Dallemagne, 1964).

The low-temperature ashed 'sulphur granules' ignited at 400 and 500° yielded 40·6 and 44·5 % of the phosphorus as pyrophosphate, respectively. These values may not represent maximum conversion because insufficient sample was available to determine the percentages formed with different temperatures and duration of heating. Studies of pyrolyzed synthetic calcium phosphates by Herman *et al.* (1961) and Fowler *et al.* (1966) showed maximum pyrophosphate formation at about 500°.

Probable sources of formation of this pyrophosphate could be $CaHPO_4$ (Gee & Deitz, 1955), octacalcium phosphate (Brown, Smith, Lehr & Frazier, 1962), and

'tricalcium phosphate hydrate' (Dallemagne, 1964; Herman & Dallemagne, 1961; Francois & Herman, 1961). According to the reactions given by Gee & Deitz (1955) a mixture of hydroxyapatite and $CaHPO_4$ with the Ca:P molar ratio of our sample (1·45) would be expected to yield 32·3% pyrophosphate; according to Brown *et al.* (1962) a mixture of octacalcium phosphate and hydroxyapatite would also produce 32·3% pyrophosphate.

The percentage of pyrophosphate formed on pyrolysis is well above that predicted on the basis of the two-component model systems listed above; it is also above that predicted assuming, for example, a three-component system containing various proportions of octacalcium phosphate, anhydrous dicalcium phosphate and hydroxyapatite to make a net Ca:P molar ratio of 1·45. However, the actual Ca:P molar ratio of the $CaO{\cdot}P_2O_5{\cdot}H_2O$ phases could be less than 1·45 due to the presence of a calcium compound(s) other than phosphate which escaped detection by our methods.

In conclusion, the chemical analyses showed that the inorganic constituents of the 'sulphur granules' examined consisted primarily of calcium phosphate which is in agreement with the work of Pine & Overman (1963). The X-ray and infrared patterns were not sufficiently descriptive to unequivocally identify the calcium phosphate phase(s). The X-ray pattern of the granules indicated poorly crystalized apatite; the infrared spectrum could be interpreted as arising from a poorly crystalized hydroxyapatite and/or a partially dehydrated or hydrolysed octacalcium phosphate, either or both mixed with anhydrous dicalcium phosphate. The formation of 44·5% pyrophosphate in the ignited granules indicated the presence of considerable acid phosphate in the original granules; both octacalcium phosphate and anhydrous dicalcium phosphate are probable sources of the pyrophosphate formed on ignition.

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Quantitative Changes in the Content of Non-Nitrogenous Compounds during Autolysis of *Aspergillus terreus*

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SUMMARY

The behaviour of certain non-nitrogenous compounds in mycelium of *Aspergillus terreus* during autolysis in culture medium was studied. The mycelium of *A. terreus* lost during autolysis 45 % of its maximum dry weight. The amount of glucose, which was present from the beginning of autolysis throughout the whole further period of incubation, continuously increased to a maximum and then sharply descended to a constant value. Xylose decreased to half of its initial concentration during the first 30 days of autolysis and then disappeared. Mannitol descended in 95 % between the 18th and the 114th day of autolysis. Seventy-four % of the fat present at the beginning of autolysis was lost during the first 138 days of autolysis. The disappearance of fat and mannitol accounted for about 48 % of the dry wt. of mycelium lost during the whole period of autolysis.

INTRODUCTION

The earliest attempts to study the chemistry of autolysis in culture of filamentous fungi were confined almost entirely to the study of the behaviour of nitrogen-containing materials present in both mycelium and culture fluid (Behr, 1930; Schmidt, 1936; Woolley & Peterson, 1937; Bohonos, Woolley & Peterson, 1942; Ritter, 1955). Very few reports have appeared which deal with the behaviour of non-nitrogenous compounds in the mycelium during the autolysis of filamentous fungi in their culture media. Prill, Wenck & Peterson (1935) studied the effect of starvation on the formation and chemical nature of the fat produced by *Aspergillus fischeri*. Smithies (1953) examined autolysis of the mycelium of *Penicillium griseofulvum*, describing the total amount of carbohydrates liberated by enzymic action during autolysis. Some investigations have been made about changes in the amounts of organic acids and sugars, but in all cases these studies have been limited to detecting the presence or absence of these compounds. When these organic acids and sugars have been estimated, this has been done by a mere visual comparison (Tandon & Chandra, 1962). Emiliani & Ucha de Davie (1962) studied the liberation of glucose from the autolysing mycelium of *A. phoenicis* in 1 % acetic acid solutions. Arima, Uozumi & Takahashi (1965) and Uozumi, Takahashi & Arima (1965) described the autolysis of *A. oryzae* and the morphological changes of mycelium which occurred. Lahoz, Reyes & Beltrá (1966) studied some chemical changes occurring in mycelium of *A. flavus* during autolysis in culture. In the present work attention has been directed towards the qualitative and quantitative changes in the content of fat, free sugars and mannitol in the mycelium of *A. terreus* during autolysis in culture.

METHODS

Organism. Aspergillus terreus Thom (our collection no. 2426) was used throughout the present study of autolysis in a liquid medium.

Chemicals. Chemicals used in the preparation of the culture medium were of analytical purity, all obtained from Probus S.A. Barcelona, Spain. Other chemicals used were either from The British Drug Houses Ltd., Poole, Dorset, England, or from Sigma Chemical Co., St Louis, Mo., U.S.A.

Culture medium. The organism was grown in Czapek-Dox medium (g./l.): glucose (anhydrous), 50; $NaNO_3$, 2·0; KCl, 0·5; $MgSO_4.7H_2O$, 0·5; KH_2PO_4, 1·0; $FeSO_4.7H_2O$, 0·01; distilled water, 1000 ml., pH 4·2. This medium was chosen as the standard medium in preference to other defined media because it contains its nitrogen as nitrate, which results in a greater 'alkaline autolysis' (Behr, 1930) accentuating the changes undergone by carbohydrates during autolysis.

Ten litres of this medium were prepared and distributed in 100 ml. amounts in Jena conical flasks (300 ml. capacity). The flasks were plugged with cottonwool and sterilized by steaming for 30 min. on three successive days. The flasks were inoculated with 1 or 2 ml. of a spore suspension obtained by gently rubbing the surface of each of 12 slopes of *Aspergillus terreus* grown on malt agar for 15 days at 24°. The inoculated flasks were incubated at 24–25° in the dark.

At regular intervals, generally 12 days, samples of five flasks were selected at random and withdrawn from the incubator. The pads of mycelium were separated from the culture liquid by filtration, pooled, washed on a Buchner funnel with distilled water, cut into small pieces and dried in an oven at 60–70° for 24 hr. Then they were dried in a desiccator (P_2O_5) to constant weight, and powdered in a small coffee mill (Turmix, model Moka-mix). The culture liquids from the five sample flasks were also pooled, and the pH value measured with Beckman pH meter, model G 2000.

An accurately weighed amount of powdered mycelium from each pooled, sample was continuously extracted in a Soxhlet apparatus with light petroleum (b.p. 50–70°) for 15–20 hr, the solvent removed by distillation (the last portions *in vacuo*) and the residue weighed. Results will be expressed as mg. component/flask. The defatted mycelium was re-extracted with diethyl ether in the same conditions as above and the resulting extract discarded.

In a typical experiment 1·0 g. air-dried, defatted mycelium was placed in a beaker and extracted twice with cold distilled water (2×100 ml.) for 1 hr with mechanical stirring. A third water extraction gave an extract which did not show any sugar spot when a portion of it after concentration was chromatographed. The first two aqueous extracts were pooled and protein removed by adding basic lead acetate (Eastham, 1949). Subsequently this treatment was found to be unnecessary, since samples of these extracts untreated with basic lead acetate gave good chromatographic separations as well as identical values for the content of total free reducing substances. A portion of this extract (100 ml.) was concentrated, under reduced pressure to final volume 10 ml. To 1 ml. of this concentrated extract, the reagents of Somogyi (1945) and Nelson (1944) were applied for the quantitative determination of total water soluble free reducing substances, by the use of a Klett–Summerson colorimeter. Al readings were compared with standards treated in the same way. Results are expressed as mg. glucose/100 g. dry wt. mycelium. From the concentrates prepared as indicated

above individual sugars and mannitol were separated, identified and estimated as described previously (Lahoz *et al.* 1966).

Total nitrogen. The assays of total nitrogen were made by the micro-Kjeldahl method.

Total phosphorus. This was determined by a modification of the method of Fiske & SubbaRow (1925).

RESULTS

The criterion of degree of autolysis we have used through this work was the loss in weight of dry mycelium. Between 24 and 27 days of incubation of the cultures autolysis set in, as indicated by a loss in mycelial dry weight. The 25th day of incubation was therefore taken as the first day of autolysis; the word 'day' in the following text refers to 'day of autolysis'. By day 114 the mycelium lost 45% of initial dry weight (Fig. 1). The pH value of the culture medium increased continuously during the first 18 days (Table 1) reaching about pH 8 at the 30 days, thereafter remaining practically constant. The nitrogen content of the mycelium steadily decreased throughout the whole period of autolysis; this decreased 54% of the initial value (Table 1). The total loss of phosphorus was 17·8 mg./flask during the autolysis, representing about 90% of the initial P in the mycelium at the beginning of autolysis.

Table 1. *The autolysis of Aspergillus terreus mycelium during incubation in Czapek-Dox medium at 25° in the dark*

Values for pH of culture filtrate, yield of mycelium, content of fat, nitrogen and phosphorus in mycelium. Autolysis began at 24–25 days of incubation.

Time of incubation (days)	Time of autolysis (days)	pH value	Yield of mycelium (g. dry wt./flask)	Content in mycelium of		
				Fat (mg./flask)	N (mg./flask)	P (mg./flask)
24	0	6·10	1·3261	295	23·0	—
27	3	6·85	1·3174	234	22·8	18·8
30	6	7·50	1·2368	229	20·5	16·0
42	18	7·75	1·0787	197	19·3	11·0
54	30	8·20	1·0050	208	19·0	7·0
66	42	8·10	0·9350	176	19·5	5·6
78	54	8·05	0·8410	150	17·6	4·2
90	66	7·70	0·8139	124	17·9	2·6
102	78	7·80	0·7863	128	17·1	2·4
114	90	7·80	0·7739	115	17·0	2·5
126	102	7·80	0·7498	117	16·4	2·2
138	114	7·80	0·7295	108	16·0	1·7
150	126	7·80	0·7464	—	—	1·2
162	138	7·80	0·7280	71	16·0	0·7
174	150	7·80	0·7012	82	10·5	0·9
186	162	7·80	0·7287	78	—	1·1

Behaviour of the mycelial fat. The content of fat at the beginning (Table 1) of autolysis, 295 mg./flask at the 0 day, decreased to a half by the 54th day, after 138 days amount of fat remained fairly constant. The total loss during autolysis being 74% of the initial content.

Behaviour of the free sugars and of the free reducing substances in the mycelium. Apart from glucose and xylose the autolysing mycelium of *Aspergillus terreus* contained no other free sugar in significant amount.

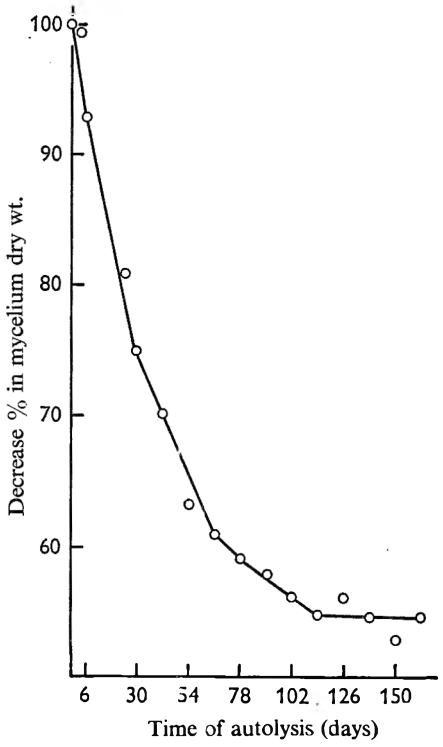

Fig. 1

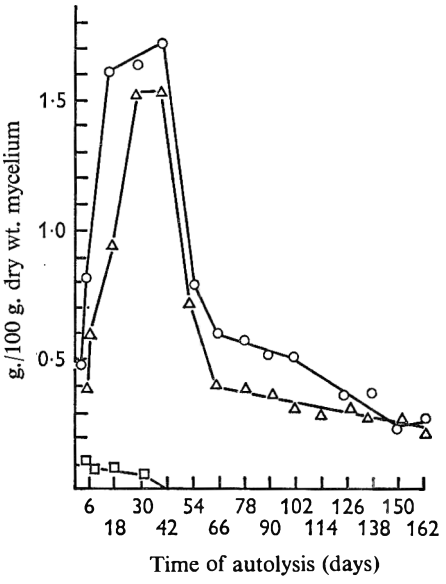

Fig. 2

Fig. 1. *Aspergillus terreus*. Change in the dry wt. of mycelium during autolysis in culture (25°, in dark).

Fig. 2. *Aspergillus terreus*. Changes of concentration of total free reducing substances, ○; glucose, △; xylose, □, in mycelium during autolysis in culture (25°, in dark).

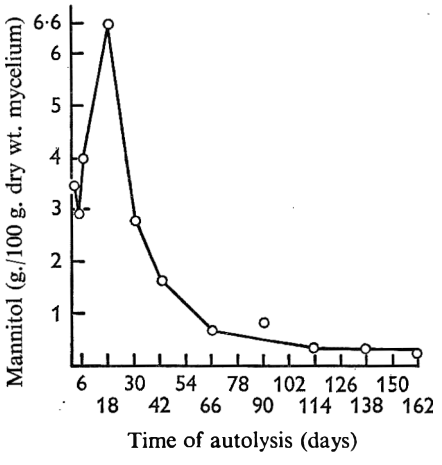

Fig. 3. *Aspergillus terreus*. Change in concentration of mannitol in mycelium during autolysis in culture (25°, in dark).

As shown in Fig. 2 there was an increase in the total amount of free reducing substances during the initial stages of autolysis. The initial values for reducing substances at the beginning of autolysis were 500 mg./100 g. dry wt. mycelium, increasing to 1600 mg./100 g. dry wt. mycelium by 18 days, 24 days later (42 days total) these reducing substances reached 1700 mg./100 g. dry wt. mycelium, followed by a sharp decrease (54–66 days) in their concentration to a final constant value. The curve for free glucose presented a very similar pattern (Fig. 2). We found an increase in the total amount of free glucose during autolysis. The initial values for the amount of glucose, 400 mg./100 g. dry wt. mycelium were nearly quadrupled by day 42, when there was a peak for total free reducing substances and glucose. Glucose decreased sharply from 42 to 66 days, then diminished at a slower rate to a constant value.

Between day 6 and day 126, glucose accounted for an average of 83 % of the total reducing power, at the end of the incubation time, day 174 to day 186, glucose accounted for the total reducing power of the aqueous extracts. Glucose was present from the beginning of autolysis through the whole period of incubation, xylose decreased half of its initial concentration (123 mg./100 g. dry wt. mycelium) during the first 30 days and then disappeared in the next 12 days.

Behaviour of mannitol

The concentration of mannitol in the mycelium of *Aspergillus terreus* during autolysis was at a minimum when autolysis began (Fig. 3). At day 18 it reached a peak (6·6 g./100 g. dry wt mycelium), decreasing then, rapidly at first, to a constant value after the day 114. Ninety-five % of the mannitol disappeared between days 18 and 114.

DISCUSSION

It is generally believed that carbohydrates present in fungal mycelia are continuously undergoing breakdown during autolysis in culture, but very few studies on carbohydrates in mycelia during autolysis of filamentous fungi are available for comparison with the present results. Our experiments indicate that carbohydrates in mycelium of *Aspergillus terreus* autolyzing in the culture fluid underwent enzymic hydrolysis in which glucose appeared, giving a concentration of glucose several times greater than in the initial stages of autolysis. Similar qualitative results were obtained by Tandon & Chandra (1962) who reported that the concentration of carbohydrates in the mycelium of *Colletotrichum gloeosporioides* decreased during autolysis.

In the autolysis of *Aspergillus phoenicis* in 1 % acetic acid solution glucose was one of the components more abundantly formed, amounting to 22 % of the dry weight of the original mycelium (Emiliani & Ucha de Davie, 1962). In our results free glucose reached much lower values (1·55 g./100 g. dry wt. mycelium) which represents only 7 % of the value for glucose released by *A. phoenicis*. However, in Emiliani & Ucha de Davie's experiments an induced autolysis took place, which in our opinion was partially due to the use of unnatural agents such as acetic acid to cause autolysis. The variety of methods used in autolysis studies partially explains the difficulties encountered in comparing different sets of results. Similar but less drastic conditions for autolysis, 'fresh mycelium' suspended in water, were used with mycelium of *Penicillium griseo-fulvum* but no reducing sugars were formed during autolysis (Smithies, 1953).

The formation of glucose in the mycelium of *Aspergillus terreus* during autolysis in

culture took place more actively in the initial stages, from day 3 or 6 to day 42 (Fig. 2), in which the highest values for glucose were formed. This recalls certain observations with yeast; Vosti & Joslyn (1954) stated that proteins and carbohydrates in yeast were involved in the early stages of autolysis.

Our experiments indicate that during autolysis fat continually disappeared from mycelium; similar results were obtained by Prill *et al.* (1935) who studied the effect of inanition on the chemical nature of the fat produced by *Aspergillus fischeri.* These workers found that the amount of fat continuously decreased, the loss being about 50% the initial value. In our experiments with *A. flavus* (Lahoz *et al.* 1966) the fat content remained constant.

A complete disappearance during autolysis of the free sugars in the mycelium of *Aspergillus terreus* seems not to take place; in the present work with an incubation period as long as 186 days, the glucose concentration at that time was greater than 200 mg./100 g. dry wt. mycelium (Fig. 2). With *A. flavus* grown in Raulin–Thom medium we have similarly found that the free sugars in the mycelium persisted up to 251 days of incubation (Lahoz *et al.* 1966).

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-eighth General Meeting at University College London, W.C. 1, *on Wednesday and Thursday* 4 *and* 5 *January* 1967. *On Wednesday the Society joined with the Pathological Society of Great Britain and Ireland for a Symposium. Abstracts of the Contributions to the Symposium on 'Mycoplasma', and of Original Papers are below.*

SYMPOSIUM: MYCOPLASMA

The Structure of Mycoplasma Cells. By S. RAZIN (*Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel*)

Mycoplasma cultures contain cells of similar size as some of the larger viruses. It is not surprising therefore that anatomical data of these minute organisms began to be known only recently, as more sophisticated electron microscopy and cell-fractionation techniques became available.

Thin sections of mycoplasmas reveal an internal cell structure very much resembling that of bacteria. A central fibrillar region represents the nucleoid, and the cytoplasmic region is filled with ribosomes. The long DNA molecule forming the mycoplasma nucleoid was liberated and resolved following rupture of the cells by the Kleinschmidt procedure (Morowitz, H. J. *et al.* (1966), *Ann. N.Y. Acad. Sci.*, in the Press). The DNA molecule has a circular appearance like that of bacteria and viruses. Although DNA molecules of the various mycoplasmas tested were of varying length, they generally were considerably shorter than those of ordinary bacteria. The limited amount of genetic material may explain the restricted metabolic and biosynthetic activity of the mycoplasmas. The DNA of several *Mycoplasma* spp. also has an extraordinarily low (23 %) guanine+cytosine content (Jones, A. S. & Walker, R. T. (1963), *Nature, Lond.* **198**, 588; Neimark, H. C. & Péne, J. J. (1965), *Proc. Soc. exp. Biol., N.Y.* **118**, 517).

The ribosomes of *Mycoplasma gallisepticum* were studied in detail (Maniloff, J. *et al.* (1965), *J. Bact.* **90**, 193). Their properties (140 A, diameter; 70·2 S sedimentation coefficient; RNA–protein ratio, 0·68) resemble those of ribosomes of other bacteria. The recent finding that the mechanism of protein biosynthesis by mycoplasmas does not differ from that of bacteria (Tourtellotte, M. E. (1966), *Ann. N.Y. Acad. Sci.*, in the Press) indicates that mycoplasma ribosomes also resemble bacterial ribosomes functionally. Thin sections of *M. gallisepticum* showed the ribosomes to be arranged in cylindrical arrays, an arrangement not found in any other mycoplasma (Domermuth, C. H. *et al.* (1964), *J. Bact.* **88**, 727; Maniloff, J. *et al.* (1965), *J. Cell Biol.* **25**, 139).

Perhaps the most pronounced anatomical feature of mycoplasmas is their lack of a cell wall. This was evident in thin sections of the organisms, and was supported by the absence of the specific bacterial cell-wall substances (Razin, S. (1963), in *Recent Progress in Microbiology*, VIII, p. 526, ed. N. E. Gibbons, Toronto: University Press). The mycoplasma cell is bounded by a single lipoprotein membrane about 80–100 A thick. Chemical analysis of membranes isolated from several mycoplasmas showed them to be composed of about 60 % protein and over 30 % of lipid. The isolated membranes could be disaggregated by detergents to subunits, which could be reaggregated to membrane-like structures upon removal of the detergent and addition of a divalent cation (Razin, S. *et al.* (1965), *Proc. natn. Acad. Sci. U.S.A.* **54**, 219). Little is known about the protein component of the membrane. Disc-gel electrophoresis of

Mycoplasma laidlawii membranes disaggregated by sodium dodecyl sulphate showed more than ten different protein bands. On the other hand, membrane proteins formed only one symmetrical sedimentation peak of about 3 S in the analytical ultracentrifuge (Rodwell, A. W. *et al.*, to be published). The lipid moiety of mycoplasma membranes consists mostly of phospholipids, glycolipids and cholesterol (Smith, P. F. (1966), *Ann. N.Y. Acad. Sci.*, in the Press; Plackett, P. *ibid.*). The mycoplasmas are unable to synthesize many of the lipid precursors, which they require for growth. Because of their dependence on an external supply of lipids, mycoplasmas are highly efficient tools for the study of lipid functions in biological membranes. Thus, cholesterol was shown to be essential for membrane integrity in parasitic mycoplasmas by restricting its supply in the growth medium (Razin, S. (1966), *Ann. N.Y. Acad. Sci.*, in the Press). Likewise, a variation of the saturated fatty acid ratio in the growth medium resulted in a similar variation in the fatty acids of the membranes of *M. laidlawii*. A change in favour of the unsaturated acids caused this mycoplasma to grow in very long filaments (Razin, S. *et al.* (1966), *J. gen. Microbiol.* **42**, 139; and (1966), *J. Bact.* **91**, 609). It seems that the ability to grow in filaments is a general property of mycoplasmas which they can manifest only when a proper and balanced supply of the essential lipids is present in the growth medium.

Antigenic Structure in the Genus *Mycoplasma*. By RUTH M. LEMCKE (*Lister Institute of Preventive Medicine, London, S.W.* 1)

Serological methods are widely used in the identification of mycoplasmas and in the diagnosis of mycoplasma infection, but they have yielded little information on the number of antigenic components present in any one species, or the location of these components in the cell.

Immunodiffusion methods using disrupted cell suspensions have indicated a multiplicity of antigenic components in every species examined. Some reports suggest the sharing of antigenic components by different species (Taylor-Robinson, D. *et al.* (1963), *J. Bact.* **85**, 1261; Taylor-Robinson, D., Fox, H. & Chanock, R. M. (1965), *Am. J. Epidemiol.* **81**, 180), whereas others consider that the majority of cross-reacting components are partly related rather than identical (Lemcke, R. M. (1965), *J. gen. Microbiol.* **38**, 91).

Two attempts have been made to work out antigenic formulae for certain species, using classical cross-absorption techniques. The formulae given by Villemot & Provost ((1959), *Revue Élev. Méd. vét. Pays trop.* **12**, 369) and Provost *et al.* ((1964) *ibid.* **17**, 23) show six antigens distributed in various combinations among six species from animals and man, but none specific to any one species. Pease ((1965), *J. gen. Microbiol.* **41**, 299), who examined strains of four species of *Mycoplasma*, found that each species had at least one specific antigenic component. Although the two reports are not comparable, it is apparent that further work is required before the validity of these antigenic formulae is accepted.

Chemical fractionation, although only applied to a limited number of species so far, has given more information on antigenic structure. *Mycoplasma mycoides* has been studied most intensively, probably because of interest in the pathogenesis of bovine contagious pleuropneumonia and in the immune response to infection and vaccination. Since 1937, data has accumulated about a specific, heat-stable polysaccharide, probably located on the cell surface. This substance has been identified as a galactan in which the predominant linkage is -6-O-β-D-galactofuranosyl-1-; it contains a small amount of lipid and has the properties of a complex hapten, but the specific determinant groups have not yet been determined (Buttery, S. H. & Plackett, P. (1960), *J. gen. Microbiol.* **23**, 357; Plackett, P., Buttery, S. H. & Cottew, G. S. (1963), *Recent Progress in Microbiology*, VIII, 535). A similar galactose-containing lipopolysaccharide complex was found in the blood and exudates of infected cattle (Gourlay, R. N. (1965), *Res. vet. Sci.* **6**, 263). Although the galactan is not toxic, it may help the organism to multiply *in vivo*; Gourlay ((1965) *loc. cit.*) found that it enhanced the virulence of *M. mycoides* when inoculated subcutaneously into susceptible cattle.

Several other mycoplasmas have been examined by Plackett *et al.* ((1963) *loc. cit.*) for the presence of specific polysaccharides, but only one was found—a glucan in bovine arthritis strains.

Recently, the antigenic structure of the human respiratory pathogen, *Mycoplasma pneumoniae*, has been investigated in several laboratories. In contrast to *M. mycoides*, the major specific component of *M. pneumoniae* is a lipid which is probably present in the cell membrane. It is a heat-stable hapten with complement-fixing activity and the ability to block growth-inhibiting and indirect-haemagglutinating antibody (Kenny, G. E. & Grayston, T. E. (1965), *J. Immunol.* **95**, 19; Prescott, B. *et al.* (1966), *J. Bact.* **91**, 2117; Sobeslavsky, O. *et al.* (1966), *ibid.* 2126; Lemcke, R. M., Marmion, B. P. & Plackett, P. (1966), *Ann. N.Y. Acad. Sci.*, in the Press). The nature of the determinant groups of the lipid hapten is still being investigated.

Examination of the other *Mycoplasma* species from man has revealed a specific heat-stable lipid only in *M. fermentans* (Kenny, G. E. (1966), *Ann. N.Y. Acad. Sci.*, in the Press). Preliminary observations on *M. hominis* (Lemcke, unpublished observations) suggest that the antigenic components involved in complement-fixation and precipitation are heat-labile, resistant to periodate and associated with the cell contents.

The mycoplasmas so far examined appear to be heterogeneous with respect to the chemical character and location of their major antigenic components.

Techniques of Mycoplasma Antibody Measurement and Identification. By D. TAYLOR-ROBINSON (*Common Cold Research Unit, Harvard Hospital, Salisbury*)

Mycoplasmas have been studied by almost every available serological technique. Some methods are better than others for quantitative measurement of antibody. Complement fixation was used first with *Mycoplasma mycoides* (Campbell, A. D. & Turner, A. W. (1936), *Bull. C.S.I.R. Aust.* **97**, 11) and mycoplasmas may be distinguished with antisera prepared against them. Complement fixation is less specific with post-infection sera and is not the most sensitive technique. Following the observation of van Herick, W. & Eaton, M. D. ((1945), *J. Bact.* **50**, 47) haemagglutination-inhibition has been used successfully in avian mycoplasma work, mainly for *M. gallisepticum* antibody measurement. The test has limited application, since apart from *M. pneumoniae* (Feldman, H. A. & Suhs, R. H. (1966), *Am. J. Epid.* **83**, 345) strains from other host species apparently do not haemagglutinate. In addition, adsorption of erythrocytes onto the surface of *M. pneumoniae* colonies and haemadsorption-inhibition have been reported (Del Guidice, R. A. & Pavia, R. (1964), *Bact. Proc.* p. 70). Again, application is limited, although the phenomenon occurs also with *M. gallisepticum* and other avian mycoplasma strains. Mycoplasmas which haemagglutinate do not necessarily exhibit colony haemadsorption. In contrast, indirect haemagglutination has wide application. However, since first employed with *M. mycoides* (Cottew, G. S. (1960), *Aust. Vet. J.* **36**, 54) it has not been used widely probably because of technical difficulties; most of the latter may be overcome by using sensitized erythrocytes stored at $-70°$ C. The method is sensitive, but results are sometimes difficult to interpret; those obtained with *M. pneumoniae* will be discussed. Agglutination was first used for species differentiation by Klieneberger (Klieneberger, E. (1938), *J. Hyg. Camb.* **38**, 458), but is of limited value for the quantitative measurement of antibody. Immunofluorescence was used extensively by Liu ((1957), *J. Exp. Med.* **106**, 455) to examine the Eaton agent before its identification as a mycoplasma. The latter development stimulated work in the human field. While immunofluorescence was found still to be of value in the diagnosis of infection, its laborious nature has precluded its widespread use. Search for other reliable techniques led to the development of the growth or metabolic inhibition (MI) test in a liquid medium; this followed the original observation of inhibition of mycoplasma growth by antibody in agar (Edward, D. G. ff. & Fitzgerald, W. A. (1954), *J. Path. Bact.* **68**, 23). MI tests are based upon the ability of mycoplasmas to reduce tetrazolium salts or break down glucose or arginine; also T-strain mycoplasmas break down urea. The requirement for a heat labile accessory factor (complement-like) is variable and will be discussed. The tests are valuable since they measure protective antibody; and they are the only means available for measuring antibody against T-strains.

The logical approach to mycoplasma identification follows these lines. First, an inquiry into the mycoplasma source. Doubt has been cast on the avian or animal host specificity of mycoplasmas, but it exists to such a degree as to be useful in identification. Likewise, knowledge that a mycoplasma is from tissue culture is helpful since only a limited number of sero-

types have been found as contaminants. Secondly, certain biochemical properties such as the break down of glucose, arginine or urea, or a biological property such as haemadsorption provide useful clues to the range of antisera required to make a definitive identification. Finally, identification is dependent upon serology. Every method so far mentioned has been used and, in addition, gel precipitation. This requires antigen concentration and possibly monospecific antisera produced by absorption. Currently, the disc growth-inhibition technique (Clyde, W. A. Jr. (1964), *J. Immunol.* **92**, 958) is most widely used. Difficulties associated with this technique will be discussed. Growth or MI tests in liquid medium are more sensitive and require less antiserum; for T-strains MI is undoubtedly the method of choice for identification.

Latent Infections of Tissue Culture by Mycoplasmas? By M. BUTLER and R. H. LEACH (*Wellcome Research Laboratories, Beckenham, Kent*)

There is evidence that some tissue culture systems can carry mycoplasma infections which may not be readily detectable by ordinary cultural methods. It has been shown (Grace *et al.* (1965), *Cancer*, **18**, 1369; Girardi *et al.* (1965), *Proc. Soc. exp. Biol. Med.* **120**, 760) that mycoplasmas in infected tissue culture can exist as minute forms (0·1 mμ) detectable only by subculture in further tissue cultures, although larger forms which grow in cell-free media may also be present concurrently. The question of latency has special importance in the interpretation of reports (Negroni, G. (1964), *Brit. med. J.* **i**, 927; Armstrong *et al.* (1965), *J. Bact.* **90**, 418; Murphy *et al.* (1965), *Cancer*, **18**, 1329; Grace *et al.* (1965), *Cancer*, **18**, 1369) in which mycoplasmas have apparently been isolated by means of tissue culture from human malignant and other diseases.

Using various tissue culture cells and mycoplasma strains the properties of deliberately infected cultures were examined. *Mycoplasma hominis* infections were easily established in HEp-2 tissue cultures, although where low doses of mycoplasma were inoculated the organism could not be re-isolated for several weeks and low-grade infections appeared to have been established. Similarly, it was not always possible to re-isolate in cell-free media the GDL strain of *M. hyorhinis* (Butler, M. & Leach, R. H. (1964), *J. gen. Microbiol.* **34**, 285; Tully, J. G. (1966), *Proc. Soc. exp. Biol. Med.* **122**, 565) from HEp-2 culture infected with this organism. The addition of HEp-2 cell extract to the medium facilitated re-isolation. From W1–38 cultures infected with the GDL strain, colonies were sometimes obtained which did not survive subcultivation to fresh cell-free media. Fluid from these W1–38 cultures passaged in HEp-2 produced characteristic cytopathic effect and mycoplasmas were reisolated in cell-free media. It would appear that in some tissue culture systems mycoplasmas may become adapted to growth in the presence of living cells which provided specific growth factors. An intracellular habitat, as reported for mycoplasmas growing in some tissue cultures (Anderson & Manaker (in the Press) quoted by Anderson *et al.* (1965), *J. Bact.* **90**, 1387; Shedden, W. I. H. & Cole, B. C. (1966), *Nature, Lond.* **210**, 868), might favour the development of an obligate parasitic relationship between the mycoplasma and the tissue cells.

In addition to direct cultivation of mycoplasmas from infected tissue cultures, other methods such as microscopy, utilizing the fluorescent antibody technique, or the Schulz–Dale technique were examined, but appeared to be insufficiently sensitive to detect the very small amounts of mycoplasma antigen which would be involved in low-grade infections.

In current work attempts have been made to produce latent infections of *M. hominis* or the GDL strain in HEp-2 and W1–38 tissue cultures by treatment of infected cultures with antibiotics, but no evidence has been obtained for induction of latency.

Cell Alterations Induced *in vitro* by Mycoplasmas. By W. RUSSELL (*National Institute for Medical Research, Mill Hill, London*)

Infection of BHK21–C13 cells by mycoplasmas of various serotypes induced cells to form colonies in agar suspension. On subculturing representative colonies most of these assumed the normal orientation and growth pattern of uninfected cells. However, some of the colonies obtained after infection with *Mycoplasma fermentans* and *M. hominis* grew with the random

orientation similar to that shown by cells transformed by polyoma and other tumour viruses. These cells grew more efficiently and released more acid into the medium than uninfected cells; no mycoplasma could be detected in them by standard techniques (Macpherson, I. A. & Russell, W. (1966), *Nature, Lond.* **210**, 1343). Transplantation studies have been made on the altered cells derived from *M. fermentans* infection and it was found that less than 10^3 cells were sufficient to produce tumours in young adult hamsters. Uninfected BHK21–C13 cells, as shown by other workers (e.g. Hale *et al.* (1965), *Brit. J. exp. Path.* **46**, 598), also produced tumours in hamsters, but under the conditions used here between 10^5 and 10^6 cells were required. In contrast however to cells transformed by tumour viruses no indication could be found of tumour or mycoplasma antigens in complement fixation or gel diffusion tests, using sera from tumour-bearing animals or from rabbits immunized with mycoplasma. Furthermore, there is no evidence of transplantation immunity and, as yet (after 8 months) there is no indication that baby hamsters inoculated with *M. fermentans* can produce tumours. Since tumours produced by the altered and normal cells are apparently identical histologically, it would appear that a mechanism of selection and stimulation may be involved in this system.

As a corollary to these investigations a study has been made of some of the biochemical and other events associated with mycoplasma infection of BHK21–C13 cells. Thus, infection of BHK21–C13 cells by *M. fermentans*, given the correct conditions, can give rise to an increased plating efficiency on glass. A number of experiments have been carried out to examine the levels of some of the enzymes concerned with nucleic acid metabolism after infection of cells with three mycoplasmas viz. *M. fermentans*, *M. orale* Type 2 and the Negroni agent. In particular, significant elevations of the levels of thymidine kinase, deoxyribonuclease and ribonuclease have been noted. Considerable amounts of these enzymes are present in extracts of the mycoplasmas themselves (cf. Razin *et al.* (1964), *J. gen. Microbiol.* **36**, 323). A study is being made of the properties of these enzymes in an effort to deduce the source of the increased enzyme activity after infection. The incorporation of tritiated thymidine and tritiated uridine is also affected to varying degrees by mycoplasma infection. It is apparent from these results that great care should be exercised in interpreting results involving changes in nucleic acid metabolism where mycoplasma contamination is a possibility.

Serological Surveys for Antibodies to *Mycoplasma* Isolated from Patients with Leukaemia.
By R. J. FALLON (*Department of Pathology, Ruchill Hospital, Glasgow, N.W.*)

Micro-organisms have long been sought as a cause of malignant disease, and for many years it has been recognized that some animal tumours could be produced as a result of virus infection. The discovery that murine and avian leukaemias could be caused by viruses has provided an extra stimulus to the search for such agents in human leukaemia as well as in other forms of malignant disease. The search for infective agents in human leukaemia has been made principally by electron microscopic examination of plasma and tissues from leukaemic patients and by attempted isolation of viruses from such materials. Various workers have reported the presence of virus-like and more recently mycoplasma-like particles in electron micrographs of material from leukaemic patients.

Attempts at virus isolation have yielded some known viruses but also some agents, at first thought to be viruses, have subsequently been shown to be mycoplasmas. These agents were isolated from material passaged in tissue cultures and in view of the frequent finding of mycoplasma as tissue-culture contaminants these findings were regarded with caution. Recently, however, mycoplasmas have been isolated directly, in cell-free media, from cases of leukaemia. With two exceptions the strains so far isolated have been shown to be known human strains, either *Mycoplasma orale* or *M. fermentans*. The exceptions, both isolated in tissue culture, are the Negroni and 880 mycoplasmas, both of which are closely related to a rodent pathogen, *M. pulmonis*.

Many patients with leukaemia have a lowered resistance to infection and it may well be that the mycoplasma isolated from cases of leukaemia are merely a manifestation of such lowered resistance. This may be accepted where the strain isolated is one commonly associated with man, however it is less easy to explain the presence of rodent strains of mycoplasma in such patients.

One way of investigating the problem further is to examine the serum of patients with leukaemia for antibodies to these mycoplasma strains and Negroni has reported (*Brit. med. J.* (1964), **i**, 927; *Proc. roy. Soc. Med.* (1966), **59**, 662) that antibodies to the agent he isolated may be demonstrated, using tissue culture techniques, in a higher proportion of sera from leukaemic patients than from non-leukaemic controls. Similarly, W. H. Murphy has reported ((1966), *Ann. N.Y. Acad. Sci.*, in the Press) that there is some correlation between antibodies to some of the strains of *M. fermentans* which he has isolated and leukaemia, but his results do not suggest a simple cause and effect relationship as there was also a correlation between leukaemia and antibodies to some viruses.

Studies have been made of the distribution of antibodies to the Negroni and 880 mycoplasmas and to human strains of mycoplasma in both leukaemic and non-leukaemic subjects in the west of Scotland. The main techniques used have been those of complement fixation and metabolic inhibition. No evidence has yet been found of complement-fixing or metabolic-inhibiting antibodies to the 880 mycoplasma in these populations and only one normal woman has been found to have metabolic-inhibiting antibodies to the Negroni mycoplasma in a survey of 393 subjects. Antibodies to some of the strains of mycoplasma isolated by Murphy (Murphy, W. H., Furtado, D. & Plata, E. (1965), *J. Am. med. Assn* **191**, 110) are also being sought using similar techniques. The significance of the failure to demonstrate antibodies to the Negroni and 880 mycoplasmas by these techniques, both in terms of its relationship to the findings reported using tissue culture, as well as to the possible distribution of these mycoplasmas in the populations surveyed is uncertain. However, it seems that infections by the Negroni and 880 mycoplasmas resulting in the production of antibodies of a type commonly produced as a result of other mycoplasmal infections are rare in the west of Scotland.

Mycoplasmas in Enzootic Pneumonia of Pigs. By R. F. W. GOODWIN and P. WHITTLESTONE (*School of Veterinary Medicine, University of Cambridge*)

Enzootic pneumonia of pigs is a very common disease in Britain; the condition also occurs in many other countries. The trend to keep pigs in larger populations, and more closely together indoors, favours the spread of the causal agent within a herd, so that most of the young stock may become infected shortly after birth. The pneumonia takes about 2 weeks to develop and usually persists for several months, during which time the affected pig is infectious to other susceptible pigs. The lesions show alveolar-cell proliferation, mononuclear-cell accumulations around the blood vessels and bronchioles and, later, progressive lymphoreticular hyperplasia. The disease is rarely fatal, except when adult animals meet it for the first time, but it has a marked economic effect; for affected animals commonly require more food to make the same live-weight gain (up to about 20 % more in controlled trials). The economic loss to the pig industry is thus very great.

The causal agent has recently been shown to be a mycoplasma, provisionally named *M. suipneumoniae* (Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1965), *Vet. Rec.* **77**, 1247): colonies were passed on solid medium to beyond the point of mechanical carry over (greater than 10^{-15}) and the final cultures induced the typical disease. The same mycoplasma had previously been grown in tissue cultures (Goodwin, R. F. W. & Whittlestone, P. (1963), *Brit. J. exp. Path.* **44**, 291) and also in liquid media (Goodwin, R. F. W. & Whittlestone, P. (1964), *Vet. Rec.* **76**, 611; (1966), *Brit. J. exp. Path.*, in the Press). Using this liquid medium, Maré, C. J. & Switzer, W. P. ((1965), *Vet. Med.* **60**, 841), induced enzootic pneumonia in pigs with fluid cultures containing coccoid organisms; from these fluids they recovered a mycoplasma which they called *M. hyopneumoniae*, but it is not known whether this mycoplasma was also capable of inducing the disease.

M. suipneumoniae is very pleomorphic: in touch preparations from lung lesions, ring and bipolar forms predominate, and these usually occur singly or in groups; in tissue cultures, the mycoplasma is usually in diffuse groups (mainly as cocci in short chains) but large ring-forms containing a single coccus-like structure are also seen; in liquid media, the main forms are cocci strung on fine branching filaments, or globular structures, usually in colonies. The size varies from about 0·3–6·0μ diameter. Sera prepared in rabbits have been used to identify

M. suipneumoniae in the fluorescent-antibody, precipitation-in-agar-gel, and growth-inhibition tests (Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1966), in preparation) and shortly a serological test might be used to diagnose the disease. It is not known, however, whether any other porcine mycoplasma will cross-react serologically with *M. suipneumoniae*. We have a number of mycoplasma isolates from enzootic pneumonia and other pneumonic conditions of pigs, only some of which induce pneumonia experimentally, and we are currently comparing them with *M. suipneumoniae*.

It is not clear whether the porcine mycoplasma isolates of Dinter, Z., Danielsson, D. & Bakos, K. ((1965), *J. gen. Microbiol.* **41**, 77), came from cases that would fall within our definition of enzootic pneumonia. *M. hyorhinis* (Switzer, W. P. (1955), *Am. J. vet. Res.* **16**, 540) and *M. granularum* (Switzer, W. P. (1964), in *Diseases of Swine*, 2nd ed., p. 498. Ed. by H. W. Dunne. Ames, Iowa, U.S.A.: The Iowa State University Press) do not seem to be specifically associated with enzootic pneumonia.

The classification of these various porcine mycoplasma within the general mycoplasma field is very confused. None of them has been compared with all the mycoplasma from other animals before being named and it is possible that some of them may be the same as other known mycoplasma. We have recently described *M. suipneumoniae* in greater detail and compared it serologically with as many other mycoplasmas as practicable (Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1966), in preparation).

A Possible Association of *Mycoplasma* with Rheumatoid Arthritis. By SHEILA M. STEWART (*City Hospital, Edinburgh*)

The possibility of an infective aetiology of rheumatoid arthritis has recently been reconsidered. There are a number of factors of the disease together with indirect experimental evidence (Duthie *et al.* (1965), in *Structure and Function of Connective Skeletal Tissue*, p. 482, Butterworths; London,) which suggest than an infective agent may play at least a partial role.

Within the last two years there have been reports of the isolation of *Mycoplasma* from rheumatoid synovial fluids (Bartholomew, L. E. (1965), *Arth. and Rheum.* **8**, 376; Jansson, E., unpublished), but the association of these organisms with the disease has not yet been established.

Evidence will be presented of the presence of granular bodies within cells from rheumatoid synovial membranes. These structures are very similar in appearance to those seen in tissue cultures artificially infected with cultures of *Mycoplasma*. They have never been seen in cells from normal synovial membranes. The failure to isolate *Mycoplasma* from these specimens may be due to their exacting nutritional requirements or the reversion of the organisms to more typical bacterial forms on cultures.

The results of metabolic inhibition tests using a strain of *M. hyorhinis* isolated from a culture of rheumatoid synovial tissue and sera from cases of rheumatoid arthritis and from non-rheumatoid controls will be reported.

ORIGINAL PAPERS

Studies on the Infection of *Bacillus subtilis* with a Defective Bacteriophage. By D. J. STICKLER and R. G. TUCKER (*Microbiology Unit, Department of Biochemistry, South Parks Road, Oxford*)

A number of strains of *Bacillus subtilis* have been found to be lysogenic, producing morphologically distinctive phage particles on induction (Stickler, D. J., Tucker, R. G. & Kay, D. (1965), *Virology* **26**, 142). Although these phases adsorb to and kill sensitive bacteria, plaque formation has not been demonstrable; the infected cells lyse about 20 min. after infection but there is no associated phage growth.

In attempts to determine why these phages fail to reproduce the interaction between the sensitive strain, *Bacillus subtilis* BS7SR, and phage ϕ 3610, produced by *B. subtilis* NCTC 3610, has been studied. Infection of growing cultures with the phage results in the rapid cessation

of protein, RNA and DNA synthesis. Respiration is impaired and a large amount of small molecular weight material is released before the completion of macroscopic lysis.

Ionesco, H., Ryter, A. & Schaeffer, P. ((1964), *Ann. Inst. Pasteur*, **107**, 764) and Seaman, E., Tarmy, E. & Marmur, J. ((1964), *Biochemistry*, **3**, 607) have also described bacillus phages that are unable to reproduce on sensitive bacteria. The latter authors found that their phage PBSX contained DNA that was similar to that of the bacterial host and suggested that the majority of the phage particles contained bacterial DNA rather than phage-specific nucleic acid. If phage ϕ 3610, like phage PBSX, contains only bacterial DNA, then the initial steps in phage infection dependent upon virus protein, namely adsorption and puncture of the cell wall might take place, but in the absence of viral-specific nucleic acid subsequent phage growth would not be possible. The situation would be similar to infection with a phage ghost, and it is of interest that the metabolic changes accompanying infection of *Bacillus subtilis* BS7SR with phage ϕ 3610 are like those found when *Escherichia coli* is infected with phage T2 ghosts (Lehman, I. R. & Herriott, R. M. (1958), *J. gen. Physiol.* **41**, 1067).

Polyribosomes in Protoplasts of *Bacillus megaterium*. By E. CUNDLIFFE (*Sub-department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Disruption of bacterial cells by mechanical methods, or by ultrasound, might be expected to shear, or otherwise degrade, polyribosomes. Consequently many previously reported 'polyribosome' preparations have consisted largely of 70S ribosomes, with variable amounts of heavier material. Preparations somewhat enriched with respect to polyribosomes have been obtained from bacterial protoplasts, which may be lysed gently by osmotic shock or by detergent action. The present paper indicates how *B. megaterium* may be rapidly and quantitatively converted to protoplasts, and reports the presence, in lysates, of polyribosome fractions containing far fewer 70s ribosomes than previously reported. Exponentially growing cultures of *B. megaterium* KM in a mineral salts/glucose/10 % sucrose medium, supplemented with 0·1 % peptone, were harvested by centrifugation and converted to protoplasts by the addition of egg white lysozyme (200 μg./ml.). The total time required, including the initial harvesting, was 10 min. or less. Such protoplasts were sensitive to osmotic shock and to detergent action.

When incubated at 37° with gentle shaking, the protoplasts entered an exponential 'growth' phase. The optical density at 600 mμ increased exponentially, ^{14}C-amino acids and ^{3}H-uridine were incorporated into TCA-precipitable material, at rates approximating to those in whole cells grown under the same conditions.

Sucrose density gradient analysis of lysates of 'exponential' protoplasts revealed a substantial enrichment with respect to polyribosomes with fewer 70s ribosomes, as compared with lysates of freshly prepared protoplasts. Brief treatment of lysates with ribonuclease degraded all the polyribosomes, giving a massive 70s peak. Actinomycin D, added to exponential protoplasts, induced a rapid breakdown of polyribosomes to monomers as shown by gradient analysis of subsequent lysates.

It is possible that exponentially growing bacteria have polyribosome profiles similar to those demonstrated in lysates of exponentially growing protoplasts.

Isocitrate lyase in *Chlorella*: Purification and the Mechanism of its Disappearance from Cells. By P. C. L. JOHN and P. J. SYRETT (*Department of Botany, University College, Gower Street, London, W.C.* 1)

The alga *Chlorella pyrenoidosa*, strain 211/8p, formed isocitrate lyase when adapted to acetate in darkness. The enzyme can be as much as 8 % of total soluble protein (100,000 *g* supernatant) (John, P. C. L. & Syrett, P. J. (1965), *Biochem. J.* **95**, 49P). It has been purified by Sephadex gel filtration and elution from DEAE-cellulose. The final preparation was homogeneous by the criteria of sedimentation, diffusion and acrylamide gel electrophoresis. The calculated molecular weight of the enzyme is 170,000. The addition of glucose to fully acetate-adapted cells of *Chlorella* was followed by a rapid fall in isocitrate lyase activity particularly in the absence of a nitrogen source; 20 % of the activity was lost in an hour

without any cell division taking place. Acrylamide gel electrophoresis showed that loss of activity was accompanied by loss of enzyme protein. Turnover of isocitrate lyase protein and other major soluble *Chlorella* proteins has been compared by measuring ^{35}S-incorporation and it has been shown that isocitrate lyase protein does not turn over more rapidly than the others. It is suggested that a metabolite produced from glucose, by combination with isocitrate lyase, produces an allosteric change in the protein which renders it liable to attack by proteinase.

The Growth and Survival of Stream-borne Bacteria in Impoverished Media. By J. W. HOPTON and K. THURAIRAJAN (*Department of Microbiology, The University, Birmingham*)

Bacteria of the genera *Pseudomonas* and *Achromobacter* generally dominate the bacterial flora which can be isolated from natural waters by conventional plating techniques. Typical faecal *Escherichia coli* is short-lived in waters, but it is thought that *Aerobacter aerogenes* and some intermediate coliform types can outlive *E. coli*.

The growth and survival of three isolates from stream waters (nos. 7, 11 and 14) and *E. coli* ML30 strain, under different conditions of deprivation, have been compared. No. 7 is an intermediate coliform, no. 11 is a typical pseudomonad and no. 14 appears to be an *Aeromonas* strain. All four organisms can grow in a basal salts solution containing glucose. Early stationary phase cells of all four organisms maintained their viability in the basal salts solution and in tris buffer. They died rapidly in distilled water and grew normally in basal salts solution containing 5 mg./l. glucose. With *E. coli* ML30 a steady-state cell population of *ca.* 10^7/ml. could be maintained in continuous culture in this same basal salts–glucose medium at dilution rate 0·1 hr^{-1} at both 25° and 37°. If the dilution rate was lowered to 0·005 hr^{-1} the steady state could still be maintained at 25° but at 37° the viable population declined. Cells of *E. coli* ML30 and no. 7, harvested from steady-state continuous cultures in basal salts–glucose (500 mg./l.) medium, maintained their viability in basal salts solution at 10° and 20° but declined at 37°.

There were striking differences in the behaviour of the organisms in sterilized stream water, canal water and lake water at 25°. No. 11 grew in all three waters, whereas *E. coli* ML30 died out in all three waters within 24 hr. No. 7 grew in stream and canal waters but declined in lake water and no. 14 remained constant in canal water and declined in lake and stream waters.

Genetic Analysis of Amidase Mutants of *Pseudomonas aeruginosa* 8602. By JANE E. BROWN and PATRICIA H. CLARKE (*Department of Biochemistry, University College London*)

Mutants of *Pseudomonas aeruginosa* 8602 have been isolated which produce amidase in the absence of inducer (C mutants); amidase with altered substrate specificity (B mutants); no detectable amidase activity (Am^- mutants). Genetic transfer can be carried out in this strain with the pseudomonad phage F116 (Holloway, B. W., Egan, J. B. & Monk, M. (1960), *Aust. J. exp. Biol. med. Sci.* **38**, 321). Transduction experiments using phage lysates from constitutive Am^+ strains as donors, and inducible Am^- strains as recipients, showed that the amidase regulator and structural genes are closely linked (Brammar, W. J., Clarke, P. H. & Skinner, A. J. (1967), *J. gen. Microbiol.*, in the Press).

Am^- strains are unable to grow on minimal agar with acetamide as the sole carbon or nitrogen source. Seven Am^- strains derived from the wild-type strain of *P. aeruginosa* were used for transduction experiments with phage F116. Am^+ transductants were found in crosses between several of these strains. Other mutants with an Am^- phenotype were derived from the constitutive strain C_{11}. These constitutive Am^- mutants were used to carry out 3-factor crosses in which the regulator mutation was used as the additional marker.

The B mutants, unlike the wild-type strain are able to grow on minimal agar with butyramide as the carbon source. The mutations in two of the constitutive B mutants, derived from the constitutive strain C_{11}, were also mapped in relation to Am^- mutations by similar 3-factor crosses. The mutant strain $C_{11}B_6$ has been treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to produce a further mutation enabling growth on valeramide as carbon source. Several mutants with this phenotype have been isolated and are being investigated.

Mutants of *Pseudomonas aeruginosa* 8602 Producing an Amidase with Altered Substrate Specificity. By P. BROWN and PATRICIA H. CLARKE (*Department of Biochemistry, University College London*)

The wild-type strain of *Pseudomonas aeruginosa* 8602 produces an aliphatic amidase (acylamido hydrolase EC 3.5.1.4) which enables it to grow on minimal agar plates with acetamide as the sole source of carbon and nitrogen. The amidase is inducible and the amide in the medium acts as both inducer and substrate. No growth occurs on minimal agar in which the carbon or nitrogen source is butyramide which is neither a substrate nor inducer of the enzyme.

Constitutive strains (C mutants) have been isolated which synthesize amidase in the absence of inducer (Clarke, P. H., Brammar, W. J. & Skinner, S. J. (1966), *Abstr. 9th. int. Congr. Microbiol*, Moscow, p. 43). We have now isolated mutants which are able to grow on minimal agar containing butyramide as a carbon source by treating the magno-constitutive strain C_{11} with the mutagenic agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These are described as butyramide or B mutants.

The amidase produced by the wild-type constitutive strains hydrolyses acetamide and propionamide and also transfers the acyl moiety to hydroxylamine to form the acyl hydroxamates. The amidase produced by the B mutants carries out these reactions and in addition can use butyramide as a substrate for both the hydrolase and transferase reactions. No significant differences have been found in the properties of the amidases produced by the wild-type strain and the constitutive strain C_{11}, and it is therefore concluded that they both produce the wild-type enzyme. The amidase produced by six B mutants cross-reacts with anti-serum to the wild-type enzyme. Using starch gel electrophoresis it was found that there was a marked difference in electrophoretic mobility at pH 8·5 between the wild-type enzyme and that produced by all six B mutants.

Strain C_{11} has been used as a source of the wild-type A amidase and strain B_6 as a source of the mutant B amidase. Both enzymes have been purified and their properties compared.

The Distribution of Lipids in *Pseudomonas aeruginosa*. By I. C. HANCOCK and PAULINE M. MEADOW (*Department of Biochemistry, University College London*)

Lipids extracted by the Folch procedure from stationary phase *Pseudomonas aeruginosa* 8602 grown in Lemco broth, constituted 20 % of the dry weight. Thin-layer chromatography showed four polar lipids: phosphatidyl ethanolamine, containing 70 % of the lipid phosphorus, and approximately equal amounts of phosphatidyl choline, phosphatidyl glycerol and a phospholipid tentatively identified as an *O*-amino acid ester of phosphatidyl glycerol. The fatty acids of the neutral and polar lipids in the extract were identified and measured by gas chromatography. The major acids were palmitic, palmitoleic, oleic, 9:10 methylene hexadecanoic and lactobacillic, the relative proportions varying from one phospholipid to another. For example, the ratio of unsaturated and cyclopropane acids to saturated acids is 1·3:1 in phosphatidyl choline and 2·3:1 in phosphatidyl ethanolamine.

Most of the cell lipids are found in the lipoprotein layer of the cell wall and in the cytoplasmic membrane and attempts were made to differentiate between them. Bacteria were extracted with reagents reported to remove lipoprotein and lipopolysaccharide and the lipid composition of the extracts and residues compared with that of whole organisms. There were no significant differences between the fatty acid compositions of any of the fractions except for the aqueous phase of a 45 % phenol extract which contains the lipopolysaccharide. Here the fatty acids found included 12:O, 10:OH, 12:OH and an unidentified acid. A particulate fraction, isolated by centrifugation at 105,000 *g* of the 30,000 *g* supernatant from sonicated bacteria, was characterized as rich in membranes by its absorption at 416 mμ and its high ATP-ase activity. Its lipid composition was indistinguishable from that of isolated cell envelopes and of whole organisms. It is concluded that the lipid composition of the lipoprotein layer of the cell wall and the cytoplasmic membrane are practically identical.

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