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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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(2) A paper should be written only when a piece of work is rounded-off. Authors should not be seduced into writing a series of papers on the same subject *seriatim* as results come to hand. It is better to wait until a comprehensive paper can be written.

(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this be necessary, and not to recapitulation.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

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Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in *J. gen. Microbiol.* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

Nomenclature and Description of Micro-organisms. The author's preference in naming micro-organisms is at present accepted provided that the designation is unambiguous and conforms with international rules of nomenclature; if desired, synonyms may be added in brackets when a name is first mentioned. Names of bacteria must conform with the Bacteriological Code of the International Committee on Bacteriological Nomenclature and the opinions issued by this International Committee (*Bacteriological Code* (1958), edited by the Editorial Board of the International Committee on Bacteriological Nomenclature and published by the Iowa State College Press, Ames, Iowa, U.S.A.). Names of algae and fungi must conform with the International Rules of Botanical Nomenclature which are considered and revised at each International Botanical Congress (published by the International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952)). Names of protozoa must conform with the International Code of Zoological Nomenclature adopted by the XVth International Congress of Zoology (published for the International Commission on Zoological Nomenclature by the International Trust for Zoological Nomenclature, London (1961)). The 1913 rules will be found in C. M. Wenyon, *Protozoology*, (1926), vol. 2 (London: Baillière Tindall and Cox). One or two small changes have been made to these rules at later International Congresses.

Useful commentaries on the taxonomy of micro-organisms will be found in the articles of the Twelfth Symposium of the Society for General Microbiology, *Microbial Classification*, edited by G. C. Ainsworth and P. H. A. Sneath (Cambridge University Press).

Micro-organisms should be designated by the names used in the works listed below. When other authorities are followed they should be cited whenever obscurity might result from their use.

Bergey's Manual of Determinative Bacteriology, 7th ed. (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Baillière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Generic Characteristics*, (1959). Baltimore, Ma., U.S.A.: The Williams and Wilkins Company.

S. T. Cowan & K. J. Steel, *Manual for the Identification of Medical Bacteria*, (1965). Cambridge University Press.

Ainsworth & Bisby's Dictionary of the Fungi, 5th ed. (1961). Kew: Commonwealth Mycological Institute.

List of Common British Plant Diseases, 4th ed. (1944), compiled by the Plant Pathology Committee of the British Mycological Society. Cambridge University Press.

Medical Research Council: Memorandum No. 23. 3rd ed. (1967). *Nomenclature of Fungi Pathogenic to Man and Animals*. London: H.M.S.O.

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Descriptions of new species should not be submitted unless an authentic specimen has been deposited in a recognized culture collection.

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Ultraviolet Induction of Chromosome Transfer by Autonomous Sex Factors in *Escherichia coli*

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SUMMARY

Ultraviolet irradiation of *Escherichia coli* K 12 bacteria, carrying an autonomous F factor or an F-prime factor, enhances the fertility of the population by increasing the number of cells which can transfer the bacterial chromosome. In contrast, under similar conditions the fertility of irradiated Hfr populations falls in proportion to the survivors. Following irradiation, the effect begins to develop after about 30 min. incubation in broth at 37°, reaches a peak at about 90 min., and thereafter slowly declines. The effect develops with similar kinetics during post-irradiation incubation in minimal medium as in broth, provided the bacteria have been minimal-grown; in the case of broth-grown cells, appearance of the effect in minimal medium is greatly delayed. A comparison of the kinetics of the effect with the growth of the population as a whole shows that the u.v.-induced donor state is not inheritable. Mitomycin C, which resembles u.v. radiation in producing DNA damage repairable by a mechanism involving excision of single-stranded fragments, also induces new donor bacteria. Other agents such as X-rays and methyl methansulphonate (MMS) do not stimulate the production of new donors but may enhance the recombination frequency since cells killed by them may continue to act as chromosome donors. The effect is not shown either by *uvr* mutants (unable to excise thymine dimers) or by *rec* mutants (unable to mediate recombination) which carry an F-prime factor. A possible mechanism is suggested whereby the excision of single-stranded fragments of the bacterial chromosome, during the repair of u.v. damage, facilitates pairing with homologous regions of the complementary sex factor strand. A recombination event, mediated by breakage and covalent bonding, then joins a free end of the excised DNA strand to the paired sex factor strand. In this way, recombination connects sex factor and chromosome by only a single strand instead of by the two strands which normally leads to insertion and the formation of an Hfr chromosome. It is postulated that such a structure can be transferred at conjugation, but is incapable of more than one cycle of replication.

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INTRODUCTION

Stimulation of recombinant formation in *Escherichia coli* K 12 crosses by ultraviolet (u.v.) irradiation of the parental bacteria was first reported by Haas, Wyss & Stone (1948). Following the discovery of sexual differentiation, Hayes (1952) found that the effect is exclusively due to an increase in the fertility of the donor (F^+ male) population; irradiation of the recipient (F^- female) parental bacteria decreases the number of recombinants in proportion to the survivors. Subsequent analysis showed that u.v. irradiation fails to increase the fertility of Hfr male bacteria, which is presumably already maximal, the number of recombinants falling with increasing dosage, in parallel with the viable count. The effect is photo-reactivable and requires incubation of the broth-grown irradiated F^+ bacteria in a rich medium for its development (Hayes, 1953). With the advent of methods of mating in liquid media, the induction by u.v. irradiation of as high as a 50-fold increase in the total number of recombinants can be achieved under optimal conditions, but this enhanced ability to generate recombinants is not inherited by the descendants of the induced F^+ bacteria (Hayes, 1960).

In this paper we redefine the phenomenon as the induction by u.v. radiation of new chromosome donors, in bacterial populations which carry an autonomous sex factor; this induction is reflected in an increase in the *absolute number* of recombinants generated, as opposed to an increase in the *frequency of recombination* (number of recombinants/100 donor bacteria) which can be due to other causes, as we shall show. We also postulate that this induction of new donors is due to an abnormal mechanism of incorporation of the sex factor into the bacterial chromosome. We present evidence that this incorporation depends on excision of single-stranded fragments of DNA during repair of irradiation damage, as well as on an act of recombination, resulting in the formation of a transmissible structure which is not inheritable and may be lethal. A model for this structure is proposed and some experiments in support of it are described.

METHODS

Strains. All *Escherichia coli* strains were K 12 derivatives; only mutant characters are designated below. The F^+, intermediate male and Hfr strains used in most comparative irradiation studies were isogenic, as follows:

F^+: W 1655.*met*$^-$*λ*$^-$*λ-r* (Lederberg & Lederberg, 1953).

Intermediate male: W 1655.F^- infected with an F-*lac*$^+$ factor derived from Hfr.B 1 (Scaife & Gross, 1963; Broda, Beckwith & Scaife, 1964; Broda, 1967).

Hfr: Hfr.B1, derived from W1655.F^+, transferring the *proB* locus as the first chromosomal marker and *lac* as the last (Broda, 1967), was used as a control strain in many experiments.

Hfr.H.*gal*$^-$ prototroph, kindly provided by Dr J. Shapiro, was used as recipient for transduction of the *uvrB* locus which is jointly transducible with the *gal* region by phage P1.

F^-: strain 242.*proA*$^-$.*lac*$^-$*str-r*, derived from strain W945 (Cavalli & Jinks, 1956) by recombination, was usually used as recipient in crosses, selection being made for *pro*$^+$ recombinants.

W1655.*met*$^-$ (Lederberg & Lederberg, 1953).

W1655.*met*$^-$*thy*$^-$ isolated by selecting for thymine requirement by the procedure of Stacey & Simson (1965).

W 1655.*uvr*$^-$ isolated by Miss C. Atkinson by selection with phage T 1 'inactivated' with u.v. light (Howard-Flanders & Theriot, 1962).

AB 2463.*rec*$^-$, derived from AB 1157.*pro*$^-$*thr*$^-$*leu*$^-$*thi*$^-$*arg*$^-$*his*$^-$*lac*$^-$*gal*$^-$*ara*$^-$*xyl*$^-$*man*$^-$-*str-r* (Howard-Flanders & Theriot, 1966).

AB 1886.*uvrA*; AB 1885.*uvrB*$^-$: both derived from AB 1157 (Howard-Flanders, Boyce & Theriot, 1966).

J 6-2.*pro*$^-$*try*$^-$*his*$^-$*lac*$^-$*str-r* (Clowes & Rowley, 1954).

In addition, use was made of an intermediate male strain [JW-6.A 1.*thr*$^-$*leu*$^-$*thi*$^-$*lac*$^-$ (F-*lac*$^-$)] isolated by Mr J. Walpole as resistant to u.v. light and also, apparently, recombination-defective.

Media. All experiments were performed in nutrient 'L' broth (Lennox, 1955) or in M 9 minimal medium supplemented with glucose and appropriate amino acids.

Platings for viable counts were made on nutrient agar and counted after overnight incubation at 37°. Recombinants were scored after 48 hr at 37° on M 9 minimal-glucose agar, supplemented according to the required selection.

Buffer was M/15 phosphate buffer at pH 7·2.

Concentrated broth: 'L' broth concentrated × 10.

Methods. Bacteria were grown and crossed in liquid medium, in screw-capped bottles attached to the periphery of a 33 rev./min. turntable in a 37° room.

Irradiation. All bacteria were irradiated after washing by filtration on a Millipore filter and resuspending in buffer.

Source of u.v. radiation: a low-pressure Hanovia 15 W. germicidal lamp used at a distance of 50 cm., giving a dose of about 4 ergs/mm.^{2}/sec.

Source of X-rays: a Phillips apparatus, yielding 1·3 Krad./min. was used. X-irradiation was done in buffer, both in the presence and in the absence of oxygen; although the viable count fell more rapidly in the presence of oxygen, there was no differential effect on chromosomal mobilization.

Recombination frequency is calculated as the number of recombinants per 100 male bacteria in the initial mating mixture. In most experiments the ratio male:female bacteria in the mating mixture was 1:10.

RESULTS

The phenomenon

The effect of u.v. radiation on the fertility of isogenic F$^+$, intermediate male (F-*lac*$^+$) and Hfr cultures is shown, in typical experiments, in Table 1. Instead of the decrease found in the total number of recombinants when the Hfr strain is irradiated, the total numbers of recombinants by irradiated F$^+$ and intermediate males is markedly increased. In these latter cases, therefore, bacteria which carry an autonomous sex factor, but do not yield recombinants, are induced to do so by u.v. irradiation. In addition, the fact that the increase in the recombination frequency is greater than the increase in the absolute number of recombinants, suggests that, unlike the case of Hfr bacteria in which the recombination frequency remains constant, at least a proportion of non-surviving male bacteria can generate recombinants. Although the absolute number of recombinants given by a u.v.-induced intermediate (F-prime) population is much greater than in the case of F$^+$ populations, the relative increase in both total number and frequency of recombinants is markedly smaller. This, presumably, is

because the irradiated intermediate male population yields a number of recombinants approximately equivalent to that observed for a corresponding Hfr population—virtually every conjugation event results in chromosome transfer, so that there is little room for further increase. It is worth noting that, in the case of intermediate males, the ratio of inheritance of distal (*his*$^+$) and proximal (*pro*$^+$) markers does not differ significantly before and after u.v. treatment.

Table 1. *A comparison of the effect of u.v. irradiation on the fertility of isogenic populations of F$^+$, intermediate male and Hfr bacteria*

One tenth vol. overnight broth cultures of F$^+$, intermediate male (F-*lac*$^+$) and Hfr male derivatives of *E. coli* K 12 strain W 1655, as well as of the F$^-$ strain 242, were transferred to fresh broth at 37° and incubated on a rotor. After 1½ hr the male culture was washed, resuspended to vol. in buffer, diluted 1 in 10 and irradiated with u.v. radiation to 40–60% survival. One tenth vol. conc. broth was added, the suspension incubated at 37° for 1 hr and then mated with the strain 242.F$^-$ suspension for 45 min. at 37°. Samples were finally diluted and plated for *pro*$^+$ recombinants. In each case the sample of the washed suspension of male bacteria, before irradiation, was enriched with conc. broth and immediately mated with the F$^-$ bacteria as control.

Type of male bacterium	Total number of recombinants per ml.			Recombination frequency		
	Before u.v.	After u.v.	Increase	Before u.v.	After u.v.	Increase
F$^+$	8×10^3	$1{\cdot}6\times10^6$	×20	$1{\cdot}7\times10^{-2}$	6×10^{-1}	×35
F-*lac*$^+$	8×10^5	$4{\cdot}0\times10^6$	×5	5	60	×12
Hfr	3×10^7	$1{\cdot}8\times10^7$	×0·6	40	40	×1·0

The absolute increase in numbers of recombinants generated by irradiated F$^+$ and intermediate (F-*lac*$^+$) bacteria could be due to: (1) an enhanced efficiency of conjugation; (2) a greater probability that cells already having an integrated sex factor will transfer their chromosome on conjugation; (3) an increased efficiency of incorporation of transferred segments into recombinant chromosomes; (4) an increase in the frequency of sex factor integration into the chromosome.

The first three possibilities are unlikely since neither the transfer of an autonomous sex factor such as F-*lac*$^+$ by intermediate male strains, nor the number of recombinants generated by Hfr strains, are increased by irradiation (see Fig. 1). The fourth possibility was therefore investigated in more detail.

The effect of u.v. dosage

The effect of increasing u.v. dosage on the number of recombinants, and on the recombination frequency, is demonstrated in Fig. 1. In the case of both F$^+$ and intermediate males, the total number of recombinants begins to rise at a u.v. dose which scarcely affects survival (>95%) and continues to do so until a survival level of about 30% is reached. Thereafter the killing effect of increasing u.v. dosage dominates the curve so that the total number of recombinants begins to fall at about the same rate as the number of survivors—the recombination frequency reaches a plateau. There are two possible explanations for the plateau. One is that the probability of the effect occurring increases with dose up to the point where the maximal amount of u.v. damage compatible with survival is sustained; thereafter new donors continue to be formed but a decreasing proportion of these can express themselves. Alternatively, the plateau could represent the dose at which the maximal possible number of chromosomal

donors have been induced and that, with increasing dosage, these decline at the same rate as the population at large, as in the case of Hfr bacteria (Fig. 1 C). Since, as we shall see, the development of the effect depends on u.v. damage while the evidence suggests that induced donor cells may be inviable, we favour the first explanation of the dose effect curve and will discuss it again later.

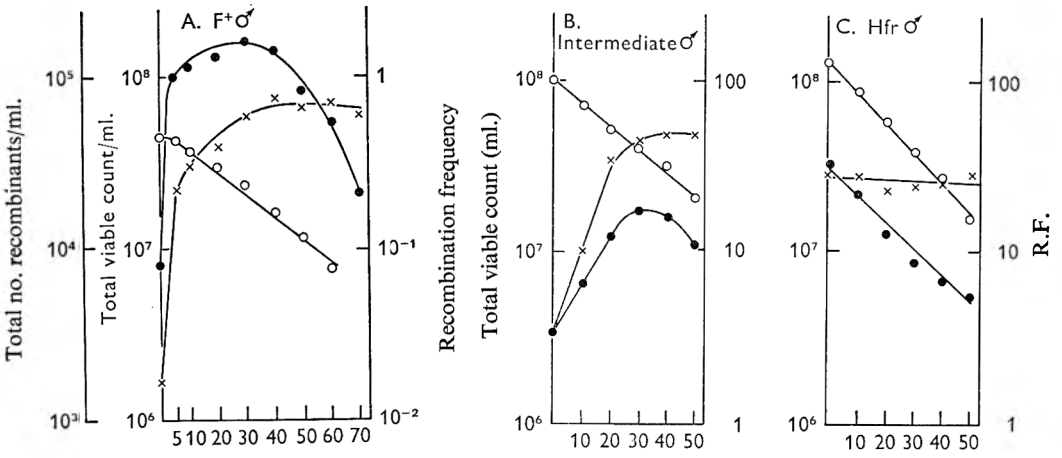

Fig. 1. Dose-response curves of total recombinants, recombination frequency and survival for three types of male bacteria irradiated with u.v. radiation. A = F^+ male (w1655.F^+). B = Intermediate male (w1655(F-*lac*$^+$)) ex HfrB1. C = Hfr male (HfrB1 ex w1655.F^+). In all cases the experiments were performed as described in Table 1, except that the u.v. dose used was varied. In A, a different u.v. lamp was used. ●—●, Total *pro*$^+$ recombinants; ○—○, total viable count; ×—×, recombination frequency (RF).

Kinetics of development of the effect

In this section we will study the development of the effect as a function of time of incubation in broth (or other media) following u.v. irradiation. The rise with time in the number of recombinants issuing from an irradiated F^+ strain is shown in Fig. 2. The total number of recombinants appears to begin to rise immediately after incubation, reaches a peak at about 1½ hr later and then slowly declines. The total viable count begins to increase exponentially after a lag of about 1 hr. In experiments with intermediate male strains, similar kinetic curves are obtained.

There are two important points about the nature of these curves. The first is whether Fig. 2 reveals the true kinetics of development of the effect following incubation in broth, since the times indicated are those at which the samples were taken for mating, which involves a further period of 45 min. in broth. It is therefore possible that the effect is not already manifest in the early samples but develops later, during the mating period. In order to distinguish these possibilities, samples of F^+ bacteria, incubated in broth for various times after irradiation (as before), were mated with female bacteria for 15 and 30 min. as well as for 45 min. It turned out that the samples removed immediately after resuspension of the irradiated bacteria in broth, and mated for only 15 min., gave the same number of recombinants as the unirradiated control. When the mating time was increased to 30 min., this sample showed a small increase in the number of recombinants. This same small increase was given by the sample removed

at 15 min. and mated for 15 min., the total time of incubation in broth again being 30 min. Thus the effect is not initiated immediately but only after 30 min. in broth, and can develop in the mating mixture.

The second point concerns the relationship of the effect to division of F^+ cells. Figure 2 appears to show that most of the new donors have been induced before the irradiated population begins to divide at about 1 hr after incubation. This would imply that the majority of new donors cannot multiply as such for if they did the number of donors, and therefore of recombinants, would double with the population

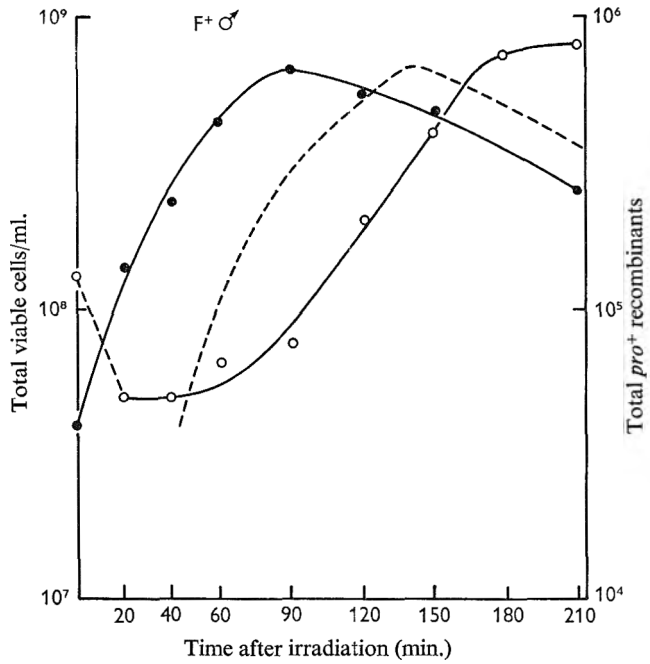

Fig. 2. The kinetics of development of the u.v. effect in an F^+ strain. The experiment was performed as described in the legend to Table 1, except that samples of irradiated F^+ suspension were removed for mating and viable count at intervals after incubation in broth at 37°. Survival immediately after irradiation = 40%. ●—●, Total *pro*$^+$ recombinants; O—O, total viable count.

as a whole. However, since the effect has now been shown to continue to develop during the 45 min. mating period in broth, the samples from which the recombinant curve is constructed should really be transposed 45 min. to the right in Fig. 2, as indicated by the interrupted line. When this is done, the rise in the number of recombinants appears to coincide with the increase in the viable count over most of the growth cycle, so that the latter part of the rise could be due to multiplication of induced donors, curtailed only by entry into the stationary phase.

There are three good reasons for believing that this interpretation is incorrect and that, in fact, induced donors do not multiply. First, viable counts show that exponentially growing F^+ bacteria stop multiplying when mixed in a 1 in 10 minority with female bacteria under the conditions of mating employed, so that the number of recombinants is properly related to the number of F^+ bacteria at the time of sampling

and not 45 min. later. Secondly, minimal-grown bacteria, after irradiation and incubation in minimal medium, may develop the maximal effect in the absence of any cellular increase (see below). Thirdly, samples of irradiated F^+ bacteria taken after 1 hr incubation in broth, diluted two-, four- and eightfold into fresh broth at 37°, and finally mated after each sample had grown up to *c.* 10^8 bacteria/ml. (at 90, 120 and 150 min. respectively), all showed the same 'plateau' recombination frequency, despite a fourfold difference in the number of generations after development of the effect. It should also be noted that, following the initiation of induction, new donors are generated at a much faster rate than are new bacteria during exponential growth.

Metabolic requirements for development of the effect

The development of the effect was investigated under various conditions of growth and in the presence of various metabolic inhibitors.

(i) *Growth in minimal medium.* F^+ and intermediate male strains grown overnight in, and subcultured to, minimal medium develop the effect with normal kinetics if incubated in the same medium, instead of in broth, after irradiation. In contrast, if broth-grown cells are irradiated and then incubated in minimal medium, supplemented with the required growth factors, the number of recombinants falls about threefold during the first hour, but then rises to many times the initial number during the second hour of incubation. We do not know to what extent the fall during the first hour is due to inability to mate as a result of the inhibition of protein synthesis which follows the 'shift-down' from broth to minimal medium (see Maaløe & Kjeldgaard, 1966). However, it is clear that recovery of the bacteria from the effects of the 'shift-down' is accompanied by delayed development of the effect.

(ii) *Inhibition of protein synthesis.* Attempts to judge whether development of the effect is inhibited by withdrawal of a required amino acid were frustrated by the fact that, under these conditions, the ability of male cells to mate falls rapidly to a low value (Hayes, unpublished data). The effects of chloramphenicol were therefore investigated. Preliminary control experiments showed that treatment of Hfr bacteria with 100 μg./ml. chloramphenicol for 30 min. has little effect on their fertility, so that a study of the action of this drug on the development of the u.v. effect seemed feasible. It turned out, however, that Hfr bacteria which have been irradiated with u.v. light show a more than tenfold diminution of fertility when treated with chloramphenicol under the same conditions, so that it is difficult to appraise the outcome of this type of experiment.

(iii) *Deprivation of energy source.* Minimal-grown bacteria incubated, after irradiation, in the same medium without glucose, develop almost the full effect. In kinetic experiments under these conditions the total number of recombinants, instead of falling after the peak (Fig. 2), remain constant for the duration of the experiment (3 hr). No increase in viable count occurs throughout this period. It seems reasonable that the development of the effect under these conditions may depend on a residual endogenous energy source. Once produced, the effect fails to decline because this energy source is depleted. No experiments were performed involving deprivation of glucose before irradiation.

Another means of energy deprivation is by the addition of 10^{-3}M-dinitrophenol (DNP) to the minimal medium, which inhibits both multiplication and chromosome transfer (Fisher, 1957). This concentration of DNP during the first 30 min. of post-

irradiation incubation reduced the effect twofold; however, the effect developed normally on a further 30 min. incubation after removal of the DNP.

(iv) *Deprivation of thymine.* A thymine-requiring mutant of strain W1655.F+, grown in minimal medium + thymine, was irradiated with u.v. light and then incubated in minimal medium without thymine for 30 min. before mating in the presence of thymine. Despite a 50% fall in the viable population from thymineless death, very nearly the same increase in the number of recombinants occurred as in the control sample, incubated in minimal medium + thymine after irradiation. In the case of u.v.-irradiated bacteria, thymineless death starts immediately after incubation, without the lag seen in unirradiated cultures. A further 40 min. of thymine starvation, yielding only 10% survivors, was also without significant effect on the absolute number of recombinants. It is concluded that a major degree of inhibition of DNA synthesis has no appreciable effect on the development of new donors.

Table 2. *The effect of various agents on the total number of recombinants, and on the recombination frequency, generated by F+ bacteria at different levels of survival*

In the experiments with u.v. radiation, Mitomycin C (MC) and X-rays, log. phase, broth-grown W 1655.F+ bacteria were used and incubated in broth of 1 hr after treatment, prior to mating with strain 242.F⁻ for 45 min. and plating for *pro*+ recombinants. In the case of 5-bromouracil uptake (BU) and thymine starvation (TS), thymine-requiring F+ bacteria were employed and, after treatment, incubated in minimal medium + thymine for one hour before mating. For methylmethansulphonate treatment (MMS) minimal medium was used throughout.

Group	Agent	Dosage	Percentage survival	Increase in total no. recombinants	Increase in recombination frequency
I	U.v.	20 sec.	68	× 16	× 23·5
		40 sec.	37	× 17·5	× 47
	MC (10γ/ml.)	5 min.	60	× 4	× 6·6
		15 min.	25	× 10	× 40
	TS	60 min.	83	× 8·4	× 10
		100 min.	36	× 5·2	× 13·2
		140 min.	8	× 1·9	× 24
	BU (40γ/ml.)	2 hr	25	× 3	× 12
II	X-rays (in O_2)	5 min.	45	× 1	× 2·2
		15 min.	5	× 0·25	× 5
		25 min.	0·1	× 0·025	× 25
	MMS (0·025M)	10 min.	40	× 0·8	× 2
		20 min.	18	× 0·6	× 3·3
		30 min.	8	× 0·3	× 3·7

Induction of the donor state by agents other than u.v. light

We tried to imitate the u.v. effect by using various other agents known to inflict damage on the chromosomal DNA. Although the type of damage produced by each agent is undoubtedly different, we looked for similarity with respect to induction of new donor bacteria in F+ and intermediate male populations. The agencies tested were X-rays, Mitomycin C (MC), methylmethansulphonate (MMS), the incorporation of 5-bromouracil (BU) into the DNA, and deprivation of thymine (TS) in a thymine-requiring mutant. These agencies fall into two clearcut groups with respect to their effect on the total number of recombinants and the recombination frequency, as is

shown in Table 2. It is clear that the agents in group I produce an increase in the absolute number of recombinants at all survival levels shown, as well as an increase in the recombination frequency. On the contrary, group II agents stimulate no increase in the absolute number of recombinants, although the percentage recombinants among the survivors rises.

The molecular mechanism of the u.v.-induction of the donor state

From the experiments so far described it is apparent that treatment with u.v. radiation and certain other agents induces the formation of new donor bacteria in F^+ and intermediate male bacterial populations. From the kinetics of induction and its decay it is evident that the induced donor state differs from the normal Hfr state in not being inheritable. This could theoretically be ascribed either to inability of the induced donor cells to divide, or to an abortive unilinear inheritance of the induced donor state. If the former, more likely, hypothesis is correct, and assuming that chromosome transfer requires an integrated sex factor, it follows that the process of induction leads to chromosomal insertion of the sex factor in such a way that the newly formed structure is capable of genetic transfer but not of replication.

In the last section we pointed out that this new type of donor is induced only by agents belonging to group I (Table 2). Many agents of this type have in common the production of DNA damage which is repaired by a process involving excision of single-stranded fragments which have suffered a lesion, so that acid-soluble nucleotides appear in the medium (Setlow & Carrier, 1964; Boyce & Howard-Flander, 1964; Boyce, 1966). As a result of this excision, single-stranded gaps are exposed in the chromosomal DNA which are repaired by new DNA synthesis using the interrupted strand as a primer and the intact, complementary strand as a template (Pettijohn & Hanawalt, 1964).

All the evidence now supports the general hypothesis that chromosome transfer at significant frequency is a consequence of insertion of a circular sex factor into the circular bacterial chromosome by a recombination event in a region of genetic homology between them (review: Scaife, 1967). At the molecular level, this homology must be due to similar base sequences on the parental DNA molecules. In order that these sequences may recognize each other so that pairing, which precedes recombination, can occur, the two strands of each DNA molecule must open up in the region of homology. The sequence of bases on each sex factor strand can then pair with the complementary chromosomal strand by hydrogen bonding. Subsequent breakage and reunion of strands presumably occurs so that, finally, both strands of the sex factor DNA are inserted into the continuity of the chromosomal DNA.

When we now reconsider the excision of DNA strands induced by u.v. light, we can regard the exposed single-stranded regions as substituting for the first step in recombination at which the DNA duplex opens. If this exposed strand should happen to possess homology for a region of the sex factor DNA, pairing with the sex factor should be greatly facilitated. On the basis of this supposition we propose a possible model which involves recombination between only a single strand of chromosomal DNA and sex factor DNA, as shown in Fig. 3.

Excision of a strand of the DNA of the bacterial chromosome in a region of sex factor homology leads to pairing of the complementary sex factor DNA strand (Fig. 3 B), followed by breakage and covalent bonding which joins a free end of the excised

strand to the paired sex factor strand (Fig. 3 C). This structure, unorthodox as it seems, appears capable of fulfilling the general requirements of the phenomenon we describe. Thus it possesses no obvious barrier to replication initiated in the sex factor continuing along the chromosome, so that transfer may be effected. On the other hand, a second replication of the chromosome, or the completion of a single replication initiated elsewhere than in the sex factor, will be blocked at the sex factor attachment site so that the cell will be inviable.

In view of the plausability of this model, let us examine some predictions which derive from it. Pre-eminent among these are the necessity for single-stranded excision and for recombination, so that the u.v. effect should not develop in *uvr*$^-$ mutant bacteria which cannot excise pyrimidine dimers (Howard-Flanders, 1964; van de Putte, van Sluis, van Dillewijn & Rörsch, 1965), nor in *rec*$^-$ mutants which are unable to mediate genetic recombination (Clark & Margulies, 1965; Howard Flanders & Theriot, 1966).

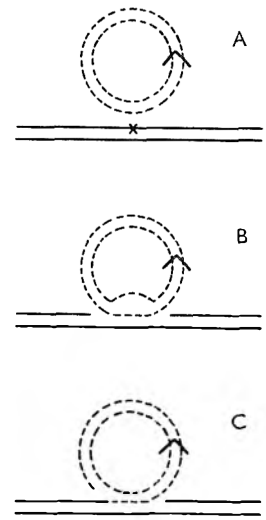

Fig. 3. A molecular model for the mechanism of induction of the donor state by u.v. radiation. The pair of interrupted lines represents the two strands of the sex factor DNA; the arrow indicates the direction of sex factor replication. The pair of continuous lines represents the two strands of chromosomal DNA.
(A) One strand of chromosomal DNA has sustained a u.v. lesion, marked x, in a region of sex factor homology. (B) The u.v. lesion, together with part of the adjacent polynucleotide strand, has been excised. The two strands of sex factor DNA have opened up in the region of homology and one has paired with the exposed complementary strand of chromosomal DNA. (C) The paired strand of sex factor DNA has been broken and rejoined to one of the free ends of the excised strand of chromosomal DNA by an act of recombination.

Experiments with rec$^+$ *and uvr*$^+$ *Escherichia coli mutants*

To examine the effect of u.v. irradiation on chromosome transfer by these mutant strains, the *rec*$^-$ strain AB 2463 and the *uvr*$^-$ strain AD 1886 were infected with an F-*lac*$^+$ factor by conjugation with W 1655 (F-*lac*$^+$). In addition, the *rec*$^-$ intermediate male derivative was also infected with the colicin factor, *colIb*, which is known to restore a high degree of u.v. resistance to this strain without affecting its recombination defect (Mr E. M. Moody, personal communication); the *uvr*$^-$ derivative was similarly

infected with *colIb*, but here no protection was observed. In general, doses of u.v. light were chosen so as to yield both survival levels and absolute doses of irradiation comparable to those found to increase the total number of recombinants with normal intermediate males (cf. Fig. 1). The results are shown in Table 3.

It can be seen that in no case did any increase in the absolute number of recombinants occur. One of a new class of recombination-deficient mutants JW-A1(F-*lac*$^-$), isolated by Mr J. Walpole, which retains almost the full wild-type degree of resistance to u.v. light, gave no recombinants with the F$^-$ strain 242, nor was it induced to do so by u.v. irradiation.

Table 3. *The effect of u.v. irradiation on the fertility of intermediate male, mutant strains unable to mediate recombination (rec$^-$) or to excise pyrimidine dimers (uvr$^-$)*

In all these crosses strain W1655.F$^-$ (*met*$^-$) was used as female, the inheritance of the male chromosomal marker, *met*$^+$, being selected.

Strain	U.v. dosage (sec.)	Percentage survival	Increase in total no. recombinants	Increase in recombination frequency
rec$^+$*uvr*$^+$(F-*lac*$^+$)	20	35	×5	×12
rec$^-$(F-*lac*$^+$)	5	4	×0·08	× 2
rec$^-$(*colIb*)(F-*lac*$^+$)	20	42	×0·9	× 2
uvr$^-$(F-*lac*$^+$)	3	60	×1·0	× 1·7
	6	4	×1·0	×25
	12	0·4	×0·25	×63
uvr$^-$(*colIb*)(F-*lac*$^+$)	3	60	×1·0	× 1·7
	6	10	×1·0	×10
	12	0·6	×0·4	×68

The frequency of recombinants given by the unirradiated *rec*$^-$(F-*lac*$^+$) strain was 150 times lower than that of the control *rec*$^+$(F-*lac*$^+$) donor. The *rec*$^-$(F-*lac*$^+$) and *uvr*$^-$(F-*lac*$^+$) strains all behaved as normal intermediate males so far as the transfer of the F-*lac*$^+$ factor was concerned. The u.v. lamps used in the experiments involving *rec*$^-$ and *uvr*$^-$ strains were not the same.

A striking feature of the experiments involving the *uvr*$^-$ donor strains is the very big increase in recombination *frequency* with u.v. dosage. It is obvious that in these strains, unlike *uvr*$^+$ strains, radiation damage which kills a high proportion of bacteria does not prevent chromosome transfer. A comparison of the proportionate reduction in the total number of recombinants in *uvr*$^-$(F-*lac*$^+$) and *uvr*$^+$ Hfr crosses shows that these are similar for any given *absolute* u.v. dose; that is, the dosage at which the total number of recombinants begins to fall is about the same in the two crosses, despite the vastly higher sensitivity of the *uvr*$^-$ donor to lethal u.v. damage. Thus the rise in the recombinant frequency in the case of the *uvr*$^-$ donor, as compared with its constancy with the Hfr donor (Fig. 1 C), must be ascribed to the fact that inability to excise pyrimidine dimers does not interfere with chromosome transfer. This view is confirmed by the finding that a *uvr*$^-$ Hfr strain, made by phage P1 co-transduction of a defective *uvrB* locus with the *gal*$^+$ gene, from strain AB1885 to a *gal*$^-$ Hfr.H strain, displays the same marked rise in recombination frequency with dosage; as before, the total number of recombinants remains constant with increasing dosage up to the dose required to reduce the number of recombinants in the control *uvr*$^+$ Hfr cross, although the proportion of *uvr*$^-$ survivors at this dose is very small. This implies that

inhibition of chromosome transfer by u.v. light is due to some effect of radiation, such as cross-linking of DNA strands, other than dimer formation.

The need for excision is substantiated by the finding that development of the u.v. effect in irradiated *uvr*$^+$ F$^+$ bacteria is inhibited by acriflavine, which prevents pyrimidine dimer excision (Harm, 1967; Setlow, 1966). Moreover, the effect is reversed by photoreactivation which acts specifically by uncoupling pyrimidine dimers (Rupert, 1961; Setlow, Carrier & Bollum, 1965). All these findings are fully compatible with the hypothesis that the u.v. effect requires both excision of damaged DNA strands as well as an act of genetic recombination, even though they do not offer direct proof of this hypothesis.

The effect of X-rays and of methylmethansulphonate (MMS) on the behaviour of u.v.-induced donor bacteria

In the model we have proposed (Fig. 3) the sex factor and the chromosome are connected by a single DNA strand, in distinction to normal chromosome donors. Despite the presumed shortness of such a single-stranded connection, we thought it possible that transfer by such a structure might prove more sensitive to the action of agents such as X-rays and MMS which are supposed to produce single-stranded breaks in DNA (see Marcovitch, 1961; Brooks & Lawley, 1961; Strauss & Wahl, 1964; Wahl, 1965), although in the case of MMS there is evidence that part, at least, of its effect is subject to a repair process based on excision (Strauss, Reiter & Searashi, 1966; Reiter, Strauss, Robbins & Marone, 1967).

To test this possibility, cultures of F$^+$ bacteria were irradiated with u.v. light and incubated in broth for 1 hr in order to induce an overwhelming majority of new donors. The bacteria were then treated for different times with either X-rays or 0·025M-MMS, and finally assayed for viability and ability to generate recombinants in crosses with female bacteria. The results were compared with those obtained with unirradiated, but otherwise similarly treated, control bacteria. Out of two experiments with MMS and three with X-rays, all gave consistent results in that the absolute number of u.v.-induced recombinants decreased at about twice the rate of those generated by bacteria unexposed to u.v. radiation. In contrast, the survival curves of the irradiated and unirradiated bacterial populations as a whole, as a function of dose of X-rays and MMS, were not significantly different. Moreover, in control experiments assessing the effects of MMS on Hfr bacteria, irradiated and unirradiated bacteria behaved similarly with respect both to survival and to the number of recombinants generated. Finally, an equivalent secondary irradiation with u.v. light, instead of with X-rays, resulted in a further increase in the total number of recombinants instead of in a fall.

In the case of X-rays, the relative decrease in the capacity for transfer of u.v.-induced donors was apparent only when the proportion of survivors exceeded about 25%.

DISCUSSION

The evidence we have presented makes it probable that the induction of donors by u.v. radiation and other agents requires recombination as well as the excision of damaged DNA strands. These induced donors differ from normal donors in two respects; the donor state in them is not inheritable, probably due to the formation of a genetic structure which can lead to transfer but cannot replicate, while the ability of the donor

bacteria to transfer their chromosome appears to be more sensitive than normal to the action of X-rays and MMS, though not of u.v. radiation. There is now little doubt that chromosome transfer by Hfr and intermediate male bacteria, and probably also by F^+ bacteria, normally depends on the double-stranded insertion of the sex factor DNA into that of the chromosome. If a physical continuity between sex factor and chromosome is indeed essential, it is difficult to envisage an alternative to the single-stranded insertion which can explain the experimental findings. A possible hypothesis that the majority of double-stranded *normal* insertions are lethal, due to disruption of some vital chromosomal function, and that the effect of u.v. irradiation is simply to increase the frequency of these normal events, does not account for absence of the effect in *uvr*$^-$ mutants, since there is no difference between the number of recombinants generated by unirradiated F^+.*uvr*$^+$ and F^+.*uvr*$^-$ populations. Thus, *faute de mieux*, we have proposed the model shown in Fig. 3.

We have seen that the total number of recombinants generated by F^+ or intermediate male populations rises with increasing dosage up to about 30% survival, after which the number of recombinants begins to fall at about the same rate as the survivors (Fig. 1). If our hypothesis is correct, that the u.v. induction of new donors is due to a facilitation of pairing between sex factor and chromosome as a result of strand excision, it follows that the effect will reach its peak at a dose which promotes the maximal degree of excision compatible with chromosome repair. This would yield just the type of dose-effect curve found.

The twofold greater sensitivity of u.v.-induced donors to relatively low doses of X-rays or MMS, as compared with normal donors, is hard to account for, even if we take the simplest view that these agents act by producing single-strand breaks in DNA. It is, however, consistent with our model since this possesses a single-stranded connection between sex factor and chromosome which should be very vulnerable to agents that produce single-strand breaks.

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REFERENCES

BOYCE, R. P. (1966). Production of additional sites of DNA breakdown in bromouracil-containing *Escherichia coli* exposed to UV. *Nature, Lond.* **209**, 688.

BOYCE, R. P. & HOWARD-FLANDERS, P. (1964). Release of ultraviolet light-induced thymine dimers from DNA in *E. coli* K 12. *Proc. natn. Acad. Sci. U.S.A.* **51**, 293.

BRODA, P. (1967). The formation of Hfr strains in *Escherichia coli* K 12. *Genet. Res., Camb.* **9**, 35.

BRODA, P., BECKWITH, J. R. & SCAIFE, J. (1964). The characterisation of a new type of F-prime factor in *Escherichia coli* K 12. *Genet. Res., Camb.* **5**, 489.

BROOKS, P. & LAWLEY, P. D. (1961). The reaction of mono- and di-functional alkylating agents with nucleic acids. *Biochem. J.* **80**, 496.

CAVALLI-SFORZA, L. L. & JINKS, J. L. (1956). Studies on the genetic system of *E. coli* K 12. *J. Genet.* **54**, 87.

CLARK, A. J. & MARGULIES, A. D. (1965). Isolation and characterisation of recombination deficient mutants of *E. coli* K 12. *Proc. natn. Acad. Sci., U.S.A.* **53**, 451.

CLOWES, R. C. & ROWLEY, C. (1954). Some observations on linkage effects in genetic recombination in *E. coli* K 12. *J. gen. Microbiol.* **11**, 250.

FISHER, K. W. (1957). The nature of the endergonic processes in conjugation in *Escherichia coli* K 12. *J. gen. Microbiol.* **16**, 136.

HAAS, F., WYSS, O. & STONE, W. S. (1948). The effect of irradiation on recombination in *Escherichia coli*. *Proc. natn. Acad. Sci., U.S.A.* **34**, 229.

HARM, W. (1967). Differential effects of acriflavine and caffeine on various ultraviolet irradiated *Escherichia coli* strains and T 1 phage. *Mutation Res.* **4**, 93.

HAYES, W. (1952). Genetic recombination in *Bact. coli* K 12: analysis of the stimulating effect of ultraviolet light. *Nature, Lond.* **169**, 1017.

HAYES, W. (1953). The mechanism of genetic recombination in *Escherichia coli*. *Cold Spr. Harb. Symp. quant. Biol.* **18**, 75.

HAYES, W. (1960). The bacterial chromosome. *Symp. Soc. gen. Microbiol.* **10**, 12.

HOWARD-FLANDERS, P. (1964). In discussion of SETLOW, R. B. (1964). Physical changes and mutagenesis. *J. cell. comp. Physiol.* **64** (Suppl. 1), 51.

HOWARD-FLANDERS, P. & THERIOT, L. (1962). A method for selecting radiation-sensitive mutants of *Escherichia coli*. *Genetics* **47**, 1219.

HOWARD-FLANDERS, P. & THERIOT, L. (1966). Mutants of *Escherichia coli* K 12 defective in DNA repair and in genetic recombination. *Genetics* **53**, 1137.

HOWARD-FLANDERS, P., BOYCE, R. P. & THERIOT, L. (1966). Three loci in *Escherichia coli* K 12 that control the excision of pyrimidine dimers and certain other mutagen products from DNA. *Genetics* **53**, 1119.

LEDERBERG, E. M. & LEDERBERG, J. (1953). Genetic studies of lysogenicity in interstrain crosses of *E. coli*. *Genetics* **38**, 51.

LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190.

MAALØE, O. & KJELDGAARD, N. O. (1966). *Control of Macromolecular Synthesis*. New York and Amsterdam: W. A. Benjamin, Inc.

MARCOVITCH, H. (1961). Do X-rays produce chromosome breakage in *Escherichia coli* K 12? *J. cell. comp. Physiol.* **58** (Suppl. 1), 107.

PETTIJOHN, D. & HANAWALT, P. (1964). Evidence for repair-replication of ultraviolet damaged DNA in bacteria. *J. molec. Biol.* **9**, 395.

REITER, H., STRAUSS, B., ROBBINS, M. & MARONE, R. (1967). Nature of the repair of methyl methane sulphonate-induced damage in *B. subtilis*. *J. Bact.* **93**, 1056.

RUPERT, C. S. (1961). Repair of ultraviolet damage in cellular DNA. *J. cell. comp. Physiol.* **58** (Suppl. 1), 57.

SCAIFE, J. (1967). Episomes. *A. Rev. Microbiol.* **21**, 601.

SCAIFE, J. & GROSS, J. D. (1963). The mechanism of chromosome mobilisation by an F-prime factor in *Escherichia coli* K 12. *Genet. Res. Camb.* **4**, 328.

SETLOW, J. K. (1966). The molecular basis of biological effects of ultraviolet irradiation and photoreactivation. In *Current Topics in Radiation Research* (Ed. by M. Ebert and A. Howard), vol. II. Amsterdam: North Holland Publishing Co.

SETLOW, R. B. & CARRIER, W. L. (1964). The disappearance of thymine dimers from DNA: an error correcting mechanism. *Proc. natn. Acad. Sci. U.S.A.* **51**, 226.

SETLOW, R., CARRIER, W. & BOLLUM, F. (1965). Pyrimidine dimers in UV-irradiated dI:dC. *Proc. natn. Acad. Sci. U.S.A.* **53**, 1111.

STACEY, K. A. & SIMSON, E. (1965). Improved method for the isolation of thymine-requiring mutants of *Escherichia coli*. *J. Bact.* **90**, 554.

STRAUSS, B. S. & WAHL, R. (1964). The presence of breaks in DNA of *B. subtilis* treated *in vivo* with the alkylating agent, methyl methane sulphonate. *Biochim. biophys. Acta* **116**, 126.

STRAUSS, B., REITER, H. & SEARASHI, T. (1966). Recovery from ultraviolet and alkylating agent-induced damage in *Bacillus subtilis*. *Radiation Res.* (Suppl. 6), 201.

VAN DE PUTTE, P., VAN SLUIS, C. A., VAN DILLEWIJN, J. & RÖRSCH, A. (1965). The location of genes controlling radiation sensitivity in *Escherichia coli*. *Mutation Res.* **2**, 97.

WAHL, R. (1965). Detection of single strand breaks in DNA alkylated *in vitro*. *Fedn. Proc., Fedn. Am. Ass. biol. Soc.* **24**, 226.

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Toxicity of Spores of *Clostridium botulinum* Strain 33A in Irradiated Ground Beef

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SUMMARY

Sterilizing doses of γ-radiation for food products (4·5 Mrad.) do not completely inactivate spore toxin or enzymes. The present study concerns spore toxin during and after irradiation of 4×10^6 spores of *Clostridium botulinum* per g. of canned ground beef irradiated to 4·5 Mrad., at temperatures of $-25°$, $0°$ and $+25°$. During subsequent incubation of irradiated samples at 30° for 10 months, periodic analyses showed no viable spores. Toxin was tested on (i) non-homogenized samples and (ii) samples homogenized in a tissue grinder.

In samples irradiated at $-25°$ and 0° and in un-irradiated un-incubated controls, 4 minimal lethal doses (m.l.d.) of toxin were found in non-homogenized samples immediately after irradiation. The toxin titre of irradiated samples gradually diminished to approximately half of its initial value at the end of 10 months at 30°. In identical sets of homogenized irradiated samples, 8 m.l.d. were found initially and the titre increased to 44 m.l.d. after 2 weeks to 4 months of incubation at 30°. Thereafter, the toxin titre slowly deteriorated to approximately 25 m.l.d. at the end of 10 months.

Samples irradiated at 25° exhibited a definite increase in toxin titre even immediately after irradiation. This seems to suggest the possibility of new toxin synthesis either during or after radiation. The toxin titre in homogenized samples reached 64 m.l.d. after 2 months at 30° and thereafter slowly diminished to 31 m.l.d. at 10 months.

Toxin titres in radiation-damaged spores were small in comparison to un-irradiated *C. botulinum* growing in beef which reached 1000 to 5000 m.l.d. in 2 days at 30°.

INTRODUCTION

The work described in this paper is based upon two main observations:

(*a*) Spores of *Clostridium botulinum* type A contain some active toxin which is highly resistant to heat (Grecz & Lin, 1966; Grecz *et al.* 1967; Lin, 1966).

(*b*) Spores which have been killed by γ-radiation (3·6 to 5·0 Mrad.) still contain active toxin (Kempe & Graikoski, 1962; Grecz & Lin, 1966). These findings indicate that the essential molecule(s) responsible for radiation survival of spores is considerably more sensitive to radiation than is spore toxin.

Cellular death from ionizing radiation is directly related to lesions in the nuclear DNA (Howard-Flanders, 1961; Szybalski & Lorkiewicz, 1962; Ginoza, 1967; Read, 1968). However, concurrently, radiation causes some inactivation of other components such as botulinum toxin (Wagenaar & Dack, 1960; Skulberg, 1965; Grecz & Lin, 1966; Grecz *et al.* 1967), enzymes, etc. The amount of damage to enzymes is not known.

It has been demonstrated that radiation-damaged spores placed into a nutrient

medium are still able to perform limited metabolic activity, e.g. they can germinate and grow into an abortive vegetative cell (Rowley, Feeherry & El-Bisi, 1968). Apparently sufficient enzymes remain active to carry out germinative processes and to synthesize new cell proteins, one of which may be botulinum toxin. In this connexion, it was of interest to study the possibility that radiation-damaged spores may be able to synthesize new toxin without showing any obvious signs of growth and multiplication. This question is of vital importance to food preservation programmes as well as to the basic understanding of metabolic capabilities of radiation-damaged spores.

METHODS

Spores of *Clostridium botulinum* strain 33A, a highly radiation-resistant strain, were incorporated into ground beef and sealed in thermal-death time (t.d.t.) cans. Approximately 4×10^6 spores of *C. botulinum* 33A were inoculated per g. of ground beef. The spores were initially heat-shocked at 80° for 10 min. in order to destroy all heat-sensitive 'vegetative' toxin and to activate spores for germination. The samples were irradiated with Co-60 to 4·5 Mrad. at a dose rate of 0·3 Mrad/hr. The temperatures during irradiation was controlled at -25^c, 0° and +25°. It required approximately 15 hr to irradiate to 4·5 Mrad. Thus spores irradiated at +25° had ample opportunity for germination and perhaps for some limited development, whereas at −25° and 0° metabolic changes were arrested. After irradiation the cans were incubated at 30° and representative samples were withdrawn for examination at regular time intervals up to 10 months. The experimental outline is presented in Table 1. A total number of

Table 1. *Experimental outline*

Temp. during irradiation	Total no. cans	Time of incubation at 30° after irradiation							
		0	1 wk.	2 wk.	4 wk.	2 mo.	4 mo.	6 mo.	10 mo.
25°	28	3	5	5	3	3	3	3	3
0°	28	3	5	5	3	3	3	3	3
−25°	28	3	5	5	3	3	3	3	3

Inoculum: 4×10^6 spores of *C. botulinum* per g. of ground beef.

28 cans was irradiated at each temperature. The number of cans examined at each time interval is indicated in Table 1. Viable counts were performed either by colony counts or by the most probable number method (Fisher & Yates, 1953) using Wynne broth (Wynne, Schmieding & Daye, 1955) or Wynne broth plus 1·5% agar as the recovery medium. Details of methods were described by Grecz, Snyder *et al.* (1965).

The amount of toxin in the samples was assayed by intraperitoneal injection of 0·2 to 1·0 ml. of appropriate dilutions into two (18 to 21 g.) white mice. Death of at least one of the animals within 4 days was considered a presumptive positive reaction; the test was confirmed with two unprotected and two protected mice, using specific type A antitoxin. The results were expressed as minimal lethal doses (m.l.d.) by the method of Wagenaar & Dack (1960), taking into account the dilution of sample and the volume injected. For example, assume that 0·4 ml. of 1/8 dilution caused death but 0·2 ml. of 1/8 had no effect, or 1/20 ($4/10 \times 1/8 = 1/20$) was toxic but 1/40 was not. Hence the sample contained 20 m.l.d. but not 40 m.l.d.

The beef samples were prepared by two methods for injection into mice:

Method 1. About 1 g. of beef was diluted 1/2, 1/4, 1/8, and so on. The meat particles were then allowed to settle and the supernatant was drawn up into a syringe and injected intraperitoneally.

Method 2. The beef samples were homogenized in a tissue grinder and appropriate dilutions were made so that homogenized beef tissue was injected with the liquid into the mouse. This was thought to be necessary because botulinum toxin is known to be absorbed on food particles (Grecz & Dack, 1963).

Table 2. *Initial toxicity of un-irradiated samples*

Initial spore inoculum: 4×10^6 spores of *C. botulinum* 33A. Initial toxicity: 4 m.l.d.

Control sample	Toxicity (m.l.d.) Non-homogenized	Homogenized beef	Spore count
1	2·7	8	$2{\cdot}0 \times 10^6$
2	4	8	$5{\cdot}81 \times 10^6$
3	2	8	$3{\cdot}47 \times 10^6$
Average	2·9	8	$3{\cdot}76 \times 10^6$

Table 3. *Toxicity of samples irradiated to 4·5 Mrad. at −25°*

Time of incubation at 30°	Average toxicity of triplicate samples (m.l.d.) Non-homogenized	Homogenized beef
0	4·0	8·0
1 week	4·0	28·4
2 weeks	3·6	44·4
1 month	3·6	37·3
2 months	3·0	35·5
4 months	2·9	36·0
6 months	2·7	32·0
10 months	2·7	26·6

RESULTS

Un-irradiated samples. The beef samples were initially inoculated with 4×10^6 spores/g. containing 4 m.l.d. of spore toxin as tested in the stock suspension of spores in phosphate buffer. After incorporation into ground beef 94% of the spores could be detected by the most probable number method (Table 2). Furthermore, approximately 3/4 of the initial amount of spore toxin could be recovered from non-homogenized samples, homogenized beef showed twice as much toxin as would be expected from intact spores.

Irradiated samples. After irradiation to 4·5 Mrad., the beef samples contained no detectable viable organisms either immediately at the start of incubation or after incubation at 30° for up to 10 months. This indicated that 4·5 Mrad. was an effective sterilizing dose and that there was no detectable repair of initial radiation damage after irradiation.

During incubation at 30° the changes in toxin titre were different depending on sample treatment during irradiation, namely; whether the samples were irradiated at −25°, 0° or +25°.

Samples irradiated at −25°. The initial toxin titre in samples γ-irradiated to 4·5 Mrad. at −25° did not decrease to a detectable degree compared with unirradiated controls (Table 3). Here the homogenized sample contained 8 m.l.d. and non-homogenized 4 m.l.d. of toxin. On incubation at 30° non-homogenized samples did not show any detectable increase in toxicity, whereas, homogenized samples increased to 28 m.l.d. after 1 week and to more than 40 m.l.d. after 2 weeks at 30°. On incubation beyond 2 weeks, there appeared to be a very definite tendency for slow toxin deterioration, perhaps analogous to toxin decline in stored cheese (Grecz, Wagenaar & Dack, 1965).

Table 4. *Toxicity of samples irradiated to 4·5 Mrad. at* 0°

Time of incubation at 30°	Average toxicity of triplicate samples (m.l.d.)	
	Non-homogenized	Homogenized beef
0	4·0	8·0
1 week	3·1	10·7
2 weeks	5·3	28·4
1 month	3·6	34·7
2 months	3·3	40·0
4 months	2·8	44·4
6 months	2·0	40·0
10 months	2·0	24·0

Table 5. *Toxicity of samples irradiated to 4·5 Mrad. at +25°*

Time of incubation at 30°	Average toxicity of triplicate samples (m.l.d.)	
	Non-homogenized	Homogenized beef
0	8·0	21·3
1 week	3·6	34·7
2 weeks	2·7	52·4
1 month	2·4	52·4
2 months	2·7	64·0
4 months	2·0	53·3
6 months	2·7	53·3
10 months	1·6	30·8

Samples irradiated at 0°. The toxicity of samples irradiated at 0° to 4·5 Mrad (Table 4) was about the same as that shown in Table 3. Furthermore, the amount of toxin detected during incubation of these two sets of samples was practically identical. This seems to suggest that the samples irradiated at non-physiological temperatures, i.e. at 0° and −25° behaved in a similar manner after and during incubation.

Samples irradiated at +25° to 4·5 Mrad. The samples behaved distinctly differently than those irradiated at −25° and 0° (Table 5).

Here it can be seen that the initial sample, before incubation contained 8 m.l.d. in the non-homogenized and 21 m.l.d. in the homogenized beef, i.e. 2–3 times as much toxin as in those samples irradiated at −25° and 0°. The data seem to indicate that toxin synthesis was initiated during irradiation at +25°. As already mentioned, the time during irradiation was 15 hr i.e. sufficient for appreciable physiological activity. On incubation at 30° the toxin titre in homogenized samples after 1 week rose to almost 35 m.l.d., after 2 weeks to 52 m.l.d. and after 2 months to 64 m.l.d. Thereafter the

toxin titre appeared to diminish slowly to a final 31 m.l.d. after 10 months. It can be seen that the peak toxin titre in these samples was considerably higher than the amount of toxin in Tables 3 and 4. Therefore, it may be suspected that new toxin, as well as new spore enzymes, may have been synthesized during irradiation of spores at +25°. These enzymes were perhaps capable of synthesizing the additional quantity of toxin shown in Table 5, as compared with samples irradiated at non-physiological temperatures. However, this increase in toxin titre is open to other interpretations than enzymic synthesis.

The final toxin titre after 10 weeks at 30° was practically the same in all three experiments, namely 27 m.l.d. (Table 3), 24 m.l.d. (Table 4), and 31 m.l.d. (Table 5).

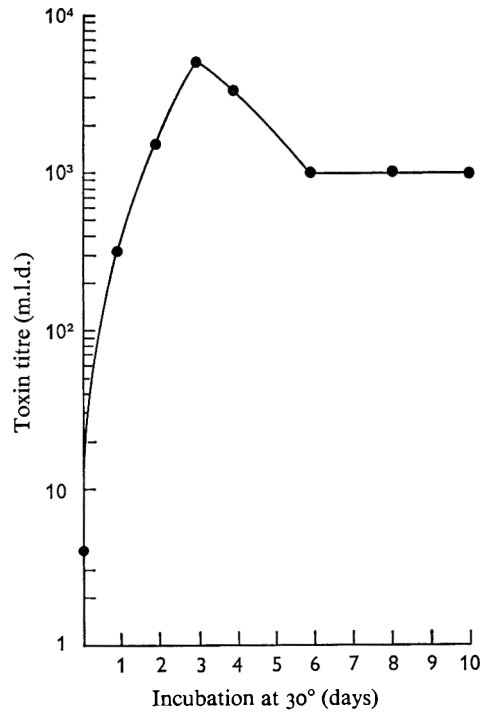

Fig. 1. Toxin production in lean ground beef by *Clostridium botulinum* 33A during incubation at 30°.

In contrast to homogenized samples, the toxin titre in non-homogenized beef: (i) showed a much lower titre in all cases, (ii) never increased on incubation, and (iii) slowly but consistently diminished starting from the beginning of the experiment.

Synthesis of toxin in ground beef. For purposes of comparison, ground beef was inoculated with 4×10^6 spores per g. and the organisms were allowed to develop at 30° (Fig. 1). A peak toxin titre of 5000 m.l.d. was reached within 3 days after which the titre dropped to 1000 m.l.d. The titre remained at 1000 m.l.d. for up to 10 days, which was the duration of the experiment.

From these data it can be concluded that the toxin titre developed by un-irradiated *Clostridium botulinum* in ground beef was approximately 100-fold higher than the peak titre developed by radiation-damaged spores.

DISCUSSION

Evidence is presented to show that although beef can be effectively sterilized by γ-radiation, existing spores may in some way introduce a significant degree of toxicity which is not necessarily detectable immediately after irradiation, but becomes evident only after 2 to 4 weeks of incubation at 30°. The cause of this increase in toxin titre is not clear. Several possibilities may be considered including: (i) new toxin synthesis by those spore enzymes which remain active after irradiation, (ii) germination of irradiated spores, (iii) slow lysis of spores at 30°, (iv) activation or fragmentation of pre-existing toxin molecules, etc.

It remains a puzzle why the progressive increase in toxin titre during storage at 30° could be detected only after homogenization of the sample, but not in unhomogenized beef. It is unlikely that the tissue homogenizer would accomplish any significant degree of spore breakage even if the radiation-damaged spores germinated and possibly grew out into vegetative forms. On the other hand, spore breakage in itself may not necessarily explain the observed increase in toxicity. Unfortunately no microscopic observations could be made in the present study because of the relatively low spore inoculum and because of interference by beef particles.

It should be emphasized that the practical significance of spore toxicity for radiation sterilization of foods appears to be of negligible importance. The amount of toxin developed in radiation-damaged spores is relatively small compared with that synthesized by un-irradiated *Clostridium botulinum* growing in ground beef. Furthermore, under natural conditions, *C. botulinum* would not be expected to be present in large numbers. However, the problem is of definite theoretical interest. It is only too obvious that there remains much ignorance about spore toxin in *C. botulinum* and particularly about the possibility of physiological activity in radiation-damaged spores, such as potential *de novo* protein synthesis, which may have been suggested by the present experiments. Solution of these problems will require many more experiments as well as more defined experimental conditions than were used in the present work.

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REFERENCES

FISHER, R. A. & YATES, F. (1953). *Statistical Tables for Biological Agricultural and Medical Research*, 4th ed., p. 49. New York: Hafner Publishing Co.

GINOZA, W. (1967). The effect of ionizing radiations on nucleic acids of bacteriophages and bacterial cells. *A. Rev. Microbiol.* **21**, 325.

GRECZ, N. & DACK, G. M. (1963). Toxin production in naturally separated liquid and solid components in preparations of heated surface ripened cheese inoculated with *Clostridium botulinum*. *J. Food Sci.* **28**, 276.

GRECZ, N. & LIN, C. A. (1966). Properties of heat resistant toxin in spores of *Clostridium botulinum* 33A. 'Symposium on Botulism'. *IX International Congress of Microbiology, Moscow*, 20–22 July 1966.

GRECZ, N., WAGENAAR, R. O. & DACK, G. M. (1965). Storage stability of *Clostridium botulinum* toxin and spores in processed cheese. *Appl. Microbiol.* **13**, 1014.

GRECZ, N., SNYDER, O. P., WALKER, A. A. & ANELLIS, A. (1965). Effect of temperature of liquid nitrogen on radiation resistance of spores of *Clostrodium botulinum*. *Appl. Microbiol.* **13**, 527.

GRECZ, N., LIN, C. A., TANG, T., SO, W. L. & SEHGAL, L. R. (1967). The nature of heat resistant toxin in spores of *Clostridium botulinum*. *Japan J. Microbiol.* **11**, 384.

HOWARD-FLANDERS, P. (1961). Factors affecting radiation injury to DNA in bacteria and bacteriophage systems. *Brookhaven Symp. Biol.* **14**, 18.

KEMPE, L. L. & GRAIKOSKI, J. T. (1962). Gamma ray sterilization and residual toxicity studies of ground beef inoculated with spores of *Clostridium botulinum*. *Appl. Microbiol.* **10**, 31.

LIN, C. A. (1966). *Heat resistant toxin in spores of Clostridium botulinum*. M.S. thesis. Illinois Institute of Technology, Chicago, Illinois.

READ, J. (1968). Repair by cells of damage caused by radiations or by radiomimetic chemicals. In *Actions Chemiques et Biologique des Radiations*. Ed. by M. Haissinsky. Paris: Masson et Cie.

ROWLEY, D. B., FEEHERRY, F. & EL-BISI, H. M. (1968). Effect of sodium chloride and irradiation on the germination and outgrowth of spores of *Clostridium botulinum* 62 A. *Bact. Proc.* G. 96.

SKULBERG, A. (1965). The resistance of *Clostridium botulinum* type E toxin to radiation. *J. appl. Bact.* **28**, 139.

SZYBALSKI, W. & LORKIEWICZ, Z. (1962). On the nature of the principal target of lethal and mutagenic radiation effects. *Abh. dt. Akad. Wiss. Berl. Med.* no. 1, 63.

WAGENAAR, R. O. & DACK, G. M. (1960). Studies on the inactivation of type A *Clostridium botulinum* toxin by irradiation with cobalt-60. *Fd Res.* **25**, 279.

WYNNE, E. S., SCHMIEDING, W. R. & DAYE, G. T., JUN. (1955). A simplified medium for counting *Clostridium botulinum* spores. *Fd Res.* **20**, 9.

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Ultrastructure of *Leptotrichia buccalis*

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SUMMARY

Thin sections of *Leptotrichia buccalis* were examined with the electron microscope. The organism showed a cell-wall profile characteristic of Gram-negative bacteria. A Gram-positive homofermentative *Lactobacillus* sp. was included in the study for comparison. The results indicate that *L. buccalis* should not be included within the Lactobacillaceae.

INTRODUCTION

The oral filamentous organism *Leptotrichia buccalis* was first adequately described by Thjötta, Hartman & Böe (1939), who looked upon this organism as a species of Gram-negative bacteria related to the fusobacteria (Böe & Thjötta, 1944). However, most later authors have been of the opinion that *L. buccalis* represents a species of Gram-positive bacteria related to the lactobacilli (Hamilton & Zahler, 1957; Gilmour, Howell & Bibby, 1961).

A clear relationship exists between the Gram reaction and the structure of the bacterial cell wall. In the electron microscope the cell wall of Gram-positive bacteria appears as a thick homogenous layer close to the plasma membrane (Murray, 1962). The cell wall of Gram-negative bacteria, on the other hand, is characterized by a varying number of thin electron-dense layers which follow more or less convoluted paths along the periphery of the cell (Murray, Steed & Elson, 1965). In the present study, the morphology of *L. buccalis* and a homofermentative *Lactobacillus* sp. have been compared in the electron microscope, with particular attention to the cell-wall morphology of these micro-organisms.

METHODS

The isolation of *Leptotrichia buccalis*, strain L 11, has been described earlier (Hofstad, 1967*a*). The bacteria were cultivated in nutrient broth enriched with 0·1 % (w/v) cysteine hydrochloride, 0·3 % yeast extract (Difco), 1 % glucose and 5 % (v/v) human ascitic fluid, and harvested by centrifugation when in the exponential phase of growth (1 to 3 days at 37°). The homofermentative *Lactobacillus* sp. strain LA 6 was isolated from human saliva; it was cultivated in Bacto-Rogosa SL broth and the bacteria were harvested by centrifugation.

Pellets of intact organisms were suspended in acetate+veronal buffered osmium tetroxide fixative, transferred to agar, and embedded in polyester resin according to the procedure suggested by Kellenberger, Ryter & Sèchaud (1958). Thin sections were

cut with glass knives, collected on carbon-coated formvar membranes, and stained on a saturated solution of uranyl acetate in 50% (v/v) ethanol in water for 60–90 min. or on 2% aqueous phosphotungstic acid for 30 min. The choice of contrasting material did not significantly influence the appearance of the structures of interest. Further reference to the staining technique has, consequently, been omitted. Specimens were examined in the electron microscope and photographed at plate magnifications of 10,000 to 30,000 diameters.

RESULTS

Leptotrichia buccalis

The organisms appeared in thin sections as circular or elongated structures; the length varied considerably depending on the plane of sectioning, while the diameter ranged from 0·6 to 0·8 μ. The concentric membranous structures surrounding the protoplasm will be referred to as outer membrane, solid membrane, and plasma membrane.

The outer membrane consisted of a double layer, about 70 Å in width (Pl. 1, fig. 1, 2, 3). The solid membrane was seen as a single electron-dense line, about 50 Å in width, following a straight-line course (Pl. 1, fig. 1, 2, 3). The plasma membrane appeared as a doublet structure which, in general, closely followed the course of the solid membrane, separated from it by a 50 Å-wide space (Pl. 1, fig. 1, 2). The various membranous structures were, in general, closely adherent to each other. However, separations of the membranes occurred occasionally between the outer and the solid membrane, and between the solid and the plasma membrane (Pl. 1, fig. 3). Invaginations of the plasma membrane, suggestive of mesosomes (Fitz-James, 1960) were encountered in a few cases (Pl. 1, fig. 3).

The homofermentative Lactobacillus sp.

The lactobacilli appeared elongated, with a diameter in transverse sections of 0·4–0·5 μ. Pairs of cells attached end to end were frequently seen (Pl. 2, fig. 4). In contrast to *Leptotrichia buccalis*, only two separate structural entities, i.e. the cell wall and the plasma membrane, could be distinguished surrounding the cytoplasm. The cell wall appeared as a homogeneous or finely granular structure, 200–300 Å in width. Its inner and outer borders were in some cells diffusely outlined, in others more distinctly defined.

The cell wall was often separated from the underlying plasma membrane by a fine space (Pl. 2, fig. 5). In most cells, however, the cell wall was closely adherent to the plasma membrane (Pl. 2, fig. 6). The external layer of the double-tracked plasma membrane was often more electron dense than the internal layer (Pl. 2, fig. 6).

Mesosomes were a prominent characteristic of this *Lactobacillus* sp. Most cells exhibited at least one such structure within each section (Pl. 2, fig. 4, 6). These formations did not affect the course of the cell wall. The cytoplasm was mostly composed of finely granular electron-dense material, while the centrally located nuclear regions displayed a less dense filamentous structure (Pl. 2, fig. 4, 5).

DISCUSSION

The cell wall profile of *Leptotrichia buccalis* is similar to that previously found in anaerobic Gram-negative organisms, such as *Veillonella* sp. (Bladen & Mergenhagen, 1964) and *Fusobacterium* sp. (Tagaki & Ueyama, 1963), and conforms to the general description of the Gram-negative cell-wall type (De Petris, 1965; Murray *et al.* 1965). Minor variations with respect to the appearance of the outer membrane, solid membrane, and plasma membrane, and the width of the spaces between these structures, are most likely related to the age of the organisms and the technique of specimen preparation. In contrast, the homofermentative *Lactobacillus* sp. examined here showed a cell wall profile typical of Gram-positive bacteria (Glauert, 1962; Murray, 1962). With regard to cell wall structure, as well as other characteristics, the homofermentative *Lactobacillus* sp. closely resembles *L. acidophilus* as described by Reyn, Birch-Andersen & Lapage (1966).

On the basis of observations of the cell-wall structure, therefore, *Leptotrichia buccalis* should be classified as a Gram-negative bacterium. This conclusion is in accordance with investigations on the chemical composition of its isolated cell walls (Hofstad, 1967*b*), which, in addition to mucopeptide, contained protein, lipid and polysaccharide. Araujo, Varah & Mergenhagen (1963) and Gustafson, Kroeger & Vaichulis (1966) have isolated endotoxins from *L. buccalis* by phenol + water extraction of whole organisms, further supporting the Gram-negative nature of this organism. Mesosomes are conspicuously present in aerobic Gram-positive bacteria (Salton, 1967). However, mesosome-like configurations, similar to those found here in a few organisms of *Leptotrichia buccalis*, but apparently separated from the plasma membrane, have been observed in strains of *Bacteroides* sp. (Bladen & Waters, 1963). The considerations above lead to the conclusion that *L. buccalis* should not be included within the Lactobacillaceae. The inclusion of the genus *Leptotrichia* in the Bacteroidaceae (Böe & Thjötta, 1944) seems more justified.

The electron microscopic work was carried out at the Institute of Anatomy, University of Bergen.

REFERENCES

ARAUJO, W. C. DE, VARAH, E. & MERGENHAGEN, S. E. (1963). Immunochemical analysis of human oral strains of Fusobacterium and Leptotrichia. *J. Bact.* **86**, 837.

BLADEN, H. A. & MERGENHAGEN, S. E. (1964). Ultrastructure of Veillonella and morphological correlation of an outer membrane with particles associated with endotoxic activity. *J. Bact.* **88**, 1482.

BLADEN, H. A. & WATERS, J. F. (1963). Electron microscopic study of some strains of Bacteroides. *J. Bact.* **86**, 1339.

BÖE, J. & THJÖTTA TH. (1944). The position of Fusobacterium and Leptotrichia in the bacteriological system. *Acta path. microbiol. scand.* **21**, 441.

DE PETRIS, S. (1965). Ultrastructure of the cell wall of *E. coli*. *J. ultrastruct. Res.* **12**, 247.

FITZ-JAMES, P. C. (1960). Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. *J. biophys. biochem. Cytol.* **8**, 507.

GILMOUR, M. N., HOWELL, A. JUN. & BIBBY, B. G. (1961). The classification of organisms termed *Leptotrichia* (*Leptothrix*) *buccalis*. I. Review of the literature and proposed separation into *Leptotrichia buccalis* Trevisan, 1879 and *Bacterionema* gen. nov., *B. matruchotii* (Mendel, 1919) comb. nov. *Bact. Rev.* **25**, 131.

GLAUERT, A. M. (1962). The fine structure of bacteria. *Br. med. Bull.* **18**, 245.

GUSTAFSON, K. L., KROEGER, A. V. & VAICHULIS, E. M. K. (1966). Chemical characteristics of *Leptotrichia buccalis* endotoxin. *Nature, Lond.* **212**, 301.

HAMILTON, R. D. & ZAHLER, S. A. (1957). A study of *Leptotrichia buccalis*. *J. Bact.* **73**, 386.

HOFSTAD, T. (1967*a*). An anaerobic oral filamentous organism possibly related to *Leptotrichia buccalis*. I. Morphology, some physiological and serological properties. *Acta path. microbiol. scand.* **69**, 543.

HOFSTAD, T. (1967*b*). An anaerobic oral filamentous organism possibly related to *Leptotrichia buccalis*. II. Composition of cell walls. *Acta path. microbiol. scand.* **70**, 461.

KELLENBERGER, E., RYTER, A. & SÈCHAUD, J. (1958). Electron microscopic study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *J. biophys. biochem. Cytol.* **4**, 671.

MURRAY, R. G. E. (1962). Fine structure and taxonomy of bacteria. *Symp. Soc. gen. Microbiol.* **12**, 119.

MURRAY, R. G. E., STEED, P. & ELSON, H. E. (1965). The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other Gram-negative bacteria. *Can. J. Microbiol.* **11**, 547.

REYN, A., BIRCH-ANDERSEN, A. & LAPAGE, S. P. (1966). An electron microscope study of thin sections of *Haemophilus vaginalis* (Gardner and Dukes) and some possibly related species. *Can. J. Microbiol.* **12**, 1125.

SALTON, M. R. (1967). Structure and function of bacterial cell membranes. *A. Rev. Microbiol.* **21**, 417.

TAGAKI, A. & UEYAMA, K. (1963). Characteristic cell wall structure of a Fusobacterium. *Jap. J. Microbiol.* **7**, 43.

THJÖTTA, TH., HARTMAN, O. & BÖE, J. (1939). A study of *Leptotrichia* Trevisan, history, morphology, biological and serological characteristics. *Avh. norske VidenskAkad, Oslo.* No. 5.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Leptotrichia buccalis*. The cytoplasm is surrounded by three concentric structures: double-tracked plasma membrane, solid membrane, and double-tracked, outer membrane. In this and the following figures, PM signifies plasma membrane, SM solid membrane, and OM outer membrane. Markers represent 0·1 μ. Magnification ×45,000.

Fig. 2. *L. buccalis*. Cell wall structure of a longitudinally sectioned cell. The solid membrane appears as a single electron-dense line which pursues an almost straight-line course. × 100,000.

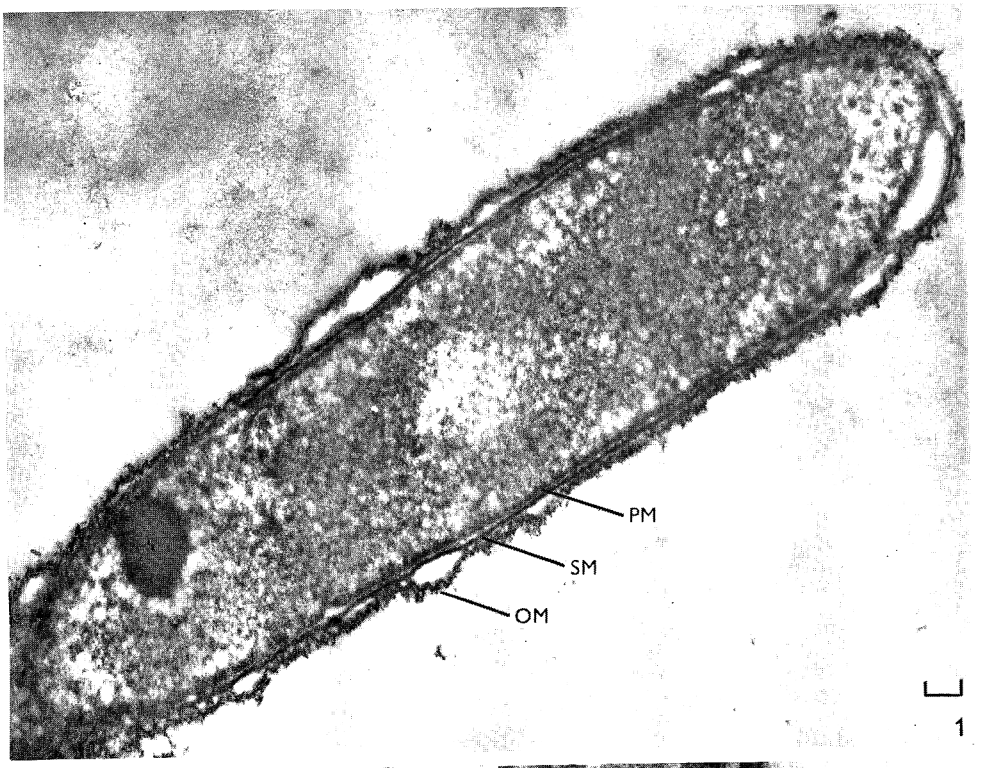
Fig. 3. *L. buccalis*. Separations of the various cell-wall components have occurred in limited areas. A mesosome-like configuration (M) can be seen near the top of the illustration. × 100,000.

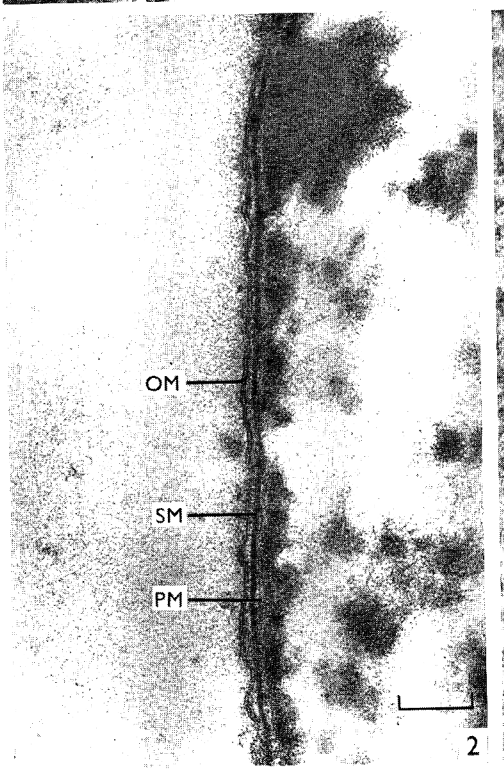
PLATE 2

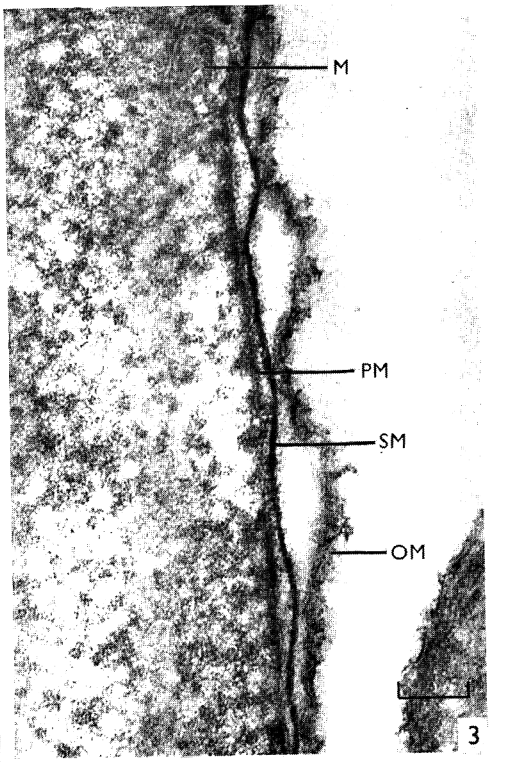
Fig. 4. Homofermentative *Lactobacillus* sp. The organisms appear as rods, often adhering end to end in pairs. The cell wall (CW) forms a thick, homogenous or granular layer immediately outside the plasma membrane. M mesosome, N nuclear region. × 100,000.

Fig. 5. Homofermentative *Lactobacillus* sp. Longitudinally sectioned cell shows separations between cell wall and plasma membrane. N nuclear region. × 100,000.

Fig. 6. Homofermentative *Lactobacillus* sp. Cross-sectioned cells with cell wall tightly adherent to the plasma membrane, except in area near lower edge of illustration. The outer layer of the plasma membrane is more electron dense than the inner layer. M mesosome. × 100,000.







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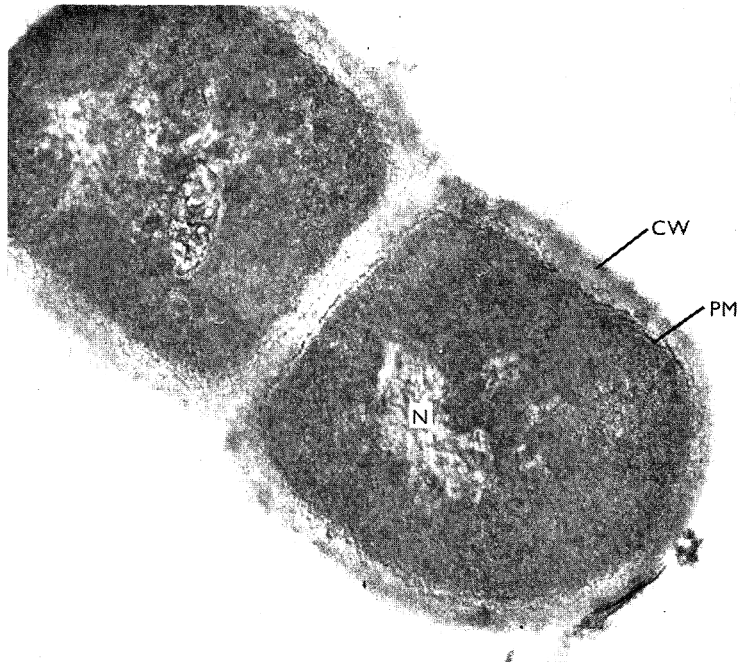
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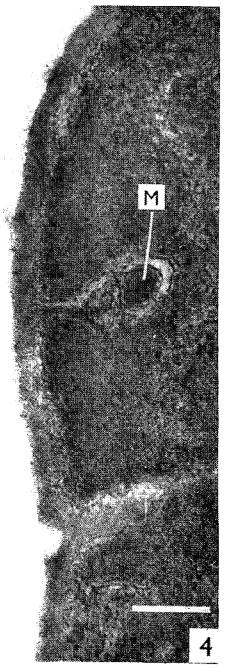
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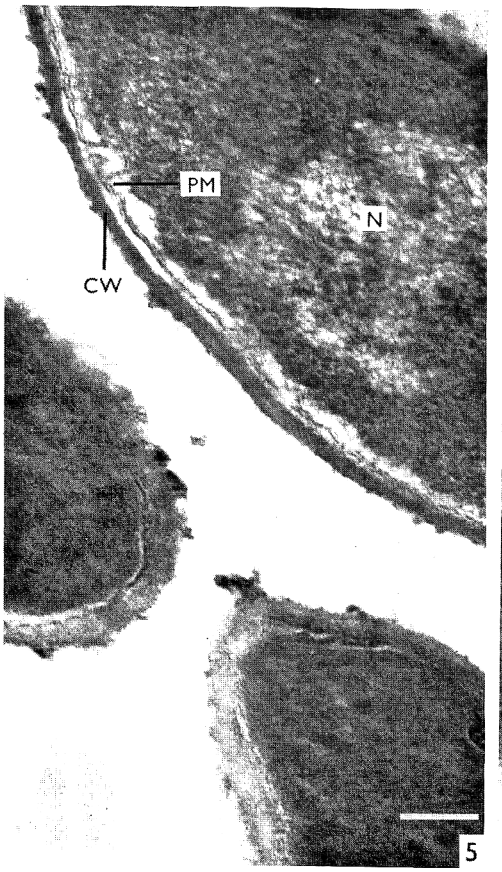
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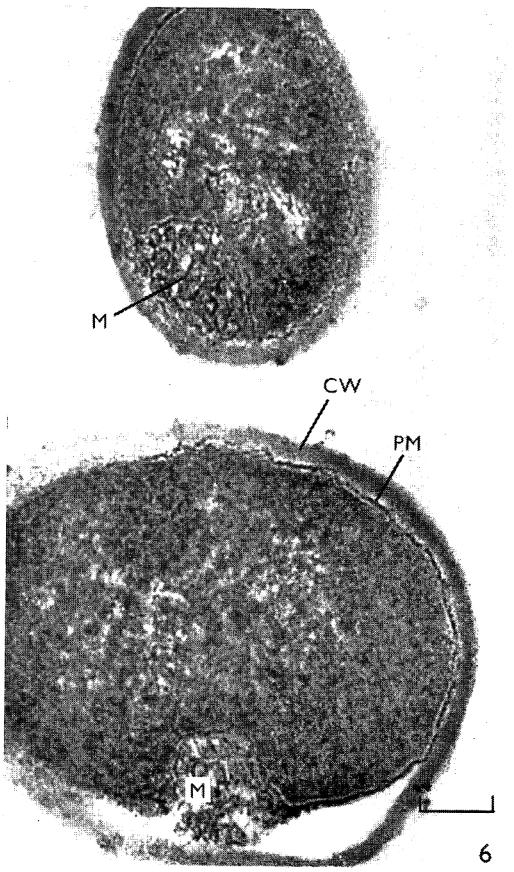
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Inhibition of Growth and Nucleic Acid Synthesis in Zinc-deficient *Mycobacterium smegmatis*

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SUMMARY

The growth of *Mycobacterium smegmatis* was inhibited by lack of zinc in a glycerol + asparagine medium depleted of trace metals but enriched with iron. Addition of zinc to this medium supported full growth of the organism. Both zinc-deficient and zinc-replete cultures had the same exponential doubling time. In zinc-deficient organisms the synthesis of RNA and DNA varied with growth rate in a manner typical for nutrient-induced decreases in a variety of organisms. Zinc-deficiency led to inhibition of nicotinamide synthesis and an increase in phosphate storage as compared with zinc-replete cultures. The pH value of zinc-deficient cultures was preponderantly alkaline. The results are discussed with reference to the metabolic role of zinc ions, and the effects of zinc deficiency is compared to the effects of iron deficiency as previously reported for the same organism.

INTRODUCTION

In a previous paper (Harris, 1967) the effect of iron deficiency on nucleic acid synthesis in submerged cultures of *Mycobacterium smegmatis* was discussed. Inhibition of growth in submerged culture of *M. smegmatis*, caused by lack of iron, is associated with a specific inhibition of DNA synthesis (Harris, 1967). Furthermore, deficiency of iron induces inhibition of DNA synthesis before inhibition of growth. Thus, in iron-deficient cultures of *M. smegmatis*, DNA is more sensitive to growth-rate changes than would be expected from the widely confirmed rules formulated by Herbert (1961) and from other work in this field (Kjeldgaard, 1961). These rules, however, were derived from studies of growth rate changes induced by changes of availability of non-mineral nutrients.

In the past, zinc-deficiency has been shown to be related to a loss of ability to metabolize carbohydrate (Webley, Duff & Anderson, 1962) which is perhaps related to the presence of zinc in many enzymes associated with carbohydrate metabolism (Stein & Fischer, 1960; Dixon & Webb, 1964; Cregolin & Singer, 1963); to distorted morphology (Webley, 1960; Herbert, 1961; Winder, O'Hara & Ratledge, 1961), and to increase in phosphate storage (Winder & Denneny, 1959; Winder & O'Hara, 1962). Comparative studies of the exponential phase of growth of *Mycobacterium smegmatis* or other organisms in zinc-replete and zinc-deficient cultures have not been made.

In the present work exponentially growing submerged *Mycobacterium smegmatis* cultures were used to determine the effect of zinc deficiency upon the growth and

nucleic acid metabolism of this organism. The conditions of growth and the methods of assay were like those used in the previous study of iron deficiency of this organism (Harris, 1967).

METHODS

Extraction procedures and assays

Mycobacterium smegmatis was grown in a chemically defined glycerol + asparagine medium depleted of trace metals and harvested as previously described (Harris, 1967). Depleted media were enriched with ferrous sulphate ($FeSO_4.7H_2O$, Analar Grade) solution to give a concentration of 2·0 μg. Fe^{2+}/ml. Zinc-replete medium was prepared by adding a solution of zinc sulphate ($ZnSO_4.7H_2O$, Analar Grade) to the iron-enriched media to give a concentration of 0·4 μg. Zn^{2+}/ml.

Materials for assay were extracted from freeze-dried samples of organisms as described by Harris (1967).

The nucleic acids were assayed spectrophotometrically and expressed in terms of phosphorus (Logan, Mannell & Rossiter, 1952; Harris, 1967).

The water and trichloroacetic acid extracts of the organism were assayed for nicotinamide by a cyanide addition reaction (Ciotti & Kaplan, 1957).

The term 'insoluble phosphate' was first used by Winder & O'Hara (1962) to describe phosphate found in the nucleic acid extracts of surface cultures of *Mycobacterium smegmatis*. In the present work, this contribution was assayed by a modified method, since it was found that when the incinerated nucleic acid extracts were not hydrolysed before phosphate assay, variable results were obtained. Unless this precaution is taken, as described below, inorganic orthophosphate is determined, but pyrophosphate is not (A. B. Harris, unpublished results). Furthermore, the absorbancy of the phosphate molybdate complex upon which the assay depends, varies with time of standing; consequently, readings were taken at a fixed time, 24 hr after mixing of the reagents with the nucleic acid extracts.

Pooled samples of the nucleic acid extracts (0·5 ml. each) were incinerated with concentrated sulphuric acid (0·1 ml.) and hydrogen peroxide (0·2 ml., 30 vol.) until fumes were seen. After cooling, water was added (1 to 5 ml.) and the mixture heated (2 hr, 100°) to hydrolyse polyphosphate formed by the incineration; the total phosphate was then determined (Le Page, 1949). From this value was subtracted phosphate contributed by the nucleic acids (Harris, 1967) to give insoluble phosphate. This phosphate is not part of the phosphate chain of the nucleic acids, in 3′, 5′ diester linkages with ribofuranose (RNA), and 2-deoxyribofuranose as found in DNA (Watson & Crick, 1953), but it is extracted with the nucleic acids.

RESULTS

Growth of Mycobacterium smegmatis

Growth departed from the exponential at about seven generation times after inoculation in zinc-replete and in zinc-deficient media. In both cultures the exponential doubling time, as with iron-deficient cultures, was 7·0 ± 0·5 hr. In zinc-deficient cultures exponential growth ceased abruptly (Fig. 1), indicative of the exhaustion of a single nutrient (Herbert, 1961), which was zinc in this case.

Synthesis of RNA in post-exponential growth of Mycobacterium smegmatis

The rate of synthesis of RNA in zinc-replete cultures of *Mycobacterium smegmatis* slowed to a greater extent than did the growth rate, as shown by the decrease in the values of P-RNA/unit dry wt organism. This result is typical of bacteria when undergoing a 'shift-down' in growth rate (Herbert, 1961). This effect was shown by *M. smegmatis* grown in zinc-replete media (Table 1). However, the content of P-RNA/dry wt organism in zinc-deficient cultures did not appreciably fall during the inhibited phase.

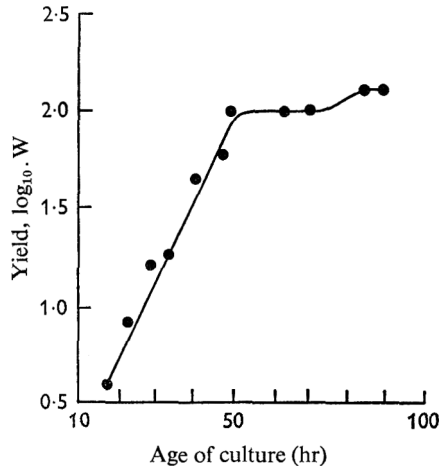

Fig. 1. Typical growth curve of *Mycobacterium smegmatis* grown with insufficient zinc in a minerally depleted glycerol + asparagine medium, at 37°, containing 2·0 μg. Fe^{2+}/ml., but no added zinc. Organisms were washed, freeze-dried, and weighed. The yield is expressed W = mg. dry wt organism from 80 ml. culture.

Synthesis of DNA during post-exponential growth of Mycobacterium smegmatis

DNA synthesis is less sensitive than RNA synthesis to growth-rate changes (Herbert, 1961). The content of P-DNA of *Mycobacterium smegmatis* grown in zinc-replete media appeared to increase during the transition from declining phase to stationary phase (Table 1). In 'shift-down' experiments the content of DNA of bacteria increases (Herbert, 1961). In the stationary phase of *M. smegmatis* grown in zinc-deficient medium the content of DNA was constant (Table 1). Thus whereas a shift-down resulting from carbon and nitrogen deficiency led to the usual DNA response in *M. smegmatis*, a shift-down resulting from zinc deficiency led to a balanced inhibition of DNA synthesis. However, a small growth of the organism persisted after the transition from the exponential phase, i.e. a slight shift-up effect was produced. This should lead to a decrease in DNA content of the organisms, but this effect, although shown in several experiments, was not marked (Table 1).

Synthesis of RNA and DNA during exponential growth of Mycobacterium smegmatis

According to Herbert (1961), an identical growth rate should lead to a similarity of chemical composition. Exponentially growing *Mycobacterium smegmatis* in zinc-replete and zinc-deficient media showed nearly identical contents of RNA and DNA

(Table 2). Furthermore, the content of RNA and DNA increased in both cultures during the exponential phase, and then decreased as the decline phase was approached. Thus, zinc deficiency affected the rate of synthesis of RNA and DNA in the same way as a shift-down induced by carbon+nitrogen deficiency, in the zinc-replete cultures (Table 2). Irrespective of the zinc-content of the media the deceleration in growth rate was anticipated by a decline in the rate of DNA and RNA synthesis, as predicated from Herbert's rules (1961). However, RNA synthesis is usually more sensitive than DNA synthesis to changes in the rate of growth (Kjeldgaard, 1961; Buetow & Levedahl, 1962). This distinction was not found with cultures of *M. smegmatis* (Table 2; Harris, 1967).

Table 1. *Yields of organism and nucleic acid content of Mycobacterium smegmatis*

Mycobacterium smegmatis was grown in 80 ml. glycerol+asparagine medium at 37°. The zinc-replete medium contained 0·4 μg. Zn^{2+}/ml. and 2·0 μg. added Fe^{2+}/ml. The zinc-deficient medium contained 2·0 μg. Fe^{2+}/ml. but no added zinc. The bacteria were harvested at the times indicated, freeze-dried, weighed, and their nucleic acids extracted.

Zinc-replete medium				Zinc-deficient medium		
Yield (mg. dry wt bacteria/ 80 ml. culture)	μg. P/mg. dry wt bacteria		Age of culture (hr)	Yield (mg. dry wt bacteria/ 80 ml. culture)	μg. P/mg. dry wt bacteria	
	P-DNA*	P-RNA*			P-DNA*	P-RNA*
192	2·70	4·95	45	102	2·3	4·5
286	2·55	5·05	50	106	2·3	5·0
285	2·55	5·27	53	118	2·3	4·5
348	2·55	4·80	56	142	2·3	5·1
442	2·70	4·87	70	148	2·1	5·0
498	3·00	4·72	75	216	2·0	4·7
562	2·85	4·40	98	226	1·8	—

* Concentrations of RNA, DNA as atoms of phosphorus (Logan, Mannell & Rossiter, 1952).

Table 2. *Yield of organism and nucleic acid content of Mycobacterium smegmatis during exponential growth*

Mycobacterium smegmatis was grown in the glycerol+asparagine medium at 37°. The zinc-replete medium contained 0·4 μg. added Zn^{2+}/ml., and 2·0 μg. added Fe^{2+}/ml. The zinc-deficient medium contained 2·0 μg. Fe^{2+}/ml., but no added zinc. The bacteria were harvested at the times indicated, freeze-dried, weighed, and their nucleic acids extracted.

Age of culture (hr)	Zinz-replete media			Zinc-deficient media		
	Yield (mg. dry wt bacteria/ 80 ml. culture)	μg. P/mg. dry wt bacteria		Yield (mg. dry wt bacteria/ 80 ml. culture)	μg. P/mg. dry wt bacteria	
		P-DNA	P-RNA		P-DNA	P-RNA
	Cultures in 160 ml. medium					
18	3·5	2·9	6·9	3·9	2·8	7·5
23	7·6	2·9	7·8	7·9	2·8	6·9
28	16·8	3·5	7·8	15·8	3·6	7·2
34	15·3	2·9	7·5	18·2	3·1	7·6
41	35·7	3·2	6·9	37·8	3·2	5·5
48	57·7	2·7	6·1	56·2	3·0	5·7

Synthesis of nicotinamide and storage of phosphate in exponential and post-exponential growth of Mycobacterium smegmatis

During exponential growth of *Mycobacterium smegmatis* the synthesis of nicotinamide (chiefly as nicotinamide adenine dinucleotide, the rest in nicotinamide adenine dinucleotide phosphate and nicotinamide mononucleotide) increased more quickly than growth rate, leading to an increase of content of nicotinamide/dry wt organism (Table 3). This result was found for cultures containing insufficient and sufficient zinc. The nicotinamide content appeared to reach a maximum just after the end of the exponential phase in both types of culture, and decreased in the decline phase (Table 3). The relationship between growth rate and phosphate storage appeared to be obscure (Table 3), there being no obvious trend in the zinc-replete cultures. The effect of zinc-deficiency, however, upon this insufficiently characterized material (Winder & O'Hara, 1962) was to increase the content as growth persisted (Table 3).

Table 3. *Nicotinamide and phosphate content of Mycobacterium smegmatis during exponential and post-exponential growth*

Mycobacterium smegmatis was grown in 80 ml. glycerine+asparagine medium at 37°. The zinc-replete medium contained 2·0 μg. Fe^{2+}/ml. and 0·4 μg. Fe^{2+}/ml. The zinc-deficient medium contained 2·0 μg. Fe^{2+}/ml. but no added zinc. The bacteria were harvested at the times indicated, freeze-dried, weighed, and their nicotinamide and phosphate extracted.

Age of culture (hr)	Content of bacteria: Nicotinamide* (μg./mg. dry wt bacteria) Zn+	Nicotinamide* (μg./mg. dry wt bacteria) Zn−	Phosphate† (μg. P/mg. dry wt bacteria) Zn+	Phosphate† (μg. P/mg. dry wt bacteria) Zn−
18	1·3	—	—	—
23	1·0	—	4·7	4·2
28	1·2	1·3	5·0	6·2
34	1·5	1·5	7·1	7·2
41	1·5	1·7	4·2	7·1
48	1·7	1·6	6·2	6·7
64	1·8	1·7	5·8	9·3
71	1·2	1·4	5·3	11·1
85	1·6	1·3	6·3	10 6
91	1·2	1·2	4·3	10·3

* The nicotinamide moiety was extracted by water and TCA (see Methods), and included contributions from NAD, NMN, NADP, as well as the reduced co-enzymes, and was estimated by a cyanide addition reaction (Ciotti & Kaplan, 1957).

† Phosphate extracted with RNA and DNA but not bound to them, and expressed in atoms of phosphorus (Logan, Mannell & Rossiter, 1952).

Changes of the pH value of cultures during growth of Mycobacterium smegmatis

Whereas the pH value of zinc-replete cultures of *Mycobacterium smegmatis* remained near neutrality throughout the exponential and decline phases, being slightly acid at the transition between the two phases, zinc-deficient cultures became progressively more alkaline throughout the exponential phase, and more so as growth inhibition persisted.

DISCUSSION

Insufficient supplies of zinc, like insufficient supplies of iron (Harris, 1967), led to an inhibition of growth of *Mycobacterium smegmatis*. However, zinc-deficiency did not lead to any specific effect upon RNA and DNA synthesis, whereas iron-deficiency does (Harris, 1967). The present work would appear to indicate that in nucleic acid metabolism, zinc has not a more primary function than has an adequate supply of carbon + nitrogen. Thus, zinc deficiency can be considered similarly to carbon + nitrogen deficiency, and the rules of Herbert (1961) apply to the same extent in zinc-replete and zinc-deficient cultures. The composition of bacterial nucleotide and the levels of pools is known to depend on growth rate (Brown, 1962). Whether the constancy of the levels of the pool in this case resulted from a relative constancy of the constituents to one another, or a fall in some with a rise in others, cannot be ascertained from these results.

Estimation of the total oxidized nicotinamide (NAD and NADP) showed that the value decreased during zinc inhibition (Table 3). A decrease in nicotinamide nucleotides has been shown to occur in surface cultures of zinc-deficient bacteria (Winder & O'Hara, 1962) though no estimate of pre-inhibition values was made. Brown (1965) found that the total concentrations of nicotinamide mononucleotides varied little despite different physiological activity in germinating pea seeds.

An inhibition of growth on *Mycobacterium smegmatis* is caused by lack of zinc in the nutrient medium. Since zinc is a constituent of many enzymes, particularly those involved in carbohydrate metabolism (see Introduction), a failure in the production of high-energy phosphate might well be caused by lack of zinc. If this is the mechanism of the effect of zinc deficiency, it might be expected that a decrease of high-energy phosphate would result. Thus, once ATP had been discharged in metabolism to give AMP, polyphosphate and/or inorganic phosphate, it would not be resynthesized, and a higher level of phosphate should be found in zinc-deficient cells. At present it is not clear to what extent the increase of storage of inorganic phosphate found in zinc-deficient cells can be attributed to a breakdown in the utilization of inorganic phosphate. Some speculation has been made for surface cultures of *M. smegmatis* (Winder & Denneny, 1959).

The chief interest of the effects of zinc deficiency in *Mycobacterium smegmatis* at present lies in its different effect from iron deficiency in the same organism (Harris, 1967). Lack of iron results in atypical responses of nucleic acid metabolism to growth shifts, zinc-deficiency is more like a carbon + nitrogen deficiency. It is concluded that mineral deficiencies cannot *a priori* be supposed to present a different class from other nutrient effects, although lack of minerals can lead to specific effects (Harris, 1967).

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REFERENCES

BROWN, E. G. (1962). The acid-soluble nucleotides of mature pea seeds. *Biochem. J.* **85**, 633.

BROWN, E. G. (1965). Changes in the free nucleotide and nucleoside pattern of pea seeds in relation to germination. *Biochem. J.* **95**, 509.

BUETOW, D. E. & LEVEDAHL, B. H. (1962). Decline in the cellular content of RNA, protein and dry weight during the logarithmic growth of *Euglena gracilis*. *J. gen. Microbiol.* **28**, 579.

CIOTTI, M. C. & KAPLAN, N. O. (1957). Procedures for determination of pyridine nucleotides. *Meth. Enzymol.* **3**, 890.

CREGOLIN, C. & SINGER, T. P. (1963). The lactic dehydrogenase of yeast. III. D(−)Lactic cytochrome *c* reductase, a zinc-flavoprotein from aerobic yeast. *Biochim. biophys. Acta* **67**, 201.

DIXON, M. & WEBB, E. C. (1964). *Enzymes*, pp. 307, 456, 464. London: Longmans.

HARRIS, A. B. (1967). Inhibition of growth and nucleic acid-synthesis in iron-deficient *Mycobacterium smegmatis*. *J. gen. Microbiol.* **47**, 111.

HERBERT, D. (1961). The chemical composition of micro-organisms as a function of their environment. *Symp. Soc. gen. Microbiol.* **11**, 39.

KJELDGAARD, N. O. (1961). The kinetics of ribonucleic acid and protein formation in *Salmonella typhimurium* during the transition between different states of balance growth. *Biochim. biophys. Acta* **49**, 64.

LE PAGE, G. A. (1949). In *Manometric Techniques and Tissue Metabolism*, 2nd ed. Ed. by W. W. Umbreit, R. H. Burris and J. F. Stauffer, p. 185. Minneapolis: Burgess Publishing Co.

LOGAN, J. E., MANNELL, W. A. & ROSSITER, R. J. (1952). A note on the determination of deoxypentose nucleic acid and pentose nucleic acid in tissue from the nervous system by ultraviolet absorption. *Biochem. J.* **51**, 480.

STEIN, E. A. & FISCHER, E. H. (1960). *Bacillus subtilis*, α-Amylase, a zinc protein complex. *Biochim. biophys. Acta* **39**, 287.

WATSON, J. D. & CRICK, F. M. C. (1953). Molecular structure of nucleic acids. *Nature, Lond.* **171**, 737.

WEBLEY, D. M. (1960). The effect of deficiency of iron, zinc and manganese on the growth and morphology of *Nocardia opaca*. *J. gen. Microbiol.* **23**, 87.

WEBLEY, D. M., DUFF, R. B. & ANDERSON, G. (1962). The metabolism of iron-, zinc- and manganese-deficient *Nocardia opaca*. *J. gen. Microbiol.* **29**, 179.

WINDER, F. G. & DENNENY, J. M. (1959). Effect of iron and zinc on nucleic acid and protein synthesis in *Mycobacterium smegmatis*. *Nature, Lond.* **184**, 742.

WINDER, F. G. & O'HARA, C. (1962). Effects of iron deficiency and of zinc deficiency on the composition of *Mycobacterium smegmatis*. *Biochem. J.* **82**, 98.

WINDER, F. G., O'HARA, C. & RATLEDGE, C. (1961). Levels of some enzymes in normal, in iron-deficient, and in zinc-deficient *Mycobacterium smegmatis*. *Biochem. J.* **80**, 21P.

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Role of Auto-inhibitors on Mycelial Growth and Dimorphism of *Glomerella cingulata*

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SUMMARY

Crowding of conidia of *Glomerella cingulata* on potato sucrose agar led to a yeast-like growth of closely opposing and sandwiched streaks whereas the outer edges of the streaks developed fluffy mycelia. In liquid cultures increasing the initial conidial inocula increased the development of conidia and correspondingly decreased the mycelia. Normally, conidia germinate to produce germ tubes which branch and develop into cottony mycelia (M phase). Crowded conditions (3000 or more conidia/mm.^{2} or about 2 million/ml.) gave rise to secondary conidia with or without short hyphae or pseudomycelia: the yeast-like cultures (Y phase). In addition to crowding, thorough aeration, protection from light radiation below 500 mμ and a temperature of incubation *c.* 21° favoured the Y phase development on appropriate media. Bicarbonate or CO_2 did not induce Y phase development. Washings and basic CH_2Cl_2 extracts of conidia (which contained auto-inhibitors) induced preferential development of conidia with concomitant decrease in mycelia. Diffusible substances associated with the conidia, in appropriate concentrations, are considered responsible for inhibition of mycelia and preferential development of Y phase.

INTRODUCTION

Glomerella cingulata produces conidia and abundant mycelia on common laboratory media. The conidia germinate readily but germination is reduced in proportion to the density of conidia; this inhibition is due to diffusible substances present in the conidia (Lingappa & Lingappa, 1966*a*). Substances which decrease, retard or inhibit the growth and development of the organism producing them may be designated as auto-inhibitors, self-inhibitors, auto-antibiotics, or inhibitory autobiotics (Lingappa & Lingappa, 1966*b*, 1967). In *G. cingulata*, inhibitors of germination were present in considerable amount in the conidia but not in the mycelia. Normally, conidia of *G. cingulata* germinate to produce extensively branching mycelia which bear conidia on aerial conidiophores or in large masses in compact acervuli. Conidia were also capable of germinating into secondary conidia with an abridged or a lack of development of mycelial phase. Colonies derived from the proliferation of secondary conidia were nonmycelial and yeast-like: *G. cingulata* was dimorphic, capable of developing mycelial (M) or yeast-like (Y) growth. Since factors inducing this dimorphism have not been reported for any other fungi, we have reported our findings on growth and dimorphism of *G. cingulata* in this paper.

METHODS

Cultures of *Glomerella cingulata* were grown in shaken Neurospora minimal medium (NM) (Difco Laboratories, Detroit, Michigan) or in potato sucrose broth (PS). Potato broth was prepared as follows: 220 g. sound, peeled and washed potatoes were sliced into 2 cm. cubes and autoclaved with 1 l. distilled water for 20 min. at 15 lb/in. and the extract was strained, while steaming hot, through Miracloth (Chicopee Mfg. Co., Milltown, N. J.) without squeezing. The volume was made up to 1 l. after addition of sucrose (5·6%, w/v). Broth made by this procedure contained *c.* 0·6% (w/v) potato solids, was very light coloured, and the preparations were reproducible. Erlenmeyer flasks (500 ml.) containing 150 ml. broth were inoculated with 10 ml. inocula (50 mg. conidia) and shaken in PS at 21° for 7–10 days. The conidia were separated from mycelia by straining the cultures through Miracloth and thoroughly rinsing the mycelia with water. Dry weights of duplicate samples of mycelia and conidia were determined. The cultures were extracted for inhibitors as previously reported (Lingappa & Lingappa, 1966*a*). Acidified conidial suspensions were extracted by shaking with light petroleum (b.p. 30–80°). The defatted slurry was made basic and extracted with $CHCl_3$. Crude bases from $CHCl_3$ extracts contained auto-inhibitors. Additional procedures are described under appropriate experiments.

RESULTS

General observation on dimorphism. Glomerella cingulata could be grown in either cottony mycelial (M) or yeast-like conidial (Y) cultures in both solid and liquid media. Whereas mycelial development was very readily obtained, the Y phase developed only under specified conditions. At 21°–25°, when cultured in PS or NM, this fungus produced both mycelia and conidia in varying proportions. Therefore we investigated the role of several of the nutritional and environmental factors that appeared to influence M or Y phase development. In some specially designed cultures, the influence of auto-inhibition on mycelial development was striking (Pl. 1). On PS agar (2%, w/v) slopes, subcultures became mycelial unless the initial conidial inocula were streaked densely, 3000 or more conidia/mm.^{2} (Pl. 1, fig. 4). On agar slopes subcultures were yeast-like on the shallow top portions and more or less mycelial on the deep ends, especially when slopes were made with larger quantities of media. Agar slopes made of 2 ml. media and densely inoculated with conidia developed yeast-like whereas cultures similarly inoculated on slopes made of 6 ml. media were mycelial (Pl. 1, fig. 4). Condensed water on the bottom of the slopes also increased mycelia. Cultures became mycelial at temperatures higher than 25° and when exposed to incandescent light or daylight.

Mat, tile, V, Z, and other designs of streak cultures were made to observe the effect of the distance between opposing streaks on development of streaked conidia on PS agar plates. Plate 1, fig. 1, 4 show that the edges of streak cultures in close opposition did not develop mycelia and remained yeast-like whereas the exposed outer edges developed cottony mycelia. The upper edge of Pl. 1, fig. 3, 1 developed only scanty mycelia and the hyphae bore terminal and lateral conidia whereas the advancing margin of streak 3 in Pl. 1, fig. 3 remained vegetative (Pl. 2, fig. 14, 15, 16). These plates suggested that some kind of intraspecific or homotypic interactions might be leading to

the development of Y phase and the inhibition of M phase. This differential development appeared to be due to concentration gradients of diffusible metabolites associated with the conidial inocula. Samples taken from cultures inoculated with dense inocula (2 million or more conidia/ml.) showed various examples of differentiation of germ tubes into secondary conidia (Pl. 2). Differentiation of germ tubes into conidia occurred as the tubes were emerging from the germinating conidia or after considerable filamentous growth as pseudomycelia. In liquid cultures the relative development of M and Y phases was quantitatively determined by separating conidia from mycelia. Consequently, differences between M and Y phase could be measured by numbers and dry weights of conidia. These procedures were used in the following experiments.

Table 1. *Influence of concentration of sugar and initial inocula on preferential development of conidia of Glomerella cingulata*

Treatments	Yields (g./l.)	
	Conidia	Mycelium
Potato extract containing 5·6% sucrose (w/v)		
1 ml. conidial inoculum*	2·53	3·16
10 ml. conidial inoculum	4·30	6·24
1 ml. conidia + 9 ml. medium†	2·85	3·10
Potato extract containing 1% sucrose (w/v)		
1 ml. conidial inoculum	2·50	1·55
10 ml. conidial inoculum	4·39	1·13
1 ml. conidia + 9 ml. medium	2·80	1·50

* Potato extracts contained 0·4% potato solids (dry wt). Inoculum contained 4 mg. conidia/ml. along with media residues.

† Supernatant medium of the inoculum, without conidia, obtained by settling conidia for 3 hr at 4°.

Influence of amount of initial inoculum. The conidia settled down in the flasks of shaken cultures of *Glomerella cingulata* when left still on shelves for 3–4 hr. The supernatent media and myceliar fragments were asceptically removed leaving about 10 ml. fluids to resuspend the settled conidia. The entire suspension of conidia of a flask was transferred to a flask containing 150 ml. PS medium and shaken for 8–10 days at 21°. This conidial culture was propagated by inoculating 10 ml. samples into fresh media to be used as conidial inocula. Cultures developed more conidia when started with 10 ml. than with 1 ml. conidial inocula (Table 1). Increasing sucrose in potato broth increased yield of both conidia and mycelia. But the relative abundance of conidia compared to mycelium could be attributed to larger inocula. Flasks inoculated with 1 ml. conidial cultures and supplemented with 9 ml. culture medium without conidia yielded slightly more conidia than those that received only 1 ml. conidial inocula, and therefore preferential development of conidia appeared to be influenced mostly, if not entirely, by the conidial inocula. The supernatant medium also had some influence. Increasing the initial conidial inocula to 2·8 g./l. (w/v) increased the formation of conidia with a concomitant decrease in mycelia (Fig. 1). This differential response to density of inocula was even more striking in potato broths containing less sucrose or none at all (Table 2).

Influence of media. Table 2 indicates that potato extract as such was the most effective for preferential development of conidia. Dehydrated potato extract was less than half as effective as freshly prepared broths. These and other results led to the improvement

of potato extracts by preparing them by autoclaving rather than by boiling over a flame. Neurospora minimal medium was a very good medium for mycelial development but not for formation of conidia.

Influence of aeration and of light. Table 3 showed that, compared to still cultures, aeration by shaking (120 strokes/min.) more than doubled the formation of conidia

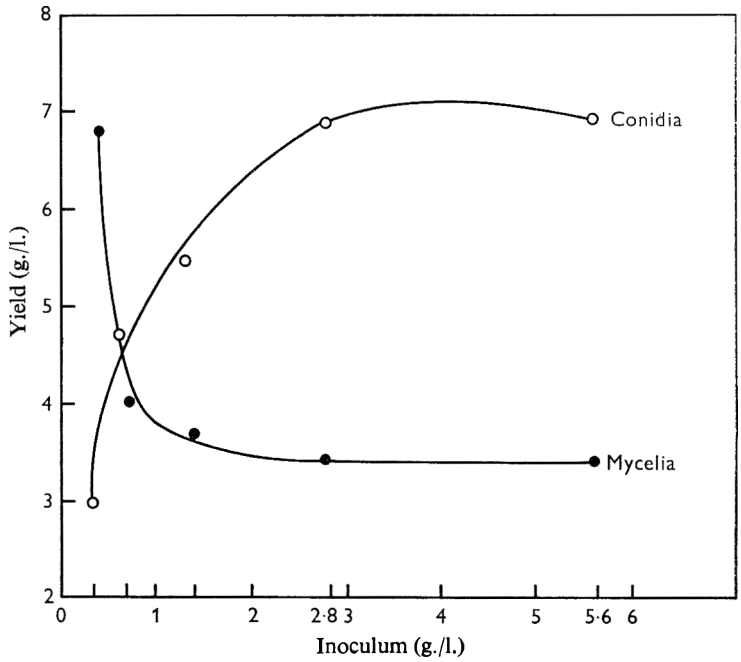

Fig. 1. Effect of increased inoculum of conidia of *Glomerella cingulata* on differential development into conidial or mycelial cultures. 150 ml. flask cultures were shaken in PS media for 8 days at 21°.

Table 2. *Influence of media on relative development of conidia and mycelium of Glomerella cingulata*

Media	Yields (g./l.) Conidia	Mycelium
A. Potato extract without additives*	3·05	0·30
Potato extract + 1 % sucrose	5·22	1·42
Potato extract + 5 % sucrose	7·53	6·70
Difco PE (0·4 % w/v in H_2O) + 5 % sucrose	2·78	2·48
Difco PE (0·8 % w/v in H_2O) + 5 % sucrose	3·30	2·83
B. Neurospora minimal medium (Difco)		
NM + 2 % sucrose	1·24	4·25
NM + 4 % sucrose	2·89	11·54
NM + 8 % sucrose	0·95	18·45
NM + 10 % sucrose	1·17	20·17

* Freshly prepared and containing 0·6 % potato solids. PE signifies dehydrated potato extract from Difco Laboratories, Detroit, Michigan. NM signifies neurospora minimal medium; above 8 % sucrose the medium became viscous and quantitative recovery of conidia became difficult.

with accompanying decrease in mycelia. Visible light promoted mycelial growth and decreased conidia. Exclusion of visible light by growing in a dark chamber, or covering with black plastic film or paper, or in dark red-coloured Ray-Sorb flasks resulted in increased development of conidia. Red-coloured flasks filter out lower wavelength light radiation but transmit red and far red lights. Lower wavelength light thus appeared to be inhibitory to development of conidia.

Table 3. *Influence of aeration and light on relative development of conidia and mycelia of Glomerella cingulata*

	Yields (g./l.)	
	Conidia	Mycelium
Shaken in clear Kimax flasks in light*	3·47	3·76
Shaken in clear Kimax flasks in dark	4·35	1·44
Still culture in Kimax flasks in dark†	1·80	7·00
Still culture in Kimax flasks in light	1·50	6·40
Still culture in Roux bottles in dark	3·28	6·70
Still culture in Roux bottles in light	2·80	6·80
Shaken in Ray-Sorb flasks‡	4·18	1·08

* Incandescent light providing *c.* 65 ft-c.

† Still cultures prepared and inoculated like other cultures but left on shelves unshaken. Still cultures in flasks differed from those in Roux bottles in having more than twice the depth of the media and only one-third the surface exposed to air, i.e. Roux bottles contained the same amount of media but in shallow layers.

‡ Kimax and Ray-Sorb are products of Owens-Illinois, Toledo, Ohio. Ray-Sorb flasks are red-coloured and transmit no light at 300 mμ, 1% at 400 mμ and 4% at 500 mμ. They were in a lighted cabinet. Yields of still Ray-Sorb flasks were similar to those of (clear) Kimax flasks.

Table 4. *Effects of addition of conidia, washings and extracts of conidia on development of conidia and mycelia of Glomerella cingulata*

	Yield (g./l.)		No. of conidia	
Materials added	Conidia	Mycelium	$\times 10^{-6}$/ml.	$\times 10^{-6}$/mg.
Medium without additives*	4·68	3·21	—	—
Medium + 300 mg. washings†	5·87	1·20	—	—
Medium + 100 mg. conidia	4·84	4·83	78	16·2
Medium + 200 mg. conidia	5·49	4·46	85	15·6
Medium + 100 mg. conidia + 20 mg. E‡	5·32	3·61	124	22·0
Medium + 100 mg. conidia + 40 mg. E	5·90	3·50	168	25·6

* Potato sucrose broth (150 ml./flask) inoculated with 40 mg. conidial inoculum and shaken at 21°.

† 20 g. freshly harvested conidia were rinsed in water and then shaken in 100 ml. water for 30 min., centrifuged, filtered, partially evaporated and added to inoculated cultures.

‡ E is the extract of 20 g. conidia obtained by CH_2Cl_2 extract of defatted conidia at pH9. This extract contained self-inhibitors of germination. Larger number of conidia was associated with smaller size, 5–8 × 6–16 μ; when produced in smaller numbers they were 10–26 μ long.

Influence of temperature. A series of flasks was inoculated with conidial inocula varying from 0·5 to 5·0 g./l. and shaken at 21° and a similar set was shaken at 30°. Those at 21° yielded increasing amounts of conidia, with corresponding decrease in mycelia, with increasing amounts of inoculum (Fig. 1): 2·8 g./l. inoculum yielded 7 g. conidia and 3·5 g. mycelia/l. at 21° but at 30° only 0·6–0·8 g. of conidia and 11–13 g.

mycelia/l. appeared at all inoculum sizes. At 32° conidia were not produced. Clearly the preferential development of conidia was modified by temperature. Measurable amounts of conidia were not produced below 18°. Therefore, all subsequent experiments were carried out in temperature controlled chambers at 21° without light which were most favourable for the development of conidial cultures.

Influence of addition of extracts of conidia. The effects of addition of conidia, washings of conidia, and crude basic CH_2Cl_2 extracts of conidia of *Glomerella cingulata* on development of M and Y phases are summarized in Table 4. Increased yields of conidia were obtained when cultures were supplemented with washings of conidia or with extracts of conidia. These additives also influenced profoundly the number and size of the conidia. These observations indicated that the formative effect of conidial density was due to substances extractable from conidia.

Table 5. *Effect of addition of potato constituents on development of conidia and mycelia of Glomerella cingulata*

Materials added	Yields (g./l.) Conidia	Mycelium
A. Effect of solanine*		
In potato sucrose broth:		
2·5 g. inoculum/l.	5·86	3·95
0·25 g. inoculum/l.	4·19	7·02
0·25 g. inoculum/l. + 10^{-3}M-solanine	3·50	8·04
In Neurospora minimal medium:		
2·5 g. inoculum/l.	1·43	3·44
0·25 g. inoculum/l.	1·02	5·10
2·5 g. inoculum/l. + 10^{-2}M-solanine	0·64	3·54
2·5 g. inoculum/l. + 10^{-3}M-solanine	1·26	5·14
0·25 g. inoculum/l. + 10^{-2}M-solanine	0·29	4·53
0·25 g. inoculum/l. + 10^{-3}M-solanine	0·98	5·81
B. Effect of ethanol extracts of potato		
Potato broth without sucrose	1·70	0·56
Potato broth with sucrose 5% (w/v)	3·35	6·86
Neurospora minimal medium	0·10	14·67
NM + 135 mg./l. ethanol extract of potato	2·30	6·70
NM + 270 mg./l. ethanol extract of potato	3·20	11·62
NM + 540 mg./l. ethanol extract of potato	0·90	13·03

* Solanine was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin.

† In experiments listed under part B of this table, flasks were inoculated with 1 ml. inoculum. Growth in potato broth media is given here to compare with that obtained in NM and in NM supplemented with the residues obtained by ethanolic extract of potato.

‡ Residue obtained by evaporation of alcohol extract of 100 g. dehydrated Difco potato extract, added into Difco Neurospora minimal medium (NM). The media contained 5% sucrose (w/v).

Role of constituents of potato extract. Potato extract was superior for conidial development of *Glomerella cingulata*, though Neurospora minimal medium gave the best vegetative growth. The materials in solvent extracts of conidia which stimulated the development of conidia may be alkaloids or related substances (Lingappa & Lingappa, 1967). Potatoes contain several alkaloids, which are readily extracted by ethanol, solanine being the best known (Cromwell, 1955). Both pure solanine and the residue obtained by drying ethanol extract of potato were separately incorporated into cultures and examined for their influence on the development of conidia in NM medium (Table 5).

Solanine was ineffective at lower concentrations and inhibitory at higher concentrations. Ethanol extractable materials from potato stimulated the development of conidia and at higher concentration inhibited the production of conidia but not mycelia. To what extent solanaceous alkaloids in the extracts were responsible for Y phase development of *G. cingulata* is not clear.

Effect of carbon dioxide and bicarbonate. In any population density-dependent interactions CO_2 may be involved. Flasks were fitted with rubber stoppers with inlet and outlet plugged glass tubes ending with soft rubber tubing. After inoculation of the freshly prepared media with conidial cultures, CO_2 was bubbled through the media and, after saturation with CO_2, the end tubes were folded, clamped and secured with tape. Below the clamps 5 mm. vertical incisions served as safety valves for any build up of gases inside the flasks. Separate experiments with 1, 10 and 50% (v/v) CO_2 showed that CO_2 concentration did not induce differential development of conidia. Well-aerated control cultures developed Y phase. To assess the role of bicarbonate, predetermined amounts of bicarbonate solution were added after inoculation using sterilizing filters in syringe holders. $NaHCO_3$ between 0·5 and 40 mM in both media did not favour the development of conidial cultures, but inhibited differentiation of conidia to a greater extent than it did the growth of mycelia.

DISCUSSION

Fungi pathogenic to man and animals which have yeast (Y) and mycelial (M) forms have been called dimorphic, having two forms (Ainsworth & Bisby, 1950). However several plant pathogenic and saprophytic fungi exhibit Y and M forms (Roberts, 1946; Lodder & Kreger-Van Rij, 1952; Cooke, 1962; Brandt, 1963; Haskins & Spencer, 1967). Therefore, a dimorphic fungus is better defined as one in which a reversible transformation from a mycelial to a nonmycelial unicellular type of growth occurs (Cochrane, 1958). Our findings present a unique case of morphogenetic response dependent on population density of initial conidial inocula. In diverse groups of organisms, various morphogenetic responses to dense populations have been attributed to CO_2. In Blastocladiales it is CO_2 and/or bicarbonate, or visible light which determine the development of the sporangial types (Cantino, 1966). CO_2 is also known to have a morphogenetic influence in *Sporotrichum schenkii* (Drouhet & Mariat, 1952), *Trichophyton mentagrophytes* (Chin & Knight, 1963), and *Mucor rouxii* (Bartnicki-Garcia, 1963). In *Blastocladiella emersonii* exogenous bicarbonate induces alternative metabolic pathways before the differentiation of sporangial types (Cantino, 1966). Incorporation of exogenous CO_2 into key metabolic intermediates and their influence on the structure of cell walls might be controlling Y and M phase development in *Mucor rouxii* (Nickerson & Bartnicki-Garcia, 1964). Exogenous incorporation of CO_2 into several metabolic intermediates is known to occur in many different groups of organisms. Our results with different concentrations of CO_2 and bicarbonate, however, did not implicate these in Y phase development of *Glomerella cingulata.*

Pine & Peacock (1958) concluded that the conversion of M phase of *Histoplasma capsulatum* into Y phase required (1) a satisfactory growth requirement of the Y phase and (2) an inhibition of growth of M phase without inhibition of conversion to Y phase. Citric acid stimulated the Y phase of *H. capsulatum* and Ca and Mg annulled this effect. Differential toxicity of citric acid to Y and M phases was also influenced by temperature

and by Ca, Mg and Zn. Differential requirements for trace metals or ability to compete for trace metals with citrate, modified by temperature, may influence development of Y or M phase of *H. capsulatum*. Langeron & Guerra (1940) described Y and M phase development of *Candida albicans* on streaked cultures similar to those illustrated in *Glomerella cingulata* in this paper. They suggested a possible role of self-inhibitors and/or depletion of essential nutrients in the development of Y phase. Fungi produce regulatory compounds that affect their growth and differentiation (Robbins, 1940; Stadler, 1952; Banbury, 1955; Bistis, 1956). Conidial development of *Colletotrichum lindemuthianum* is enhanced by extracts of mycelia and conidia (Mathur, Barnett & Lilly, 1950). In *G. cingulata*, filtrates of perithecial cultures inhibited conidial development and filtrates of conidial cultures inhibited perithecial development (Wheeler, 1956). A hormonal substance produced in the culture media was responsible for induced selfing in *G. cingulata* (Driver & Wheeler, 1955). Endogenous inhibitors were not responsible for decreased production of tyrosinase by the mycelial cultures of *G. cingulata* grown on increasing amounts of media (Sussman, Coughey & Strain, 1955). A morphogenetic staling substance in the cultures of *Fusarium oxysporum* which in higher concentrations inhibited spore germination and induced autolysis has been described (Robinson & Park, 1965). In *G. cingulata* we have reported the existence of self-inhibitors of germination of conidia in the washings and extracts of conidia (Lingappa & Lingappa, 1966*a*, *b*). When similar washings and extracts of conidia were added, cultures developed into predominantly Y phase; the active materials were non-gaseous since the extracts were dried in a rotary evaporator at 15 mm. pressure. This and other results reported here indicated that some diffusible products of metabolism associated with the conidia of *G. cingulata* induced its Y phase development. Possible non-gaseous metabolic products that may be expected in the washings and extracts of conidia are the various carboxylic, amino or fatty acids and compounds rich in SH groups (Nickerson & Bartnicki-Garcia, 1964). The conidia were extracted to remove lipids and the extracts contained basic materials (Lingappa & Lingappa, 1967) which were ninhydrin negative. Sulphur was not detected in sodium fusion products of the extracts prepared according to Shriner, Fuson & Curtin (1956). Therefore, carboxylic, amino or fatty acids or SH compounds are not primarily responsible for the Y phase development. These extracts contained germination inhibitors but whether or not the same germination inhibitors were inducing dimorphism is not known. The crude ethanolic extracts of potato induced some preferential development of Y phase of *G. cingulata* which might be due to Solanium alkaloids, but the effect due to washings and extracts of conidia could not be due to this source because the conidia were washed free of all media. Development of Y phase from the conidia of *G. cingulata* took place by differentiation of either the tips of germ tubes, or of lateral branches of pseudomycelia, or by direct budding, or by development of constriction of hyphae into monilial chains. Direct conversion of macroconidia of *H. capsulatum* into yeast cells by polar and non-polar budding, by formation of yeast cells from the tip of hyphae and by forming chains of monilial cells from hyphae, have been described by Pine & Webster (1962). Turian & Matikian (1966) also described budding of conidia and constriction and severence of preformed aerial hyphae in Neurospora. Functionally Y and M phases differ conspicuously in several respects including pathogenicity (Mackinnon, 1940). There is considerable evidence that the Y and M phases differ in their susceptibility to exogenous inhibitors (Pine & Peacock, 1958; Brandt, 1963;

Jillson & Nickerson, 1948). Smut, rust and Taphrina fungi are mycelial in their host tissue and yeast-like in their development outside their host plants. Sporidia of *Cronartium ribicola* produced secondary sporidia up to six successive generations on water agar but on susceptible host needles the sporidia produced germ tubes to infect and develop the parasitic mycelial phase (Bega, 1960). The tendency to develop the Y phase in many filamentous fungi seems advantageous in tiding over a brief period of unfavourable conditions during vegetative development and in greater dissemination (Clinton & McCormick, 1919). Specific exogenous or endogenous factors may play a role in these developmental responses which have great survival value to the organism. Specific metabolites accumulating in the culture media are known to induce sporulation in several fungi (Trione, Leach & Mutch, 1966). Recently, we have isolated two auto-inhibitors from the cultures of *Candida albicans*, phenethylalcohol and tryptophol, which may be playing a role in dimorphic development of *C. albicans* (Lingappa *et al.* 1968). The conidial extracts of *G. cingulata* contain auto-inhibitors of germination; whether the same inhibitors inhibit M phase and induce Y phase are not clear.

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REFERENCES

AINSWORTH, G. C. & BISBY, G. R. (1950). *A Dictionary of the Fungi.* Kew, Surrey: The Commonwealth Mycological Institute.

BANBURY, G. H. (1955). Physiological studies in the Mucorales III. The zygotropism of zygophores of *Mucor mucedo* Brefeld. *J. exp. Bot.* **6**, 235.

BARTNICKI-GARCIA, S. (1963). Symposium on biochemical bases of morphogenesis in fungi. III. Mold-Yeast dimorphism of Mucor. *Bact. Rev.* **27**, 293.

BEGA, R. V. (1960). The effect of environment on germination of sporidia in *Cronartium ribicola*. *Phytopathology* **50**, 61.

BISTIS, G. (1956). Sexuality in *Ascobolus stercorarius*. I. Morphology of the ascogonium; plasmogamy; evidence for a sexual hormonal mechanism. *Am. J. Bot.* **43**, 389.

BRANDT, W. H. (1963). Dimorphism and interactions between the Oakwilt fungus and associated fungi. *Plant dis. Rep.* **47**, 579.

CANTINO, E. C. (1966). Morphogenesis in aquatic fungi. In *The Fungi*, vol. 2. New York, London: Academic Press.

CHIN, B. & KNIGHT, S. G. (1963). Stimulation of glucose metabolism in *Trichophyton mentagrophytes* during incubations in increased carbon dioxide tensions. *J. gen. Microbiol.* **30**, 121.

CLINTON, G. P. & MCCORMICK, F. A. (1919). Infection experiments of *Pinus strobus* with *Cronartium ribicola*. *Bull. Conn. agric. Exp. Stn.* **214**, 428.

COCHRANE, V. W. (1958). *Physiology of Fungi.* New York: John Wiley.

COOKE, W. B. (1962). A taxonomic study in the 'Black Yeasts'. *Mycopathol. Mycol. Appl.* **17**, 1.

CROMWELL, B. T. (1955). The Alkaloids. In *Modern Methods of Plant Analysis*, vol. 4. Berlin: Springer-Verlag.

DRIVER, C. H. & WHEELER, H. E. (1955). A sexual hormone in Glomerella. *Mycologia* **47**, 311.

DROUHET, E. & MARIAT, F. (1952). Étude des facteurs déterminant le développement de la phase levure de *Sporotrichum schenkii*. *Annls. Inst. Pasteur, Paris* **83**, 506.

HASKINS, R. H. & SPENCER, J. F. T. (1967). *Trichosporonoides oedocephalis* gen., n. sp. *Can. J. Bot.* **45**, 515.

JILLSON, O. F. & NICKERSON, W. J. (1948). Mutual antagonism between pathogenic fungi. Inhibition of dimorphism in *Candida albicans*. *Mycologia* **40**, 369.

LANGERON, M. & GUERRA, P. (1940). Orientation de la filamentisation des champignons levuriformes cultivés sur lames gélossées. *Annls Parasit. h. comp.* **17**, 580.

LINGAPPA, B. T. & LINGAPPA, Y. (1966*a*). The nature of self-inhibition of germination of conidia of *Glomerella cingulata*. *J. gen. Microbiol.* **43**, 91.

LINGAPPA, B. T. & LINGAPPA, Y. (1966*b*). Significance of inhibitory autobiotics of micro-organisms. *Abstr. 9th int. Congr. Microbiol., Moscow*, p. 223.

LINGAPPA, B. T. & LINGAPPA, Y. (1967). Alkaloids as self-inhibitors of fungi. *Nature, Lond.* **214**, 516.

LINGAPPA, B. T., PRASAD, M., LINGAPPA, Y., HUNT, D. F. & BIEMANN, K. (1969). Phenethyl alcohol and tryptophol: Autoantibiotics produced by the fungus *Candida albicans*. *Science, N.Y.* **163**, 192.

LODDER, J. & KREGER-VAN RIJ, N. J. W. (1952). *The Yeasts. A Taxonomic Study*. Amsterdam: New Holland Publishing Co.

MACKINNON, J. E. (1940). Dissociation in *Candida albicans*. *J. infect. Dis.* **66**, 59.

MATHUR, R. S., BARNETT, H. L. & LILLY, V. G. (1950). Sporulation of *Colletotrichum lindemuthianum*. *Phytopathology* **40**, 104.

NICKERSON, W. J. & BARTNICKI-GARCIA, S. (1964). Biochemical aspects of morphogenesis in algae and fungi. *A. Rev. Plant. Physiol.* **15**, 327.

PINE, L. & PEACOCK, C. L. (1958). Studies on the growth of *Histoplasma capsulatum*. IV. Factors influencing conversion of the mycelial phase to the yeast phase. *J. Bact.* **75**, 167.

PINE, L. & WEBSTER, R. E. (1962). Conversion in strains of *Histoplasma capsulatum*. *J. Bact.* **83**, 149.

ROBERTS, C. (1946). A comparative study of *Torulopsis pulcherrima* and *Traphrina deformans* in culture. *Farlowia* **2**, 345.

ROBBINS, W. J. (1940). Effect of extracts of Phycomyces upon its development. *Am. J. Bot.* **27**, 559.

ROBINSON, P. M. & PARK, D. (1965). The production and quantitive estimation of a fungal morphogen. *Trans. Br. mycol Soc.* **48**, 561.

SHRINER, R. L., FUSON, R. C. & CURTIN, D. Y. (1956). *The Systematic Identification of Organic Compounds. A Laboratory Manual*, 4th ed. New York: John Wiley.

STADLER, D. R. (1952). Chemotropism in *Rhizopus nigricans*: the staling reaction. *J. cell. comp. Physiol.* **39**, 449.

SUSSMAN, A. S., COUGHEY, P. & STRAIN, J. C. (1955). Effect of environmental conditions upon tyrosinase activity in *Glomerella cingulata*. *Am. J. Bot.* **42**, 810.

TRIONE, E. J., LEACH, C. M. & MUTCH, J. T. (1966). Sporogenic substances isolated from fungi. *Nature, Lond.* **212**, 163.

TURIAN, G. & MATIKIAN, N. (1966). Conidiation of *Neurospora crassa*. *Nature, Lond.* **212**, 1067.

WHEELER, H. E. (1956). Sexual versus asexual reproduction in *Glomerella*. *Mycologia* **48**, 349.

EXPLANATION OF PLATES

PLATE 1

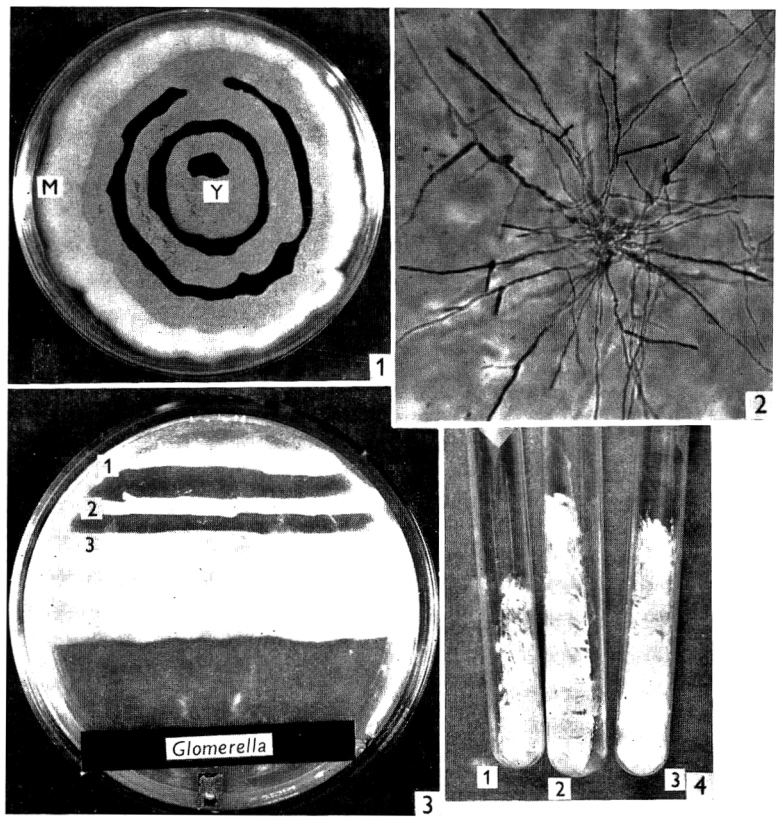
Auto-inhibition of mycelia and development of Y phase of *Glomerella cingulata*. Cultures were grown on potato sucrose agar at 21°.

Fig. 1. Circularly streaked conidia developed no mycelia along the opposing edges of the streaks whereas the edge of the outermost streak developed advancing cottony mycelia (M). The rest of the streaks proliferated into masses of secondary conidia (Y) on a bed of pseudomycelia (acervuli?). 9 days. Half actual size.

Fig. 2. Mycelial colony developing from conidia, showing branching hyphae. 24 hr × 50.

Fig. 3. Development of Y phase in sandwiched streak culture. 1. Slight mycelia developed from the outer edge but none along the inner edge opposing the second streak. 2. Sandwiched streak opposed by streaks 1 and 3 developed into Y phase. 3. Streak culture continued to develop Y phase throughout except along exposed edge which developed extensive cottony mycelia. 14 days. Three-fifths actual size.

Fig. 4. Conidia were densely streaked on PS agar slopes made with 2 ml. medium (1), 6 ml. medium (2), and 6 ml. medium inoculated sparsely with conidia (3). Culture on slope 1 was yeast-like, on slope 2 partly mycelial, but on slope 3 abundantly mycelial especially on the deep bottom portion of the slope. Half actual size.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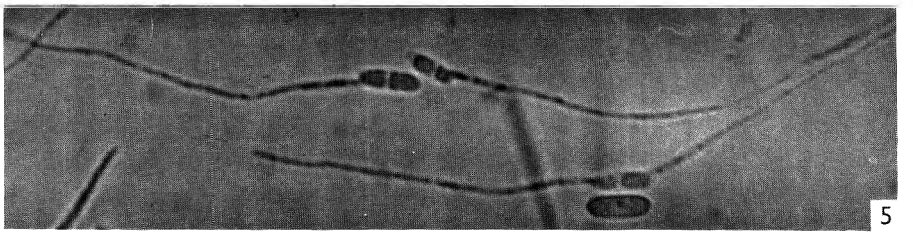
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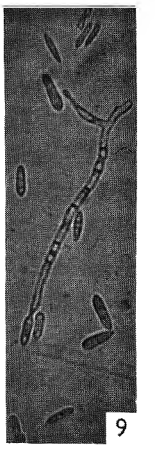
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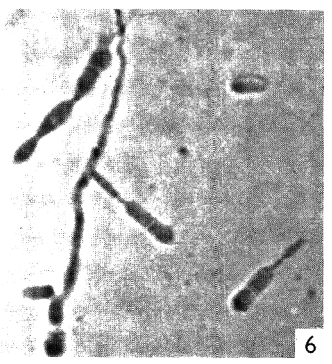
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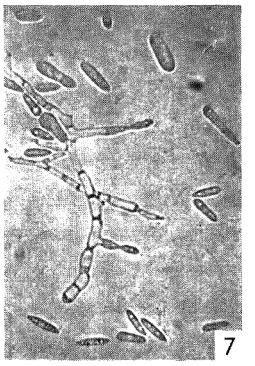
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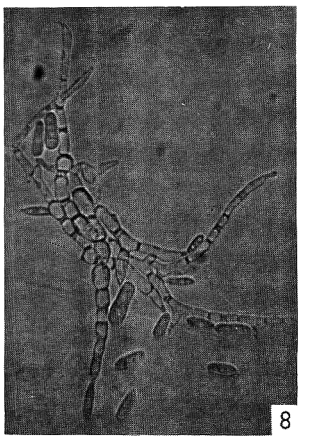
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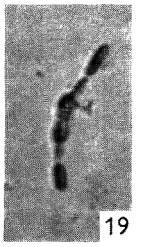
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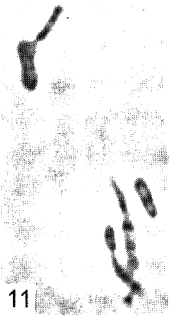
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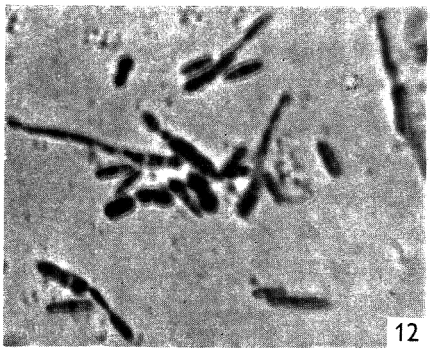
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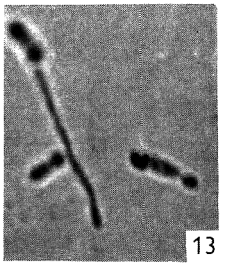
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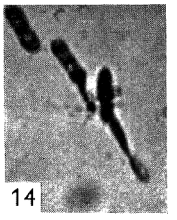
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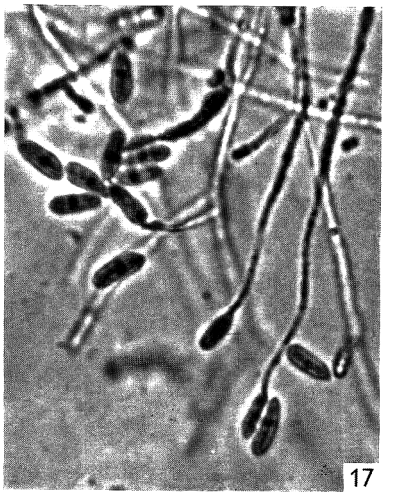
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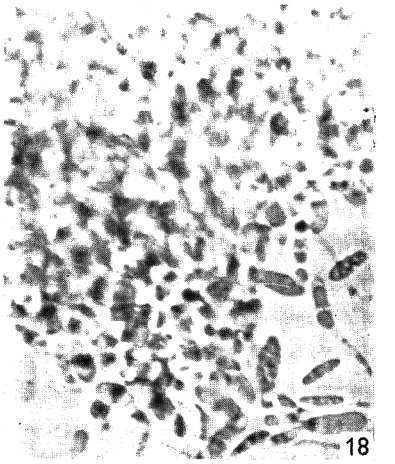
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PLATE 2

Developmental stages in Y and M phases of *Glomerella cingulata*.

Fig. 5. Development of germtubes in cultures that gave rise to cottony mycelia. × 350.

Fig. 6. Budding conidia. × 350.

Fig. 7–9. Pseudomycelia and secondary conidia. × 350.

Fig. 10–15. Formation of secondary conidia. × 350.

Fig. 16. Margin of mycelial growth of Pl. 1, fig. 3, showing vegetative growth. × 350.

Fig. 17. Margin of inhibited mycelial growth of Pl. 1, fig. 3, showing hyphal tips differentiating conidia 48 hr after germination. × 350.

Fig. 18. Masses of conidia in the Y phase cultures. Pl. 1, fig. 1, Y. × 350.

Fig. 19. Bipolar budding of conidium. × 350.

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A Comparative Study of the Mitochondrial Structure of Petite Strains of *Saccharomyces cerevisiae*

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SUMMARY

A respiratory-competent strain of *Saccharomyces cerevisiae* (1 D) had mitochondria of diverse morphology. A spontaneous petite strain and petites induced by acriflavine, ultraviolet-radiation or phenethyl alcohol showed similar mitochondrial diversity to the parent strain, but in all the petites except the spontaneous one there were fewer mitochondrial profiles showing cristae. In another respiratory-competent strain (11) the mitochondria were of uniform morphology. A phenethyl alcohol-induced petite derived from this strain again showed fewer cristate mitochondrial profiles. There was no evidence of the grossly aberrant and lamellate mitochondrial profiles observed in strain 1 D and its petites. Each of the cultures obtained from an ascospore tetrad from a 1 D × 11 cross was induced to form petites with phenethyl alcohol; all showed a significant reduction in the proportion of mitochondrial profiles with cristae. Two of them showed the mitochondrial diversity characteristic of parent strain 1 D. Other features of mitochondrial morphology appear to be under nuclear control and are strain-dependent. All the petites were cytoplasmic mutants and lacked cytochromes *a* and *b*.

INTRODUCTION

In certain facultatively anaerobic yeasts, including *Saccharomyces cerevisiae*, a group of mutants may be produced, known as 'petites' (Ephrussi, Hottinguer & Chimenes, 1949). These mutants have defective mitochondria and are therefore unable to grow on non-fermentable substrates. A characteristic of petite yeasts is the absence of cytochrome *a* and usually also of cytochrome *b*. The petite condition is normally inherited cytoplasmically ($\rho-$ petites), but also arises by mutation of nuclear genes ($p-$ petites). Glucose represses the formation of mitochondrial cristae in respiratory-competent yeast (see Marchant & Smith, 1968), and also of cytochromes *a* and *b* (see Roodyn & Wilkie, 1968).

Isolated mitochondria have DNA, ribosomal and transfer RNA, and are able to incorporate amino acids into an insoluble protein fraction. However, the exact relationship between mitochondrial DNA and mitochondrial structure remains unclear. It has not yet been established that mitochondrial DNA is directly involved in specifying mitochondrial protein. The morphology of mitochondria, even in $\rho-$ cells, could still be largely dictated by nuclear genes. The petite condition can be induced by a wide range of mutagens, and the question arises whether these mutagens result in the same $\rho-$ phenotype, even in yeasts of different genotype.

An initial investigation, by Yotsuyanagi (1962), of acriflavine-induced $\rho-$ petites,

revealed structural alterations in the mitochondria, whereas *p* petites had mitochondria similar to those found in respiratory-competent yeasts. Both categories of petite lacked cytochrome *a*. The yeasts were grown in 4% glucose medium; during the early phase of growth mitochondria were few in number and had poorly developed cristae, presumably because of glucose repression. Yeasts from late stationary phase had larger mitochondria, often containing lamellations of the inner membrane. A major difference between $\rho+$ and $\rho-$ cultures was that the former alone possessed well-defined cristate mitochondria throughout late log. phase.

Avers, Pfeffer & Rancourt (1965) have also examined acriflavine-induced petites of *Saccharomyces cerevisiae* from structural and histochemical viewpoints. Instead of cytochrome *a* spectra, cytochrome oxidase activity was measured. Using a medium with 1% glucose, they found that mitochondria in petites completely lacking in cytochrome oxidase were similar to those described by Yotsuyanagi (1962) from logarithmic phase $\rho-$ cultures: i.e. the mitochondria were non-cristate. On the other hand, yeasts in which cytochrome oxidase activity was low, appeared to have mitochondria indistinguishable from the wild type (Avers, Pfeffer & Rancourt, 1965; Avers, Rancourt & Lin, 1965).

Petites of *Saccharomyces cerevisiae* produced by *p*-nitrophenol treatment, grown under unspecified conditions on glucose, showed few and lamellate mitochondria (Osumi & Katoh, 1967).

Bowers, McClary & Ogur (1967) have demonstrated the importance of glucose repression in these studies on mitochondrial structure. A petite strain, of unspecified origin, grown on 2% glucose had non-cristate mitochondrial profiles. The same mutant grown in a chemostat with a limiting low concentration of glucose showed mitochondria indistinguishable from the wild type.

In the present study glucose repression was avoided by using melibiose, a fermentable but non-repressing substrate (Reilly & Sherman, 1965). We have compared petites induced by various mutagens on a single strain, and by one mutagen (phenethyl alcohol) on a set comprising two parental strains and their resultant tetrad progeny. We have looked for the mitochondrial peculiarities reported in petite yeasts by other workers, i.e. lamellate cristae or lack of cristae, and by counting profiles, have examined whether these features are consistently more frequent in a range of petites. These features were also examined for evidence of nuclear, as opposed to cytoplasmic, inheritance.

METHODS

Yeast strains. Haploid strains 11 and 1D of *Saccharomyces cerevisiae* were crossed on minimal medium. The resultant diploid cells were sporulated, and ascospores dissected with a de Fonbrune micromanipulator. One tetrad, 2A–D, was selected for the present investigation. Mating type and growth requirements were as follows:

Parents:	1D—a/*ad*, *leu*, *lys*
	11 —α/ad_2
Progeny:	2A—a/*leu*, *lys*
	2B—α/*ad*, *leu*, *lys*
	2C—a/ad_2
	2D—α/*ad*

Cultural conditions. The following media were used. YEPS: 1 % Difco yeast extract, 2 % Difco Bacto-peptone, 2 % glucose. YEPM, YEPG: as for YEPS but with 2 % melibiose or 4 % glycerol as carbon source. PDM: petite differential medium, YEPG with addition of 0·2 % glucose. MMS: minimal medium of Wickerham (1946), containing salts, vitamins and glucose only. SPM: sporulating medium of 0·3 % raffinose, 0·2 % sodium acetate. Media were solidified with 2 % agar; incubation temperature was 30°.

Liquid cultures were shaken at 30° in 250 ml. flasks containing 100 ml. melibiose medium. The yeasts were harvested in late log. phase: 24 hr for $\rho+$ cells, 48 hr for $\rho-$.

Petites. Petite colonies were recognized by their small size on PDM. To distinguish true petites from the small, slow-growing, respiratory-competent colonies which arose after treatment with ultraviolet radiation or phenethyl alcohol (PEA), suspected petite colonies from the PDM plates were suspended in water and dropped on to YEPG plates. Only those clones which showed no signs of growth after a week on the glycerol plates were selected for further examination. Suspected $\rho-$ strains were confirmed by crossing with a $\rho+$ strain, where the resulting zygotes were respiratory-competent and sporulated to give all $\rho+$ progeny, and by crossing with another $\rho-$ strain where the zygotes were not respiratory-competent and did not sporulate.

Spontaneous petites, comprising about 2 % of the population, were isolated from melibiose plates.

Ultraviolet-induced petites were obtained by exposing plated yeasts, at a density of 200/plate, for 1 min. to a Phillips 6 W TUV lamp, at 10 cm. The kill was about 80 %, with 20 % petites among the survivors.

Acriflavine petites were induced by adding acriflavine 5 μg./ml. to YEPS medium, giving 100 % induction.

PEA petites were obtained from colonies dropped out on to YEPS plates containing 0·1 % phenethyl alcohol (Eastman Chemical Co.). The population was found to vary from 90 % $\rho-$ to 90 % $\rho+$, depending on the strain.

Cytochrome determination. Yeasts grown in melibiose medium were harvested and washed twice in 2 % sodium dithionite solution, to reduce the cytochromes. The yeasts were resuspended in an equal volume of the dithionite solution and placed in the 1 cm. cuvette of a Unicam SP 800 split-beam recording spectrophotometer. Spectra were examined at room temperature, against a blank consisting of several sheets of filter paper; this blank had no absorption peaks in the 300 to 700 mμ range.

Electron microscopy. Yeasts from the end of the logarithmic growth phase were harvested, washed twice in distilled water and then fixed in 1·5 % aqueous $KMnO_4$ for 4 hr at room temperature. Permanganate was the preferred fixative in this study as it gives reasonable fixation of membranous structures in intact *Saccharomyces cerevisiae*. After fixation and washing, the yeasts were centrifuged and left in 1 % aqueous uranyl acetate for 18 hr. The pellets were washed in water, dehydrated in tertiary butanol mixtures (Johansen, 1940) and embedded in Araldite. Sections were cut with a diamond knife on a LKB Ultratome and stained with saturated uranyl acetate in 50 % ethanol (Gibbons & Grimstone, 1960) and then with lead citrate (Reynolds, 1963). The specimens were examined and photographed in a Siemens Elmiskop 1 or a Zeiss EM 9 at 60 kV.

RESULTS

Cytochrome analysis. Respiratory-competent cultures of *Saccharomyces cerevisiae* strains 11 and 1D showed absorption peaks due to cytochrome *a* at 600 mμ, *b* at 560 and 530 mμ and *c* at 550 and 520 mμ. All the ρ− strains showed only cytochrome *c*. Spectra from the two ρ+ parents and six PEA-induced ρ− clones are reproduced in Fig. 1. The 1D ρ− and 2D ρ− spectra have very small maxima at 600 mμ, equivalent to a cytochrome *a* content less than 5% that of the ρ+ strains, and none of the ρ−

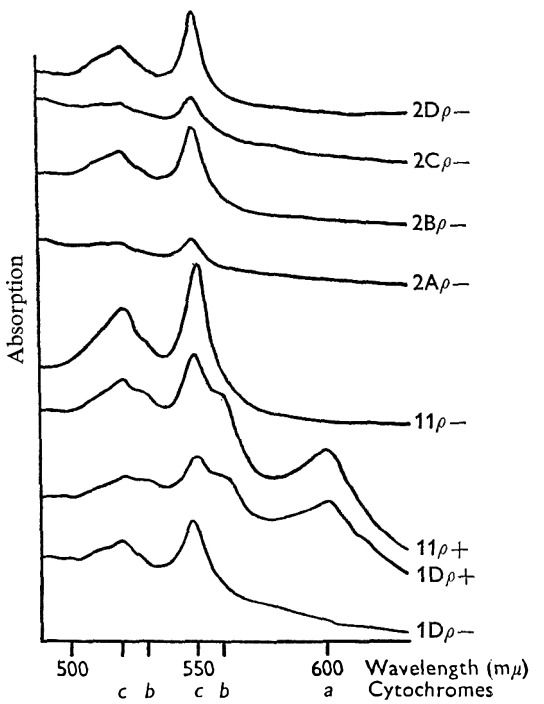

Fig. 1. Cytochrome absorption spectra of respiratory-competent (ρ+) and phenethyl-alcohol-induced petite (ρ−) strains of *Saccharomyces cerevisiae*.

spectra have the *b* shoulder at 560 mμ. The cytochrome *c* peaks in the ρ− tetrad appear to have segregated 2:2 between 2A and 2C (low) and 2B and 2D (high). In the ρ+ parent strains, cytochrome *c* peaks are higher in 11 than in 1D.

Electron microscopy. We were concerned, in this investigation, with the question whether the petite mutation results in mitochondria which are either visibly aberrant or non-cristate. Mitochondrial profiles were therefore divided into three classes: cristate, non-cristate and aberrant (lamellate or distorted). As the sections were of standard thickness it was possible to compile significant data on the relative frequency of cristae in mitochondrial profiles.

Effect of various mutagens on strain 1D. In thin sections of respiratory-competent yeasts of *Saccharomyces cerevisiae* strain 1D grown to late logarithmic phase in 2%

melibiose, there was great variation in mitochondrial size and shape (Pl. 1, fig. 1 to 4). Cristae were absent from some profiles but were extensively developed in the majority. In a few instances the mitochondria had a lamellate appearance.

The spontaneous petite of strain 1 D was not distinguishable from the parental strain on the basis of its mitochondrial structure except for somewhat higher numbers of lamellate mitochondria (Pl. 1, fig. 5, 6). All other features were the same as the parental type.

Strain 1 D petites induced with acriflavine had slightly fewer mitochondria in each section than the parent strain (average of two profiles per section as opposed to three per section). Some of the mitochondria were grossly aberrant but none was lamellate. A good proportion of the mitochondria had well-defined cristae (Pl. 2, fig. 7). The rest of the organism was of similar cytology to the parent strain.

Table 1. *Enumeration of normal and aberrant mitochondria in respiratory-competent* ($\rho+$) *and petite* ($\rho-$) *strains of Saccharomyces cerevisiae*

Strain	Mutagen	No. of mitochondrial profiles examined	% cristate mitochondrial profiles*	% completely non-cristate mitochondrial profiles	% grossly aberrant or lamellate mitochondrial profiles
1 D $\rho+$	—	84	73	21	6
1 D $\rho-$	Spontaneous	66	70	20	10
1 D $\rho-$	Ultraviolet	37	54	46	0
1 D $\rho-$	Acriflavine	51	55	37	8
1 D $\rho-$	Phenethyl alcohol	49	55	27	18
11 $\rho+$	—	101	95	5	0
11 $\rho-$	Phenethyl alcohol	50	60	40	0
2A $\rho-$	Phenethyl alcohol	20	65	35	0
2B $\rho-$	Phenethyl alcohol	40	52	40	8
2C $\rho-$	Phenethyl alcohol	31	55	45	0
2D $\rho-$	Phenethyl alcohol	15	47	33	20
Summation of above results					
Parental types		185	85	12	3
Petites		359	58	35	7

* No assessment was made of reductions in the amount of cristal membranes: a profile showing any internal membrane was counted as cristate.

In the petite of strain 1 D induced with PEA, mitochondria were well developed, often with extensive cristae (Pl. 2, fig. 8). Lamellate mitochondria were quite frequently observed (Pl. 2, fig. 9). An additional feature of these yeasts was a proliferation of the endoplasmic reticulum to yield a discontinuous system throughout the cytoplasm (Pl. 2, fig. 10).

The ultraviolet-induced petite of strain 1 D (Pl. 2, fig. 11) had poorly defined mitochondrial profiles, but nevertheless some of them were clearly cristate.

Effect of PEA on various strains of Saccharomyces cerevisiae. Two parent strains of *S. cerevisiae* were used: 1 D and 11. The appearance of strain 1 D and its PEA-induced petite have already been described above. A petite strain was induced from 11 by PEA treatment. Petites were also produced by PEA treatment of an 11 × 1 D ascospore tetrad (2A–2D). The mitochondria of strain 11 respiratory-competent type were larger and had better developed cristae than strain 1 D (Pl. 2, fig. 12, Pl. 3, fig. 13); no lamellate or aberrant mitochondria were observed. Other features of the two strains were identical. The five PEA-induced petite strains from 11 and the cross with 1 D all showed similar mitochondrial morphology (Pl. 3, fig. 14 to 18). The major difference between the petite strains and the parent strains was the reduction in the number of cristae in each mitochondrial profile. Also, as in the other petites, an increased proportion of the mitochondrial profiles showed no cristae. There were a number of grossly aberrant mitochondria in two of these petites (2 B and 2 D), but no lamellate ones were observed in any of the strains derived from parent strain 11.

An analysis of the mitochondrial profiles found in sections of parental and petite strains of *S. cerevisiae* is given in Table 1.

DISCUSSION

By using a fermentable but non-repressing carbohydrate such as melibiose it has been possible to investigate the mitochondrial structure of *Saccharomyces cerevisiae* petites without the complication of glucose repression. Melibiose is a disaccharide of glucose and galactose but the concentration of the repressing metabolite apparently does not reach a critical value during fermentation. Although respiration is absent in petite yeasts, glucose repression could still be an important factor by interfering with the biogenesis of the mitochondria.

The mitochondrial cytochromes *a* and *b* were absent from petite strains, whatever the means of mutagenesis. This is exactly the situation which occurs when respiratory-competent yeasts are treated with chloramphenicol, erythromycin and similar antibiotics which are believed to specifically inhibit protein synthesis in the mitochondria (Clark-Walker & Linnane, 1966, 1967; Huang, Biggs, Clark-Walker & Linnane, 1966).

The petite yeasts show changes in their mitochondrial structure, but as the mitochondria of *Saccharomyces cerevisiae* are so varied even in the respiratory-competent type, care has to be taken in the interpretation of these changes. For one thing, it is evident that in respiratory-competent strains there is variation in the appearance of mitochondria from one strain to another. Petites produced from these strains show a similar variation in mitochondrial structure. However, it is possible to detect definite trends in the structural changes accompanying the petite mutation (see Table 1). In strain 1 D there was a decrease in the number of mitochondrial profiles with cristae in all the petites except the spontaneous petite. This may mean that the spontaneous petite arose from some minimal change whereas the mutagens caused multiple lesions. Strain 11 showed no aberrant or lamellate mitochondrial profiles either in the parental type or in its petite. The reduction in the number of cristate profiles was similar to the strain 1 D petites. The apparent lack of cristae in a given profile does not necessarily imply that the mitochondrion as a whole is non-cristate, but it shows that there is at least a decrease in the amount of inner membrane.

These results are in contrast with those of Avers, Pfeffer & Rancourt (1965) and Yotsuyanagi (1962) who reported extensive loss of cristae and disorganization in the mitochondria of cytoplasmic petites. It may be significant that these two groups both used glucose media thereby permitting glucose repression. Our results are more in agreement with those of Bowers *et al.* (1967) who avoided repression by using a chemostat with low levels of glucose and found well-defined cristate mitochondria in a tricarboxylic acid mutant of *Saccharomyces*.

The petites produced from the 1D × 11 cross had a similar reduction of cristate profiles to the parent strain petites. A feature of interest here, however, is the 2:2 segregation for aberrant mitochondria. This could be evidence of nuclear control over mitochondrial structure, even in the petite condition.

One of the aims of the present investigation was to compare petites produced by PEA with those induced by other mutagens. PEA is known to prevent the replication of bacterial DNA (Lark & Lark, 1966) and a similar interference with replication of mitochondrial DNA may be responsible for PEA-induction of petites. Of the six PEA petites examined, five were indistinguishable from the acriflavine or ultraviolet-induced petites. The sixth, from strain 1D, showed a large amount of membrane distributed throughout many of the organisms. Any abnormalities in the PEA-induced petite mitochondria, apart from the reduction in cristal material, do not seem significant when compared with the inherent variation in mitochondrial structure in respiratory competent ($\rho+$) cells of strain 1D. We have not therefore been able to detect any specific effect of PEA on mitochondrial structure, which differs from any of the other mutagens.

Thus it can be concluded that the main result of the change from $\rho+$ to $\rho-$ is a reduction in the amount of mitochondrial cristal membranes. Contrary to previous authors, we do not find any characteristically $\rho-$ abnormality which we would consider reliable enough to be used as a morphological marker for the cytoplasmic inheritance of mitochondrial characters. On the contrary, what mitochondrial peculiarities we have found seem to be under nuclear control.

There are limitations in comparative work on the ultrastructure of petite and normal mitochondria using the methods described in this paper. It is hoped, however, to improve the resolution of mitochondrial variation by examining isolated mitochondrial fractions.

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REFERENCES

AVERS, C. J., PFEFFER, C. R. & RANCOURT, M. W. (1965). Acriflavine induction of different kinds of 'petite' mitochondrial populations in *Saccharomyces cerevisiae*. *J. Bact.* **90**, 481.

AVERS, C. J., RANCOURT, M. W. & LIN, F. H. (1965). Intracellular mitochondrial diversity in various strains of *Saccharomyces cerevisiae*. *Proc. natn. Acad. Sci. U.S.A.* **54**, 527.

BOWERS, W. D., MCCLARY, D. O. & OGUR, M. (1967). Electron microscopy of a tricarboxylic acid mutant of *Saccharomyces*. *J. Bact.* **94**, 482.

CLARK-WALKER, G. D. & LINNANE, A. W. (1966). *In vivo* differentiation of yeast cytoplasmic and mitochondrial protein synthesis with antibiotics. *Biochem. biophys. Res. Commun.* **25**, 8.

CLARK-WALKER, G. D. & LINNANE, A. W. (1967). The biogenesis of mitochondria in *Saccharomyces cerevisiae*. A comparison between cytoplasmic respiratory-deficient mutant yeast and chloramphenicol-inhibited wild type cells. *J. Cell Biol.* **34**, 1.

EPHRUSSI, B., HOTTINGUER, H. & CHIMENES, A. M. (1949). Action de l'acriflavine sur les levures. I. La mutation 'petite colonie'. *Annls Inst. Pasteur, Paris* **76**, 351.

GIBBONS, I. R. & GRIMSTONE, A. V. (1960). On flagellar structure in certain flagellates. *J. biophys. biochem. Cytol.* **7**, 697.

HUANG, M., BIGGS, D. R., CLARK-WALKER, G. D. & LINNANE, A. W. (1966). Chloramphenicol inhibition of the formation of particulate mitochondrial enzymes of *Saccharomyces cerevisiae*. *Biochem. biophys. Acta* **114**, 434.

JOHANSEN, D. A. (1940). *Plant Microtechnique*, 1st ed. New York: McGraw-Hill.

LARK, K. G. & LARK, C. (1966). Regulation of chromosome replication in *E. coli*: a comparison of the effects of phenethyl alcohol with those of amino-acid starvation. *J. molec. Biol.* **20**, 9.

MARCHANT, R. & SMITH, D. G. (1968). Membranous structures in yeasts. *Biol. Rev.* **43**, 459.

OSUMI, M. & KATOH, T. (1967). Oxygen-induced formation of mitochondrial membrane matrix in respiration-deficient yeasts. *Jap. Wom. Univ. J.* **14**, 67.

REILLY, C. & SHERMAN, F. (1965). Glucose repression of cytochrome a synthesis in cytochrome-deficient mutants of yeasts. *Biochim. biophys. Acta* **95**, 640.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208.

ROODYN, D. B. & WILKIE, D. (1968). *The Biogenesis of Mitochondria*. London: Methuen.

WICKERHAM, L. J. (1946). A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. *J. Bact.* **57**, 293.

YOTSUYANAGI, Y. (1962). Études sur le chondriome de la levure. II. Chondriomes des mutants a deficience respiratoire. *J. Ultrastruct. Res.* **7**, 141.

EXPLANATION OF PLATES

PLATE 1

Fig. 1 to 4. Sections of respiratory-competent cells of *Saccharomyces cerevisiae* parent strain 1D, showing variations in mitochondrial morphology. ×47,000; 32,000; 26,000; 54,000.

Fig. 5, 6. Spontaneous petite of strain 1D. Mitochondrial profiles are numerous and range from normal cristate forms to lamellate types. ×45,000; 50,000.

PLATE 2

Fig. 7. Acriflavine-induced petite of strain 1D, showing some cristate and some non-cristate mitochondrial profiles. ×23,000.

Fig. 8 to 10. Phenethyl alcohol (PEA-)induced petite of strain 1D, showing essentially normal cristate mitochondrial profiles (fig. 8), a lamellate profile (fig. 9) and proliferation of endoplasmic reticulum (fig. 10). ×40,000; 50,000; 32,000.

Fig. 11. Ultraviolet-induced petite of strain 1D. The mitochondrial profiles are poorly defined, but some are distinctly cristate. ×25,000.

Fig. 12. Respiratory-competent yeast of parent strain 11, with regular, well-defined, cristate mitochondrial profiles. ×60,000.

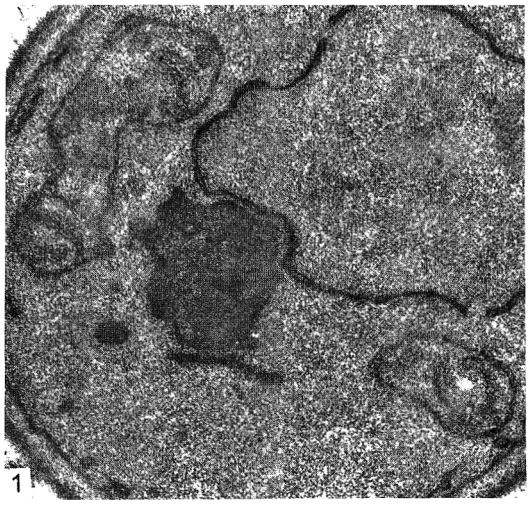
PLATE 3

Fig. 13. Respiratory-competent parent strain 11. ×49,000.

Fig. 14. PEA-induced petite of strain 11, illustrating reduction in the number of cristae in the mitochondrial profiles. ×50,000.

Fig. 15, 16. PEA-induced petite of strain 2B (resulting from 11 × 1D cross). Some aberrant mitochondria (Fig. 15) and some normal cristate profiles (Fig. 16) are visible. ×17,000; 72,000.

Fig. 17, 18. PEA-induced petite of strain 2D (resulting from 11 × 1D cross), showing aberrant but basically cristate mitochondrial profiles. ×45,000; 45,000.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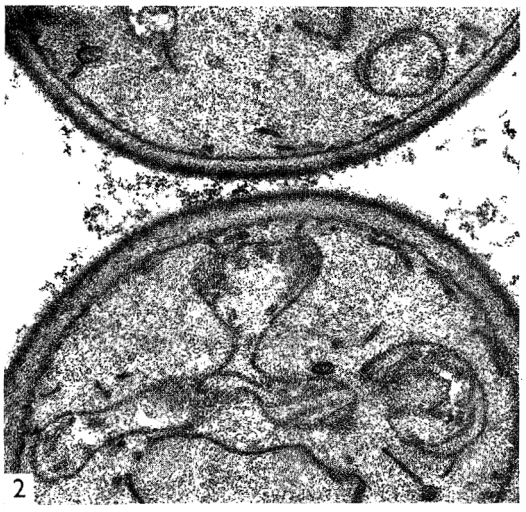
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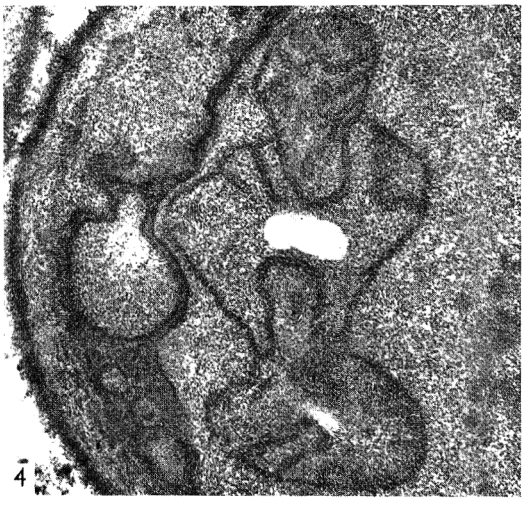
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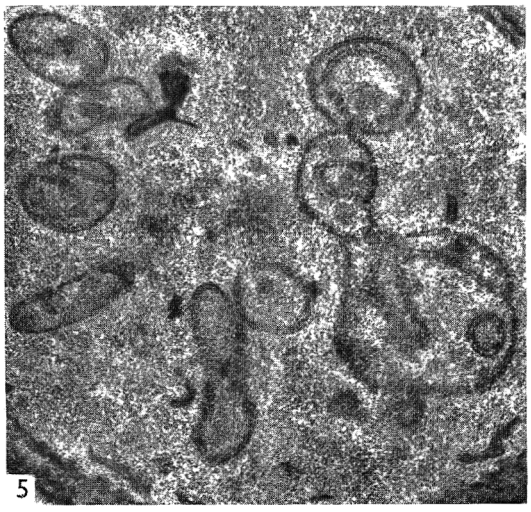
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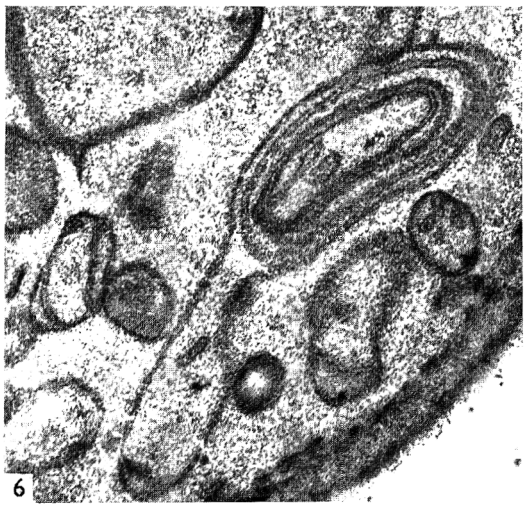
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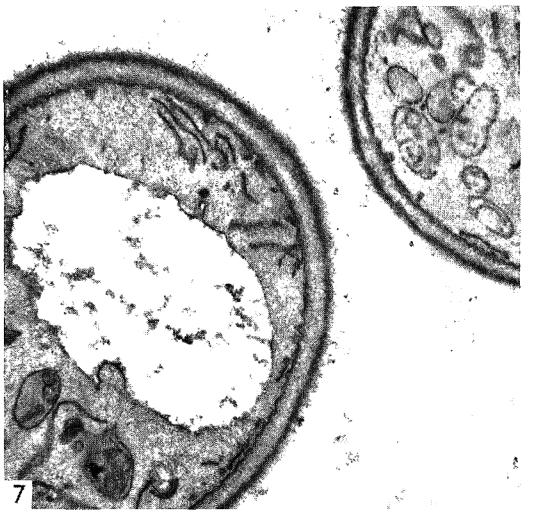
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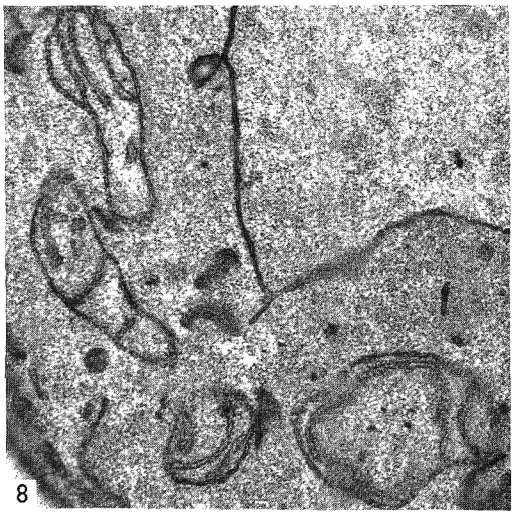
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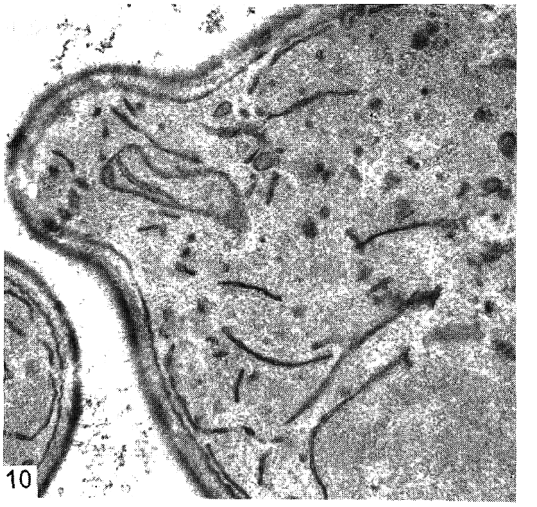
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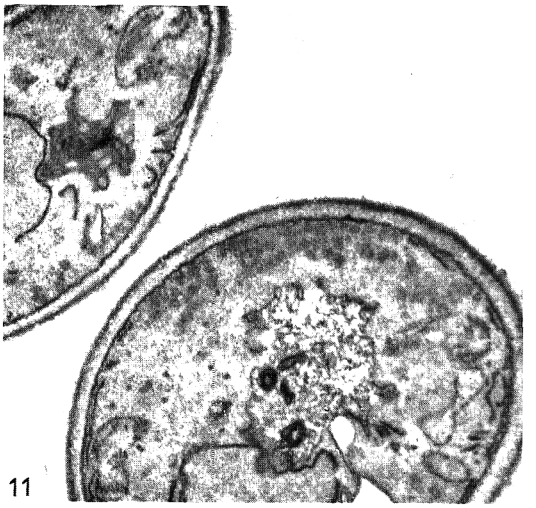
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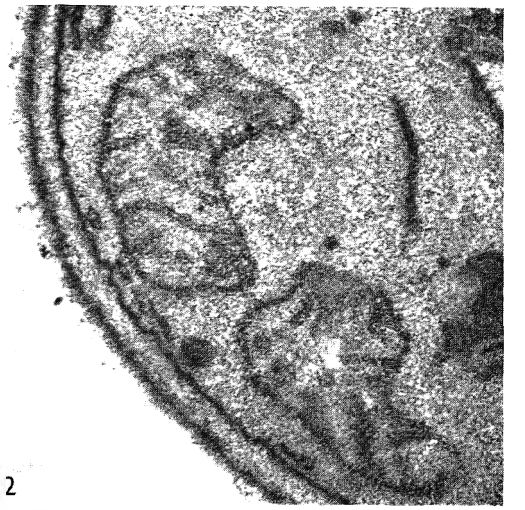
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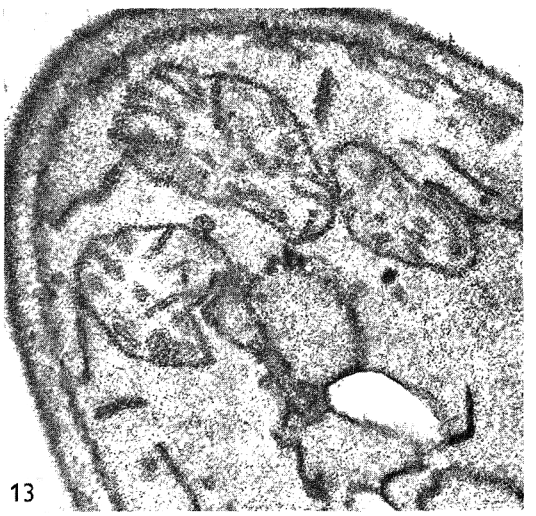
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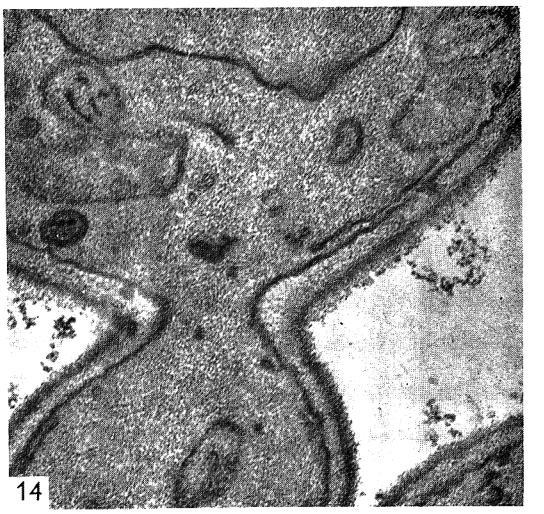
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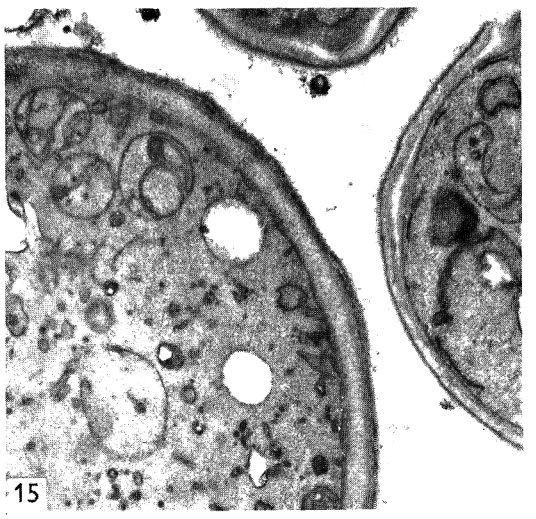
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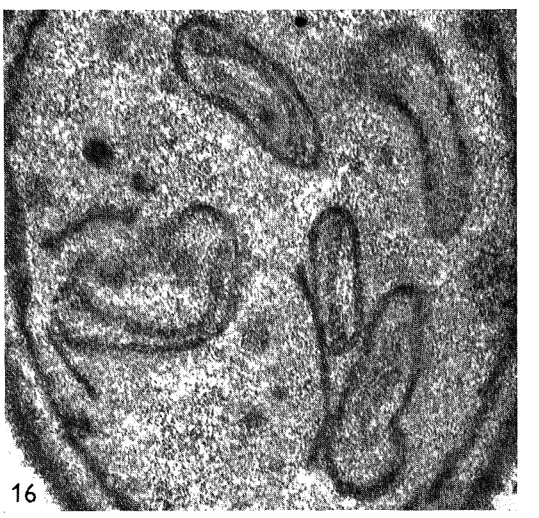
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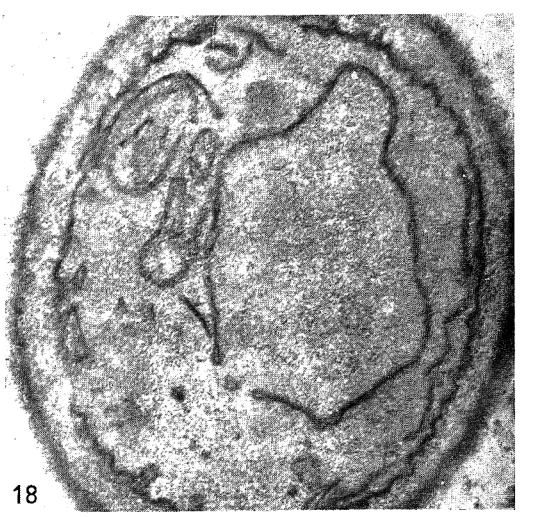
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Abnormal Growth of Tissues Infected with *Plasmodiophora brassicae*

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SUMMARY

Clubroot formation on *Sinapis alba* plants infected with *Plasmodiophora brassicae* was not prevented by application of 2,3,5-triiodobenzoic acid or α-naphthylphthalamic acid (inhibitors of polar transport of auxins in plants) to the hypocotyls. However, the fresh weight of club tissue produced on treated plants was lower than on untreated plants. This difference in weight was of the same order as the difference in total fresh weights of treated and untreated plants. Explants of tumour tissue from two Brassica species infected with *P. brassicae*, unlike explants from healthy roots, produced callus on a tissue culture medium without growth substances. Callus growth from infected tissues depended on the presence of active vegetative plasmodia in the cells. Established clones of *Brassica rapa* (Golden Ball) callus infected with vegetative plasmodia of *P. brassicae*, unlike healthy callus clones, grew well on a series of media deficient in one or more growth substances. Growth of infected Golden Ball callus on medium without growth substances was prevented by the tryptamine antagonist xylamidine tosylate and by the monoamine oxidase inhibitor iproniazid. Inhibition was annulled by including auxins or coconut milk in the medium. The relevance of these findings to abnormal growth of Brassica roots infected with *P. brassicae* is discussed.

INTRODUCTION

Disturbance of the phytohormone levels in host tissues is frequently an important consequence of infection of plants by many obligate and other fungal parasites (Brian, 1967). Changes in the levels of indoleacetic acid (IAA) and gibberellins are often involved (Sequeira, 1963; Shaw, 1963; Bailiss & Wilson, 1967). Moreover, there is evidence that cytokinins are implicated in the disease syndromes resulting from rust and powdery mildew infections (Brian, 1967). The possible importance of such disturbances in understanding the nature of obligate parasitism has been discussed by Thrower (1965), Heitefuss (1966) and Brian (1967).

Tumorous growth of roots and hypocotyls, resulting from cellhypertrophy and hyperplasia, is the most striking feature of Brassica plants infected with *Plasmodiophora brassicae*. Changes in the phytohormone levels of the diseased tissues are clearly involved. Indeed, Katsura, Egawa, Toki & Iishii (1966) have detected higher than normal levels of two growth substances, one thought to be IAA, in clubroot galls on *Brassica rapa* roots, and Williams (1966) has correlated cell and nuclear volumes of *B. oleracea* var. *capitata* cells, infected with *P. brassicae*, with the quantity of parasite within those cells. Williams suggested that the stimulus for cell division and

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cell hypertrophy in clubroot galls lies within the parasitized cells and is not transferred to adjacent cells. Experiments are reported here which test the possibility that *P. brassicae* infected roots act as sinks for growth substances produced in the aerial parts of the plants, and tests are described where callus cultures, derived from roots of *Brassica* spp. infected with *P. brassicae*, were grown on tissue culture media deficient in one or more growth substances.

METHODS

Organisms. Plasmodiophora brassicae strain S (Ingram, 1969) was used throughout. Infected and healthy plants of *Brassica oleracea* var. *capitata* (Early Drumhead cabbage) and *B. rapa* (Balmoral turnip) were grown in soil according to the method of Ingram (1969). The procedure used for growing healthy and infected plants of *Sinapis alba* is described below. The clones of *B. rapa* (Golden Ball turnip) callus used in tissue culture growth experiments had been cultured continuously on coconut milk medium (see below) for 9 months. Uninfected callus clones had been derived from surface-sterilized roots of healthy plants and infected callus clones had been derived from 5-week-old *P. brassicae* clubs and manipulated to contain only the vegetative plasmodial stage of the parasite life-cycle (Ingram, 1969). A further Golden Ball callus clone (GBA 4), which at the time of the experiments did not contain *P. brassicae*, was also 9 months old and had been established from a clubroot gall. Plasmodia had been present in the tissues for 3 months following callus initiation, but had then been lost from the cells.

2,3,5-Triiodobenzoic acid (TIBA) and α-naphthylphthalamic acid (NPA) experiments. Sinapis alba seeds were germinated in sterilized soil at 20° in a growth cabinet (Ingram, Knights, McEvoy & McKay, 1968) operated on alternating 8 hr light and 16 hr dark periods (*Sinapis alba* does not flower in short days). Seedlings were transplanted singly, after 7 days, to 3·5 inch pots containing sterilized soil or soil infected with 10^8 spores of *Plasmodiophora brassicae* per g. Hypocotyls of experimental plants were ringed, 3 days after transplanting, with lanolin paste containing TIBA (0·4 and 0·8 %) or NPA (0·4 %). Hypocotyls of control plants were ringed at the same time with pure lanolin paste. Lanolin rings were renewed every 5 days and all treatments were replicated 10 times. Experiments were harvested 28 days after transplanting the seedlings and growth was assessed by measuring the height of plants from cotyledons to apex, counting the number of internodes and by fresh weights. The upper green parts of plants, and the roots or clubs (including hypocotyls) were weighed separately. Before weighing, roots and clubs were washed free of soil and blotted dry. In one experiment the effects of TIBA and NPA on flowering of *Sinapis alba* was determined by growing plants in alternating 16 hr light and 8 hr dark periods.

Tissue culture experiments. Tissue cultures were incubated at 26°, in a growth room lit by a single, 100 W. tungsten lamp, on a basal medium of salts, micronutrients, vitamins, sucrose and agar (Ingram, 1969) with the following additives: A, no additives, B, α-naphthylacetic acid (NAA) 0·5 mg./l. + kinetin 1·0 mg./l.; C, kinetin 1·0 mg./l.; D, NAA 0·5 mg./l.; E, coconut milk 150 ml./l. + 2,4-dichlorophenoxyacetic acid (2,4-D) 6·0 mg./l. + NAA 0·1 mg./l.; F, coconut milk 150 ml./l.; G, 2,4-D 6·0 mg./l. + NAA 0·1 mg./l. The pH of all media was adjusted to 5·5 with NaOH or HCl before autoclaving at 115° for 10 min.

Tissue culture initiation tests. 1·0 $mm.^3$ inocula taken from surface, sterilized 6-week-

old clubs or healthy roots of *Brassica oleracea* var. *capitata* (Drumhead) and *B. rapa* (Balmoral) were incubated singly on 15 ml. of medium A or medium E (see above) contained in 25 mm. × 150 mm. Pyrex glass test tubes. Tests were also done using 0·5 cm. root tip segments from healthy, 7-day-old sterile seedlings of *B. oleracea* var. *capitata* (Drumhead) as inocula. Histological techniques used in these tests, surface sterilization procedures and preparation of sterile seedlings have been described by Ingram (1969).

Growth tests using established clones of Brassica rapa (Golden Ball) callus. The growth of healthy and *Plasmodiophora brassicae* infected callus of *B. rapa* (Golden Ball) was compared on media A, B, C, D, E, F, and G (see above). Explants (1·5 $mm.^3$) of callus were transferred to basal medium for 1 week before commencement of the tests and then singly to 15 ml. batches of the test media contained in 25 mm. × 150 mm. culture tubes. Growth was normally assessed visually and by fresh and dry weights after eight 1-week transfers on the test media. The growth of Golden Ball callus clone GBA 4 tissue was compared, using techniques similar to those described above, on media A and E.

Experiments with xylamidine tosylate (XT) and isonicotinic acid-2-isopropyl hydrazide (iproniazid). The effects of XT and iproniazid on growth of infected *Brassica rapa* (Golden Ball) callus on media A, E, F and G (see above) was investigated using 100 ml. Erlenmeyer flasks containing 20 ml. medium inoculated with three 1·5 $mm.^3$ explants of callus (maintained on basal medium for 1 week before commencement of the experiments). XT was added to sterile media, having first been dissolved in a little 95 % (v/v) ethanol in water and then taken up in a large volume of distilled water and sterilized by membrane filtration. Control media, which did not contain XT, were supplemented with ethanol equivalent to that in the XT solution. Iproniazid was also added to sterile media, having first been dissolved in distilled water and sterilized by membrane filtration. In some experiments media were also supplemented with tryptamine hydrochloride (dissolved in water, sterilized by membrane filtration); with a amino acid mixture based on an analysis of the amino acids of coconut milk done by Tulecke, Weinstein, Rutner & Laurencot (1961) (dissolved in water and added before autoclaving); with Difco pure Casamino acids (added as a solid before autoclaving); and with 1,3-diphenylurea (added before autoclaving). In all experiments calluses were transferred to fresh medium each week and growth was assessed by fresh and dry weights.

RESULTS

Effects of 2,3,5-triiodobenzoic acid (TIBA) and α-naphthylphthalamic acid (NPA) on the development of clubroot in intact plants of Sinapis alba

Both TIBA and NPA interfere with the polar transport of auxins in plants (Niedergang-Kamien & Skoog, 1956; Morgan, 1964; Keitt & Baker, 1966). TIBA may operate by immobilizing auxin, thus reducing the amount available for transport (Winter, 1967*a*; Goldsmith, 1968).

TIBA and NPA had similar and marked effects on the vegetative growth of *Sinapis alba* plants maintained in infected and sterilized soil (Pl. 1, fig. 1). Compared with untreated control plants these effects were: height was reduced by over 50 % (Tables 1, 2); leaves were reduced in area and were darker green than normal; dormancy of cotyledonary and axillary buds was broken and treated plants had two to six branches;

total fresh weight was reduced by as much as 60% (Tables 1, 2); an abnormal swelling of the hypocotyls developed immediately above the point of application of TIBA or NPA, while the region below this point had a much smaller diameter than control hypocotyls; and the onset of flowering by plants grown in long days was delayed by 7 to 10 days, while those flowers which did develop were dwarfed. The number of internodes produced by *S. alba* plants (normally between 10 and 13 internodes per plant) was not affected by TIBA or NPA.

Table 1. *The effect of 2,3,5-triiodobenzoic acid (TIBA) on Sinapis alba plants grown for 35 days in sterilized soil and in soil infected with resting spores of Plasmodiophora brassicae (short days)*

Club and root weights as percentages of the mean total fresh weights of plants are given in parentheses (weights and heights are the means of ten replicates).

Treatment	Mean fresh weights (g.) Tops	Clubs or roots	Mean heights (cm.)
Sterilized soil			
Pure lanolin	18·3±0·8	1·0±0·1 (5·2%)	17·3±0·8
0·4% TIBA	7·3±0·6	0·2±0·02 (2·2%)	9·6±0·5
0·8% TIBA	6·7±0·4	0·1±0·02 (2·0%)	9·4±0·6
Infected soil			
Pure lanolin	10·2±0·4	2·2±0·4 (16·6%)	15·1±0·9
0·4% TIBA	4·6±0·5	0·9±0·2 (16·2%)	8·1±0·5
0·8% TIBA	4·6±0·4	1·1±0·1 (18·9%)	8·2±0·4

Concentrations of TIBA is given as % (w/w) in lanolin paste.

Table 2. *The effect of α-naphthylphthalamic acid (NPA) on Sinapis alba plants grown for 35 days in sterilized soil and soil infected with resting spores of Plasmodiophora brassicae (short days)*

Club and root weights as percentages of the mean total fresh weights of plants are given in parentheses (weights and heights are the means of ten replicates).

Treatment	Mean fresh weights (g.) Tops	Clubs or roots	Mean heights (cm.)
Sterilized soil			
Pure lanolin	16·9±0·5	1·1±0·04 (6·1%)	22·1±0·7
0·4% NPA	5·0±0·8	0·1±0·04 (2·8%)	8·9±4·4
Infected soil			
Pure lanolin	11·0±0·6	2·8±0·3 (20·2%)	17·2±1·0
0·4% NPA	3·5±0·4	0·6±0·1 (15·1%)	8·6±0·7

Concentration of NPA is given as % (w/w) in lanolin paste.

The fresh weight of *Plasmodiophora brassicae* clubs formed on TIBA-and NPA-treated plants grown in infected soil was much lower than the weight of clubs formed on control plants (Tables 1 and 2). In the case of TIBA treated plants this difference in weight, however, was of the same order as the difference between the total fresh weights of treated and control plants. Clubroot tissue accounted for 16·6% of the mean total weight of control plants compared with 16·2 and 18·9% of the mean total weights of plants treated with 0·4 and 0·8% TIBA in lanolin respectively. In the case of NPA-treated plants clubroot tissue accounted for 20·2% of the mean total weight of control plants and for only 15·1% of the mean total weight of plants treated with

0·4 % NPA in lanolin. However, it is notable that both TIBA and NPA led to marked reductions in the percentage fresh weights of healthy roots of uninfected plants, compared with controls (Tables 1 and 2).

Callus culture initiation by clubroot tissues on basal medium

Tissue culture initiation by 1·0 mm.^{3} explants of 5-week-old clubroot tissue and 5-week-old healthy root tissue of *Brassica oleracea* var. *capitata* (Drumhead) and of *B. rapa* (Balmoral) was compared on basal medium (A) and on coconut milk-2,4-D-NAA medium (E) (Table 3 and Pl. 1, fig. 2). The clubroot tissues formed callus after 4 to 7 days' incubation on both media. This growth continued for 4 to 6 weeks, then darkening of the tissues occurred and growth ceased. On medium E new callus formed on the degenerate tissues after a short time and continued to develop uninterrupted (Ingram, 1969). On basal medium, however, roots began to form immediately on about 50 % of the apparently dead calluses and continued to grow for approximately 4 weeks, then organized root growth ceased. Those roots which were already formed became disorganized and further growth was in the form of slow growing callus which, in the case of Balmoral, was maintained for 18 months on basal medium.

Table 3. *Callus initiation by healthy and Plasmodiophora brassicae infected Brassica rapa (Balmoral Turnip) root tissue explants on basal medium and on coconut milk medium*

Where appropriate, the mean fresh weight of calluses is given in mg. Callus growth: —, no growth; +, slow growth; ++, good growth; +++, vigorous growth.

	Callus growth			
	Healthy		Infected	
Days on medium	Basal medium	Coconut milk medium	Basal medium	Coconut milk medium
7	—	+	++	++
18	—	+	+++ (29·5 ± 5·7)*	+++ (57·9 ± 12·5)†
50	—	+++ (50·1 ± 6·6)‡	Callus degenerate, roots developing	Callus degenerate
50 >	—	Continuing vigorous callus growth	Callus growth from roots continuing slowly	Regrowth of callus, continuing slowly

* Mean of 18 replicates. † Mean of 14 replicates. ‡ Mean of 20 replicates.

Tissue cultures could not be initiated easily from roots of healthy soil grown Drumhead plants because of contamination by endogenous micro-organisms. However, those tissue inocula which were contamination free did not form callus on basal medium, although vigorous callus growth did take place on medium E. Further, 1·0 cm. long root tip segments from healthy, sterile Drumhead seedlings continued to develop as roots on basal medium and only gave rise to callus on medium E. Healthy Balmoral root tissue explants did not form callus on basal medium, but on medium E callus began to develop slowly after 1 week and proliferated rapidly, without interruption, after 7 weeks.

Sections of Drumhead and Balmoral tissues grown on basal medium showed that

the development of *Plasmodiophora brassicae* within the tissues was similar to the development of the parasite in infected Brassica tissues grown on medium E (Ingram, 1968). At the time of tissue culture initiation most plasmodia in the explants were at an advanced stage of development, with many nuclei. Callus produced on the inocula did not become infected with the parasite immediately, and infected cells did not divide. Callus decline coincided with resting spore formation in the cells of the explant region of the tissue mass. Roots which formed on the cultures became infected with vegetative plasmodia after the resting spores had germinated *in situ* to give primary (root hair) plasmodia and zoosporangia first. Disorganization of the roots to give callus tissue commenced when vegetative plasmodia reappeared in the tissues. Balmoral callus grown on basal medium for 12 months contained all stages of the *P. brassicae* life-cycle.

Growth of Brassica rapa (Golden Ball) callus, infected with vegetative plasmodia, on media deficient in growth substances

Callus growth from explants of 9-month-old, established *Brassica rapa* (Golden Ball) callus infected with vegetative *Plasmodiophora brassicae* plasmodia and from healthy callus explants of this species was compared on basal medium and on basal medium supplemented with one or more growth substances (media A to G). The results of these tests are summarised in Table 4 and Pl. 2, fig. 3 and 4.

Table 4. *Mean fresh and dry weights of callus produced by explants of healthy and Plasmodiophora brassicae plasmodia infected callus of Brassica rapa (Golden Ball Turnip) on basal and other media*

	Weight of callus (mg.)			
	Healthy*		Infected†	
Supplements to basal medium	Fresh weight	Dry weight	Fresh weight	Dry weight
None	No growth	No growth	116·5 ± 23·6	15·8 ± 8·5
1·0 mg./l. kinetin + 0·5 mg./l. NAA	76·2 ± 6·1	8·4 + 1·1	216·6 ± 27·9	24·7 ± 2·6
1·0 mg./l. kinetin	No growth	No growth	140·5 ± 23·5	16·4 ± 2·5
0·5 mg./l. NAA	87·7 ± 15·8‡	8·6 ± 1·7‡	174·3 ± 23·9	19·4 ± 2·0
6·0 mg./l. 2,4-D + 0·1 mg./l. NAA + 150 ml./l. coconut milk	411·7 ± 15·3	36·0 ± 1·3	409·1 ± 13·6	38·2 ± 1·5
150 ml./l. coconut milk	82·2 ± 10·1‡	7·7 ± 1·1‡	349·3 ± 36·2	34·4 ± 2·4
6·0 mg./l. 2,4-D + 0·1 mg./l. NAA	124·1 ± 8·7	15·3 ± 1·0	179·2 ± 20·5	16·0 ± 2·2

* Ten replicates. † Five replicates. ‡ One to six short roots formed on each explant.

Growth of healthy callus. Explants of healthy Golden Ball callus tissue did not continue to grow after transfer to basal medium (A). However, some callus growth did occur on basal medium supplemented with both NAA 0·5 mg./l. and kinetin 1·0 mg./l. (B), although no growth occurred on basal medium supplemented with kinetin alone (C), while on basal medium supplemented with NAA alone (D) callus growth was poor and 1 to 6 short roots formed on each explant. On medium containing 2,4-D 6·0 mg./l., NAA 0·1 mg./l. and coconut milk (E) the explants produced a large amount of firm, healthy callus, and on medium containing 2,4-D and NAA but no coconut milk (G), callus growth was firm and healthy, although the bulk of tissue produced was only about half that produced on medium E. On medium supple-

mented with coconut milk alone (F) callus growth was very poor and 1 to 6 short roots formed on each explant.

Growth of infected callus. Explants of Golden Ball callus tissue infected with vegetative plasmodia of *Plasmodiophora brassicae* were able to continue callus growth on all media tested, and roots were never produced. On basal medium (A) growth was slow and variable but always took place. Growth from the explants was rather better on medium containing NAA 0·5m g./l. and kinetin 1·0 mg./l. (B), while on media containing kinetin alone (C) or NAA alone (D) callus growth was about the same as on basal medium. As with healthy tissues, good callus growth occurred on medium containing 2,4-D 6·0 mg./l., NAA 0·1 mg./l. and coconut milk (E). Unlike healthy tissues, however, infected tissue explants grew almost as well on basal medium supplemented with coconut milk alone (F) as on medium E, while on medium containing 2,4-D and NAA but no coconut milk (G) growth was similar in bulk to that produced by healthy tissues on the same medium.

In one experiment the possibility that growth of infected tissues on the growth substance deficient media may have been supported by auxins or kinins carried over from the previous culture medium was tested. The results showed that callus growth continued without diminution for 16 1-weekly transfers on all of the test media.

Growth of clone GBA 4 callus on basal medium. Explants of clone GBA 4 of *Brassica rapa* (Golden Ball) callus, which had once contained *Plasmodiopthora brassicae*, did not make any growth when they were transferred to basal medium (A). The tissue was capable of good growth on coconut milk-2,4-D-NAA medium (E). There was no evidence that the GBA 4 tissues had any ability to grow on growth factor deficient media.

Experiments with xylamidine tosylate

Xylamidine tosylate (XT) antagonizes the peripheral effects of 5-hydroxytryptamine in isolated rat uterus and in various *in vivo* tests with rats (Copp, Green, Hodson, Randall & Simm, 1967). It also antagonizes the tryptamine-stimulated growth of detached apices of Avena coleoptiles (Winter, 1967*b*). The effect of XT on proliferation of the infected *Brassica rapa* (Golden Ball) callus on basal medium was investigated.

Effective concentration. Growth of explants of infected Golden Ball callus tissue was compared on basal medium (A) supplemented with 0·0, 5·0, 10·0, 20·0 and 40·0 mg./l. XT (Table 5). Callus growth from the explants occurred on unsupplemented basal medium as in previous experiments and growth was not inhibited by 5·0 mg./l. XT. However, XT was slightly inhibitory to growth at the 10·0 mg./l. and 20·0 mg./l. concentrations, while at the 40·0 mg./l. concentration callus growth was prevented completely.

Reversal of inhibition. Proliferation of infected Golden Ball callus tissue explants was compared on media A, E, F and G (see above), unsupplemented or supplemented with 50 mg./l. XT. The results of these tests are summarized in Table 6 and Pl. 2. fig. 5. Growth of the callus on basal medium (A) was prevented completely by the presence of XT. However, XT had no marked inhibitory effect on callus proliferation on medium containing 2,4-D, NAA and coconut milk (E) or coconut milk alone (F), On medium containing 2,4-D and NAA, but no coconut milk (G), callus development was retarded to some extent by XT, although explants did produce some new tissue which, at the end of the experiment (6 weeks), was beginning to proliferate vigorously.

Fresh hand sections of Golden Ball callus tissue grown on media containing XT gave no evidence that *Plasmodiophora brassicae* plasmodia in the cells had been killed.

Effect of other compounds on xylamidine tosylate inhibited growth. 1,3-diphenylurea (DPU), a constituent of coconut milk, is stimulatory to cell division in carrot root tissue explants when 2·0 mg./l. are included in a basal medium supplemented with 200 mg./l. casein hydrolysate (Shantz & Steward, 1955). However, DPU (2·0 mg./l.), Casamino acids (200 mg./l.) and an amino acid mixture (Tulecke *et al.* 1961), alone and in combination, did not reverse the inhibition of growth of infected Golden Ball callus on basal medium (A) containing 50·0 mg./l. XT. There was some slight indication in another experiment that DPU at a concentration of at least 20·0 mg./l., in combination with 200 mg./l. Casamino acids, was partially effective in reversing the XT inhibition, but no consistent results were obtained.

Table 5. *The mean weights of 15 replicate calluses of Brassica rapa (Golden Ball Turnip), infected with Plasmodiophora brassicae plasmodia, after 5 weeks of growth on basal medium supplemented with various concentrations of xylamidine tosylate*

Concentration of xylamidine tosylate (mg./l.)	Weight of callus (mg.)	
	Fresh	Dry
0	66·0 ± 6·0	7·3 ± 0·8
5·0	66·1 ± 7·0	8·4 ± 1·0
10·0	54·3 ± 6·5	6·4 ± 0·8
20·0	43·3 ± 6·3	5·9 ± 1·1
40·0	No growth	No growth

Table 6. *Mean fresh and dry weights of 10 replicate calluses of Brassica rapa (Golden Ball Turnip), infected with Plasmodiophora brassicae plasmodia, after 6 weeks of growth on basal and other media, unsupplemented and supplemented with 50 mg l. xylamidine tosylate.*

Additives to basal medium	Mean weight of calluses (mg.)			
	Without xylamidine tosylate		With xylamidine tosylate	
	Fresh	Dry	Fresh	Dry
None	96·2 ± 11·6	13·1 ± 1·7	No growth	No growth
150 ml./l. coconut milk + 6·0 mg./l. 2,4-D + 0·1 mg./l. NAA	323·8 ± 13·3	27·6 ± 1·1	328·7 ± 12·5	31·2 ± 9·6
150 ml./l. coconut milk	279·0 ± 25·6	32·7 ± 4·6	266·0 ± 28·5	32·1 ± 3·8
6·0 mg./l. 2,4-D + 0·1 mg./l. NAA	115·5 ± 12·1	12·6 ± 1·0	42·9 ± 4·2*	6·1 ± 4·4*

* Growth poor, but improving by end of experiment.

Tryptamine hydrochloride at 2·5, 5·0, 10·0, 20·0 and 40·0 mg./l. was tested for its ability to prevent inhibition of infected Golden Ball callus growth on basal medium (A) by XT. Although the experiment was repeated several times no evidence was obtained that inhibition was prevented by this compound at any of the concentrations listed.

Experiments with iproniazid

Amine oxidase inhibitors prevent the formation of indoleacetic acid from tryptamine (Perley & Stowe, 1966) and inhibit tryptamine-stimulated growth of excised Avena coleoptile apices (Winter, 1966). Iproniazid is an inhibitor of monoamine oxidase in animal tissue (Zeller & Sarker, 1962). Growth of explants of *Brassica rapa* (Golden Ball) callus, infected with vegetative *Plasmodiophora brassicae* plasmodia, was compared on media A, E, F and G (see above) all supplemented with 0·0, 50·0 or 100·0 mg./l. iproniazid (Table 7). At the 50 mg./l. concentration iproniazid was not markedly inhibitory to growth of infected callus explants on any of the media tested. However, at the 100 mg./l. concentration iproniazid inhibited callus growth completely on basal medium (A) but only slightly on media containing coconut milk and auxins, alone and in combination (E to G).

Table 7. *Mean fresh and dry weights of 10 replicate calluses of Brassica rapa (Golden Ball Turnip), infected with Plasmodiophora brassicae plasmodia, after 6 weeks of growth on basal and other media, unsupplemented and supplemented with iproniazid*

Additives to basal medium	Concentration of iproniazid (mg./l.)	Mean weights of calluses (mg.)	
		Fresh	Dry
None	0	91·9 ± 6·6	10·4 ± 0·9
	50	63·7 ± 6·8	9·8 ± 1·2
	100	No growth	No growth
150 ml./l. coconut milk + 6·0 mg./l. 2,4-D + 0·1 mg./l. NAA	0	180·4 ± 15·4	17·0 ± 0·7
	50	180·1 ± 15·8	15·5 ± 1·3
	100	141·9 ± 17·4	14·1 ± 2·0
150 ml./l. coconut milk	0	177·5 ± 17·1	19·9 ± 2·4
	50	150·7 ± 13·9	16·8 ± 1·3
	100	157·9 ± 10·9	15·7 ± 1·3
6·0 mg./l. 2,4-D + 0·1 mg./l. NAA	0	82·2 ± 11·9	7·9 ± 1·1
	50	80·2 ± 4·9	7·8 ± 0·7
	100	54·8 ± 16·1	6·0 ± 1·2

DISCUSSION

Sandstedt & Schuster (1966) suggested that gall formation in plants infected with *Meloidogyne incognita* may occur because this nematode enables the infected tissues to retain and use auxin which might otherwise be transported elsewhere in the plant. The possibility that *Plasmodiophora brassicae* might act as a sink for growth substances produced in the aerial parts of infected plants was tested by ringing *Sinapis alba* plants growing in infected soil with 2,3,5-triiodobenzoic acid (TIBA) and α-naphthylphthalamic acid (NPA). Both compounds have been reported to prevent the polar transport of auxins in plant tissues, although there is no direct evidence that they have this effect in *S. alba.*

The experiments did indicate that the distribution of auxins, and possibly gibberellins, in *Sinapis ala* was affected by TIBA and NPA, resulting in such marked changes in the treated plants as reduced internode length and breaking of apical dominance. The formation of clubs on the *S. alba* plants was not prevented by the TIBA or NPA treatments, although the fresh weight of both club tissue and root tissue produced on treated plants was considerably lower than on untreated plants. Similar results were obtained when TIBA was applied to the hypocotyls of infected *Brassica oleracea* var.

capitata (Drumhead) plants in experiments not reported here. Neither the TIBA experiments nor the NPA experiments provided any clear evidence that *Plasmodiophora brassicae* infected root tissues of *S. alba* are able to act as a sink for growth substances produced in the aerial parts of the plant.

A probable explanation for the high levels of auxin found in clubbed roots of *Brassica rapa* by Katsura *et al.* (1966) is that growth substances are synthesized by *Plasmodiophora brassicae*, or by the host tissues in response to this pathogen, at or very close to the infection site. This probability is strongly supported by the demonstration that tissue explants from clubbed roots of *B. oleracea* var. *capitata* (Drumhead) and *B. rapa* (Balmoral), unlike tissue explants from uninfected roots of these species, were able to form callus on a medium which did not contain growth substances. Callus growth on this basal medium depended upon the presence of vegetative plasmodia in the cells. During the period when only resting spores were present callus growth was replaced by the formation of organized roots, indicative of a shift in the balance of growth substances in the callus mass. Roots on the infected tissues became disorganized and callus formation was resumed as soon as resting spores germinated *in situ* and vegetative plasmodia reappeared in the cells. These observations are in keeping with an unpublished observation of the author that callus tissues of many uninfected Brassica species initiate roots when transferred from a medium containing auxin and kinetin to a basal medium.

It appeared from the results discussed above that the presence of vegetative plasmodia of *Plasmodiophora brassicae* in Brassica tissues renders them independent of an exogenous supply of auxin and kinin for disorganized growth. This conclusion was further borne out in the experiments where growth of established, uninfected and plasmodially infected *Brassica rapa* (Golden Ball) callus was compared on a series of media deficient in one or more growth substances. Infected calluses were able to continue growth when transferred to a medium without growth substances, whereas uninfected calluses were not. There was little difference in the growth of infected and uninfected calluses on medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthylacetic acid (NAA) and coconut milk. However, the growth of infected tissues was markedly better on medium which did contain auxin and kinetin or auxin and coconut milk than on basal medium. Basal medium supplemented with coconut milk alone supported infected callus proliferation to the same extent as basal medium supplemented with both coconut milk and auxin. In contrast, medium containing coconut milk alone did not induce proliferation of uninfected callus, although a few root initials were formed. The possibility must be considered that the coconut milk contained auxin precursors available only to *P. brassicae* and not to the host tissues or, alternatively, that nutrients stimulatory to growth of the parasite were present. Tryptophan was not detected in an analysis of the amino acids of the coconut milk used in the experiments (C. Wheeler, using a Beckman 'Unichrom' autoanalyser—personal communication), and tryptamine is unlikely to have been present since the coconut milk had twice been autoclaved at 115° for 10 min. at the time of the experiments. However, Shantz & Steward (1955) have isolated the cell division factor 1,3-diphenylurea (DPU) from coconut milk. Tulecke *et al.* (1961) identified a number of nutrients in coconut milk, any of which could have been stimulatory to *P. brassicae*. These nutrients included a number of organic acids: sugars such as sucrose, glucose, fructose, sorbitol; inositol; vitamins; and a wide range of amino acids.

It is notable that uninfected *Brassica rapa* (Golden Ball) calluses were capable of some growth on basal medium supplemented with NAA alone, and grew quite well on medium containing NAA and 2,4-D but no coconut milk, indicating that an exogenous supply of kinin was not esssntial for uninfected tissue proliferation. The experiment with Clone GBA 4 of *B. rapa* (Golden Ball), a callus clone from which *P. brassicae* had been lost, did not provide any evidence that the parasite has any ability to transform its host's cells to be independent of exogenous growth substances as is the case with the Crown Gall disease caused by *Agrobacterium tumefaciens* (Braun & Lipetz, 1966).

That the pathway to indoleacetic acid (IAA) was involved in the growth of *Brassica rapa* (Golden Ball) callus on basal medium was strongly suggested by the xylamidine tosylate (XT) and iproniazid experiments. Both XT, an antagonist of tryptamine stimulated growth in plants, and the monoamine oxidase inhibitor iproniazid prevented growth of the infected callus on basal medium, but not on basal medium supplemented with coconut milk and auxin, alone or in combination. It is interesting that coconut milk alone was more efficient in preventing the inhibition of infected callus growth by XT and iproniazid than was auxin alone. Although DPU is known to be present in coconut milk (Shantz & Steward, 1955), this compound did not have any marked ability to prevent the inhibition of infected callus growth by XT in the basal medium, even when it was used in combination with Casamino acids. (An explanation for this result could be that it was difficult to obtain good dispersion of DPU in the tissue culture media.) Another possible reason for the efficiency of the coconut milk in preventing the inhibitory effects of XT and iproniazid is that it contained auxins or auxin precursors which were not susceptible to XT or iproniazid and were, therefore, available to the infected tissues for growth.

Wightman (1962) suggested that indoleacetonitrile (IAN) is an important intermediate in the biogenesis of IAA from tryptophan in cabbage. He was unable to name any of the intermediates in the formation of IAN itself, but did rule out the possibilities that tryptamine and indoleacetaldehyde were involved. Moreover, the mustard oil glucoside, glucobrassicin, has been isolated from cabbages (Gmelin & Virtanen, 1961) and is thought to be a precursor of IAN and a storage form of auxin in Brassicas (Andersen, 1966). It is likely that these findings for cabbage could apply equally to other *Brassica* spp. such as *Brassica rapa.* Although it is known that xylamidine tosylate does prevent the conversion of tryptamine to IAA in Avena (Winter, 1967*b*), nothing is known regarding the specificity or the exact site of action of this compound. Further experiments where specific precursors of IAN and IAA, and other inhibitors of the enzymes involved in the biosynthesis of these compounds, are fed to *Plasmodiophora brassicae*-infected or uninfected Brassica tissues, or to isolated but living *P. brassica* plasmodia, could define more precisely the route by which auxins are synthesised in infected tissues. That the biosynthetic pathway involved is different from the pathway followed in uninfected tissues is possible.

I wish to thank Professor P. W. Brian for advice and discussions, Dr D. G. Morgan for α-naphthylphthalamic acid, Dr H. F. Hodson for xylamidine tosylate, Dr C. Wheeler for carrying out an analysis of the amino acids of coconut milk, the Agricultural Research Council for financial support, and Mrs C. Breen for technical assistance.

REFERENCES

ANDERSEN, A. S. (1966). *Auxin physiology of glucobrassicin.* Ph.D. thesis, University of Iowa.

BAILISS, K. W. & WILSON, I. M. (1967). Growth hormones and the creeping thistle rust. *Ann. Bot., Lond., N.S.* **31**, 195.

BRAUN, A. C. & LIPETZ, J. (1966). The use of tissue cultures in phytopathology. In *Cells and Tissues in Culture.* Ed. by E. N. Willmer, vol. III, p. 691. London: Academic Press.

BRIAN, P. W. (1967). Obligate parasitism in fungi. *Proc. R. Soc.* B, 168, 101.

COPP, F. C., GREEN, A. F., HODSON, H. F., RANDALL, A. W. & SIMM, M. F. (1967). New peripheral antagonists of 5-hydroxytryptamine. *Nature, Lond.* **214**, 200.

GMELIN, R. & VIRTANEN, A. I. (1961). Glucobrassicin, the precursor of 3-indolylacetonitrile, ascorbigen and SCN^- in *Brassica oleracea* species. *Acta chem. fenn.* **34**, 15.

GOLDSMITH, M. H. M. (1968). The transport of auxin. *A. Rev. Pl. Physiol.* **19**, 347.

HEITEFUSS, R. (1966). Nucleic acid metabolism in obligate parasitism. *A. Rev. Phytopath.* **4**, 221.

INGRAM, D. S. (1969). Growth of *Plasmodiophora brassicae* in host callus. *J. gen. Microbiol.* **55**, 9.

INGRAM, D. S., KNIGHTS, B. A., MCEVOY, I. J. & MCKAY, P. (1968). Studies in the Cruciferae. Changes in the composition of the sterol fraction following germination. *Phytochemistry* **7**, 1241.

KATSURA, K., EGAWA, H., TOKI, T. & ISHII, S. (1966). On the plant hormones in the healthy and Plasmodiophora infected roots of *Brassica rapa* var. *neosuguki* Kitam. (1). *Ann. Phytopath. Soc. Japan* **32**, 123.

KEITT, G. W. & BAKER, R. A. (1966). Auxin activity of substituted benzoic acids and their effect on polar auxin transport. *Pl. Physiol. Lancaster* **41**, 1561.

MORGAN, D. G. (1964). Influence of α-naphthylphthalamic acid on the movement of indolyl-3-acetic acid in plants. *Nature, Lond.* **207**, 476.

NEIDERGANG-KAMIEN, E. & SKOOG, F. (1956). Studies on polarity and auxin transport in plants. I. Modification of polarity and auxin transport by triiodobenzoic acid. *Physiologia Plant.* **9**, 60.

PERLEY, J. E. & STOWE, B. B. (1966). On the ability of *Taphrina deformans* to produce indoleacetic acid from tryptophan by way of tryptamine. *Pl. Physiol. Lancaster* **41**, 234.

SANDSTEDT, R. & SCHUSTER, M. L. (1966). The role of auxins in root-knot nematode-induced growth on excised tobacco stem segments. *Physiologia Plant.* **19**, 960.

SEQUEIRA, L. (1963). Growth regulators in plant disease. *A. Rev. Phytopath.* **1**, 5.

SHANTZ, E. M. & STEWARD, F. C. (1955). The identification of compound A from coconut milk as 1,3-diphenylurea. *J. Am. chem. Soc.* **77**, 6351.

SHAW, M. (1963). The physiology and host-parasite relations of the rusts. *A. Rev. Phytopath.* **1**, 259.

THROWER, L. B. (1965). Host physiology and obligate fungal parasites. *Phytopath. Z.* **4**, 319.

TULECKE, W., WEINSTEIN, L. H., RUTNER, A. & LAURENCOT, H. J. (1961). The biochemical composition of coconut water as related to its use in plant tissue culture. *Contr. Boyce Thompson Inst. Pl. Res.* **21**, 115.

WIGHTMAN, F. (1962). Metabolism and biosynthesis of 3-indoleacetic acid and related indole compounds in plants. *Can. J. Bot.* **40**, 689.

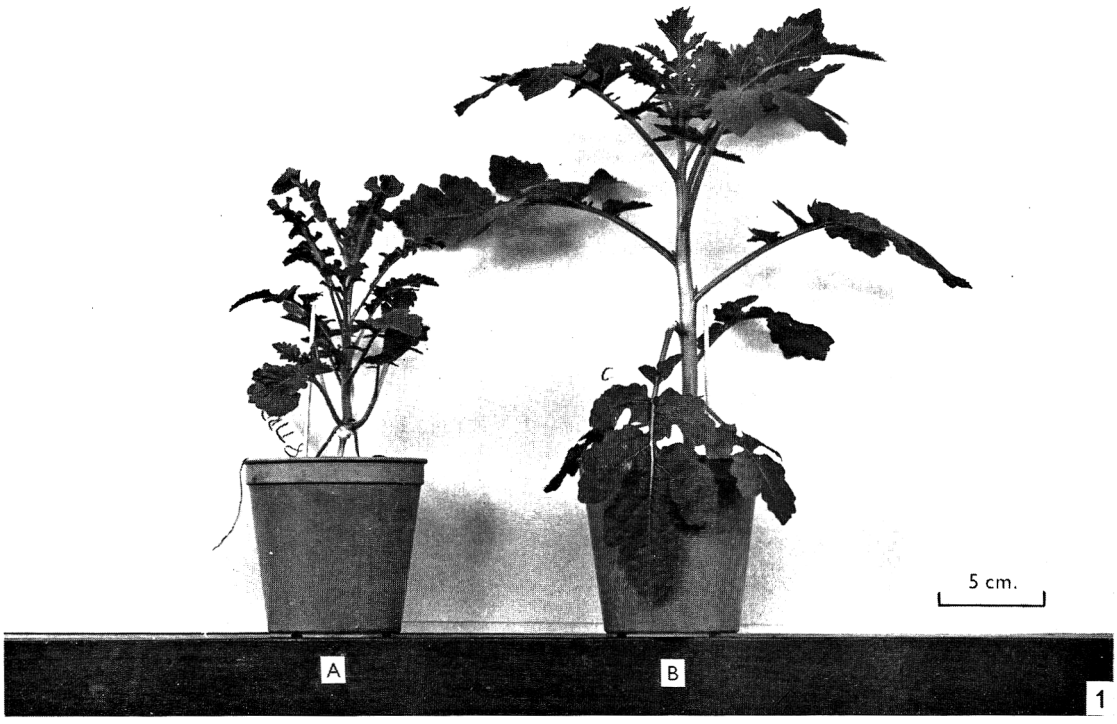
WILLIAMS, P. H. (1966). A cytochemical study of hypertrophy in clubroot of cabbage. *Phytopathology* **56**, 521.

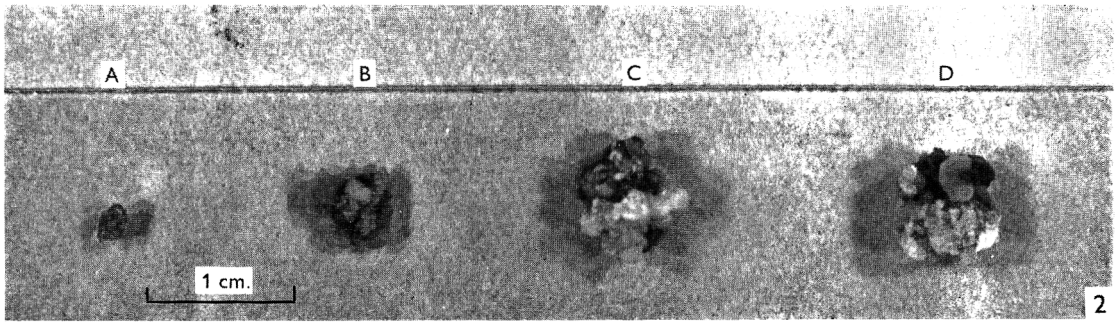
WINTER, A. (1966). A hypothetical route for the biogenesis of IAA. *Plana* **71**, 229.

WINTER, A. (1967*a*). The promotion of the immobilization of auxin in Avena coleoptiles by triiodobenzoic acid. *Physiologia Plant* **20**, 330.

WINTER, A. (1967*b*). Antagonism of tryptamine stimulated growth in apices of *Avena* coleoptile by xylamidine tosylate. *Nature, Lond.* **216**, 801.

ZELLER, E. A. & SARKER, S. (1962). Amine oxidases. XIX. Inhibition of monoamine oxidase by phenylcyclopropylamine and iproniazid. *J. biol. Chem.* **237**, 2333.





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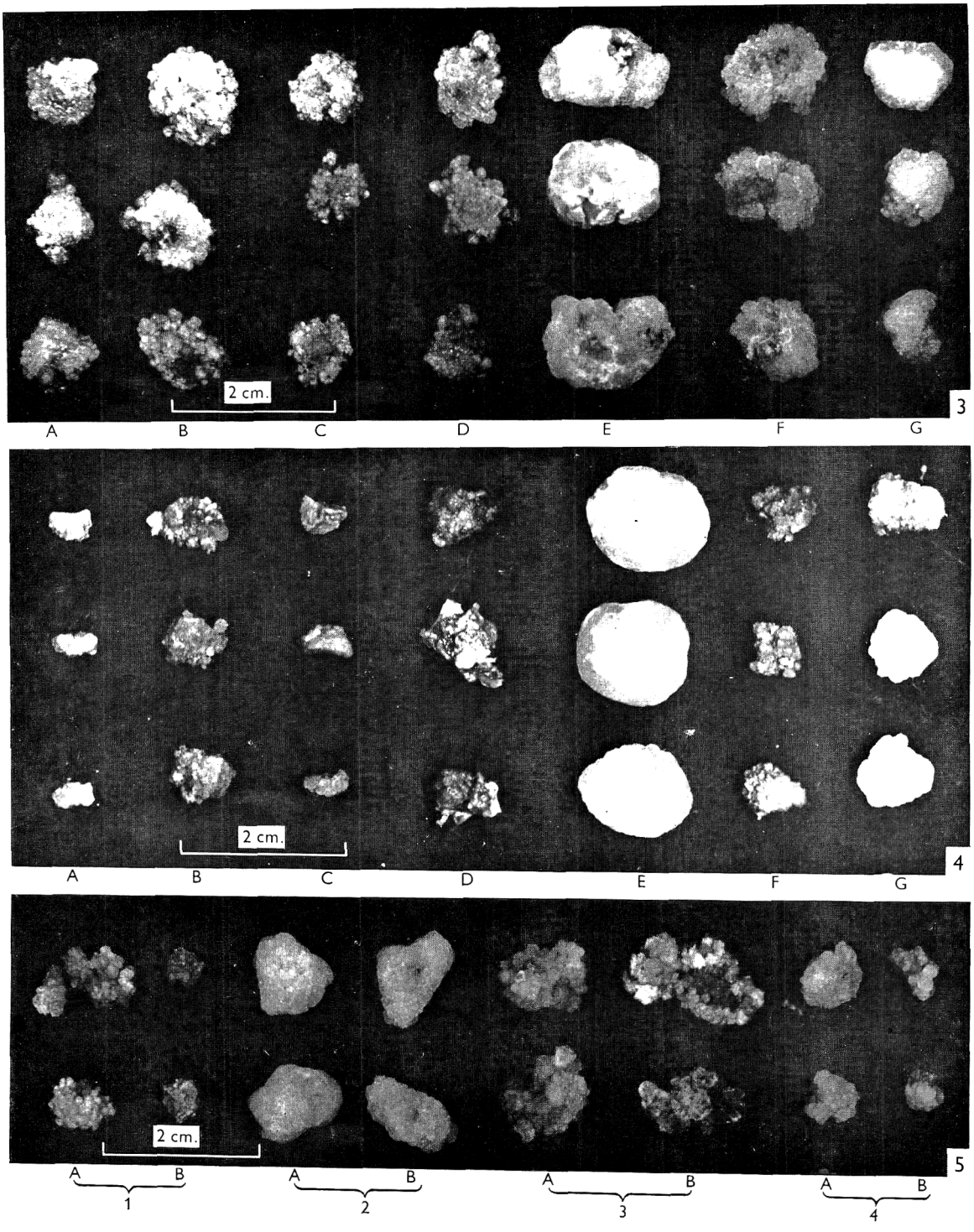

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. The effect of 2,3,5-triiodobenzoic acid (TIBA) on *Sinapis alba* after 35 days of growth in alternating 16 hr dark and 8 hr. light periods. Hypocotyls of the plants were ringed at 7 days with: A, TIBA, 0·8 % in lanolin; B, pure lanolin.

Fig. 2. Callus formed by explants of tissue from an uninfected root and a *Plasmodiophora brassicae* clubbed root of *Brassica rapa* (Balmoral Turnip) after 21 days incubation on basal medium (no growth substances) and on coconut milk medium (2,4-dichlorophenoxyacetic acid 6·0 mg./l. + α-naphthyacetic 0·1 mg./l. + coconut milk 150 ml./l.). A, Uninfected tissue on basal medium; B, uninfected tissue on coconut milk medium; C, infected tissue on basal medium; D, infected tissue on coconut milk medium.

PLATE 2

Fig. 3, 4. Three replicate examples each of *Brassica rapa* (Golden Ball Turnip) callus infected with vegetative plasmodia of *Plasmodiophora brassicae* (Fig. 3) and uninfected Golden Ball callus (Fig. 4) after 8 weeks of growth on basal medium supplemented as follows: A, no supplements; B, α-naphthylacetic acid (NAA) 0·5 mg./l. + kinetin 1·0 mg./l.; C, kinetin 1·0 mg./l.; D, NAA 0·5 mg./l. E, 2,4-dichlorophenoxyacetic acid (2,4-D) 6·0 mg./l. + NAA 0·1 mg./l. + coconut milk 150 ml./l.; F, coconut milk 150 ml./l.; G, 2,4-D 6·0 mg./l. + NAA 0·1 mg./l.

Fig. 5. Two replicate examples each of *Brassica rapa* (Golden Ball Turnip) callus infected with; vegetative plasmodia of *Plasmodiophora brassicae* after 8 weeks of growth on unsupplemented media (A) and on media supplemented with xylamidine tosylate 50·0 mg./l. (B). 1, Basal medium; 2, basal + 2,4-dichlorophenoxyacetic acid (2,4-D) 6·0 mg./l. + α-naphthylacetic acid (NAA) 0·1 mg./l. + coconut milk 150 ml./l; 3, basal + coconut milk 150 ml./l.; 4, basal + 2,4-D 6·0 mg./l. + NAA 0·1 mg./l.

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Inhibition of Genetic Transformation in *Bacillus subtilis* by Phenethyl Alcohol

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SUMMARY

Phenethyl alcohol (PEA) has been found to obstruct genetic transformation in *Bacillus subtilis*. A concentration of 0·3 % (v/v) PEA prevents the development of competence and destroys predeveloped competence without causing a measurable decrease in viable cells. Treatment of competent cells with 0·3 % PEA prior to their exposure to transforming deoxyribonucleic acid (DNA) causes the cells to become incapable of irreversibly incorporating DNA. Treatment of competent cells which have irreversibly incorporated DNA with 0·3 % PEA causes a reduction in the rate of transformation. These observations suggest that PEA affects the ability of competent cells to irreversibly incorporate DNA and also affects the cells' ability to express the DNA in the form of transformed cells once the DNA is irreversibly incorporated. The possible nature of the effect of PEA on competent cells is discussed.

INTRODUCTION

Phenethyl alcohol (PEA) has been shown to be inhibitory to Gram-negative (Lilley & Brewer, 1953) and Gram-positive (Berrah & Konetzka, 1962; Mendelson, 1965) bacteria, and animal cells (Zahn *et al.* 1966; Leach *et al.* 1964; Bruchosvky, Mak & Till, 1967; Bruchovsky & Till, 1967; Rosenkranz, Mednis, Marks & Rose, 1967; Plagemann, 1968).

The nature of the inhibitory action of PEA on bacteria has been studied extensively, but the results have been somewhat contradictory. Berrah & Konetzka (1962), Treick & Konetzka (1964), and Lark & Lark (1966) report that PEA blocks deoxyribonucleic acid (DNA) synthesis. Rosenkranz, Carr & Rose (1964, 1965*a*, *b*) and Rosenkranz *et al.* (1967) attribute PEA action to an inhibition of ribonucleic acid (RNA) synthesis. Lester (1965) and Silver & Wendt (1967) found that PEA affected the permeability of bacterial cells. Silver & Wendt (1967) associate the permeability change with a limited breakdown of the cytoplasmic membrane. Plagemann (1968) concludes that PEA affects a major process of growth control in rat hepatoma cells, and he suggests that this effect may be the result of PEA acting on the cytoplasmic membrane.

We tested the effect of PEA on genetic transformation of *Bacillus subtilis* 168 and found the compound to be very inhibitory. This same observation was made independently and almost simultaneously in another laboratory (Leach & Richardson, 1966). We undertook an investigation of the nature of the effect of PEA on *B. subtilis* transformation and obtained the data presented in this report.

METHODS

Organisms. Bacillus subtilis 168 *ind*, obtained from Dr W. Romig, University of California at Los Angeles, was used as the recipient. *Bacillus subtilis* 168 *ind str-r*, obtained by u.v.-induction, served as the non-radioactive transforming DNA donor. To prepare tritium-labelled transforming DNA, we used *B. subtilis* 168 *ind thy str-r*, a strain that was acquired by transforming *B. subtilis* 168 *ind thy* (Farmer & Rothman, 1965), obtained from Dr F. Rothman, Brown University, Providence, Rhode Island, with DNA obtained from *B. subtilis* 168 *ins str-r*. Cultures were maintained as spore stocks on potato extract agar (Thorne, 1962). Each week that vegetative cells were needed they were prepared by an inoculum from the spore stock on Antibiotic Medium 3. All incubation of the growing organisms was at 37°.

Media. The salts medium (MS) of Spizizen (1958) was used as the base for all defined media and for dilution blanks. Growth medium contained MS plus 0·5% glucose and 5·0 μg./ml. L-tryptophan. Transformation medium (TM) contained MS, 0·5% glucose, 5·0 μg./ml. L-tryptophan, 10^{-4} M-ferrous ammonium sulphate, and 0·01% acid hydrolysed casein. Growth medium, with and without L-tryptophan, additionally supplemented with 0·005% acid hydrolysed casein, was used as a plating medium. Difco-Brain Heart Infusion (BHI) and Difco-Antibiotic Medium 3 were also used as plating media. All plating media contained 1·5% agar.

Extraction of DNA. Deoxyribonucleic acid was extracted according to a modification of the procedure of Marmur (1961).

Procedure for attaining competence. Growth medium was inoculated with a loopful of vegetative *Bacillus subtilis* 168 *ind* and incubated until the culture reached an optical density at 600 mμ of about 0·490 on the Spectronic 20 spectrophotometer. Then the culture was monitored until 1·0 ml. added to 5·0 ml. of TM would adjust the optical density to 0·100. The TM, freshly inoculated at that rate, was divided into 5·0 ml. samples to allow easy sampling at close intervals. The samples were incubated in a gyrotory shaker and at desired intervals aliquots were removed and assayed for viable organisms and for frequency of transformation.

Transformation assay. The frequency of transformation was assayed by exposing cells to DNA in transformation tubes containing 0·3 ml. cells, 0·3 ml. DNA dissolved in Marmur's (1961) saline citrate, and 2·4 ml. MS containing 0·5% glucose. When phenethyl alcohol (PEA) was added to transformation tubes, the volume of MS containing 0·5% glucose was adjusted to allow a final transformation tube volume of 3·0 ml. The frequency of transformation was calculated by dividing the number of transformants appearing after a period of DNA uptake by the viable cell titre at the beginning of the uptake period. Organisms were exposed to DNA for 30 min. unless otherwise indicated. After being exposed to DNA, organisms were removed from the transformation tubes and were impinged upon membrane filters. The impinged organisms were washed with about 50 ml. MS to stop DNA incorporation (and to remove PEA when present). In some experiments, DNA incorporation was stopped with deoxyribonuclease (DNase), but organisms were still impinged upon membrane filters and washed. DNase treatment consisted of exposing organisms and DNA to 50·0 μg./ml. of DNase and 5·0 μmde-$MgSO_4$/ml. for 5·0 min. Membrane filters with impinged, washed organisms were transferred to streptomycin-free plates and incubated 4 hr before the membranes were transferred to plates containing

dihydrostreptomycin sulphate 450 μg./ml. Colonies arising from transformed organisms were counted after 24 hr on BHI, or after 36 to 48 hr on L-tryptophan-free growth medium containing 0·005 % acid hydrolysed casein. Viable count plating was done at the time the organisms were added to transformation tubes. When transformants were plated to BHI, viable count plating was done on Antibiotic Medium 3, otherwise viable count plating was done on growth medium containing 0·005 % acid hydrolysed casein. DNA was used at saturating concentrations in all transformation experiments. When radioactive DNA incorporation was measured, DNase-treated organisms were washed 3 times in ice-cold MS. The washed cells were then dissolved in 0·5 ml. N-NaOH and 14 ml. scintillation fluid (Lin, Mosteller & Hardesty, 1966).

Preparation of tritium-labelled transforming DNA

Bacillus subtilis 168 *ind thy str-r* was routinely grown in MS containing 0·5 % glucose, L-tryptophan 40 μg./ml., 0·005 to 0·01 % acid hydrolysed casein, thymidine 50·0 μg./ml., and aminopterin 10 to 100 μg./ml. Methyl-tritiated thymidine (sp.act. 2·0 C/mM) was added at concentrations of 50 mC/l.

RESULTS

We elected to test PEA on *Bacillus subtilis* transformation at a concentration which would permit some growth, but at a reduced rate. As can be seen from Fig. 1, 0·3 % (v/v) PEA was found to be partially inhibitory; therefore, this concentration was used in transformation studies.

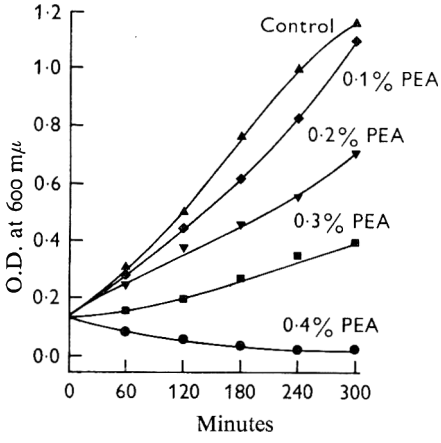

Fig. 1

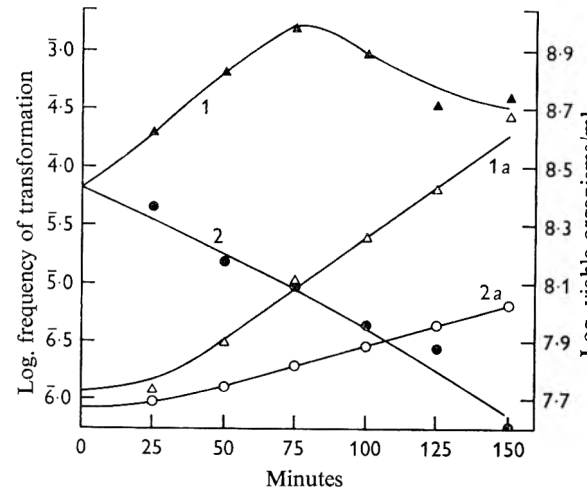

Fig. 2

Fig. 1. Effect of various concentrations of PEA on growth. Organisms were transferred from growth medium to transformation medium (TM) as if competence were to be induced. The freshly inoculated TM was then divided into five aliquots. To four of the aliquots the indicated concentrations of PEA were added. Growth was measured turbidimetrically.

Fig. 2. Effect of 0·3 % PEA on competence development. Organisms were transferred from growth medium to TM and the freshly inoculated TM was immediately divided into two parts. To one part PEA was added to a final concentration of 0·3 %. The other part served as a PEA-free control. At the intervals indicated both cultures were assayed for viable count and frequency of transformation. The marker transformed was *str-r*. Curve: 1, frequency of transformation, control; 1*a*, viable organisms control; 2, frequency of transformation, PEA added; 2*a*, viable organisms, PEA added.

When organisms were allowed to develop competence in TM according to our procedure, a competence development pattern like the control-frequency of transformation line in Fig. 2 was observed. When 0·3 % PEA was added to TM along with the inoculum (at 0 time), competence failed to develop and in fact organisms lost competence during the course of partially inhibited growth. The competent organisms present at the beginning of PEA exposure in Fig. 2 were reduced 100-fold during the period of the experiment.

It is clear from the data in Fig. 2 that PEA blocked transformation. To test the effect of PEA on highly competent organisms (pre-developed competence), 0·3 % PEA was added 60 min. after inoculation, the time just prior to that at which maximum competence was anticipated. The organisms were left in the presence of PEA for

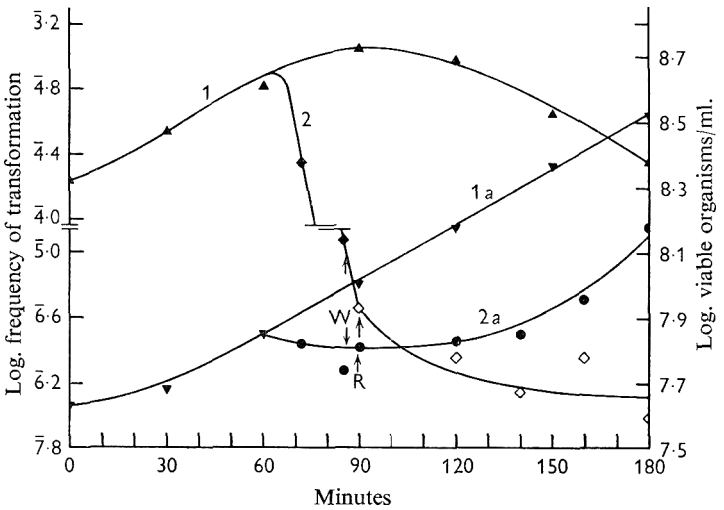

Fig. 3. Effect of removal of PEA from competent organisms exposed to 0·3 % PEA for 25 min. 0·3 % PEA was added to one-half of a culture which had been in TM for 60 min. Twenty-five min. later the organisms in PEA were washed on a membrane filter (point W in figure) and resuspended in fresh TM prewarmed to about 37° (point R in figure). The other half of the 60 min. culture served as a PEA-free control and was not washed. The two cultures were assayed for viable count and frequency of transformation at the intervals indicated. The marker transformed was *str-r*. Curve: 1, frequency of transformation, control; 1*a*, viable organisms, control; 2, frequency of transformation, PEA added and PEA removed: 2*a*, viable organisms, PEA added and PEA removed.

25 min., after which the PEA was removed by washing on a membrane filter. The PEA-treated, washed organisms were resuspended in fresh TM and re-incubated. The frequency of transformation and viable organisms were assayed during the entire treatment period, and the results appear in Fig. 3. The data in Fig. 3 corroborate the data in Fig. 2. Phenethyl alcohol stopped the development of competence and destroyed predeveloped competence at an inhibitor level that did not kill but actually permitted some growth. The competent organisms which had been treated with PEA did not regain competence for at least 90 min. after PEA was removed by washing. The effect of PEA on competent organisms was very rapid. Competence increase was stopped immediately upon addition of PEA, and after only 5–6 min. competence was lost at an exponential rate until PEA was removed.

Fox (1957), Lerman & Tolmach (1957), Young & Spizizen (1961), Levine & Strauss (1965), and Kammen, Beloff & Canellakis (1966) have shown that the number of cells transformed per given amount of transforming DNA is proportional to the length of exposure of the cells to the DNA. This relationship makes it possible to measure DNA uptake by scoring for transformants after various periods of exposure of organisms to transforming DNA.

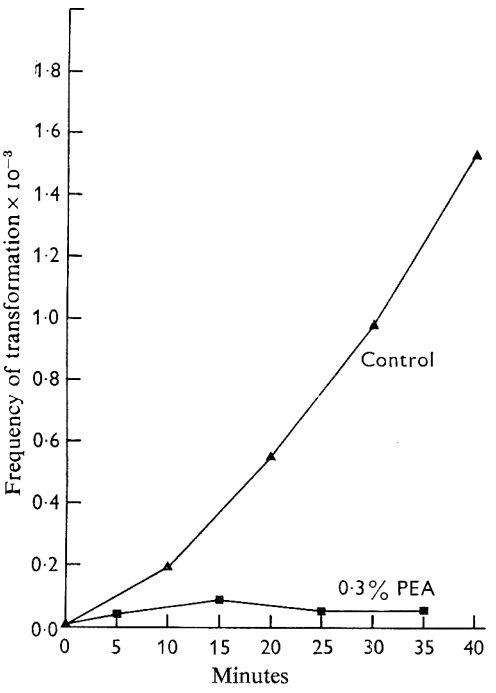

Fig. 4

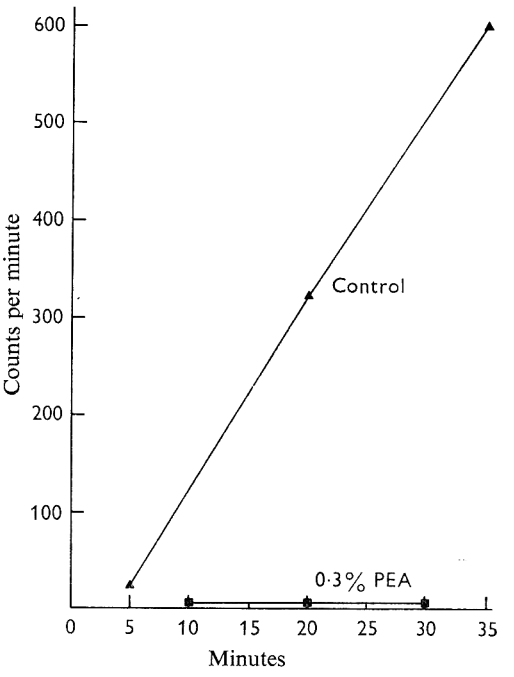

Fig. 5

Fig. 4. Effect on the frequency of transformation when 0·3 % PEA is added to competent organisms with DNA. Ninety min. competent organisms were added to transformation tubes. At the time of organism and DNA addition, 0·3 % PEA was added to one-half of the tubes (0·3 % PEA in figure). The other half of the tubes served as PEA-free controls (control in figure). Samples were taken at the times indicated and assayed for frequency of transformation. The frequency of transformation was calculated by dividing the number of transformants at each sampling point by the viable organism titre at the time of addition of the organisms to the transformation tubes. The marker transformed was *str-r*.

Fig. 5. Effect of 0·3 % PEA on the uptake of tritium-labelled DNA. 9·0 ml. aliquots of 90 min. competent organisms in TM were mixed with 1·0 ml. tritium-labelled DNA. The conditions of PEA exposure and sampling were identical to those described in Fig. 4. After the samples were assayed for frequency of transformation, the remaining organisms were washed, dissolved in NaOH, and assayed for radioactivity. The control and 0·3 % PEA curves in the figure correspond to the curves in Fig. 4.

Because PEA acts so rapidly (Fig. 3) it could be added to competent organisms along with transforming DNA and organisms were rendered non-transformable before any appreciable number were transformed. This permitted us to devise experiments to show that PEA blocked irreversible DNA uptake (incorporation).

Competent *Bacillus subtilis* 168 *ind* were exposed to a saturating concentration of DNA and the number of transformants appearing after various periods of DNA

exposure is shown by the 'Control' in Fig. 4. The experiment was repeated with cells to which 0·3 % PEA had been added with transforming DNA (0·3 % PEA, Fig. 4). The control shows that the number of transformants was proportional to time of exposure to DNA; therefore, the experiment measured DNA uptake. The absence of transformation in organisms treated with PEA suggests that PEA obstructed transformation by blocking DNA uptake by competent organisms. This assumption is valid only if all reactions in transformation subsequent to DNA uptake can occur without inhibition. Since it is possible that PEA blocks process(es) occurring after uptake (see Table 2) more direct evidence for uptake inhibition is required than the absence of transformation in the presence of PEA.

Such evidence was obtained from an experiment identical to the one described in Fig. 4, but using tritium-labelled transforming DNA. In this experiment, both the frequency of transformation pattern and the radioactive DNA uptake pattern were determined, but since the former was essentially identical to that in the previous figure, only the radioactive DNA uptake data are plotted in Fig. 5. It is evident from Fig. 5 that PEA blocked DNA uptake by competent *Bacillus subtilis*.

Fox & Hotchkiss (1957) consider DNA uptake (incorporation) to proceed in two steps. Competent organisms first bind DNA in a reversible fashion. Reversibly bound DNA can be removed from organisms by DNase and/or by washing. As a second step, some reversibly bound DNA becomes irreversibly bound. Irreversibly bound DNA cannot be removed from competent organisms by treatment with DNase or by washing.

Table 1. *Reversible and irreversible incorporation of* ^3H *DNA by competent and non-competent organisms*

Cell type	PEA addition	Incorporation (c.p.m. 10^7 viable organisms) Reversible (without DNase)	Ir-reversible (with DNase)	Frequency of transformation
90 min. Competent organisms	None (control)	116	18	$3{\cdot}05 \times 10^{-3}$
	0·3 % with DNA	91	2·0	$4{\cdot}64 \times 10^{-5}$
Non-competent organisms	None	22	0·1	$6{\cdot}4 \times 10^{-6}$

Bacteria were exposed to radioactive DNA for 30 min. One half of each system was assayed for radioactivity before being treated with DNase; the other one half after. DNase-treated organisms were also assayed for frequency of transformation. The marker transformed was *str-r*.

The Fox & Hotchkiss (1957) model of the incorporation of transforming DNA was used to show that PEA blocks irreversible but not reversible incorporation of DNA by *Bacillus subtilis*. Competent organisms were exposed to tritium-labelled DNA in the presence and in the absence of 0·3 % PEA. One-half of the organisms in each system were assayed for radioactive DNA incorporation without being treated with DNase (reversible incorporation), and the other half were assayed for radioactive DNA incorporation after DNase treatment (irreversible incorporation). The DNase-treated organisms were also assayed for frequency of transformation, and the results of the experiment appear in Table 1. The data in Table 1 show that PEA blocked

irreversible incorporation but did not block reversible incorporation. The non-competent control was included in the experiment to demonstrate that higher levels of reversible incorporation were a consequence of competence.

The data thus far presented indicate that PEA blocked transformation in *Bacillus subtilis* by rendering competent organisms incapable of irreversibly incorporating DNA. DNA. The data in Table 1 suggest that PEA affected the competent organisms rather than the transforming DNA, but the data in Fig. 1 to 4 could be explained by an effect of PEA on the DNA rather than the competent organism. Rosenkranz *et al.* (1965*a*) and Richardson & Leach (1967) were unable to demonstrate an interaction between PEA and DNA, and Leach & Richardson (1966) found that exposing transforming DNA to 0·5 % PEA for 2 hr did not affect its transforming ability. This published data confirms the implication of the data in Table 1 that PEA affects the competent organism and not transforming DNA.

Table 2. *Decrease in potential transformants compared to decrease in viable organisms and loss of incorporated ^3H DNA following treatment of potential str-r transformants with 0·3 % PEA*

Treatment	Number of transformants ($\times 10^6$)	Viable organisms ($\times 10^{-7}$)	Frequency of transformation ($\times 10^{-2}$)	Incorporated DNA (c.p.m.)
a	0·68	8·5	0·80	539
b	0·045 (6·6 %)*	6·0 (71 %)*	0·075 (9·4 %)*	489 (91 %)*

(*a*) 90 min. competent organisms exposed to ^3H DNA for 30 min.
(*b*) 90 min. competent organisms exposed to ^3H DNA for 30 min., then exposed to 0·3 % PEA for 30 min.
* Percentage of values before PEA treatment.

To determine if PEA affected the competent organism only by making it impermeable to DNA, organisms with irreversibly incorporated tritium-labelled DNA were exposed to 0·3 % PEA for 30 min. The frequency of transformation of the organism was measured before and after PEA treatment as was the amount of radioactive DNA irreversibly incorporated (Table 2). Table 2 reveals that the frequency of transformation of organisms which have already incorporated DNA was also lowered by treatment with 0·3 % PEA, indicating that simple irreversible incorporation of DNA was not the only facet of transformation affected by PEA. Examination of Table 2 reveals that under the conditions of the experiment a decrease in viable organisms was also observed. The viable count decrease was only 29 %, indicating that random loss of competent organisms could not be responsible for the 93 % reduction in number of transformants. Although there was a 93 % reduction in the number of transformants and a 29 % decrease in the number of viable organisms, there was only a 9 % decrease in the amount of DNA which had been irreversibly incorporated prior to the PEA treatment. This retention of radioactive DNA was compatible with the observation that the cell envelope of PEA-treated organisms was impermeable to DNA.

The experiments described in Fig. 2 to 4 were also performed with the *ind*+ marker. The results of the *ind*+ marker experiments were almost identical to the results in Fig. 2 to 4.

DISCUSSION

Phenethyl alcohol renders competent *Bacillus subtilis* organisms unable to irreversibly incorporate transforming DNA and thereby blocks transformation. In addition, organisms which have incorporated DNA prior to PEA treatment transform at a frequency that is only 9·4% of an untreated control. Whether both consequences of PEA action are the result of a single effect on competent organisms is not clear but the information presented here offers some basis for conjecture.

At least two modes of PEA action could account for a single inhibitory effect on competent organisms. One such mode of action would be a selective killing of competent organisms by PEA. Although 0·3% PEA is only bacteriostatic to a growing population as a whole, it could be bacteriocidal to the competent organisms in the population. The greater decrease in the number of transformants than in the viable count is consistent with the hypothesis of selective killing of competent organisms. However, the smaller decrease in frequency of transformation experienced when bacteria which had incorporated DNA were treated with PEA suggests that some process other than simple killing of competent organisms is involved. A second mode of PEA action which would explain both consequences of PEA treatment would be an impermeability of PEA-treated competent organisms to transforming DNA. Competent *Bacillus subtilis* incorporate more DNA than is immediately used to produce transformants (Bodmer & Ganesan, 1964; Pene & Romig, 1964) and prior to its integration, the irreversibly incorporated DNA is 'stored', either outside the membrane (Kammen, Wojnar & Canellakis, 1966) or in association with the membrane (Erickson & Braun, 1968; Urban 1968). It can be assumed that 'stored' DNA serves as a source of DNA for integration for the 3–5 hr during which *B. subtilis* organisms remain competent (Nester & Stocker, 1963; Nester, 1964; Kammen, Wojnar & Canellakis, 1966). If this occurs, the frequency of transformation could be lowered by merely making irreversibly incorporated, 'stored' DNA unavailable for subsequent integration. It is the making of 'stored' DNA unavailable for integration, for instance by fixing the DNA in its proposed membrane associated 'storage' site, that could be the mode of PEA action. The 'storage' of DNA is probably associated with irreversible incorporation so that an inhibition of 'storage' would probably be reflected in an inhibition of irreversible incorporation also.

Of the many possible explanations for the effect of PEA on transformation described here, the most plausible hypothesis suggests a modification of the membrane that results in impermeability to newly added DNA and the trapping of 'stored' DNA. The membrane has been involved in 'storage' of incorporated DNA, and PEA affects the cytoplasmic membrane; therefore, an effect of PEA on the cytoplasmic membrane of the competent *Bacillus subtilis* could be responsible for our observations. In our laboratory we have been unsuccessful in demonstrating DNA fixation in membranes isolated and purified from bacteria treated with PEA after irreversibly incorporating DNA; however, it is recognized that isolated cell membranes could be expected to react different from the *in vivo* situation.

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REFERENCES

BERRAH, G. & KONETZKA, W. A. (1962). Selective and reversible inhibition of the synthesis of bacterial deoxyribonucleic acid by phenethyl alcohol. *J. Bact.* **83**, 738.

BODMER, W. F. & GANESAN, A. T. (1964). Biochemical and genetic studies of integration and recombination in *Bacillus subtilis* transformation. *Genetics* **50**, 717.

BRUCHOVSKY, N. & TILL, J. E. (1967). Effects of β-phenethyl alcohol on mouse L cells in suspension culture. I. Reversible inhibition of cell proliferation and effects on the uptake of labelled precursors of nucleic acid and protein. *Molec. Pharmacol. (U. S.)* **3**, 124.

BRUCHOVSKY, N., MAK, S. & TILL, J. E. (1967). Effects of β-phenethyl alcohol on mouse L cells in suspension culture. II. Effects on the cell division cycle. *Molec. Pharmacol (U. S.)* **3**, 133.

ERICKSON, R. & BRAUN, W. (1968). Relationship between transformation frequencies of different markers and the stage of DNA replication of recipient cells. *Bact. Proc.* p. 60.

FARMER, J. L. & ROTHMAN, F. (1965). Transformable thymine-requiring mutant of *Bacillus subtilis*. *J. Bact.* **89**, 262.

FOX, M. S. (1957). Deoxyribonucleic acid incorporation by transformed bacteria. *Biochim. biophys. Acta* **26**, 83.

FOX, M. S. & HOTCHKISS, R. D. (1957). Initiation of bacterial transformation. *Nature, Lond.* **179**, 1322.

KAMMEN, H. O., BELOFF, R. H. & CANELLAKIS, E. S. (1966). Transformation in *Bacillus subtilis*. I. Role of amino acids in stabilization of transformants. *Biochim. biophys. Acta* **123**, 39.

KAMMEN, H. O., WOJNAR, R. J. & CANELLAKIS, E. S. (1966). Transformation in *Bacillus subtilis*. II. The development and maintenance of the competent state. *Biochim. biophys. Acta* **123**, 56.

LARK, K. G. & LARK, C. (1966). Regulation of chromosome replication in *Escherichia coli*: A comparison of the effects of phenethyl alcohol treatment with those of amino acid starvation. *J. molec. Biol.* **20**, 9.

LEACH, F. R. & RICHARDSON, A. (1966). Inhibition of *Bacillus subtilis* transformation by phenethyl alcohol. *Fedn Proc. Fedn Am. Socs exp. Biol.* **25**, 735.

LEACH, F. R., BEST, N. H., DAVIS, E. M., SANDERS, D. C., & GIMLIN, D. M. (1964). Effect of phenethyl alcohol on cell culture growth. I. Characterization of the effect. *Expl. Cell Res.* **36**, 524.

LERMAN, L. S. & TOLMACH, L. J. (1957). Genetic transformation. I. Cellular incorporation of DNA acompanying transformation in *Pneumococcus*. *Biochim. biophys. Acta* **26**, 68.

LESTER, G. (1965). Inhibition of growth, synthesis, and permeability in *Neurospora crassa* by phenethyl alcohol. *J. Bact.* **90**, 29.

LEVINE, J. S. & STRAUSS, N. (1965). Lag period characterizing the entry of transforming deoxyribonucleic acid into *Bacillus subtilis*. *J. Bact.* **89**, 281.

LILLEY, B. D. & BREWER, J. H. (1953). The selective antibacterial action of phenylethyl alcohol. *J. Am. pharm. Ass. (Scientific ed.)* **42**, 6.

LIN, S. Y., MOSTELLER, R. D. & HARDESTY, B. (1966). The mechanism of sodium fluoride and cycloheximide inhibition of haemoglobin biosynthesis in the cell-free reticulocyte system. *J. molec. Biol.* **21**, 51.

MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. molec. Biol.* **3**, 208.

MENDELSON, N. H. (1965). β-phenethyl alcohol inhibition of *Bacillus subtilis*. *Bact. Proc.* p. 17.

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

NESTER, E. W. & STOCKER, B. A. B. (1963). Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis*. *J. Bact.* **86**, 785.

PENE, J. J. & ROMIG, W. R. (1964). On the mechanism of genetic recombination in transforming *Bacillus subtilis*. *J. molec. Biol.* **9**, 236.

PLAGEMANN, P. G. W. (1968). Phenethyl alcohol: reversible inhibition of synthesis of macromolecules and disaggregation of polysomes in rat hepatoma cells. *Biochim. biophys. Acta* **155**, 202.

RICHARDSON, A. G. & LEACH, F. R. (1967). The effect of phenethyl alcohol on the competent *Bacillus subtilis* cell. *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**, 865.

ROSENKRANZ, H. S., CARR, H. S. & ROSE, H. M. (1964). Phenethyl alcohol and messenger RNA. *Biochem. biophys. Res. Commun.* **17**, 196.

ROSENKRANZ, H. S., CARR, H. S. & ROSE, H. M. (1965*a*). Phenethyl alcohol. I. Effect on macromolecular synthesis of *Escherichia coli*. *J. Bact.* **89**, 1354.

ROSENKRANZ, H. S., CARR, H. S. & ROSE, H. M. (1965*b*). Phenethyl alcohol. II. Effect on thymine-requiring *Escherichia coli*. *J. Bact.* **89**, 1370.

ROSENKRANZ, H. S., MEDNIS, A., MARKS, P. A. & ROSE, H. M. (1967). Metabolic effects of phenethyl alcohol on mammalian cells. *Biochim. biophys. Acta* **149**, 513.

SILVER, S. & WENDT, L. (1967). Mechanism of action of phenethyl alcohol: Breakdown of the cellular permeability barrier. *J. Bact.* **93**, 560.

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

THORNE, C. B. (1962). Transduction in *Bacillus subtilis*. *J. Bact.* **83**, 106.

TREICK, R. W. & KONETZKA, W. A. (1964). Physiological state of *Escherichia coli* and the inhibition of deoxyribonucleic acid synthesis by phenethyl alcohol. *J. Bact.* **88**, 1580.

URBAN, J. E. (1968). *The effects of phenethyl alcohol on genetic transformation of Bacillus subtilis*. Ph.D. thesis; The University of Texas at Austin.

YOUNG, F. E. & SPIZIZEN, J. (1961). Physiological and genetic factors affecting transformation of *Bacillus subtilis*. *J. Bact.* **81**, 823.

ZAHN, R. K., TIESLER, E., HEICKE, B., HANSKE, W., FORSTER, W., HOLLSTEIN, H. & WALTER, H. (1966). Cellular division and cellular volume distribution in the presence of 2-phenyl-ethanol and some of its derivatives. *Nature, Lond.* **212**, 293.

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Growth of Rumen Bacteria on Plant Cell Wall Polysaccharides

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SUMMARY

Three strains of *Butyrivibrio fibrisolvens* were isolated from the rumen contents of cattle feeding on red (*Trifolium pratense* L.) or white (*T. repens* L.) clover. The substrates used in these isolations were plant hemicellulose fractions other than simple insoluble xylan. The strains showed some differences in their ability to grow on various plant polysaccharides and to secrete polysaccharases specific to these polymers. The same type of rumen contents yielded, on polygalacturonic acid media, a strain of *Lachnospira multiparus* which grew only on pectin and secreted as sole polysaccharase a polygalacturonase. Only one of the three *B. fibrisolvens* strains grew vigorously on polygalacturonic acid and its polygalacturonase appeared to be different to that of the *L. multiparus*.

INTRODUCTION

The main polysaccharides of pasture plant cell walls are cellulose and xylan and many studies have been made on the action of rumen bacteria on these compounds. Thus strains of *Butyrivibrio fibrisolvens*, *Bacteroides succinogenes*, *B. ruminocola*, *Ruminococcus* spp. and *Clostridium lochheadii* from the rumen have been shown to utilize one or both of these polymers (see Hungate, 1966). Other polysaccharides associated with plant cell walls, but usually present in smaller amounts, include pectin and polymers containing galactose, mannose, rhamnose and uronic acids as well as the pentoses, arabinose and xylose. The insoluble xylan commonly used in the isolation of rumen hemicellulose fermenters lacks, for instance, the complex galactose-rich branched, hemicellulose-B type polymers and it is likely that previous isolations of xylan-fermenting bacteria have missed fermenters of 'branched-B' type polysaccharides. The action of rumen bacteria on these latter compounds does not appear to have been investigated to any extent except in studies using preparations of mixed bacteria (Gaillard, Bailey & Clarke, 1965). We have now prepared galactose-rich fractions ('branched-B' hemicellulose of Gaillard, 1965) from clover and grass in sufficient amounts for cultural studies and, using media containing these polysaccharide fractions, we have isolated bacteria from the rumens of cattle. Bacteria have also been isolated from similar rumen contents using media containing free pentose and pectin polygalacturonic acid respectively.

The bacterial strains growing on these hemicellulose fractions would be expected to produce a mixture of extra- or intracellular carbohydrases capable of hydrolysing each polysaccharide. The mixture of carbohydrases produced is of interest not only in understanding the bacterial breakdown of the polymers, but also as a possible tool in

examining polysaccharide structure. The fermentation properties, occurrence of intracellular carbohydrases and other characteristics of these bacteria have, therefore, been examined and form the subject of this paper.

METHODS

Animals. Two dry cows fitted with permanent rumen cannulae were used. One cow (no. 35) was stall-fed red clover (*Trifolium pratense* L.) hay, while the other (no. 51) grazed a pasture of mixed ryegrass (*Lolium perenne* L.) + white clover (*T. repens* L.).

Carbohydrates. Monosaccharides, disaccharides and soluble starch were the usual commercial sugars. Laminarin, larch galactan, lichenin, araban and polygalacturonic acid (P.G.A.) were from Koch-Light Ltd. Insoluble xylan was from Mann Research Laboratories, New York, citrus pectin from Sigma Chemical Co., St Louis, U.S.A., and guaran from an unknown commercial source. Isolichenin was donated by Dr D. H. Hutson. Finely ground cellulose was prepared from cotton wool by the pebble-milling technique of Hungate (1950). Wheat flour pentosan (water soluble arabino-xylan) was prepared from wheat flour (Howard, 1957), barley β-glucan from barley meal (Parrish, Perlin & Reese, 1960) and lupin galactan from blue lupin seeds (Hirst, Jones & Walder, 1947). Chromatograms of acid hydrolysates (N-H_2SO_4, 2 hr, 100°) of the two latter polysaccharides showed the presence of small amounts (1 to 2 %) of pentoses as well as the expected glucose or galactose. A similar acid hydrolysate of the polygalacturonic acid showed no sign of any neutral sugars but a hydrolysate of the citrus pectin contained both galactose and arabinose (estimated > 1 to 2 %).

Hemicellulose fractions were prepared from ryegrass (*L. perenne* L.) leaves, corn (*Zea mais* L.) stems and red clover (*T. pratense* L.) stems as follows. Ground, freeze-dried plant tissue was extracted either by successively boiling with ethanol (80 %, v/v), water and ammonium oxalate (0·5 %, w/v) or by boiling with neutral detergent (Gaillard, 1966) to give a cell wall (hemicellulose + cellulose − lignin) residue. Hemicellulose was dissolved from this residue by overnight extraction with potassium hydroxide under nitrogen (10 %, w/v). Hemicellulose-A xylan was removed from the 10 % KOH filtrate by acidification with acetic acid to pH 4·5 and centrifugation, after which the supernatant was dialysed and freeze-dried to give hemicellulose-B. This hemicellulose-B was fractionated (Gaillard, 1965) by dissolving in strong (S.G. 1·3) calcium chloride and treating with iodine-potassium iodide solution to give ultimately 'linear-B' arabino-xylan and 'branched-B' heteroglycan. (The monosaccharide compositions of these fractions are given in Table 3.)

Bacteria. Bacteria were isolated from rumen contents by the anaerobic method of Hungate (1950). All manipulations were done in a stream of CO_2 freed of O_2 by passage over reduced copper turnings at 460°. Inoculation of the original roll-tube series and subsequent transfers were made through the rubber bung with 1 ml. hypodermic syringes.

The medium used for isolation, growth and subsequent maintenance of the isolates had the following composition: mineral salt solution, 10 ml.; centrifuged rumen fluid, 20 ml.; distilled water, 70 ml.; $NaHCO_3$, 0·5 g.; cysteine-HCl, 0·02 g.; resazurin, 0·001 g.; $(NH_4)_2SO_4$, 0·1 g.; tryptone (Difco), 0·1 g. Specific carbohydrates, 0·05–0·10 g. were added to this basic medium and agar, 1·1 % (w/v), was added when required. The salt solution contained (g./100 ml.): K_2HPO_4, 1·5 g.; NaCl, 6·0 g.; $MgSO_4.7H_2O$,

0·05 g.; $CaCl_2$, 0·05 g. Centrifuged rumen fluid was obtained by centrifuging strained rumen contents for 20 min. at 10,000 *g*.

Maintenance of cultures. Cultures in current use were maintained on the medium on which they were isolated, subculturing being carried out every 3 days. Stock cultures were maintained on agar at $-70°$.

Fermentation of carbohydrates. The basal medium was the same as that used for the isolation of the bacteria. Fermentation of the carbohydrate substrates was tested in the basal medium with 0·05 % (w/v) added carbohydrate. The inoculum for each test was one drop of a 24 hr culture in the basic liquid medium with glucose. Growth was assessed after 24 and 48 hr incubation at 37°.

Fermentation products. Cultures for fermentation analysis were grown in 150 ml. medium (250 ml. round-bottomed flasks) under an atmosphere of 100 % CO_2. The medium used was the basic medium with 0·1 % (w/v) glucose as carbon source. After incubation for 48 hr the cells were removed by centrifugation and the medium deproteinized by precipitation with $ZnSO_4$ and $Ba(OH)_2$.

Volatile fatty acids were quantitatively determined after steam distillation in a Markham (1942) apparatus by both gas-liquid chromatography on the apparatus of James & Martin (1952) and column partition chromatography on Celite (Swim & Utter, 1957). Lactic acid was measured by the colorimetric method of Barker & Summerson (1941). A mixture of lactic and succinic acids was also separated by chromatography on celite and after measuring lactic acid in the mixture the succinic acid concentration obtained by difference. Ethanol was determined by Conway's (1957). micro-diffusion method

Bacterial extracts. Bacterial cells from cultures (500 ml.) grown in 1 l. round-bottom flasks were harvested by centrifugation (20,000 *g*) and disrupted with Ballotini beads (no. 14) in citrate buffer (0·1 M, pH 6·0) in a Nossal (1953) shaker. After removal of cell debris by centrifugation (20,000 *g*) the supernatant (5 to 10 ml. from 2 to 5 g. wet wt of cells) was dialysed at 0° for 24 hr against the same citrate buffer. A portion of the centrifuged, cell-free culture fluid was also similarly dialysed.

Carbohydrase activity. Enzyme digests containing bacterial extract (0·2 ml.) or dialysed cell-free culture fluid (0·5 ml.), citrate buffer (0·1 M, pH 6·0, 0·2 ml.) and polysaccharide (2 to 4 mg.) were incubated for 24 hr at 37° under toluene and then analysed for liberated sugars by paper chromatography. Chromatograms were developed with ethyl acetate + water + pyridine (2 + 2 + 1) and sprayed with aniline hydrogen phosphate (Howard, 1957) reagent.

RESULTS

Characterization of the isolates cultured

Four anaerobic bacteria (PC/101, 102, 103 and 104) were isolated on four substrates (arabinose, corn 'branched-B', clover 'branched-B' and P.G.A.). These bacteria were the only ones which grew on the substrates used, although only one attempt was made to isolate bacteria on each substrate. The characteristics of the bacteria isolated are given below. The standard fermentation reactions are shown in Table 1.

Isolate PC/101. This organism was isolated from cow 35 at a dilution of 10^{-8} on a medium containing arabinose. It was a Gram-negative, motile, curved rod, 1·0 to 3·5 $\mu \times 0{\cdot}5\ \mu$, occurring singly, in pairs and short chains. In arabinose roll-tubes it

grew as a diffuse, rhizoid colony, 0·5 to 1·0 mm. in diameter. Growth in arabinose or glucose liquid medium was in the form of a flocculent sediment.

Glucose was fermented with the production of gas and butyric, acetic, formic and lactic acids in the ratio 2:1:2:3.

Isolate PC/104. This organism was isolated from cow 35 at a dilution of 10^{-8} on a medium containing 'branched-B' heteroglycan from corn. It was a Gram-negative, motile, curved rod, similar to isolate PC/102 and measured 1·0 to 3·5 $\mu \times 0{\cdot}5\ \mu$. Growth in xylan or glucose liquid medium was in the form of small ropey clumps which flocculated as the culture aged.

Table 1. *Carbohydrates fermented by strains of rumen bacteria*

A positive sign indicates that growth occurred. A negative sign indicates that no growth occurred. All strains failed to grow on lactic acid, mannitol and glycerol.

	Bacterial isolates			
Carbohydrate	PC/101	PC/104	PC/103	PC/102
Glucose	+	+	+	+
Galactose	+	+	+	−
Maltose	+	+	+	−
Mannose	+	−	+	−
Xylose	+	+	+	−
Arabinose	+	+	+	−
Fructose	+	+	+	+
Sucrose	+	+	+	+
Cellobiose	+	+	+	+
Melibiose	+	+	+	−
Starch	+	+	+	−

Glucose was fermented with the production of gas, and volatile acid, predominately butyric. A small amount of lactic acid was also produced.

Isolate PC/103. This organism was isolated from cow 51 at a dilution of 10^{-8} on a medium containing 'branched-B' heteroglycan from red clover. It was a Gram-negative, motile, curved rod, 1·0 to 3·7 $\mu \times 0{\cdot}5\ \mu$, occurring singly, in pairs and chains up to 60 μ in length. Growth in xylan liquid medium was flocculent.

Butyric acid together with acetic, formic and lactic acids, was produced from glucose. A small amount of gas was also produced.

Isolate PC/102. This organism was isolated from cow 35 at a dilution of 10^{-9} on a medium containing polygalacturonic acid (P.G.A.). It was a weakly Gram-positive, motile rod, usually curved, 1·5 to 3·0 $\mu \times 0{\cdot}3\ \mu$, occurring singly and in pairs with occasional long chains. Colonies on glucose agar were filamentous and about 1 mm. in diameter. The end-products produced from glucose were predominantly acetic and lactic acids. Propionic and succinic acids were not produced. Some CO_2 was formed.

Identity of the isolates cultured

Isolates PC/101, 103, 104 which were morphologically identical were identified as strains of *Butyrivibrio fibrisolvens* (Bryant & Small, 1956*a*). All strains produced butyric, acetic, formic and lactic acids, the proportions varying with each strain but butyric acid always being produced in large amounts. The standard carbohydrate

fermentation pattern of each strain was the same except that isolate PC/104 did not ferment mannose.

Isolate PC/102 was identified as a strain of *Lachnospira multiparus* (Bryant & Small, 1956*b*) differing from the type strain in that acetic acid was the major product and only traces of formic acid and ethanol were formed.

Growth of the isolated bacteria on polysaccharides. Each organism was inoculated into tubes of the standard medium containing various plant polysaccharides as carbohydrate source. After incubation for 48 hr at 37° growth was assessed visually. The results obtained are summarized in Table 2.

Table 2. *Growth of isolated rumen bacteria on plant polysaccharides*

Standard media contained added polysaccharide (0·05 %) and inoculated tubes were incubated for 48 hr at 37°. Growth was graded on the basis of: −, no growth; ±, doubtful growth; +, ++, moderate and heavy growth respectively; n.t., not tested because of lack of substrate.

	Bacterial isolates			
Polysaccharide	PC/101	PC/104	PC/103	PC/102
Xylan	++	++	++	−
Wheat flour pentosan	++	++	++	−
Grass 'branched-B' heteroglycan	++	++	n.t.	−
Clover 'branched-B' heteroglycan	n.t.	+	++	−
Laminarin	±	−	−	−
Lichenin	−	+	++	±
Barley β-glucan	(+)*	(++)*	++	−
Cellulose	−	−	−	−
Lupin galactan	±	+	+	−
Larch galactan	+	+	−	−
Guaran	−	−	+	−
Polygalacturonic acid	−	−	++	++

* Possibly growth on pentosan impurity.

Hydrolysis of polysaccharides by bacterial extracts and culture fluids

Cell extracts prepared from the organisms grown on various carbohydrates were tested for polysaccharase activity against a range of plant polysaccharides. The results are summarized in Table 3. With one exception the carbohydrase activity demonstrated involved the liberation of monosaccharides by cell extracts prepared from cells harvested and disrupted without any attempt to exclude oxygen. The exception was the polygalacturonase activity of *Butyrivibrio fibrisolvens* (PC/103). While chromatograms of digests containing P.G.A. and cell extract from *Lachnospira multiparus* (strain PC/102) showed a strong spot corresponding to galacturonic acid indicative of polygalacturonase this was not the case with similar extracts of the butyrivibrio. When the butyrivibrios were, however, harvested with the exclusion of oxygen and incubated intact under toluene with P.G.A. galacturonic acid was liberated. No sign of such a hydrolysis could be obtained with extracts or cell-free culture fluid even though the organisms were extracted as far as possible in the absence of oxygen. A digest containing P.G.A. with culture fluid, extract and debris combined did, however, show the liberation of a possible digalacturonic acid but no free galacturonic acid.

Dialysed cell-free culture fluids from the cultures were also tested for polysaccharase

Table 3. *Hydrolysis of polysaccharides by cell extracts from isolated rumen bacteria*

Digests containing cell extract (0·2 ml.), citrate buffer (0·1 M, ph 6·0, 0·2 ml.) and polysaccharide (2 mg) were incubated at 37° for 24 hr and analysed for liberated sugars by paper chromatography. Visual assessment of sugar spot intensities was used to grade hydrolysis on the following basis: −, no hydrolysis; +, weak intensity spots with incomplete hydrolysis; ++, strong intensity spots suggesting complete hydrolysis. With heteropolysaccharides ++ alone indicates all constituent monosaccharides liberated, monosaccharides named in brackets indicates only these sugars liberated. Ara = arabinose, Xyl = xylose, Gluc = glucose, Gal = galactose, Mann = mannose, GalUr = galacturonic acid, Uronic = unknown uronic acid.

Polysaccharide	Monosaccharide components (ratio)	PC/101 grown on glucose	PC/104 grown on			PC/103 grown on			PC/102 grown on P.G.A.
			Grass 'branched-B'	Lupin galactan	Barley-β-glucan	Red clover 'branched-B'	Lupin galactan	P.G.A.	
Xylan	Xyl, Ara (10:1)	++	++	−	−	+ (Ara)	+ (Ara)	+	−
Wheat flour pentosan	Xyl, Ara (4:1)	++	++	++	+ (Ara)	+ (Ara)	+ (Ara)	+	−
Grass and red clover 'Linear-B' arabino-xylan	Xyl, Ara, Gluc (8:1:1)+ Uronic acid	++	+ I	I I	.	.	.	.	−
Araban	Ara	+	++	++	++	++	++	++	−
Grass 'branched-B' heteroglycan	Xyl, Ara, Gal, Uronic (12:4:1·4:1)	+	++	.	.	.	.	−	−
Red Clover 'branched-B' heteroglycan	Xyl, Ara, Gal, Uronic (1:1·6:2·0:1·2)	+ (Ara)	+ (Ara)	+ (Ara)	++	+ (Ara)	++	+ (Xyl)	−
Laminarin	Gluc	−	+	−	−	−	−	−	−
Barley, β-glucan	Gluc	−	−	−	−	−	+	+	−
Lichenin, cellulose isolichenin, starch	Gluc	−	−	−	−	−	−	−	−
Lupin galactan	Gal	−	−	++	−	+	++	−	−
Larch galactan	Gal	−	−	−	−	−	−	−	−
Guaran	Gal, Mann (1:2)	−	+ (Gal)	−	−	−	−	—	−
Polygalacturonic acid	GalUr	−	−	−	−	−	−	−	++

. = not tested

activities. Apart from an occasional liberation of trace amounts of arabinose (from araban and arabino-xylan) these culture fluids did not liberate any mono- or oligosaccharides from the polysaccharides. Enzyme results appeared anomalous in one case. Although organisms PC/101 and PC/104 appeared to grow on barley β-glucan the extracts liberated pentoses but no glucose from this polysaccharide suggesting that these organisms were only growing on the pentosan impurity.

DISCUSSION

Although an exhaustive search for fermenters of hemicellulose 'branched-B' was not made, the fact that the only two isolates obtained on hemicellulose 'branched-B' in the present work were strains of *Butyrivibrio fibrisolvens*, an organism commonly isolated on xylan, suggests that there is no specific fermenter of the 'branched-B' polymer and that its fermentation is shared among the common xylan fermenting species. Growth of the three *B. fibrisolvens* strains on various polysaccharides and the activities of the polysaccharases extracted from them do, however, point to strain differences. It may be, therefore, that some strains of *B. fibrisolvens* are better able to attack the 'branched-B' polysaccharide than many of the xylan-fermenting strains. It is perhaps relevant to the xylan-fermenting abilities of *B. fibrisolvens* that the three strains always produce pentosanases regardless of the substrate they are grown on, whereas the production of enzymes hydrolysing seed galactan required growth on this polysaccharide.

Pectin is closely associated with hemicellulose and rumen bacteria able to ferment pectin include strains of *Bacteroides succinogenes*, *B. ruminicola*, *Butyrivibrio fibrisolvens*, *Succinivibrio dextrinosolvens* and *Lachnospira multiparus* (see Hungate, 1966). Of these organisms *L. multiparus* has been observed (Bryant *et al.*, 1960) to be abundant in the rumens of cattle fed on legume forages which contain more pectin than grasses, so that it was not surprising that the rumen contents of our cow, fed clover containing 5 to 6% pectin, yielded on the P.G.A. substrate a strain of *L. multiparus*. Carbohydrate, enzyme and growth studies suggest that the strain was highly specific for pectin with a normal polygalacturonase. The only one of the three Butyrivibrio isolates which grew on the P.G.A. was that (PC/103) isolated on the uronic acid-rich hemicellulose 'branched-B' from clover. Growth of this organism on P.G.A. was luxuriant after 48 hr but the pectinases appeared to be different to the Lachnospira polygalacturonase particularly in being difficult to extract.

The present study of hemicellulose fermentation has been confined to four rumen bacteria isolated on media containing specific carbohydrates, these being the only bacteria isolated on these substrates at a single attempt. Other studies have shown that rumen bacteria isolated for example on cellulose (Dehority, 1967) are able to hydrolyse xylan and it may well be that some of these organisms can ferment plant hemicellulose 'branched-B' type polysaccharides. It is likely though that the bacteria isolated directly from the rumen contents using specific carbohydrate substrates are those most actively responsible for the breakdown of these substrates in the rumen. *Butyrivibrio fibrisolvens* has long been accepted as an important organism in the rumens of cattle, particularly with regard to the fermentation of xylan (Hungate, 1966). That it was the only organism isolated on the 'branched-B' fractions is significant from this point of view. The differences in substrate specificity between the three strains studied

are significant with regard to the highly successful adaptation of a single species to fill several niches in a complex nutritional environment.

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REFERENCES

BARKER, S. W. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535.

BRYANT, M. P. & SMALL, N. (1956*a*). The anaerobic monotrichous butyric acid-producing rod-shaped bacteria of the rumen. *J. Bact.* **72**, 16.

BRYANT, M. P. & SMALL, N. (1956*b*). Characteristics of two new genera of anaerobic curved rods isolated from the rumen of cattle. *J. Bact.* **72**, 22.

BRYANT, M. P., BARRENTINE, B. F., SYKES, J. F., ROBINSON, I. M., SHAWVER, C. V. & WILLIAMS, L. W. (1960). Predominant bacteria in the rumen of cattle on bloat provoking ladino clover pasture. *J. Dairy Sci.* **43**, 1435.

CONWAY, E. J. (1957). *Microdiffusion Analysis and Volumetric Error*, 4th ed. (rev.). London: Crosby Lockwood and Son Ltd.

DEHORITY, B. A. (1967). Rate of isolated hemicellulose degradation and utilization by pure cultures of rumen bacteria. *J. appl. Microbiol.* **15**, 987.

GAILLARD, B. D. E. (1965). Comparison of the hemicelluloses from plants belonging to two different plant families. *Phytochemistry* **4**, 631.

GAILLARD, B. D. E. (1966). Calculation of the digestibility for ruminants of roughages from the contents of cell wall constituents. *Neth. J. Agric. Sci.* **14**, 215.

GAILLARD, B. D. E., BAILEY, R. W. & CLARKE, R. T. J. (1965). The action of rumen bacterial hemicellulases on pasture plant hemicellulose fractions. *J. agric. Sci.* **64**, 449.

HIRST, E. L., JONES, J. K. N. & WALDER, W. O. (1947). The constitution of the galactan from *Lupinus albus* seeds. *J. chem. Soc.* p. 1225.

HOWARD, B. H. (1957). Hydrolysis of the soluble pentosans of wheat flour and *Rhodymenia palmata* by ruminant micro-organisms. *Biochem. J.* **67**, 643.

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

HUNGATE, R. E. (1966). *The Rumen and its Microbes*, p. 70. London and New York: Academic Press.

JAMES, A. T. & MARTIN, A. J. P. (1952). Gas-liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. *Biochem. J.* **50**, 679.

MARKHAM, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. *Biochem. J.* **36**, 790.

NOSSAL, P. M. (1953). A mechanical cell disintegrator. *Aust. J. exp. Biol. med. Sci.* **31**, 583.

PARRISH, F. W., PERLIN, A. S. & REESE, E. T. (1960). Selective enzymolysis of poly-β-D-glucans and the structure of the polymers. *Can. J. Chem.* **38**, 2094.

SWIM, H. E. & UTTER, M. F. (1957). Isotopic experimentation with intermediates of the tricarboyxlic acid cycle. *Meth. Enzymol.* **4**, 584.

J. gen. Microbiol. (1969), **56**, 87–97
Printed in Great Britain

Temperature-sensitive DNA Synthesis in a Mutant of *Bacillus subtilis*

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SUMMARY

The properties of a temperature-sensitive mutant of *Bacillus subtilis* in which DNA synthesis is specifically and reversibly inhibited above 40° are described. When incubated at 45° there was no detectable breakdown of DNA in the mutant and the synthesis of deoxynucleoside triphosphates was normal. The temperature-sensitive element affects DNA synthesis *in vivo* but not *in vitro* and (unlike an initiator) it appears to be needed for DNA synthesis throughout the replication cycle.

INTRODUCTION

In the hope that biochemical studies of mutants with abnormal DNA synthesis might reveal new features of the replication mechanism, some temperature-sensitive mutants of *Bacillus subtilis* were isolated and examined. This report describes the properties of one mutant in which DNA synthesis, but not other macromolecular synthesis, ceases at 45°.

METHODS

Chemicals. Nucleosides, nucleotides, nucleoside triphosphates and bromodeoxyuridine were from Sigma Chemicals Ltd., London. Tritiated thymidine (methyl-T, 5 Ci/m-mole), [^{14}C]thymine 58 mCi/m-mole, [^{14}C]thymidine 36 mCi/m-mole, [5-^{3}H] uridine 3·5 Ci/m-mole, [^{14}C]leucine 34 mCi/m-mole and carrier free $H_3{}^{32}PO_4$ were from the Radiochemical Centre, Amersham, Bucks., England. Schwartz BioResearch Inc., New York, supplied [^{3}H]TTP, 4·8 Ci/m-mole. Amino acids were from British Drug Houses Ltd., chloramphenicol from Allen and Hanbury, London.

Bacteria. Mutant strains of *Bacillus subtilis* were obtained by courtesy of the following: 168 *ind*$^-$ *thy*$^-$ (J. Farmer and F. Rothman); 168 *ind*$^-$ (C. Anagnostopoulos).

Spores were grown at 30° on Schaeffer agar plates and were prepared and purified as described by Yoshikawa (1965).

Media. Difco Antibiotic Medium no. 3 (16 g./l.) was used for routine culture and plating and is referred to throughout as broth. Salts medium was Spizizen minimal medium (Anagnostopoulos & Spizizen, 1961) supplemented when necessary with thymidine 5 or 10 μg./ml., L-tryptophan 50 μg./ml. and casein hydrolysate (Oxoid) 200 μg./ml.

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For some experiments a rich defined medium (A.S. medium) was used which consisted of minimal salts medium supplemented with amino acids as in the spore germination medium of Donnellan, Nags & Levinson (1964). It was made as follows: 2 ml. concentrated minimal salts medium (Vogel & Bonner, 1956) plus 5 ml. 10% (w/v) glucose was diluted to 100 ml. with a solution containing L-valine 0·2 g. L-arginine HCl 0·5 g., L-leucine 1·0 g., L-threonine, 0·5 g., L-serine 0·63 g. L-glutamine 4·7 g., L-alanine 1·25 g., L-asparagine 1·5 g./l. The concentrated salts, the glucose and amino acid mixture were autoclaved separately. Thymidine (5 μg./ml.) and tryptophan (50 μg./ml.) were added to the medium when required.

For experiments on ^{32}P-labelled nucleotide pools, bacteria were grown in 1% Difco peptone. A sample of the batch of peptone used was analysed for total phosphate after digestion with perchloric acid. The 1% (w/v) peptone medium contained 0·77 μ-mole phosphate per ml. Thymine-requiring strains grew well in this peptone; additional thymine was not needed.

Assays of DNA, RNA and protein synthesis. The incorporation of TCA-insoluble [^{3}H] or [^{14}C]thymidine, [5-^{3}H]uridine and [^{14}C]leucine was taken to be a measure of the synthesis of DNA, RNA and protein respectively. Duplicate 0·2 ml. samples were taken from the labelled culture at intervals. The samples were frozen immediately in acetone + solid CO_2 and stored at $-20°$. At a convenient time (generally the following day) they were allowed to melt and 3 ml. cold 6% (w/v) TCA was added. After standing at 4° for not less than 20 min. the samples were filtered on 2 cm. Oxoid membrane filters, washed five times with cold TCA, twice with methylated spirit, placed in counting vials and allowed to dry at room temperature. The radioactivity was measured in a Beckman scintillation counter.

Cell extracts for in vitro assays. About 1 g. (wet weight) of bacteria growing exponentially at 30° in 2 l. of broth was collected by centrifugation. The packed organisms were washed once with 0·02 M-phosphate buffer (pH 7) suspended in 20 ml. of the same buffer and incubated for 1 hr. at 30° with 1 mg. lysozyme. The lysate was then centrifuged at 10° for 1 hr at 38,000 *g*. The supernatant fluid was decanted and frozen in CO_2 + acetone and stored at $-20°$.

Assay of DNA polymerase in vitro. Cell extract (0·1 ml. 5 to 8 mg. protein/ml.) was added to 0·9 ml. of 0·01 M-tris HCl buffer (pH 7·2) containing 1·6 μ-mole Na ATP, 2·5 μ-mole $MgSO_4$, 85 μm-mole dAMP, dCMP and dGMP, 7·0 μm-mole [^{3}H]TTP (0·5 Ci/m-mole) and 80 μg native *Bacillus subtilis* DNA, prepared by the method of Marmur (1961) from *B. subtilis* strain 168 *ind.*$^-$. Incubation was in stoppered tubes at 30° or 45°. Samples of 0·025 ml. were removed at intervals on to filter-paper discs (Bollum, 1959) washed five times with 6% TCA, twice with methylated spirit, dried and the radioactivity counted.

Analysis of nucleoside triphosphate pool. (1) [^{3}H]thymidine triphosphate: cultures of ts 230 (2×10^7 cells/ml.) in 10 ml. broth were labelled for 10 min. at 30° or 45° with [^{3}H]thymidine (2 μCi/ml.) after growth at 30° or after one hr at 45°. The organisms were filtered on 2 cm. membrane filters and washed rapidly with 0·5 ml. broth to remove extracellular [^{3}H]thymidine. The filters were covered with 0·8 ml. cold 6% (w/v) TCA and the organisms were suspended by stirring with a fine glass rod. After 30 min. at 4°, the suspension was centrifuged and the supernatant fluid extracted six times with about 3 ml. diethyl ether to remove TCA. Remaining acidity was neutralized with one drop of 0·1 M-trisodium citrate solution. Carrier TMP and TTP

(0·5 mg. of each) was added and 0·1 ml. of this solution was applied to paper strips (Whatman 3 MM) for electrophoresis according to Smith (1955) in 0·05 M-citrate buffer (pH 3·75). After 2 hr. at 24 V./cm. the strips were dried and the u.v.-absorbing regions marked. The strips were then cut into 0·5 cm. sections and their radioactivities determined.

(2) ^{32}P nucleoside triphosphates: the techniques used were basically those of Randerath & Randerath (1964) and Neuhard & Munch-Petersen (1966). Two volumes of 10 ml. were taken from an exponentially growing culture of ts 230 in 1 % peptone at a density of 3×10^7 bacteria/ml. One volume was incubated at 30° and the other at 45° for 30 min., then 0·1 mC $H_3{}^{32}PO_4$ (carrier-free) was added to each culture, to give a final specific activity of 13 μCi/μ-mole phosphate. Incubation was continued at 30° and 45° for 15 min., then the organisms were filtered on 2 cm. membrane filters (Millipore, 0·45 μ) and washed rapidly with 1 ml. of peptone to remove extracellular $^{32}PO_4$. The membrane filters were placed in flat-bottomed tubes containing 1 ml. distilled water, frozen immediately in a CO_2 + acetone bath and stored at −40°.

To extract the ^{32}P-labelled nucleotides, 3 ml. cold 6 % (w/v) TCA was added to the frozen filtered bacteria, followed by 0·01 ml. of a mixture containing 3 μ-mole/ml. of ATP, UTP, GTP, CTP, dATP, TTP, dGTP, dCTP. Then 0·01 ml. M-potassium phosphate buffer, pH 7·0, was added, the mixture was allowed to thaw and kept at 4° for 20 min. before it was passed through a millipore filter (pore size 0·45 μ) and the filtrate was extracted six times with 3 ml. diethyl ether. The extract was made neutral with 0·05 ml. M-NH_4OH and incubated for a few minutes with shaking at 45° to remove traces of ether. It was then freeze-dried. The residue was dissolved in 0·2 ml. distilled water and stored at −40°.

Thin-layer plates (20 × 20 cm.) of polyethylene-imine cellulose were prepared according to Randerath & Randerath (1964). The ^{32}P-labelled extracts (0·005 ml.) were spotted on to the plates together with 5 μl. of a mixture containing 3 μ-mole/ml. of each ribo- and deoxyribo-nucleoside triphosphate. Plates were developed in the solvent system described by Neuhard & Munch-Petersen (1966): in the first dimension, M-acetic acid + M-lithium chloride: in the second dimension, 3 M-ammonium acetate + 5 % (w/v) boric acid, the pH value of the mixture being adjusted to 6·5 with ammonia. After development in the first solvent the plates were dried in a current of cold air and washed for 15 min. in methanol to remove lithium chloride. After the second development, the plates were dried in warm air and examined by u.v. radiation in a dark room. The position of the u.v. absorbing spots was marked and traced on to thin white paper. The plates were then taped to X-ray film (Kodak 'Kodirex' E.P. 6·5 × 8·5) and exposed for 24 hr. Sometimes a longer exposure was needed. If the autoradiograph was satisfactory, the spots previously located by u.v. absorption were excised from the thin-layer plate and their radioactivity counted. The excision was done with a scalpel, after first moistening the spot (ringed in pencil) with a drop of amyl alcohol. Correspondence between the carrier (u.v.-absorbing) and radioactive spots was exact.

Pulse labelling of DNA. The retention of tritiated thymidine in bacteria pulse-labelled at 30° then shifted to 45° was measured as follows: a suspension of strain ts 230 growing in 10 ml. Spizizen salts medium with thymidine 10 μg./ml. and tryptophan 50 μg./ml. was filtered at a density of 3×10^7 bacteria/ml. and suspended in 5 ml. medium without thymidine.

After 3 min. aeration at 30°, 10 μCi thymidine (18·6 Ci/m-mole) were added and aeration continued for 2 min. The bacteria were then collected on a membrane filter, washed and suspended in 16 ml. salts medium. The suspension was divided into four and incubated with and without thymidine (50 μg./ml.) at 30° and 45°. Samples of 0·2 ml. were taken at intervals and the TCA-soluble radioactivity determined.

Isolation of temperature sensitive mutants. Bacillus subtilis ind⁻ thy⁻, grown to a density of 10^7 bacteria/ml. in 100 ml. salts medium, was treated for 2 hr at room temperature with *N*-nitro-*N*¹-nitroso-guanidine 20 μg./ml. A plate count at this stage showed approximately 10% survival. The mutagen was removed by membrane filtration and the bacteria were suspended in broth and kept at 4° overnight. Next morning the culture was revived in broth at 22°, then transferred to 10 ml. salts medium with 50 μg./ml. trytophan, 0·05% (w/v) casein hydrolysate and 20 μg./ml. bromodeoxyuridine. It was then incubated for 1½ hr at 45° (many cells lysed at this stage). The remaining bacteria were collected on a membrane filter, suspended in 10 ml. broth and aerated overnight at 4°. Suitable dilutions were plated to determine the viable count, which was 10^5 bacteria/ml. Seventy-five broth plates were spread, to give about 70 colonies per plate. After 2 days at 22°, replica plates were made (75 plates at 45°, 75 at 22°) and colonies growing at 22° but not at 45° were picked, streaked, then tested again for temperature sensitivity. Of 5000 colonies examined, twenty proved to be temperature sensitive mutants. Six of these showed restricted DNA synthesis at 45°. The most marked effect was in the strain numbered ts 230 and this was chosen for detailed study.

RESULTS

Temperature dependence of DNA synthesis. Figure 1 shows the uptake of [^{3}H]thymidine at 37°, 45° and 49° by the temperature sensitive mutant (a) and the parent strain (b). The bacteria were prelabelled before the temperature shift by growth for more than ten generations in [^{3}H]thymidine-A.S. medium.

RNA and protein synthesis. The uptake of [^{14}C]leucine/[^{3}H]thymidine (Fig. 2) and [5-^{3}H]uridine/[^{14}C]thymine (Fig. 3) was followed in simultaneous labelling experiments. RNA and protein synthesis continued in the absence of DNA synthesis for about 2 hr after the temperature was raised to 45°. In agreement with this observation, bacterial mass, as judged by turbidity, increased about sixfold during this time.

Morphology, viability and cell division. No unusual change in morphology was seen during the first 30 min. after raising the temperature of a log.-phase broth culture to 45°. Between 30 min. and 1 hr the bacteria began to lose motility and to grow in long chains, each organism becoming elongated. After 2 hr they had a contorted appearance and swollen regions could be seen, generally at one end of a bacterium but occasionally at both ends, or in the middle. On further incubation (3 to 5 hr) lysis occurred.

Although the total count increased eightfold during incubation at 45° in broth (Fig. 4) the viable count started to decrease after about half an hour (Fig. 5). In minimal salts medium the behaviour of the mutant was similar to that in broth, but there was less division, elongation and swelling.

Although there was rapid death of vegetative organisms at 45°, there was no loss of viability of ts 230 spores when kept at 45° in distilled water for 3 hr.

The utilization of exogenous thymidine. The contorted filaments formed by ts 230

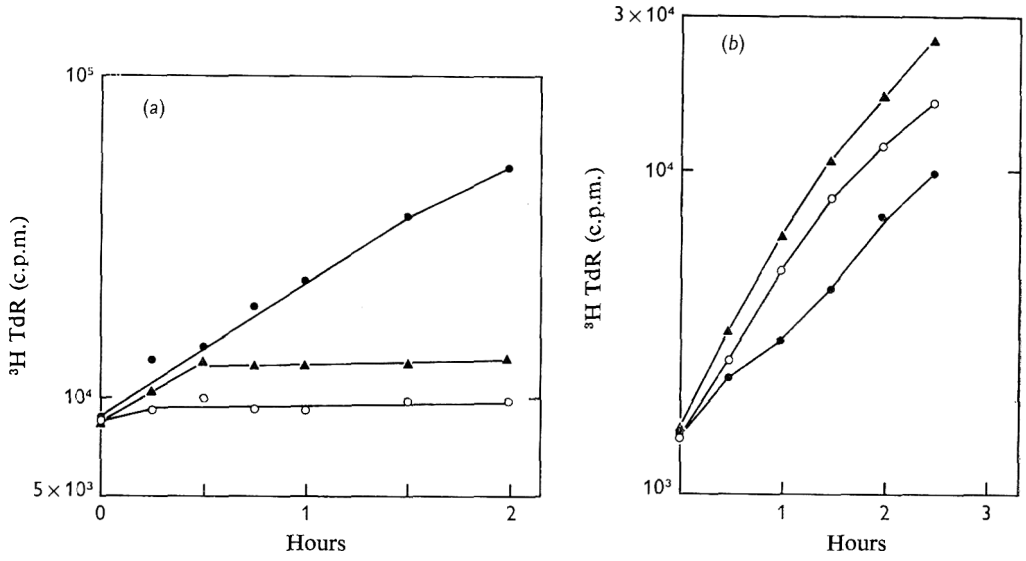

Fig. 1. DNA synthesis in ts 230 (*a*) and 168 ind$^-$ thy$^-$ (*b*). Log.-phase bacteria growing in A.S. medium at 30° were diluted and transferred to 37°, 45° or 49° when the density was 5×10^6 bacteria/ml. 37°, ● ●; 45°, ▲ ▲; 49°, ○ ○.

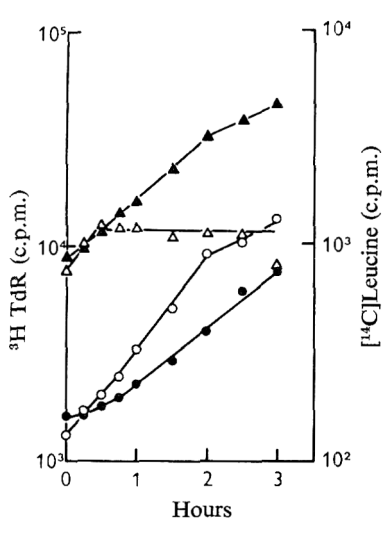

Fig. 2

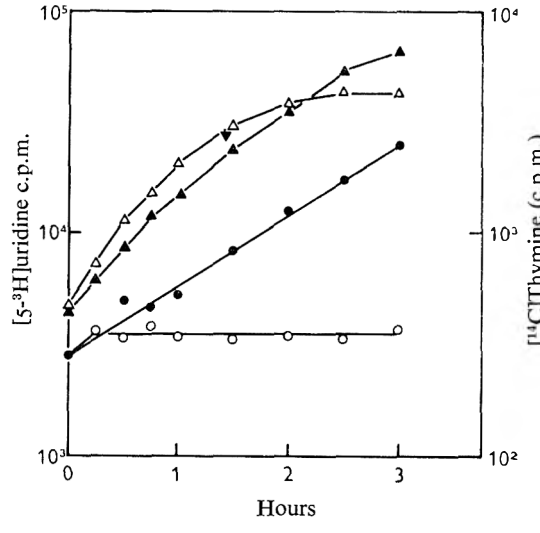

Fig. 3

Fig. 2. Protein and DNA synthesis in ts 230. Cultures prelabelled by growth for many generations in A.S. medium containing [^{14}C]leucine and [^{3}H]thymidine were transferred from 30° to 45° and the simultaneous uptake of leucine and thymidine was measured. The medium contained 0·1 μCi/ml. [^{14}C]leucine and 1·0 μCi/ml. [^{3}H]thymidine with 5 μg./ml. carrier thymidine. Bacterial density at temperature shift 5×10^6/ml. [^{14}C]Leucine 32°, ● ●; [^{14}C]-leucine 45°, ○ ○; [^{3}H] TdR 32°, ▲ ▲; [^{3}H]TdR 45°, △ △.

Fig. 3. RNA and DNA synthesis in ts 230. Bacteria grown for many generations in A.S. medium containing [5-^{3}H]uridine and [^{14}C]thymine were shifted from 30° to 45° and the subsequent uptake of ^{3}H and ^{14}C measured. The medium contained 0·1 μCi/ml. [^{14}C]thymine plus 5 μg./ml. carrier thymine and 1·0 μCi/ml. [5-^{3}H]uridine. Bacterial density at temperature shift was 5×10^6/ml. [^{14}C]Thymine 30°, ● ●; [^{14}C]thymine 45°, ○ ○; [5-^{3}H]uridine 30°, ▲ ▲; [5-^{3}H]uridine 45°, △ △.

at 45° appeared to be similar to those produced by the parent strain of *Bacillus subtilis* when incubated in a rich but thymineless medium (e.g. spore germination medium, Donnellan *et al.* (1964)). This suggested that the temperature sensitive lesion in ts 230 might be one which caused failure either to take up thymidine or to convert it to thymidine triphosphate. To test this possibility, ts 230 was grown in broth for 1 hr at 45°, then tritiated thymidine (2 μc/ml.) was added for 10 min. The bacteria were rapidly collected on membrane filters and the TCA-soluble extract was examined

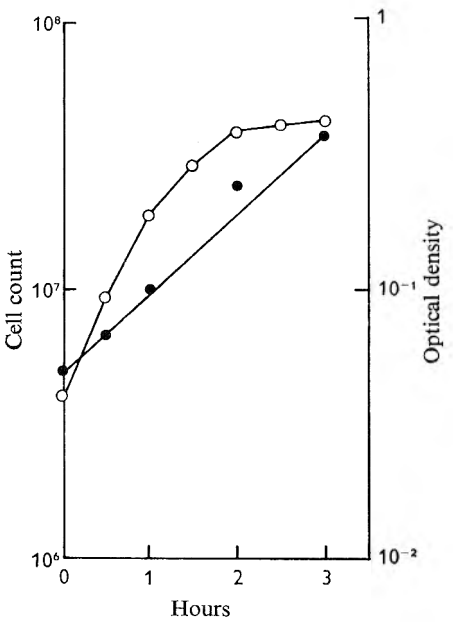

Fig. 4

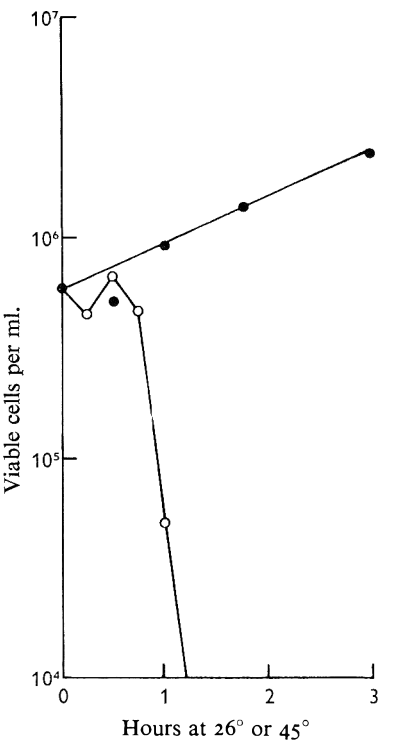

Fig. 5

Fig. 4. Increase of bacterial number and turbidity of ts 230 incubated in broth at 45°. Samples were removed at intervals, shaken with glass beads to disperse aggregates and counted in a Petroff Hauser chamber. Optical density 660 mμ, ○ ○; bacterial count, ● ●.

Fig. 5. Survival of ts 230 incubated in broth at 45°. Suitable dilutions were made and 0·1 ml. of cultures incubated at 26° and 45° were spread on broth plates. Colonies were counted after 2 days at room temperature. Viability at 26°, ● ●; viability at 45°, ○ ○.

by paper electrophoresis to check for the conversion of [^{3}H]thymidine to [^{3}H]thymidine triphosphate. About 70 % of the radioactivity on the strip was located in the TTP band (Fig. 6).

Synthesis of nucleoside triphosphates. Cultures of ts 230 and the non-temperature-sensitive parent strain 168 *thy*$^-$ *ind*$^-$ growing exponentially at 30°, were labelled with ^{32}P as follows: (*a*) ts 230 at 30°, shifted to 45° for 30 min., labelled with $H_3{}^{32}PO_4$ for 15 min. (*b*) ts 230 at 30° labelled with $H_3{}^{32}PO_4$ for 15 min. at 30°, (*c*) 168 *thy*$^-$ *ind*$^-$ at 30° shifted to 45° for 30 min., labelled with $H_3{}^{32}PO_4$ for 15 min. at 45°. After labelling, the bacteria were rapidly collected on membrane filters. Acid-soluble extracts were

prepared and analysed by thin-layer chromatography (see Methods). The radioactive regions located on the chromatographs by autoradiography corresponded exactly to the u.v.-absorbing regions of the carrier (non-radioactive) nucleoside triphosphates. The distribution of radioactivity among the triphosphates is given in Table 1.

It is evident that the pool of nucleoside triphosphates in the mutant held at 45° did not differ significantly from that of the controls (the wild-type at 45° or the mutant at 30°). None of the nucleoside triphosphates present in the controls was absent from the 45° extract; and there were no additional spots, indicative of abnormal nucleotides, on the 45° autoradiograph.

Table 1. *Distribution of radioactivity on chromatograms of nucleoside triphosphates in extracts of ts 230 (c.p.m. ^{32}P).*

	ATP	UTP	GTP	CTP	dATP	TTP	dGTP	dCTP
Ts 230 30°	8477	6504	3656	2764	673	537	379	878
Ts 230 45°	15031	5086	2370	5758	1247	881	702	726
168 ind⁻ thy⁻ 30°	10236	3256	1778	1370	427	1146	439	480
168 ind⁻ thy⁻ 45°	13455	9068	2597	1815	1360	1247	807	571

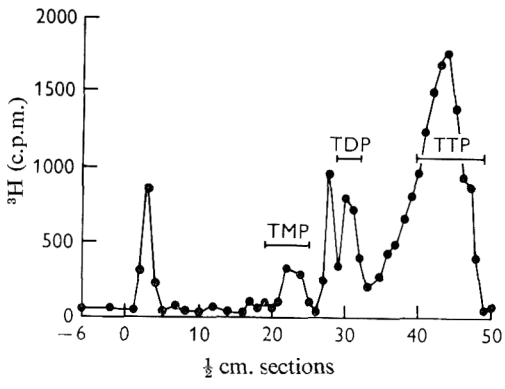

Fig. 6

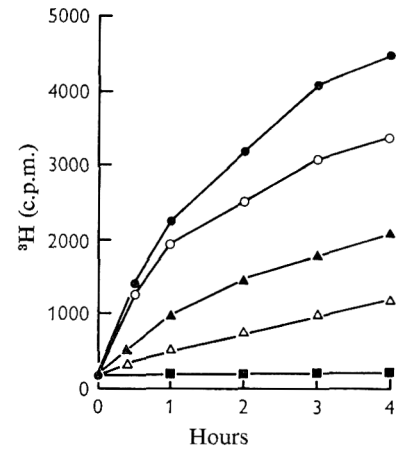

Fig. 7

Fig. 6. Conversion of [^{3}H]thymidine to TMP, TDP, and TTP by ts 230. Bacteria were incubated 1 hr at 45° then labelled for 10 min. at 45° with [^{3}H]thymidine. Distribution of the radioactivity on an electrophoretogram of the TCA-soluble extract is shown. Lines (⊢⊣) show the position of u.v. absorbing bands of non-radioactive TMP, TDP and TTP added to the extract.

Fig. 7. DNA polymerase and deoxynucleotide kinase activity in extracts of ts 230 and 168 ind⁻ thy⁻. The reaction mixtures contained 560 μg./ml. ts 230 protein or 825 μg./ml. 168 ind⁻ thy⁻ protein, 7 μm-mole/ml. ^{3}HTTP (82,000 c.p.m./μm-mole) and other components as given in Methods. The ordinate shows the TCA-soluble radioactivity of 0·025 ml. samples. ts 230 extract incubated at 30°, △ △; ts 230 extract incubated at 45°, ○ ○; 168 ind⁻ thy⁻ extract incubated at 30°, ▲ ▲; 168 ind⁻ thy⁻ extract incubated at 45°, ● ●; 168 ind⁻ thy⁻ extract incubated at 45°; no ATP, ■ ■.

DNA polymerase tested in vitro. Figure 7 shows the incorporation of [^{3}H]TTP into DNA when cell-free extracts of ts 230 and of the parent strain were supplied with [^{3}H]TTP plus deoxynucleotides and ATP. The DNA polymerase was not inactivated by prolonged incubation at 45°. Similar results (not shown) were obtained when the

enzymes were extracted from organisms which had already been incubated for 1 hr at 45°.

Stability of ts 230 DNA at 45°. No loss of TCA-soluble label was observed either from generally labelled or pulse-labelled organisms: samples of $2{\cdot}5 \times 10^6$ bacteria labelled throughout their DNA remained at 5590 ± 130 c.p.m. over 180 min. at 45°. Bacteria pulse-labelled with tritiated thymidine of high specific activity (see Methods) also retained their TCA-insoluble label when incubated at 45°: samples of 4×10^5 bacteria taken between 15 and 60 min. stayed at $12{,}435 \pm 330$ c.p.m.; when incubation at 45° was in medium without thymidine, the count rate stayed at $13{,}030 \pm 360$ c.p.m.

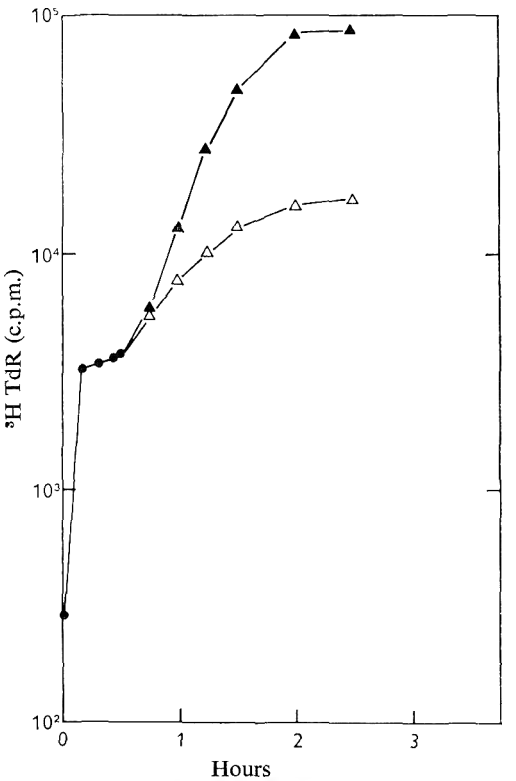

Fig. 8

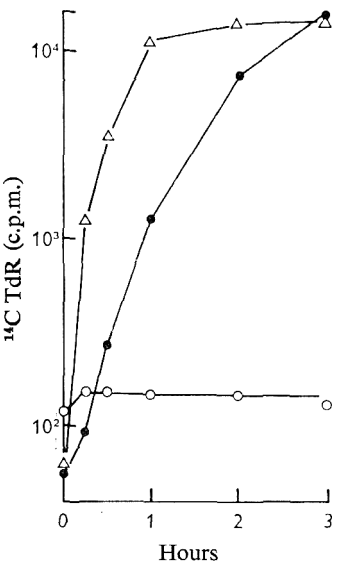

Fig. 9

Fig. 8. Resumption of DNA synthesis at 30° after inhibition at 45°. Log.-phase ts 230 bacteria at a density of 5×10^6/ml. were incubated in A.S. medium (5 μg./ml. TdR) with 2·0 μCi ^{3}H-TdR/ml. for 30 min. at 45°, then divided into two and transferred to 30°. To one half was added 50 μg./ml. chloramphenicol. No chloramphenicol, ▲ ▲; with chloramphenicol, △ △; 30 min. at 45°, ● ●.

Fig. 9. Resumption of DNA synthesis after thymine starvation at 45°. Log.-phase cultures (10 ml.) of ts 230 and 168 ind$^-$ thy$^-$ were grown in broth at 30° until the optical density at 600 mμ was 0·73. The bacteria were collected on membrane filters, washed, and suspended at a density of 5×10^7/ml. in Spizizen salts medium supplemented with 20 μg/ml. tryptophan and 0·04 % casein hydrolysate; they were then aerated at 45° for 15 min. Tubes containing 5 ml. broth and 0·1 μc/ml. [^{14}C]thymidine were inoculated with 0·5 ml. of the thymine-starved ts 230 or 168 ind$^-$ thy$^-$ suspension and were incubated as follows: (*a*) 168 ind$^-$ thy$^-$ at 45°, △ △; (*b*) ts 230 at 45°, ○ ○; (*c*) ts 230°, ▲ ▲. Duplicate 0·25 ml. samples were withdrawn at intervals to determine the incorporation of [^{14}C] thymidine.

Reversibility of the block in DNA synthesis. When a log.-phase culture of ts 230 is shifted from 30° to 45° DNA synthesis is stopped within 30 min. If the temperature is then lowered to 30°, DNA synthesis starts again after a short lag. That the resumption of DNA synthesis does not require the synthesis of new protein is shown by the response in the presence of chloramphenicol (Fig. 8).

Thymine starvation at 45°. The inhibition, at temperatures above 40°, of DNA synthesis in ts 230 could be the result of progressive inactivation of an essential enzyme during the first 15 min. of incubation. If so, 15 min. at 45° in the absence of thymidine would inactivate the enzyme, and subsequent restoration of thymine at 45° would not produce any further DNA synthesis. On the other hand, if the limited amount of DNA synthesis at 45° was the result of the organism's inability to initiate new cycles of replication at that temperature, DNA synthesis should be observed after thymine was restored and it should continue until all cycles, interrupted at the time of thymine starvation, were completed.

An experiment to distinguish between these possibilities is shown in Fig. 9. DNA synthesis after thymine starvation for 15 min. at 45° was zero if thymine was restored at 45°. Control experiments in which thymine was restored to the temperature-sensitive mutant at 30° or to the parent strain at 45°, showed that the 15 min. period of thymine starvation at 45° did not itself abolish the capacity for DNA synthesis when thymine was restored.

DISCUSSION

Bacterial mass and number increase and RNA and protein synthesis continue when ts 230 is incubated at 45°. A non-specific lesion, for example, a change of membrane permeability or a defect in energy metabolism, can therefore be ruled out as a possible cause of the block in DNA synthesis. Of the many possible ways in which a specific block in DNA synthesis could occur, some can be eliminated on the evidence given, while others can be rated as more probable or less probable.

The morphological changes and the loss of viability after a lag period when ts 230 was incubated at 45° were reminiscent of thymineless death in the parent strain. However, the *ts* mutant could form TTP from externally supplied thymidine even after 1 hr at 45° (Fig. 6). Thin layer chromatography of the acid-soluble extracts of ^{32}P-labelled organisms confirmed that the ts mutant was still able to synthesize TTP after 45 min. at 45°, and further showed that the synthesis of dATP, dGTP and dCTP and the ribonucleoside triphosphates was normal at 45°. The temperature-sensitive step in ts 230 is therefore not in the biosynthetic pathways leading to nucleoside triphosphates. The continued activity, at 45°, of the deoxynucleotide kinases in the DNA polymerase assay (Fig. 7) supports this conclusion.

Since the DNA polymerase appeared to function normally at 45° *in vitro*, the mutant is not defective in the ability either to produce DNA substrates or to polymerize them. The defect may be due to inability of the organism's DNA to direct the synthesis of new DNA. Such a situation might be caused by double-strand breaks (Cairns & Davern, 1966) or it might arise if the initiation of new cycles of replication was prevented. These possibilities have been explored in the experiments on the stability of ts 230 DNA at 45° and on the effects of thymine starvation at 45°.

There was no appreciable release of acid soluble nucleotides from bacteria prelabelled for several generations with tritiated thymidine when they were incubated at

45°. Bacteria pulse-labelled at the replicating fork similarly showed no evidence of breakdown of the DNA after a temperature shift. This shows that there was no gross breakdown (e.g. by an uncontrolled deoxyribonuclease) at this temperature. But the production of a small number of single-strand breaks would not have been detected. It is unlikely that double-strand breaks occur in view of the reversibility of the block in DNA synthesis (Fig. 8). There is evidence from studies on the effects of X-rays that *Escherichia coli* cannot repair double-strand breaks in its DNA (Kaplan, 1966). In *Bacillus subtilis*, which is not much more resistant to X-irradiation than *E. coli*, the effect of double-strand breaks would similarly be expected to be irreversible.

If single-strand breaks are the cause of inhibition of DNA synthesis, two possibilities may be considered: (1) single-strand breaks do not occur in the course of normal DNA function, but in ts 230 there is an aberrant enzyme which produces breaks at temperatures above 40°; (2) single-strand breaks do occur and are necessary for normal DNA functions; in ts 230 these breaks are repaired efficiently at 30° but inefficiently or not at all above 40°.

Neither of these possibilities can be discounted at present and to rule them out completely would require very refined experiments. However, it is by no means certain that the presence of single-strand breaks in DNA would necessarily prevent its replication. Indeed, unless the entire bacterial chromosome rotates when the DNA is replicated (and this seems mechanically difficult) a small number of single-strand breaks would be a prerequisite for replication. One might also expect that if breaks were not repaired they would be extended by exonuclease action. Prolonged exonuclease action should lead to appreciable loss of labelled thymine from the DNA but this did not take place.

The increase of DNA after a temperature shift to 45° in A.S. medium (50% in Fig. 1) is close to that expected (40%) for the completion of a cycle of replication and failure to initiate a new cycle. But if ts 230 had a temperature-sensitive initiator, replication cycles which could be completed at 45° should also be completed at 47° or 49°, the same amount of DNA being synthesized at each temperature. This was not found: the amounts of DNA synthesized decreased with increasing temperature (Fig. 1*a*). Further evidence against the theory that ts 230 is an initiator mutant comes from the experiment illustrated in Fig. 9: the bacteria were held for 15 min. at 45° in conditions (absence of thymine) where DNA synthesis was impossible. Synthesis of DNA did resume when thymine was restored at 30°, but not when it was restored at 45°. If the temperature-sensitive lesion was one which allowed DNA synthesis to continue to the end of a cycle, those replication cycles already in progress at the time of the temperature shift should have been completed when thymine was restored. But it is clear that they were not completed. Thus the evidence does not support the hypothesis of a temperature-sensitive initiator; rather, it suggests that some element which is continuously needed for DNA synthesis throughout the replication cycle becomes inactivated above 40° at a rate which increases with temperature.

In the experiments described, all the known components of the DNA replication system have been tested for possible temperature sensitivity, but none of them has proved to be abnormal. The results of the *in vitro* assay of ts 230 DNA polymerase must be interpreted with caution in view of the evidence of de Waard, Paul & Lehman (1965), that not all of the ts mutations mapping in the locus for T4 DNA polymerase

gave enzymes that were inactive *in vitro*. Thus the possibility remains that DNA polymerase may be abnormal in ts 230, present methods of assay being inadequate to detect the abnormality. It now seems very probable that the polymerase responsible for replicating the bacterial chromosome acts at a site on the cell membrane and forms part of a replicative complex through which the DNA duplex passes as it is unwound and copied. Such a complex would include a number of elements, any one of which could be temperature-sensitive *in vivo* without giving any sign of abnormal sensitivity *in vitro*. Our present hypothesis is that ts 230 is a representative of one such class of mutants.

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REFERENCES

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

BOLLUM, F. J. (1959). Thermal conversion of non-priming DNA to primer. *J. biol. Chem.* **234**, 2733.

CAIRNS, J. & DAVERN, C. I. (1966). Effect of ^{32}P decay upon DNA synthesis by a radiation-sensitive strain of *E. coli*. *J. molec. Biol.* **17**, 418.

DONNELLAN, J. E., NAGS, E. H. & LEVINSON, H. S. (1964). Synthetic media for *B. subtilis* spores. *J. Bact.* **87**, 332.

KAPLAN, H. S. (1966). DNA-strand scission and loss of viability after X-irradiation of normal and sensitized bacterial cells. *Proc. natn. Acad. Sci. U.S.A.* **55**, 1442.

MARMUR, J. (1961). A procedure for the isolation of DNA from micro-organisms. *J. molec. Biol.* **3**, 208.

NEUHARD, J. & MUNCH-PETERSEN, A. (1966). Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of *E. coli* during thymine starvation. *Biochim. biophys. Acta* **114**, 61.

RANDERATH, K. & RANDERATH, E. (1964). Ion exchange chromatography of nucleotides on poly-ethyleneimine cellulose thin layers. *J. Chromatog.* **16**, 111.

SMITH, J. D. (1955). The electrophoretic separation of nucleic acid components. In *The Nucleic Acids*. Ed. by E. Chargaff & J. N. Davidson, vol. 1. New York: Academic Press Inc.

VOGEL, H. J. & BONNER, D. M. (1956). Acetylornithinase of *E. coli*: partial purification and some properties. *J. biol. Chem.* **218**, 97.

DE WARD, A., PAUL, A. V. & LEHMAN, I.R. (1965). The structural gene for DNA polymerase in bacteriophages T4 and T5. *Proc. natn. Acad. Sci. U.S.A.* **54**, 1241.

YOSHIKAWA, H. (1965). DNA synthesis during germination of *B. subtilis*. *Proc. natn. Acad. Sci. U.S.A.* **53**, 1476.

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Effect of Polysorbate 80 on Cell Leakage and Viability of *Pseudomonas aeruginosa* Exposed to Rapid Changes of pH, Temperature and Tonicity

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SUMMARY

Leakage of 260 mμ-absorbing cell constituents from *Pseudomonas aeruginosa* was enhanced with increasing concentrations of Polysorbate 80 up to a characteristic maximum at 0·125%. This relationship applied at 4°, 18° or 37° at bacterial concentrations between 1×10^8 and 9×10^8/ml. and for three strains of this organism, but did not apply with *Escherichia coli*. Uptake of a fluorescent dye was also greatest at 0·125% Polysorbate 80. Increasing concentration of NaCl in the suspending liquid progressively decreased leakage. In buffer between pH 5·7 and 9·5, the low degree of leakage varied insignificantly. Polysorbate-treated organisms consistently leaked more readily and also showed greater percentage viability losses on rapidly changing the pH, temperature or NaCl concentration of the suspending solution. There was little correlation between loss of viability and leakage. There was a correlation between loss of viability and the magnitude of the sudden change in any of the colligative properties of the organism's environment. Uptake of Polysorbate from aqueous solution at 20° was rapid. These results supported the hypothesis that Polysorbate 80 had an immediate effect on the permeability barrier of the cell.

INTRODUCTION

Brown & Richards (1964) showed that the non-ionic surface-active agent polyoxyethylene sorbitan mono-oleate (Polysorbate 80) enhanced the antibacterial activity of benzalkonium chloride, chlorhexidine diacetate and polymyxin-B sulphate. Polysorbate 80 acted synergistically with these agents on growth-rate inhibition and on lysis of *Pseudomonas aeruginosa* and *Escherichia coli*: this non-ionic agent alone had no observable effect on these phenomena.

The present work tests the hypothesis that Polysorbate 80 alters cell permeability. This was assessed by measuring the effect of Polysorbate 80 on the leakage of 260 mμ(nm.)-absorbing substances (Salton, 1951; Newton, 1953; Hugo & Longworth, 1964), on entry of a fluorescent dye (Newton, 1954) and on viability of bacteria under stress. Gorrill & McNeil (1960) and Farrell & Rose (1968) have reported the lethal effects of cold shock on *Pseudomonas aeruginosa*. This paper reports work on the effects of rapid changes of each of several colligative properties of the organisms environment. A brief report of part of this work has appeared previously (Brown & Winsley, 1966).

METHODS

Organisms and cultural conditions. The test organism was *Pseudomonas aeruginosa* NCTC 6750. Stationary-phase pseudomonads were obtained by incubation at 37° for 20 hr in batch culture in nutrient broth (Oxoid, No. 1: Oxo Ltd., London, S.E. 1).

Cell leakage experiments. Stationary-phase organisms were harvested by centrifugation at 20° (M.S.E. refrigerated centrifuge, 14 Spenser St., London, S.W. 1.) at 2500 *g*, washed twice in 0·5 M-NaCl solution and finally suspended at 10^8 to 10^9 bacteria/ml. (0·06 to 0·6 mg. dry wt bacteria/ml.) in various aqueous solutions, including NaCl, tris + maleate buffer (Geigy, 1962), Polysorbate 80 (Crillet 4, Croda Ltd., Cowick Hall, Snaith, Goole, Yorkshire) and distilled water alone. Leakage was assessed by centrifuging at 20° samples of the suspensions at 2500 *g* for 20 min. and measuring the extinction at 260 mμ (nm.) of the supernatant fluid with a Unicam S.P. 500 spectrophotometer, corrections being made for the extinction of the suspending solutions.

Effect of Polysorbate 80 on the optical effect of the organisms. The light-scattering properties of bacteria in suspension (measured as extinction at 600 mμ) alter with changes in environmental osmotic pressure; the ability of bacteria to show this alteration (the optical effect) may be regarded as a measure of the integrity of the osmotic barrier of the bacteria (Mager, Kuczynski, Schatzberg & Avi-Dor, 1956; Postgate & Hunter, 1962).

Stationary-phase pseudomonads were grown in broth containing 0–0·175% Polysorbate 80; the bacteria from each culture were then washed and finally suspended separately in plain nutrient broth at a standard concentration; 0·05 ml. samples of each washed suspension were added to 3 ml. quantities of either nutrient broth or aqueous NaCl solutions (0, 0·1 or 0·5 M). After 2 min. at 20°, the extinction of each suspension was read at 600 mμ against a reference cell containing the appropriate suspending liquid.

Effect of Polysorbate 80 on cell permeability to 1-anilinonaphthylamine-8-sulphonic acid (ANS). Newton (1954) used the dye *N*-tolyl-α-naphthylamine-8-sulphonic acid (TNS) to demonstrate the polymyxin-induced changes in the permeability of *Pseudomonas aeruginosa*. In the presence of polymyxin, the dye was able to penetrate to protein-containing parts of the organism, there to form a fluorescent conjugate. This technique was modified to investigate the effect of contact with Polysorbate 80 on cell permeability to a similar dye, ANS (Kodak Ltd., London).

Stationary-phase pseudomonads were cultured in nutrient broth containing 0–0·2% Polysorbate 80; the bacteria from each culture were washed and finally suspended separately at a standard concentration in aqueous 0·5 M-NaCl solution. A control suspension, completely permeable to ANS, was produced by heating at 100° for 30 a sample of the washed suspension grown in polysorbate-free broth.

One ml. samples of the heated and unheated suspensions were each mixed with 3 ml. of 0·5 M-NaCl solution plus 2 ml. of 10^{-3} M-ANS in 0·5 M-NaCl solution. Appropriate bacteria-free blanks were also set up. The maximum fluorescence of each mixture in excitation light at 436 mμ was measured in an Aminco-Bowman Spectrophotofluorimeter at 18°. Each suspension was re-read as above after removal of the bacteria by centrifugation at 2500 *g*.

Viability experiments. A stationary phase culture was divided into two parts; the bacteria from one part were harvested, washed and suspended as above in saline,

while bacteria from the other part were treated in the same way but nutrient broth replaced the saline. Total (chamber) counts were made (coefficient of variation = 4%) on both final suspensions and showed that numbers differed insignificantly. Samples (0·1 ml.) of the saline suspension were added to 99·9 ml. of various suspending liquids held at various temperatures. Viability after dilution was assessed on 0·5 ml. samples taken at intervals, diluted in the same liquid and at the same temperature as that of the sample, and finally plated in triplicate on 10 ml. nutrient agar (Oxoid) spread plates, which were then incubated for 24 hr at 37° (coefficient of variation = 4·3%). The initial 100% viability was taken as that of the saline suspension, diluted immediately in saline at 20°; the viability of the broth suspension was assessed after dilution with broth at 20°.

Uptake of Polysorbate 80. Pseudomonads, harvested and washed in saline as above, were suspended at 5×10^8 bacteria/ml. in aqueous polysorbate 80 solutions at 20°. Samples, removed at intervals, were centrifuged to remove the bacteria. The supernatants were assayed for residual polysorbate by method II of Stevenson (1954).

Surface-tension measurements. The surface tension of solutions of Polysorbate 80 in either water or nutrient broth was assessed by using a de Noüy tensiometer with a 1 cm. diameter platinum wire ring in contact with the liquid surface. Readings for each solution were made at 1 min. intervals up to 10 min. at 18°.

RESULTS

Effect of Polysorbate 80 on cell leakage

Figure 1 shows the leakage patterns from bacteria grown in nutrient broth containing from zero to 0·15% Polysorbate 80, washed, and suspended finally in water at 20°. The 260 mμ-absorbing constituents leaked out at rates decreasing with storage time, but increasing with concentration of polysorbate in the original culture: 0·13% polysorbate allowed the maximum leakage rate; above this concentration the rate decreased. The peak leakage concentration appeared to be 0·125%, which is shown in Fig. 2: the leakage in 3 hr into water at three temperatures was measured from bacteria grown in contact with 0 to 0·25% polysorbate. The relatively high leakage at 4° correlated with the reported sensitivity of *Pseudomonas aeruginosa* to rapid cooling in water (Gorrill & McNeil, 1960). The process of regulation of leakage may be an energy-requiring process (Hamilton, 1968) involving an enzyme system, which is inactivated by low temperatures (Meynell, 1958) or rapid cooling (Farrell & Rose, 1967).

The same relationship between leakage rate and polysorbate concentration occurred with bacteria grown in the absence of polysorbate, but stored for 3 hr at 20° in aqueous polysorbate solutions. The results were essentially the same as in Fig. 2 and the peak leakage concentration was again 0·125%. This value was much higher than the critical micelle concentration (C.M.C.) of Polysorbate 80 in water (*c.* 0·001%: Vidal-Paruta & King, 1964), but nevertheless within a concentration range (0·05% to 0·3%) producing greatest surface-tension depression, either in water or broth solution. Repeating this experiment separately with concentrations of 1×10^8, 5×10^8 and 9×10^8 bacteria/ml. did not significantly alter the peak leakage concentration value of polysorbate 80. This unexpected result could be explained in terms of lack of sensitivity of the technique to detect small changes in the peak leakage concentration. Alternatively, polysorbate

micelles present may be of such a shape and size as to be able to affect more than one bacterium at the higher concentrations. Thus, to affect the permeability properties of the bacteria, polysorbate required neither an environment of salts (cf. the action of polymyxin, Newton, 1953) nor actively dividing bacteria. The leakage experiments were repeated using laboratory strains *Pseudomonas aeruginosa* CST 64 and *P. aeruginosa* OSU 64. The peak leakage concentration and the pattern of leakage was the same for each strain, but the characteristic leakage rates differed. In contrast, leakage under these conditions from *E. coli* showed lesser leakage rates with a less well-marked peak at a lower polysorbate concentration.

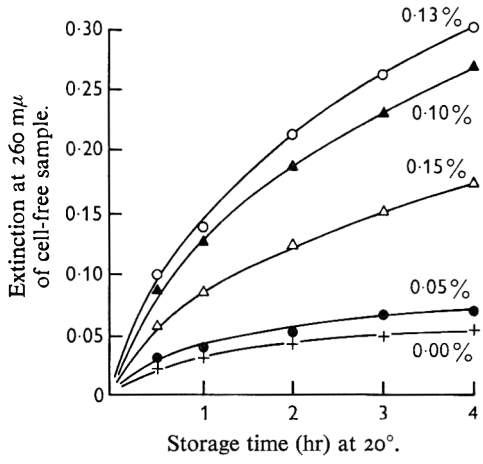

Fig. 1

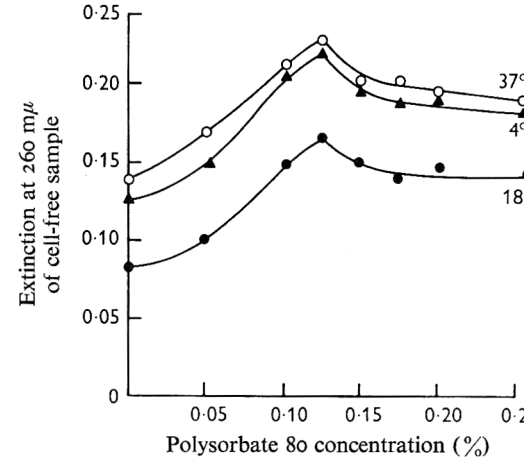

Fig. 2

Fig. 1. 260 mμ leakage during storage in water at 20° of washed pseudomonads grown in presence of Polysorbate 80 (0 to 0·15 %).

Fig. 2. 260 mμ leakage during storage in water at 4°, 18° and 37° of washed pseudomonads grown in presence of Polysorbate 80 (0 to 0·25 %). Same relationship with a peak at 0·125 % occurs for organisms grown in the absence of polysorbate but suspended in solutions as above.

Increasing the NaCl concentration in the suspension liquid of bacteria, grown without or with 0·1 % polysorbate, progressively inhibited the leakage (Fig. 3). However, in each saline solution the bacteria previously exposed to polysorbate leaked more rapidly than the controls. These saline solutions ranged in pH from 5·8 (water) to 6·2 (2 M-NaCl). There was relatively little leakage after storage for 4 hr at 20° in tris-maleate buffer between pH 5·7 and 9·5. At each pH the leakage was about the same as occurred after 4 hr in 0·5 M-NaCl (Fig. 3). In every case polysorbate-exposed cells showed greater leakage.

Effect of Polysorbate 80 on the Optical Effect of the Organisms

Previous contact with polysorbate did not significantly alter the pronounced optical effect of the organisms at various NaCl concentrations. There was no optical effect with heat killed organisms.

The observed lack of influence on the optical effect by polysorbate may have been because there was relatively little viability loss by this treatment with NaCl solutions

(Postgate & Hunter, 1962) and any alteration by polysorbate to the functioning of the cell's osmotic barrier was slight.

Effect of Polysorbate 80 on the permeability to ANS

The fluorescence of the bacteria, and therefore the amount of dye/protein conjugate, increased with polysorbate concentration in the growth medium up 0·125 % (Table 1). That fluorescence depended on the presence of bacteria and not only on leaked contents was shown by the 'after centrifugation' values which exceeded only slightly that of the blanks. Heat-killed bacteria were readily permeable to the dye. It seems unlikely that polysorbate would allow entry into the bacterium of ANS but cause no change in the optical effect. A possible explanation is that polysorbate alters the cell wall/membrane structure and allows uptake of ANS and combination with protein at this site.

Table 1. *Uptake of fluorescent dye by Pseudomonas aeruginosa after growth in broth with Polysorbate 80*

The figures represent fluorescent-intensity as a percentage of intensity of a heated suspension

Polysorbate in broth culture (%)	Fluorescence intensity	
	Before centrifugation	After centrifugation
0	7·4	1·16
0·01	8·4	1·09
0·1	9·0	1·16
0·125	10·4	1·20
0·2	10·0	1·20
Heated bacteria	100	—
Broth control	1·11	1·11
Blank control	1·11	1·11

Uptake of Polysorbate 80 by the pseudomonads

A chemical assay (Stevenson, 1954) of polysorbate was used to study uptake by bacteria; a spectrophotometric assay for this substance, utilizing a u.v. extinction peak at 234 mμ (Parker, Barnes & Bradley, 1966), gave inconsistent results, influenced by the maker's batch of polysorbate. Uptake by organisms from aqueous polysorbate solutions was very rapid (Fig. 4) and was complete in 5 min., the minimum time taken to separate organisms by centrifugation. Although quicker separation of organisms and suspending solution was achieved by Millipore (Millipore (U.K.) Ltd., Heron House, 109 Wembley Hill Road, Wembley, Middlesex) membrane filtration, the uptake of polysorbate by the membrane was not found to be satisfactorily predictable.

Effect of Polysorbate 80 on viability

Gorrill & McNeil (1960) reported loss of viability with *Pseudomonas aeruginosa* suddenly exposed to various aqueous solutions. Figure 5 shows a similar phenomenon occurring after sudden exposure to aqueous sodium chloride solutions. Solutions of 2 M, 0·05 M and 0·02 M were also used, but have been omitted from the figure for clarity. The typical response involved an initial swift drop, followed by a more gradual one: 0·2 M and 0·5 M-NaCl caused similar, minimal losses of viability (Fig. 6).

In each case organisms previously exposed to polysorbate showed greater viability losses. 0·5 ml. of 4 M-NaCl solution spread over and absorbed by an agar plate was not inhibitory to the growth of organisms spread subsequently over the plate. Comparison with the viability of bacteria washed and suspended in broth showed that the washing process with 0·5 M-saline had reduced viability by about 20 %.

Figure 7 illustrates a similar phenomenon. Organisms, washed and suspended in 0·5 M-NaCl solution at 20°, were suddenly exposed to 0·5 M-NaCl solution at

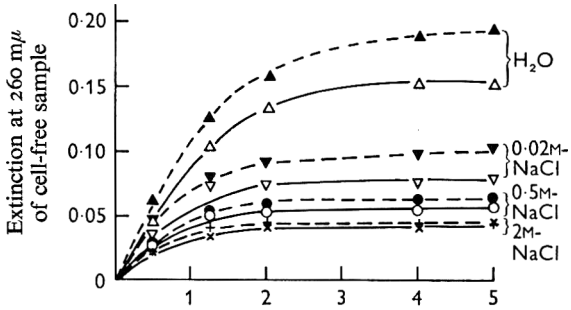

Fig. 3

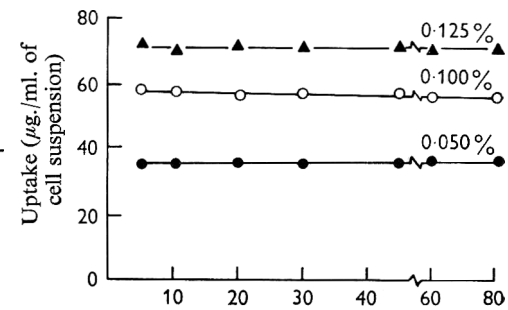

Fig. 4

Fig. 3. 260 mμ leakage during storage at 20° in water or aqueous sodium chloride solutions of washed pseudomonads grown in presence or absence of Polysorbate 80. ——, Organisms grown in broth; – – –, organisms grown in broth + 0·1 % Polysorbate 80.

Fig. 4. Uptake at 20° of Polysorbate 80 from its aqueous solutions (0·05, 0·10 or 0·126 %) by suspensions of washed pseudomonads grown in absence of Polysorbate 80.

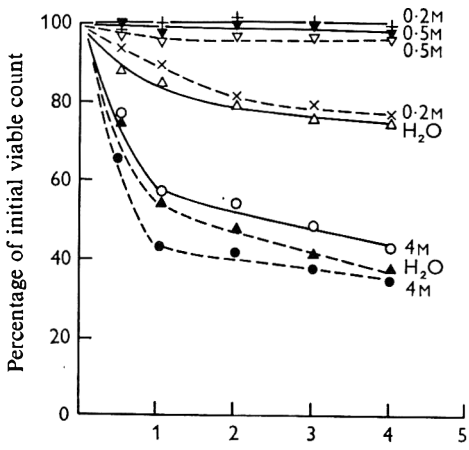

Fig. 5

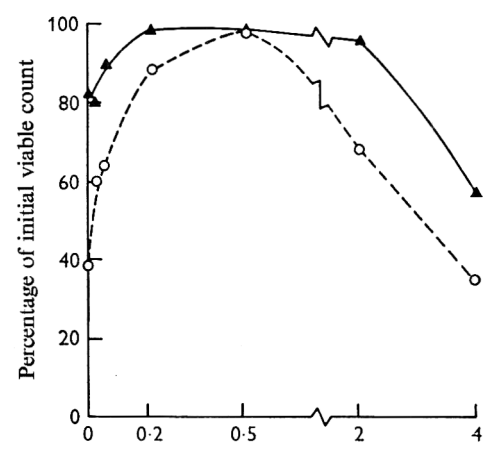

Fig. 6

Fig. 5. The effect at 20° on the viability of washed pseudomonads, grown in presence or absence of Polysorbate 80, of sudden exposure to and storage in water or aqueous sodium chloride solutions (0·2, 0·5 or 4 M).——, Organisms grown in broth; – – –, organisms grown in broth + 0·1 % Polysorbate 80.

Fig. 6. The effect on the viability of washed pseudomonads grown in presence or absence of Polysorbate 80, of sudden exposure to and storage for 1 hr at 20° in various sodium chloride solutions or in water. ——, Organisms grown in broth; – – –, organisms grown in broth + 0·1 % Polysorbate 80.

temperatures between 20° and 40°. Viabilities were assessed on samples after 30 min., 2 hr and 4 hr storage of organisms exposed during growth to 0·1 % polysorbate and of controls, Greatest kill correlated with the greatest sudden temperature increase.

A similar experiment involved organisms shocked by sudden exposure to tris-maleate buffer at between pH 5·7 and 8·8 (Fig. 8). Least loss of viability occurred at pH 7·4 and, in each case, organisms previously exposed to polysorbate were more susceptible.

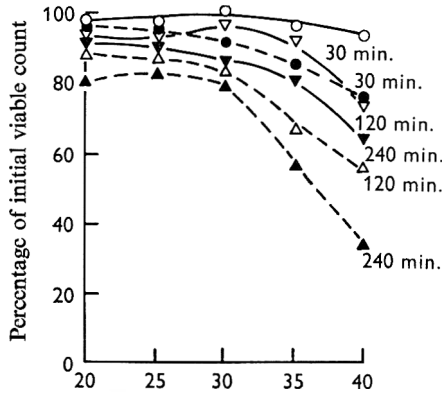

Fig. 7

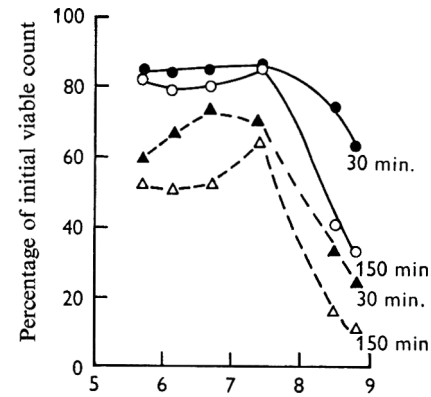

Fig. 8

Fig. 7. The effect on the viability of washed pseudomonads, grown in presence or absence of Polysorbate 80, of sudden exposure to and storage for 30, 120 or 240 min. in 0·5 M-sodium chloride solution at various temperatures. ——, Organisms grown in broth; – – –, organisms grown in broth + 0·1 % Polysorbate 80.

Fig. 8. The effect on viability of washed pseudomonads grown in presence or absence of Polysorbate 80, of sudden exposure to and storage for 30 and 150 min. in tris-maleate buffer at 20°. ——, Organisms grown in broth; – – –, organisms grown in broth + 0·1 % Polysorbate 80.

DISCUSSION

The loss by leakage of constituents, including the 260 mμ-absorbing purine and pyrimidine compounds, from bacteria exposed to surface-active agents is well known, and the pattern, showing a peak leakage concentration of agent, has been reported for several cationic agents (Salton, 1951; Newton 1953; Hugo & Longworth, 1964). In the present work, a non-ionic agent has caused a similar effect. With *Pseudomonas aeruginosa* the characteristic peak leakage concentration of the agent was the same at temperatures of 4°, 18°, 20° and 37° and at three concentrations of organisms. Contact with the agent during growth of the pseudomonads in broth or during subsequent storage in aqueous polysorbate solutions gave the same leakage patterns (Fig. 2). Polysorbate did not alter the optical effect and this result suggests that there is only a relatively subtle effect on the osmotic barrier of these bacteria. The growth rate of *P. aeruginosa* does not vary in nutrient broth containing polysorbate within the concentration range used in this work, suggesting an absence of any gross change in the osmotic barrier. The technique of using penetration of ANS showed contact with 0·125 % polysorbate to cause greatest uptake (Table 1) and this concentration of the surfactant in fact also caused the maximum outflow of 260 mμ-

absorbing substances (Fig. 2). Hugo & Longworth (1964) suggested that chlorhexidine, especially above its peak leakage concentration, caused extensive denaturation of the protein and gross cytoplasmic disorganisation. Similar protein-precipitating effects occurred with a polyalkylbiguanide (Davies & Field, 1967). These effects may possibly have contributed to both loss of viability and the fall-off in leakage observed, but it is unlikely, since non-ionics have little denaturing action on proteins (Putnam, 1953). The uptake of polysorbate is presumably via hydrogen bonding: Hamilton (1968) considered that the adsorption by organisms of membrane-active antibacterial agents was via a relatively non-specific physico-chemical phenomenon. Once bound, polysorbate alters the permeability properties of the organisms and increased leakage was observed immediately after adding the surfactant. These results indicated a direct physical action, rather than one involving permease-inhibition or stimulation of autolytic enzymes, and are consistent with the hypothesis that polysorbate has an immediate effect on the organisms permeability regulation system. Such a physical effect may involve interference by the surfactants with the packing or molecular organization within the membrane or membrane/wall envelope of these Gram-negative organisms.

Cell-membrane disorganization would allow an enhanced interchange of molecules and ions between the internal and external environments of the bacterium. Non-ionic agents caused reduced electrostatic resistance and increased cation permeability of reconstituted lipid-membrane preparations (Seufert, 1965). Thus, loss of viability may be the consequence of two processes: (i) the loss of vital cell constituents, including purine and pyrimidine compounds, which reflects degradation of cellular RNA (Holden, 1958; Postgate, 1967), (ii) gain of substances normally not present or in strictly limited amounts.

The outflow of 260 mμ-substances was decreased when the organisms were suspended in saline or buffer solutions (Fig. 3). Other pseudomonads also showed less lysozyme-induced damage to the wall/membrane structure when suspended in saline solutions (Shively & Hartsell, 1964). However, viability losses in these solutions were still marked and therefore no correlation existed between degree of kill and loss of 260 mμ substances. There was a correlation between degree of kill and the magnitude of the sudden change in any of the colligative properties of the organism's environment. Although this type of effect is well known, especially with cold shock (Gossling, 1958; Meynell, 1958; Gorrill & McNeil, 1960), previous exposure to polysorbate aggravated the viability loss for any given abrupt change.

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REFERENCES

BROWN, M. R. W. & RICHARDS, R. M. E. (1964). Effect of Polysorbate 80 on the resistance of *Pseudomonas aeruginosa* to chemical inactivation. *J. Pharm. Pharmac.* **16** (Suppl.), 51T.

BROWN, M. R. W. & WINSLEY, B. E. (1966). The effect of Polysorbate 80 on cell leakage of *Pseudomonas aeruginosa*. *J. gen. Microbiol.* **45**. iv.

DAVIES, A. & FIELD, B. S. (1967). The bactericidal action of biguanides. *Biochem. J.* **106**, 46.P

FARRELL, J. & ROSE, A. H. (1967). Temperature effects on micro-organisms. In *Thermobiology*. Ed. by A. H. Rose, p. 142. London: Academic Press.

FARRELL, J. & ROSE, A. H. (1968). Cold shock in a mesophilic and a psychrophilic Pseudomonad. *J. gen. Microbiol.* **50**, 429.

GEIGY, (1962). *Documenta Geigy Scientific Tables*, 6th ed., p. 314. Manchester: Geigy Pharmaceutical Publishing Co. Ltd.

GORRILL, R. H. & MCNEIL, E. M. (1960). The effect of cold diluent on the viable count of *Pseudomonas pyocyanea*. *J. gen. Microbiol.* **22**, 437.

GOSSLING, B. S. (1958). The loss of viability of bacteria in suspension due to changing the ionic environment. *J. appl. Bact.* **21** (2), 220.

HAMILTON, W. A. (1968). The mechanism of the bacteriostatic action of tetrachlorosalicylanilide: a membrane-active antibacterial compound. *J. gen. Microbiol.* **50**, 441.

HOLDEN, J. T. (1958). Degradation of intracellular nucleic acid and leakage of fragments of *Lactobacillus orabirosus*. *Biochem. biophys. Acta* **29**, 667.

HUGO, W. B. & LONGWORTH, A. R. (1964). Some aspects of the mode of action of chlorhexidine. *J. Pharm. Pharmac.* **16**, 655.

MAGER, J., KUCZYNSKI, M., SCHATZBERG, G. & AVI-DOR, Y. (1956). Turbidity changes in bacterial suspensions in relation to osmotic pressure. *J. gen. Microbiol.* **14**, 69.

MEYNELL, G. G. (1958). The effect of sudden chilling on *Escherichia coli*. *J. gen. Microbiol.* **19**, 380.

NEWTON, B. A. (1953). The release of soluble constituents from washed cells of *Pseudomonas aeruginosa* by the action of polymyxin. *J. gen. Microbiol.* **9**, 54.

NEWTON, B. A. (1954). Site of action of polymyxin on *Pseudomonas aeruginosa*: antagonism by cations. *J. gen. Microbiol.* **10**, 491.

PARKER. M. S., BARNES, M. & BRADLEY, T. J. (1966). The use of the Coulter Counter to detect the inactivation of preservatives by a non-ionic surface-active agent. *J. Pharm. Pharmac.* **18**, 103S.

POSTGATE, J. R. (1967). In *Advances in Microbiol Physiology*. Ed. by A. H. Rose and J. F. Wilkinson. Vol. 1, pp. 1. London: Academic Press.

POSTGATE, J. R. & HUNTER, R. (1962). The survival of starved bacteria. *J. gen. Microbiol.* **29**, 233.

PUTNAM, F. W. (1953). In *Proteins*. Ed. by H. Neurath and K. Bailey. Vol. 1B, p. 817. New York: Adademic Press.

SALTON, M. R. J. (1951). The adsorption of cetyltrimethylammonium bromide by bacteria, its action in releasing cellular constituents and its bactericidal effects. *J. gen. Microbiol.* **5**, 391.

SEUFERT, W. D. (1965). Induced permeability changes in reconstituted cell membrane structures. *Nature, Lond.* **207**, 174.

SHIVELY, J. M. & HARTSELL, S. E. (1964). Bacteriology of the pseudomonads. II. Chemical treatments affecting the lytic response. *Can. J. Microbiol.* **10**, 911.

STEVENSON, D. G. (1954). Assay methods for non-ionic surface-active agents. *Analyst, Lond.* **79**, 504.

VIDAL-PARUTA, M. R. & KING, L. D. (1964). Critical micelle concentration of non-ionic surfactants in water and carbon tetrachloride. *J. pharm. Sci.* **53** (10), 1217.

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The Effect of Synthetic Detergent on the Determination of the Molecular Weight of a Carotenoid-glycoprotein from *Sarcina flava*

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SUMMARY

Membranes from *Sarcina flava* were solubilized by using 1 % (v/v) Lubrol L and a water-soluble detergent-rich carotenoid glycoprotein complex isolated and purified. Homogeneity of the complex was indicated on the ultracentrifuge but molecular weight determinations made on a membrane osmometer indicated that the molecular weight values obtained in the presence of detergent may depend on the presence or absence of salts.

INTRODUCTION

The use of synthetic detergents for the solubilization of protein complexes or of membranes is widespread (Smith, 1941; Salton & Netschey, 1965; Salton, 1967; Bailey, 1968). Since it is sometimes impossible to remove detergent from solubilized material, molecular weights have been determined in the presence of detergent (Wolken & Schwertz, 1956). The carotenoids of *Sarcina flava* are localized in the membrane (Strang, 1968) and it is likely that the dihydroxy C_{50} carotenoid (Thirkell, Strang & Chapman, 1967) becomes an integral part of the structure of the membrane fraction. This work was to attempt to isolate and purify the complex so formed and to determine its molecular weight.

METHODS

Growth and harvesting of bacteria; preparation of carotenoid-glycoprotein. Sarcina flava (NCTC 7503) was grown in nutrient broth (Oxoid Ltd.) at 34° and harvested after 3 days. The membrane fraction was isolated by the method of Salton & Freer (1965) and solubilized by repeated extractions with 1 % Lubrol L (I.C.I. Ltd.) in 0·1 M-tris + maleate buffer, pH 7·2. After centrifugation at 100,000 *g* for 30 min. the clear yellow supernatant fluid was exhaustively dialysed against tap water followed by distilled water. The carotenoid-glycoprotein was purified by repeated precipitation with $(NH_4)_2SO_4$ followed by dialysis.

Ultracentrifuge for homogeneity. Solutions in distilled water or 0·1 M-KCl were exhaustively dialysed against their respective solvents for more than 48 hr. Concentrations were determined by dry weight and ash, and by refractometry.

Molecular weight determinations. Osmometry was used on both solutions in a high-speed Hewlett–Packard membrane osmometer equipped with B 19 membranes (Schliecher & Schüll). In each case, reduced osmotic pressures were plotted against concentration.

RESULTS

The complex contained carotenoid (indicated by spectrum and by reaction with $SbCl_3$ (Morton, 1942), sugar(s) (by the method of Whistler & Wolfrom, 1962) and a peptide (amino acid analysis). Sedimentation in distilled water or 0·1 M-KCl after 180 min. at a nominal speed of 59,780 rev./min. gave symmetrical schlieren patterns indicating a single compound characterized by a S^0_{20} value of $1{\cdot}49 \times 10^{-13}$ sec.

Reduced osmotic pressure measurements recorded in the presence or absence of KCl were plotted against concentration (Fig. 1). Correction for non-ideality of solution was carried out by extrapolation to zero concentration and the results fitted into the general formula:

$$\overline{M}n = \frac{RT}{(\pi/c)_{c\to 0}},$$

where RT was corrected for the density of the solvents. From the figure, an $\overline{M}n$ value of $2{\cdot}28 \times 10^4$ was obtained for the preparation in distilled water, and $6{\cdot}20 \times 10^4$ for the preparation in 0·1 M-KCl. The shapes of the curves are strongly suggestive of association of macromolecules.

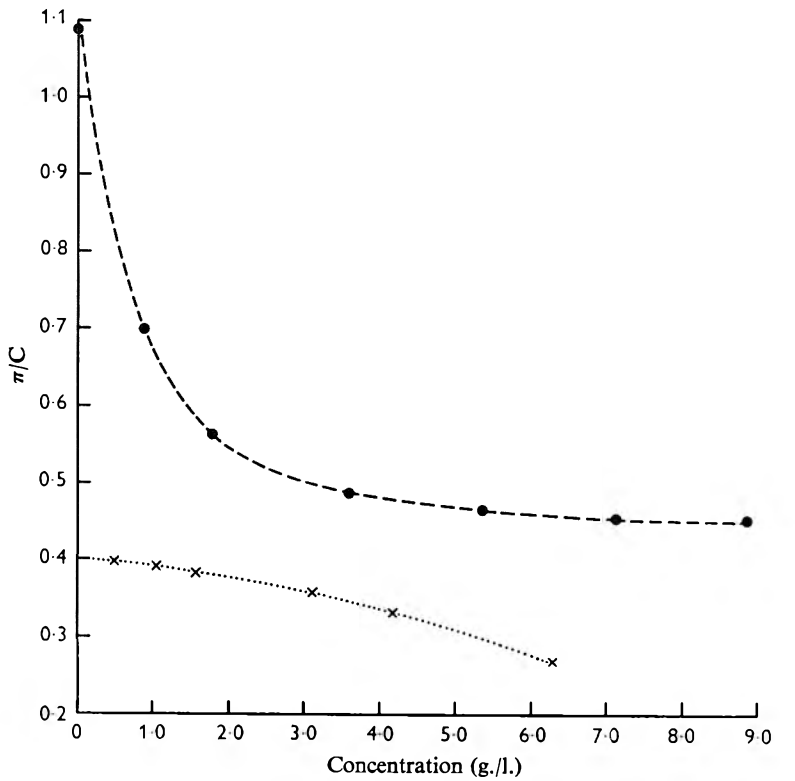

Fig. 1. π/C against c for (a) complex in distilled water (● – – ●), (*b*) complex in 0·1 M-KCl (× --- ×).

DISCUSSION

Addition of salt to a detergent-rich solution appears to bring about association of macromolecules, increasing micelle size to a point where the solubility properties of the solution tend towards those of the detergent itself. This view is supported by the discrepancy of $\overline{Mn}$ values recorded on the osmometer. Molecular weights determined in the presence of detergent may also depend on the technique used and the concentration of solution. Any molecular weight values determined in the presence of high concentrations of detergent may be open to doubt.

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REFERENCES

BAILEY, J. L. (1968). Characterization of pigment-protein complexes related to systems I and II. *Int. Congr. Photosyn. Res.*, Freudenstadt.

MORTON, R. A. (1942). *Absorption spectra Applied to Vitamins and Hormones.* London: A. Hilgar.

SALTON, M. R. J. (1967). Struture and composition of bacterial membranes. *Protides biol. Fluids* **15**, 279.

SALTON, M. R. J. & FREER, J. H. (1965). Composition of the membranes isolated from several Gram-positive bacteria. *Biochim. biophys. Acta* **107**, 531.

SALTON, M. R. J. & NETSCHEY, A. (1965). Physical chemistry of isolated membranes. *Biochim. biophys. Acta* **107**, 539.

SMITH, E. L. (1941). The chlorophyll-protein compound of the green leaf. *J. gen. Physiol.* **24**, 565.

STRANG, R. H. C. (1968). *Some investigations into the Sarcina bacteria.* Ph.D. Thesis, St Andrews University.

THIRKELL, D., STRANG, R. H. C. & CHAPMAN, J. R. (1967). The pigments of *S. flava*: a new series of C_{50} carotenoids. *J. gen. Microbiol.* **49**, 157.

WOLKEN, J. J. & SCHWERTZ, F. A. (1956). Molecular weight of algal chloroplastin. *Nature, Lond.* **177**, 136.

WHISTLER, R. L. & WOLFROM, M. L. (1962). *Methods in Carbohydrate Chemistry*, vol. 1, p. 388, New York and London: Academic Press.

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DNA Base Composition and Taxonomy of Phytopathogenic and Other Enterobacteria

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SUMMARY

Using lysates and purified DNA, base ratios were determined by the buoyant density ultracentrifugation and the absorbance-temperature transition techniques; the correspondence was very high between the two methods. The DNAs from a large number of Erwinia species, mostly phytopathogenic and nonphytopathogenic, but including some 'Erwinia-like' bacteria from animal sources and from other genera of the family Enterobacteriaceae were studied. The DNA base ratios of these bacteria fell mainly in the range 50–58 % GC, consistent with their taxonomic placement in the family Enterobacteriaceae. Certain assemblages of erwinias could be grouped on the basis of GC content into clearly differentiated clusters, which, accidentally or otherwise, correlated neatly with certain existing nomenclatural groupings. It was not possible, from the GC values alone, to decide whether the present multiplicity of Erwinia species can be justified as separate species, or whether and how they might be combined into fewer species, or whether and how they might be demoted to some infraspecific level. Indeed, it is not clear whether the genus *Erwinia*, defined as it is solely on the basis of plant habitat, should be maintained or whether its species might not, just as rationally, be distributed throughout other genera of the family Enterobacteriaceae. Classifications of the genus *Erwinia* on over-all phenotypic similarity result in a small number of specific taxa which, on the basis of the present study, are seen to contain organisms of different GC contents. In the present state of the art, one can only be agnostic about the amount of such differences in GC content which corresponds to separate taxa or to a particular categoreal level (generic, specific, infraspecific) in the present hierarchical taxonomic scheme.

INTRODUCTION

The genus *Erwinia* was proposed originally by a committee (Winslow *et al.* 1917) with the candid admission that there was insufficient information for a more rational nomenclature: '...the plant pathogens...seem to us perhaps to deserve distinct generic rank. They resemble the proteus group in their Gram-negative reaction, general growth characters, tendency toward slime formation, and generally negative indole reaction, but their fermentative powers are less vigorous and the exact biochemical changes they produce in carbohydrates are unknown. We suggest that all

the peritrichate plant pathogens be for the present grouped under a new genus to be called *Erwinia*...'.

Erwin Frink Smith, the pioneer phytobacteriologist who was commemorated by this act, stated, quite resolutely (cf. Jones, 1939, p. 43): 'I have no sympathy with those who would...lump everything indiscriminately...the species I have mentioned ought not be put into one genus (*Erwinia*) simply because they are plant pathogens.' His advice went unheeded, and the causal agent of fire blight of pear—the first disease of plants proven to be caused by bacteria (Burrill, 1882)—is today the type species (*Erwinia amylovora*) of the genus *Erwinia*. This impractical nomenclatural concept—in which taxa of phytopathogenic bacteria are segregated from other bacteria solely upon the basis of plant pathogenicity—has persisted to the present time. In *Bergey's Manual* (1957) the genus *Erwinia* is placed in the family Enterobacteriaceae, fenced off from its probable affinities not only by the nomenclatural barrier, but also by the insularity ensuing from the completely separate developments of plant and animal bacteriology.

Early in the twentieth century several bacterial soft-rot diseases of plants were studied and ascribed causally to several different bacterial 'species' which usually were named in accordance with the host plant from which they had been isolated. The genus *Erwinia* was sufficiently vague in concept to accommodate these soft-rot bacteria. Despite the early comparative studies (Jones, 1901; Harding & Morse, 1909; Jennison, 1923) which showed the essential similarity of most soft-rot bacteria to the one first described—now called *Erwinia carotovora*—a multiplicity of species names was used, with various definitions, but most frequently based on the curious 'new host–new species' notion (Starr, 1959; Stolp, Starr & Baigent, 1965) However, it is clear that the usual soft-rot bacteria are as different from *E. amylovora* as, for example, *Enterobacter cloacae* is from *Shigella dysenteriae*. Non-phytopathogenic yellow-pigmented bacteria are common on plants and some of these have been referred to the genus *Erwinia* (Billing & Baker, 1963; Graham & Hodgkiss, 1967). Finally, a group of yellow-pigmented bacteria from animals and humans is thought to be related to *Erwinia* (Muraschi, Friend & Bolles, 1965; von Graevenitz & Strouse, 1966; Whitcomb, Shapiro & Richardson, 1966; Graham & Hodgkiss, 1967; W. H. Ewing, personal communication; I. J. Slotnick, personal communication).

We have undertaken a comprehensive study of the genus *Erwinia* and related bacteria, including investigations on such aspects as nutrition (Starr & Mandel, 1950); end-products of glucose dissimilation in *E. amylovora* (Sutton & Starr, 1959), in *E. carotovora* (Kraght & Starr, 1952), and in other erwinias (in preparation); uronic acid catabolism (Kilgore & Starr, 1959), pectolytic enzymes (Kraght & Starr, 1953; Starr & Moran, 1962; Nasuno & Starr, 1966; Moran, Nasuno & Starr, 1968*a*, *b*); pigment formation (Starr, Cosens & Knackmuss, 1966); bacteriophage and bacteriocin relationships (in preparation); pathogenicity (Starr, 1947; Starr, Cardona & Folsom, 1951; in preparation). In this report, we present the DNA base ratios of representative cultures of Erwinia species and other enterobacteria. These data, together with the other information becoming available about these organisms, should eventually provide the basis for clarification of the taxonomic relationships of these phytopathogenic and other enterobacteria.

METHODS

Organisms. The majority of the phytopathogenic bacteria were obtained from the International Collection of Phytopathogenic Bacteria (ICPB) maintained at the Department of Bacteriology, University of California, Davis. Many of these cultures are also accessioned in the National Collection of Plant Pathogenic Bacteria, Harpenden, Herts., England. The strains were chosen to be representative of the broadest possible range of geographical sources, host plants, and date of isolation. 'Erwinia-like' bacteria isolated mainly from plant materials were provided by E. Billing (Billing & Baker, 1963) and D. C. Graham (Graham & Hodgkiss, 1967); those isolated from American white-tail deer, humans and leafhoppers were provided by T. F. Muraschi (Muraschi *et al.* 1965), I. J. Slotnick, R. F. Whitcomb (Whitcomb *et al.* 1966), W. H. Ewing, and A. von Graevenitz (von Graevenitz & Strouse, 1966). Other enterobacteria came from the American Type Culture Collection (ATCC); these were selected to represent designated type or neotype cultures as far as these are available. Further details about the cultures are given in Tables 1–3.

For clarity in this presentation, we will use the genus and species designations under which the culture was received. Several trivial names are used here in accordance with present practices of workers in this field: 'erwinia', meaning any organism referred to the genus *Erwinia* with no connotation regarding origin (plant, animal) or phytopathogenicity; 'Erwinia-like' bacteria, meaning definitely non-phytopathogenic erwinias from plants or animals; 'pectobacteria', meaning pectolytic (i.e. soft-rot) members of the genus *Erwinia*; 'true-Erwinia', meaning the non-pectolytic phytopathogens (*E. amylovora* and other non-pectolytic species) of the genus *Erwinia*; 'enterobacteria', meaning any member of the family Enterobacteriaceae, including all of the foregoing categories.

Deoxyribonucleic acid isolation and analysis. Lysates of bacteria for CsCl buoyant density ultracentrifugation were prepared from slant cultures, as previously described (Mandel, 1966). Purified samples of DNA were prepared by the method of Marmur (1961) from organisms from 2 l. cultures grown in Penassay broth (Difco) at 30° with rotary agitation. These samples also were analysed in the ultracentrifuge for CsCl buoyant density, and their melting temperatures in 0·15 M-NaCl containing 0·015 M-sodium citrate at pH 7·0 were determined by changes in extinction at 260 mμ (nm.) (Mandel & Marmur, 1968). The DNA base ratios were estimated from mean buoyant densities by means of the equation of Schildkraut, Marmur & Doty (1962), and from the T_m by means of the equation of Marmur & Doty (1962). Each DNA sample was analysed at least twice by CsCl buoyant density equilibrium centrifugation, and many were additionally examined by the extinction/temperature transition-technique. Following the usual convention, the DNA base ratios are expressed as molar percentages of guanine+cytosine ('GC content') in terms of the total base content.

RESULTS AND DISCUSSION

The GC contents of cultures ascribed to the genus *Erwinia* and to other genera of the Enterobacteriaceae are presented in Tables 1–3. Table 1 contains the buoyant densities and calculated GC contents of the DNAs from the cultures of phytopathogenic erwinias, obtained by analysis of lysates. Table 2 presents the buoyant densities and

Table 1. *Buoyant density in CsCl and GC content of DNA in lysates of cultures of Erwinia species and other phytopathogenic Enterobacteriaceae*

	Strain (ICPB)	Mean density (g./cm.^{3})	GC (mole %)
E. amylovora	EA 11	1·7125	53·6
	EA 131	1·713	54·1
	EA 137	1·7127	53·8
	EA 146	1·713	54·1
	EA 162	1·713	54·1
	EA 167	1·7125	53·6
	EA 169	1·713	54·1
E. ananas	EA 101	1·7125	53·6
	EA 133	1·712	53·1
	EA 180	1·713	54·1
	EA 181	1·7125	53·6
E. aroideae	EA 13	1·711	52·0
	EA 14	1·712	53·1
	EA 144	1·710	51·0
E. atroseptica	EA 112	1·7103	51·3
	EA 153	1·712	53·1
E. carnegieana	EC 185	1·710	51·0
	EC 187	1·7098	50·8
	EC 183	1·7107	51·7
	EC 189	1·7103	51·3
	EC 189	1·710	51·0
	EC 191	1·710	51·0
	EC 221	1·710	51·0
	EC 222	1·7105	51·5
	EC 223	1·710	51·0
E. carotovora	EC 105	1·710	51·0
	EC 131	1·7115	52·6
	EC 138	1·711	52·0
	EC 150	1·7115	52·6
	EC 153	1·7107	51·7
	EC 208	1·7113	52·3
f.sp. *parthenii*	EC 115	1·7115	52·6
E. cassavae	EC 11	1·714	55·1
E. chrysanthemi	EC 16	1·714	55·1
	EC 175	1·714	55·1
	EC 183	1·715	56·1
	EC 205	1·716	57·1
Corn stalk-rot pathogen	EM 107	1·7125	53·6
	EM 108	1·713	54·1
	EM 109	1·712	53·1
	EM 110	1·7125	53·6
	EM 111	1·7125	53·6
	EM 111 mut	1·7125	53·6
	EM 112	1·713	54·1
	EC 209	1·7125	53·6
	EC 210	1·7125	53·6
	EC 210 mut	1·7125	53·6
E. cypripedii	EC 155	1·713	54·1
	EC 160	1·7135	54·6
E. cytolytica	EC 207	1·715	56·1
E. dieffenbachiae	ED 102	1·714	55·1
	ED 103	1·715	56·1

Table 1 (*cont.*)

	Strain (ICPB)	Mean density (g./cm.^{3})	GC (mole %)
E. dissolvens	ED 105	1·7125	53·6
	ED 106	1·716	57·1
	ED 109	1·7165	57·6
	ED 110	1·714	55·1
	ED 111	1·7147	55·8
	ED 112	1·716	57·1
	ED 113	1·7155	56·6
	ED 114	1·716	57·1
E. mangiferae	EM 101	1·7135	54·6
E. milletiae	EM 102	1·714	55·1
	EM 113	1·713	54·1
	EM 114	1·714	55·1
E. nigrifluens	EN 104	1·715	56·1
E. nimipressuralis	EN 1	1·714	55·1
E. oleraceae	EO 1	1·7095	50·5
E. quercina	EQ 101	1·714	55·1
	EQ 102	1·7135	54·6
E. rhapontici	ER 1	1·710	51·0
	ER 102	1·712	53·1
	ER 106	1·712	53·1
E. rubrifaciens	ER 103	1·711	52·0
	ER 104	1·7115	52·6
	ER 105	1·7115	52·6
E. salicis	ES 4	1·7103	51·3
	ES 102	1·7105	51·5
E. solanisapra	ES 101	1·710	51·0
E. tracheiphila	ET 5	1·711	52·0
	ET 102	1·7095	50·5
	ET 106	1·709	50·0
'*Aerobacter aerogenes*' (banana strain)	3151	1·715	56·1
'*Bacterium stewartii*'	SS 11	1·714	55·1
	SS 102	1·7135	54·6
'*Xanthomonas uredovorus*'	XU 103	1·712	53·1
	XU 104	1·712	53·1

calculated GC contents of the DNAs in the lysates of the cultures of non-phytopathogenic Erwinia-like bacteria. Table 3 presents comparative calculations of the GC contents determined both in CsCl and by thermal denaturation on purified samples of DNA extracted from representative Erwinia cultures and reference cultures of other enterobacteria. Table 4 summarizes tests of statistical significance of pairwise differences in densities between selected sets of Erwinia species; there is no necessity to equate such statistically significant differences with taxonomic significance.

The data have been applied to several purposes. Where the cultures have already been assigned to a specific taxon, the uniformity of the taxon has been tested with regard to the uniformity of GC contents. Various proposals for combining existing taxa have been examined in the light of the GC data. The wisdom of referring the non-phytopathogenic Erwinia-like bacteria to the family Enterobacteriaceae has been evaluated in terms of the GC values. The homogeneity of the family Enterobacteria-

ceae, with the various erwinias included, is scrutinized. The rationality of maintaining a genus *Erwinia* is considered. Since a rather large number of DNAs were examined by both buoyant density and transition temperature techniques, comparisons are made between the two methods (Table 3).

Table 2. *Buoyant density in CsCl and GC content of DNA in lysates of non-phytopathogenic 'Erwinia-like' bacteria*

Source or Name	Strain (ICPB)	Mean density (g./cm.^{3})	GC (mole %)
Plant materials (Billing)	2650–651	1·714	55·1
	2650–653	1·713	54·1
	2650–656	1·7145	55·6
	2650–658	1·712	53·1
	2650–661	1·714	55·1
	2650–662	1·7135	54·6
Erwinia lathyri	EL 101 B	1·714	55·1
	EL 102	1·714	55·1
	EL 103	1·714	55·1
	EL 104	1·7115	52·6
	EL 105	1·712	53·1
	EL 106	1·7165	57·7
	EL 107	1·713	54·1
Erwinia herbicola	2553	1·714	55·1
	2554	1·713	54·1
	3161	1·714	55·1
	3162	1·712	53·1
	3163	1·714	55·1
'*Xanthomonas trifolii*'	XT 109	1·7145	55·6
	XT 110	1·7145	55·6
Deer (Muraschi)	2948	1·7149	56·0
	2949	1·714	55·1
	2950	1·715	56·1
Leafhopper (Whitcomb)	2973-L	1·7115	52·6
	2973-S	1·7125	53·6
Man (Slotnick)	2858-A	1·7145	55·6
	2858-B	1·714	55·1
Man (Muraschi)	2953	1·7155	56·6
	2956	1·713	54·1
Man (von Graevenitz)	2984	1·719	60·2
	2986	1·7185	59·7
	2987	1·715	56·1
Man (Ewing)	2992	1·714	55·1
	3080	1·7145	55·6
'*Bacterium typhi flavum*'	3164	1·714	55·1
	3165	1·714	55·1

The proposalof Waldee (1945) to separate the soft-rot bacteria (as a genus *Pectobacterium*) from the rest of the genus *Erwinia* is neither supported nor confirmed by the GC data. The assortments within these two groups, the pectobacteria and the true-Erwinia, as reviewed by Graham (1964), can now be assessed in terms of GC contents. *Erwinia amylovora* (53·9% GC) could accommodate *E. cassavae* and *E. mangiferae*. The DNAs of *E. salicis* and *E. tracheiphila*, on the other hand, are 4% to 5% lower in GC content than those of *E. amylovora* and its relatives. In addition,

the yellow-pigmented erwinias (Dye, 1963; Graham & Hodgkiss, 1967)—'*Xanthomonas uredovorus*', *E. ananas*, *E. lathyri*, and '*Bacterium stewartii*'—as well as many of the yellow Erwinia-like bacteria, have GC contents not unlike the *E. amylovora* group and lower than the Pseudomonadaceae genera in which some of them had earlier

Table 3. *Melting temperatures, buoyant densities in CsCl, and calculated GC contents of purified DNA samples from representative Erwinia and other Enterobacteriaceae*

Name	Strain	T_m (°C)	GC (mole %)	Buoyant density (g./cm.^{3})	GC (mole %)
Erwinia amylovora	ICPB EA 11	91·0	53·0	1·7125	53·6
E. aroideae	ICPB EA 14	91·5	54·0	1·712	53·1
E. carotovora	ICPB EC 138	91·2	53·4	1·711	52·0
E. chrysanthemi	ICPB EC 16	92·1	55·6	1·714	55·1
E. dissolvens	ICPB ED 106	92·7	57·1	1·716	57·1
E. lathyri	ICPB EL 103	92·2	55·9	1·714	55·1
E. milletiae	ICPB EM 102	92·0	55·4	1·714	55·1
E. nigrifluens	ICPB EN 104	92·5	56·6	1·715	56·1
E. nimipressuralis	ICPB EN 1	92·0	55·4	1·714	55·1
E. oleraceae	ICPB EO 1	90·4	51·5	1·7095	50·5
E. rhapontici	ICPB ER 1	90·2	51·1	1·710	51·0
E. salicis	ICPB ES 4	90·8	52·5	1·7103	51·3
E. tracheiphila	ICPB ET 106	90·9	52·7	1·709	50·0
Escherichia aurescens	ATCC 12814	90·3	51·3	1·710	51·0
E. coli	K-12 Hfr G6	90·4	51·5	1·710	51·0
E. coli	ATCC 11775	90·5	51·7	1·710	51·0
E. coli	ATCC 12814	90·3	51·3	1·710	51·0
Enterobacter aerogenes	ATCC 13048	91·8	54·9	1·712	53·1
E. aerogenes	ATCC 13882	92·1	55·6	1·715	56·1
E. alvei	ATCC 23280	89·2	48·7	1·707	48·0
E. cloacae	ATCC 13047	92·0	55·4	1·7135	54·6
E. lipolyticus	ATCC 14460	91·4	53·9	1·7115	52·6
Klebsiella pneumoniae	ATCC 13883	92·9	57·6	1·7123	53·4
K. edwardsii subsp. *edwardsii*	ATCC 13886	92·5	56·5	1·7155	56·6
K. edwardsii subsp. *atlantae*	ATCC 13887	92·6	56·9	1·715	56·1
K. rhinoscleromatis	ATCC 13884	92·7	57·1	1·7145	55·6
Citrobacter freundii	ATCC 8090	90·9	52·7	1·7115	52·6

Table 4. *Tests of statistical significance of pairwise differences in bouyant densities of the DNAs from selected sets of Erwinia species*

	$x \pm \sigma$	n	t	D.F.	p (sign ignored)
E. carnegieana	1·7100 ± 0·0008	22	3·78	37	0·001
E. carotovora	1·7111 ± 0·0009	17			
E. amylovora	1·7128 ± 0·0007	16	6·6	18	0·001
E. salicis	1·7105 ± 0·0006	4			
E. chrysanthemi	1·7148 ± 0·0009	8	8·45	29	> 0·001
'Corn stalk rotters'	1·7126 ± 0·0005	23			
E. amylovora	1·7128 ± 0·0007	16	0·034	22	< 0·5
E. ananas	1·7125 ± 0·0005	8			
E. carotovora	1·7111 ± 0·0009	17	0·874	21	0·4
E. aroideae	1·7110 ± 0·0011	6			

been included (see De Ley, 1968, for an independent study on this point). The Erwinia-like organisms isolated from American white-tail deer (Muraschi), from man (Ewing, Slotnick, Muraschi, and one of the three strains sent by von Graevenitz), from leaf-hoppers (Whitcomb), and the non-phytopathogenic plant Erwinia-like organisms received from Billing and from Graham have GC contents which are also similar to the *E. amylovora* range, and these are all similar to the data for Klebsiella and certain Enterobacter cultures. Two of the three erwinia strains isolated from humans by von Graevenitz & Strouse (1966) have higher GC contents (60·2 %, 59·7 % GC) than any other Enterobacteriaceae cultures examined in this study.

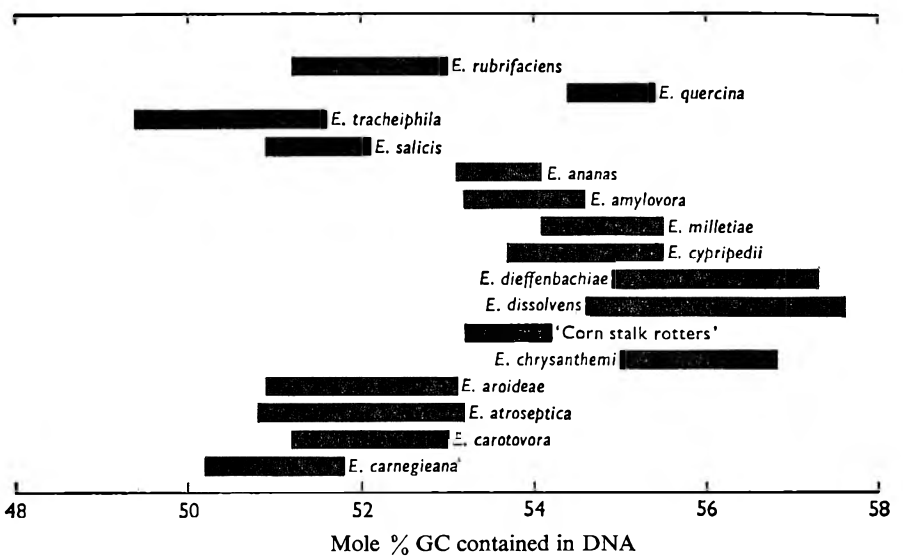

Fig. 1. The GC contents of the DNAs of representative Erwinia species. The values plotted are means ± one standard deviation.

Turning to the soft-rot erwinias, the pectobacteria, *Erwinia carotovora* (52·1 % GC) does not display any tendency to discrete GC contents for its relatives *E. aroideae*, *E. solanisapra*, *E. oleraceae*, and *E. atroseptica*, thus justifying the current practice of lumping these into one species. *Erwinia chrysanthemi* is characterized by 56 % GC and, on this basis, cannot be considered to be identical with *E. carotovora* (52·1 % GC). The several organisms rotting the stalks of corn plants (A. Kelman, unpublished) are significantly different (i.e. statistically so; Table 4) in their GC contents (53·7 % GC) from the *E. carotovora* (52·1 % GC), *E. chrysanthemi* (56 % GC), and *E. dissolvens* (56·3 % GC) cultures; they might form a taxon separate from these three, but whether at the specific or infraspecific level remains to be decided on other grounds. Similarly, the representatives of *E. carnegieana* (51·1 % GC) are distinct in their GC contents, as compared to the other soft-rot erwinias, and might be viewed as a separate taxon; here again, the hierarchical level cannot be decided from these data alone.

The other named strains which have been examined in this study fall into the range of 50–58 % GC values, typical of the other members of the family Enterobacteriaceae (Marmur, Falkow & Mandel, 1963; Hill, 1966; Rosypal & Rosypalová, 1966). The

extremes are reached with *Enterobacter alvei* ATCC 23280 (48·0 % GC) and the aforementioned human erwinia strain from von Graevenitz, ICPB 2984 (60·2 % GC).

Based on a study of over-all phenotypic similarity, Martinec & Kocur (1963) have suggested that all the species presently in the genus *Erwinia* might be reduced to two species: *E. carotovora* and *E. amylovora* (and var. *salicis*). In a similar study with over 200 cultures and using some 80 traits, Dye (1968) has recommended reduction to five species: *E. amylovora* (and var. *salicis*, var. *tracheiphila*, var. *nigrifluens*, var. *quercina*), *E. herbicola* (and var. *ananas*), *E. uredovorus*, *E. stewartii*, *E. carotovora* (and var. *atrospetica*, var. *chrysanthemi*, var. *cypripedii*, var. *rhapontici*). Since following these recommendations would lead to taxa (specific and infraspecific) which the present DNA data show would be composed of organisms with statistically different GC contents (Table 4), we are at a loss to decide on the wisdom of these suggestions. In support of this view, the computer sorting of Lockhart & Koenig (1965) and Krieg & Lockhart (1966) shows no intensification of sorting when the S-values which they report for Erwinia species are plotted versus our data on their GC contents. Indeed, the range of link-sorting is less than the range of GC values throughout the family Enterobacteriaceae; hence, the traits used in the generation of these computer analyses are probably of little value in discriminating taxa at the generic level and below. The data may, of course, also mean that a species which is based on high over-all phenotypic similarity can include individual specimens with genetic make-ups varying over a range corresponding to several per cent in GC content.

We are not now prepared to equate discrete differences in GC contents with discrete taxa (see Rosypal & Rosypalová, 1966). In the present state of the art, who is to say what particular amount of difference in DNA base ratios or in DNA homologies justifies separating one taxon from another? We can not now be other than agnostic about the amount of such differences which would be required to place taxa in any particular position in the hierarchial taxonomic categories. All we can now say with assurance is that certain assemblages of erwinias can be grouped on the basis of GC contents into clearly differentiated clusters, and that these clusters—accidentally or otherwise—correlate neatly with certain existing nomenclatural groupings.

There is no compelling evidence from the GC data that the Erwinia group, either whole or in parts, comprises a taxon or taxa separable at the generic level from the other genera of the Enterobacteriaceae. Indeed, from Fig. 1 it will be seen that a case could be made for distributing the erwinias throughout the other genera of the family Enterobacteriaceae. Broadly based comparative studies now in progress are designed to clarify this matter; hence, no nomenclatural recommendations will be made here.

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REFERENCES

BILLING, E. & BAKER, L. A. E. (1963). Characteristics of Erwinia-like organisms found in plant material. *J. appl. Bact.* **26**, 58.

Bergey's Manual of Determinative Bacteriology. (1957). 7th ed. Ed. by R. S. Breed, E. G. D. Murray and N. R. Smith. Baltimore: Williams and Wilkins Co.

BURRILL, T. J. (1882). The bacteria: an account of their nature and effects, together with a systematic description of the species. *Illinois Ind. Univ., Report of the Board of Trustees* **11**, 93.

DE LEY, J. (1968). DNA base composition of yellow Erwinia strains. *Antonie van Leeuwenhoek* **34**, 257.

DYE, D. W. (1963). The taxonomic position of *Xanthomonas uredovorus* Pon *et al.* 1954. *N.Z. Jl Sci.* **6**, 146.

DYE, D. W. (1968). A taxonomic study of the genus *Erwinia*. *1st. Int. Congr. Pl. Path.* (London, July 1968), Abstracts of Papers, 51.

GRAEVENITZ, A. VON, & STROUSE, A. (1966). Isolation of Erwinia spp. from human sources. *Antonie van Leeuwenhoek* **32**, 429.

GRAHAM, D. C. (1964). Taxonomy of the soft-rot coliform bacteria. *A. Rev. Phytopath.* **2**, 13.

GRAHAM, D. C. & HODGKISS, W. (1967). Identity of Gram negative, yellow pigmented, fermentative bacteria isolated from plants and animals. *J. appl. Bact.* **30**, 175.

HARDING, H. A. & MORSE, W. J. (1909). The bacterial soft rots of certain vegetables. *Tech. Bull. N.Y. St. agric. Exp. Stn.* **11**, 251.

HILL, L. R. (1966). An index to deoxyribonucleic acid base compositions of bacterial species. *J. gen. Microbiol.* **44**, 419.

JENNISON, H. M. (1923). Potato blackleg with special reference to the etiological agent. *Ann. Mo. bot. Gdn.* **10**, 1.

JONES, L. R. (1901). A soft rot of carrot and other vegetables caused by *Bacillus carotovorus*. *Rep. Vermont. agric. Exp. Stn* **13**, 299.

JONES, L. R. (1939). Biographical memoir of Erwin Frink Smith 1854–1927. *Biogr. Mem. natn. Acad. Sci.* **21**, 1.

KILGORE, W. W. & STARR, M. P. (1959). Catabolism of galacturonic and glucuronic acids by *Erwinia carotovora*. *J. biol. Chem.* **234**, 2227.

KRAGHT, A. J. & STARR, M. P. (1952). Fermentation of galacturonic acid and glucose by a strain of *Erwinia carotovora*. *J. Bact.* **64**, 259.

KRAGHT, A. J. & STARR, M. P. (1953). Pectic enzymes of *Erwinia carotovora*. *Archs. Biochem. Biophys.* **42**, 271.

KRIEG, R. E. & LOCKHART, W. R. (1966). Classification of enterobacteria based on overall similarity. *J. Bact.* **42**, 1275.

LOCKHART, W. R. & KOENIG, K. (1965). Use of secondary data in numerical taxonomy of the genus *Erwinia*. *J. Bact.* **90**, 1638.

MANDEL, M. (1966). Deoxyribonucleic acid base composition in the genus *Pseudomonas*. *J gen. Microbiol.* **43**, 273.

MANDEL, M. & MARMUR, J. (1968). Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Meth. Enzym.* **12**, 195.

MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. molec. Biol.* **3**, 208.

MARMUR, J. & DOTY, P. (1962). Determination of the base composition of dexoyribonucleic acid from its thermal denaturation temperature. *J. molec. Biol.* **5**, 109.

MARMUR, J., FALKOW, S. & MANDEL, M. (1963). New approaches to bacterial taxonomy. *A. Rev. Microbiol.* **17**, 329.

MARTINEC, T. & KOCUR, M. (1963). A taxonomic study of the genus *Erwinia*. *Folia. Publ. Fac. Nat. Sci. J. E. Purkynye Univ.* (Biologia), **4**, 1.

MORAN, F., NASUNO, S. & STARR, M. P. (1968*a*). Extracellular and intracellular polygalacturonic acid *trans*-eliminases of *Erwinia carotovora*. *Archs Biochem. Biophys.* **123**, 298.

MORAN, F., NASUNO, S. & STARR, M. P. (1968*b*). Oligogalacturonide *trans*-eliminase of *Erwinia carotovora*. *Archs Biochem. Biophys.* **125**, 734.

MURASCHI, T. F., FRIEND, M. & BOLLES, D. (1965). *Erwinia*-like microorganisms isolated from animal and human hosts. *Appl. Microbiol.* **13**, 128.

NASUNO, S. & STARR, M. P. (1966). Polygalacturonase of *Erwinia carotovora*. *J. biol. Chem.* **241**, 5298.

ROSYPAL, S. & ROSYPALOVÁ A. (1966). Genetic, phylogenetic and taxonomic relationships among bacteria as determined by their deoxyribonucleic acid base composition. *Folia. Publ. Fac. Nat. Sci. J. E. Purkynye Univ.* (Biologia) **7**, 1.

SCHILDKRAUT, C. L., MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. molec. Biol.* **4**, 430.

STARR, M. P. (1947). The cause of bacterial root and stem disease of guayule. *Phytopathology* **37**, 291.

STARR, M. P. (1959). Bacteria as plant pathogens. *A. Rev. Microbiol.* **13**, 211.

STARR, M. P. & MANDEL, M. (1950). The nutrition of phytopathogenic bacteria. IV. Minimal nutritive requirements of the genus *Erwinia*. *J. Bact.* **60**, 669.

STARR, M. P. & MORAN, F. (1962). Eliminative split of pectic substances by phytopathogenic soft-rot bacteria. *Science, N.Y.* **135**, 920.

STARR, M. P., CARDONA, C. & FOLSOM, D. (1951). Bacterial fire blight of the raspberry. *Phytopathology* **41**, 915.

STARR, M. P., COSENS, G. & KNACKMUSS, H.-J. (1966). Formation of the blue pigment indigoidine by phytopathogenic Erwinia. *Appl. Microbiol.* **14**, 870.

STOLP, H. STARR, M. P. & BAIGENT, N. L. (1965). Problems in speciation of phytopathogenic pseudomonads and xanthomonads. *A. Rev. Phytopath.* **3**, 231.

SUTTON, D. D. & STARR, M. P. (1959). Anaerobic dissimilation of glucose by *Erwinia amylovora*. *J. Bact.* **78**, 427.

WALDEE, E. L. (1945). Comparative studies of some peritrichous phytopathogenic bacteria. *Iowa St. Coil. J. Sci.* **19**, 435.

WHITCOMB, R. F., SHAPIRO, M. & RICHARDSON, J. (1966). An Erwinia-like bacterium pathogenic to leafhoppers. *J. Invertebr. Path.* **8**, 299.

WINSLOW, C. E. A., BROADHURST, J., BUCHANAN, R. E., KRUMWIEDE, C. JUN., ROGERS, L. A. & SMITH, G. H. (1917). The families and genera of the bacteria. Preliminary report of the committee of the Society of American Bacteriologists on characterization and classification of bacterial types. *J. Bact.* **2**, 505.