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Ploidal Inheritance in the Slime Mould *Dictyostelium discoideum:* Haploidization and Genetic Segregation of Diploid Strains

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SUMMARY

Diploid and metastable strains of *Dictyostelium discoideum* were cultivated in liquid medium for extended periods of exponential growth. A progressive increase occurred in the proportion of cells yielding stable haploid clones. The haploidization of a doubly heterozygous diploid yielded the expected segregant classes.

INTRODUCTION

Dictyostelium discoideum is a cellular slime mould. The extraordinary capacity of this group for multicellular organization has been studied by many workers. The development of a suitable chromosome staining procedure (Wilson, 1953; Wilson & Ross, 1955; Ross, 1960; Sussman, R.R. 1961) has focused interest on its cytogenetics. Examination of mitotic figures revealed the existence of haploid and diploid amoebae (Wilson & Ross, 1957; Ross, 1960) and the ploidal composition of a culture was found to be stable and clonally inherited (Ross, 1960; Sussman & Sussman, 1962). Three general types of strains have been encountered so far: (i) stable haploid, containing a negligible proportion of diploid derivatives; (ii) stable diploid, containing a negligible proportion of haploid derivatives; (iii) metastable strains containing appreciable proportions of both. The ploidal compositions of these strains are maintained during serial subcultures and clonal re-isolations. Properties of the three types of strains, including size distributions of spores and myxamoebae and certain morphogenetic capacities, were described by Sussman & Sussman (1962). They also specified some conditions under which each of the types could be isolated from the others. The present paper is concerned with a more detailed examination of the conditions of cultivation which permit the haploidization of stable diploid and metastable strains.

METHODS

Organisms. Two stable diploid strains of *Dictyostelium discoideum*, RA and H-1, and one metastable stock, I-2A, were used.

Cultivation. Mass and clonal cultures were maintained on SM agar in association with *Aerobacter aerogenes* (Sussman, 1951). Many of the growth studies were made in liquid media of two kinds, the first being a suspension of washed *A. aerogenes* in M/60 phosphate buffer (Gerisch, 1960), the second a growing culture of *A. aerogenes* in a nutrient broth (Sussman, 1961*a*). Twenty ml. volumes in Erlenmeyer flasks

were shaken (200 cycles/min. with a 1·5 in. stroke) at 22°. Growth was measured by total and viable cell counts.

Size distributions of spores. Spores from haploid strains of *Dictyostelium discoideum* are regular ellipsoids. Spores from diploid strains are much more irregular and often banana, boomerang or serpentine in shape. In both cases, the major diameter is an accurate measure of cell size (Sussman & Sussman, 1962). Sizing was done with wet mounts under an oil-immersion objective, with an ocular micrometer. Spore lengths are given as micrometer divisions (1 division = 0·83 μ).

Chromosome counts. The staining procedure involves fixation in Carnoy's fluid and exposure to aceto-orcein after acid hydrolysis. Metaphase chromosomes are immediately and clearly visible under phase contrast and to some extent under bright field. (See Sussman, R. R. 1961, for photomicrographs.)

RESULTS

Criteria used to detect ploidal composition

The fundamental criterion by which the ploidal varieties have been defined is, of course, the number of chromosomes viewed in metaphase. The haploid number in *Dictyostelium discoideum* is 7. Thus, of several hundreds of countable figures in strains RA and H-1, none was haploid, while haploid stocks derived later on from these showed no diploid metaphase figures out of several hundred examined. Strain I–2A has on all occasions shown a preponderance (*c.* 90 %) of diploid chromosome sets. Other metastable strains display a preponderance of haploid cells (Sussman & Sussman, 1962).

The size distributions of both spores and myxamoebae could be strictly correlated with ploidy (Sussman & Sussman, 1962). This correlation made it possible to diagnose the ploidy of a clone by microscopic inspection of its spores. The criteria used are: (*a*) the incidence of spores big enough ($d_m \geqslant 16$ div.) or small enough ($d_m \leqslant 8$ div.) to be at the high extreme of the diploid or the low extreme of the haploid size distributions; (*b*) the mean major diameters of the populations; (*c*) the spore shapes. Many spot checks made by inspection of mitotic figures in the course of this and a previous investigation (Sussman & Sussman, 1962) support the validity of this method of diagnosing ploidy.

The haploidization of strain RA

Twenty ml. of nutrient broth inoculated with RA myxamoebae at an initial density of 10^4 cells/ml. and 1 ml. of a 24 hr. bacterial broth culture were incubated with shaking at 22°. Plate-grown cells experience a lag, but liquid-grown amoebae transferred from the log phase do not. Figure 1 shows the growth kinetics of two such cultures maintained in continuous exponential growth for 47 and 40 generations respectively by serial passage. The generation time was initially about 4·0 hr. but speeded up to 3·3 hr. after several passages. Samples were plated at intervals in replicate to yield clones. The ploidal compositions of these were scored by spore size determinations and occasionally by chromosome counts. Early samples yielded nothing but diploid clones, but later samples gave rise to progressively increasing proportions of haploid clones. Figure 2 is a summary of data for the two cultures mentioned previously. These are from a total of four experiments and show between

them the greatest variation. In one, haploids were detected after only 8 generations, and by 30 generations diploids were undetectable. In the other, haploid clones were not observed until after 20 generations and diploid clones were still found after 45 generations. In the four experiments, all of the clones examined were characteristic of either the stable haplophase or diplophase on the basis of spore size. Figure 3 shows the size distributions of spores from 10 diploid and from 16 haploid clones chosen at random from one of the experimental plates. They resemble the distributions previously obtained for diploid and haploid stocks.

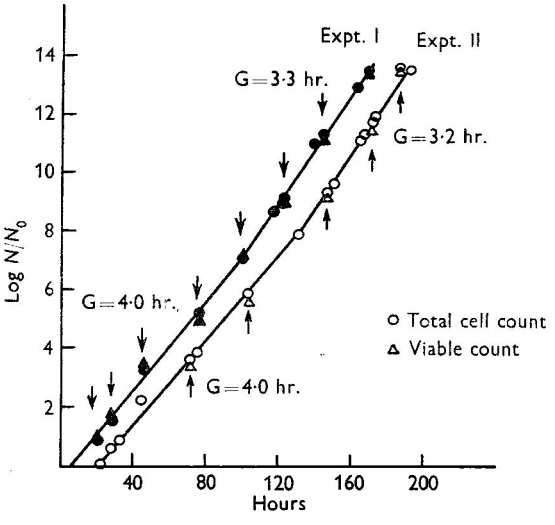


Fig. 1

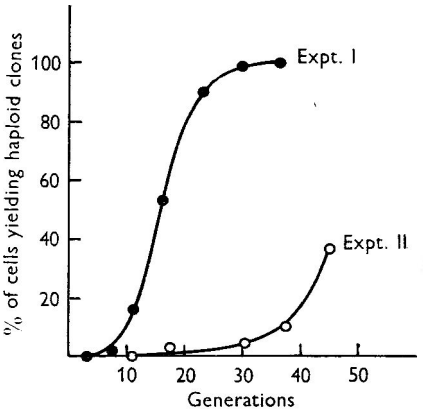


Fig. 2

Fig. 1. Growth kinetics of *Dictyostelium discoideum*, strain RA. 'G' refers to generation times. The arrows designate the times at which samples were withdrawn for plating in order to score ploidal composition.

Fig. 2. Appearance of cells capable of yielding haploid clones in cultures of *Dictyostelium discoideum* strain RA. Clones were scored for spore size and chromosome counts in samples taken at the times designated by the arrows in Fig. 1.

Selective advantage of the haploid segregants of strain RA

In both of the curves shown in Fig. 1, a significant increase in growth rate occurred during the period of cultivation. This suggested that the shift from diplophase to haplophase might have been partly caused by haploid segregants growing faster than their diploid progenitors. Several of the haploid clones to which strain RA has given rise during the sustained exponential growth in liquid culture were replated to ensure clonal homogeneity and then grown in liquid medium in the same manner as described above. All grew significantly faster than the parental RA stock. Figure 4 summarizes the data for one isolate, strain 44–14. A reconstitution experiment was made to see whether the faster growth rate of the haploid segregants could account for the ploidal shift observed in Fig. 2. Two liquid cultures were started, one containing strain RA only; and the other a mixture of RA and 44–14 initially in a ratio of 86:14. Samples were plated for clonal analysis of spore size at intervals during sustained exponential growth. Figure 5 shows that in the control culture, cells capable of giving rise to haploid clones were detected in the controls after 18

generations and reached about 40 % of the total by 33 generations. Therefore, if the haploid segregant grew at the same rate as its diploid parent when mixed with it, the experimental culture which contained 14 % haploids at zero time would have been expected to yield the same curve as the control but displaced upward from it by 14 % (the lower dashed line in Fig. 5). If, on the other hand, the haploid segregant could grow faster than its diploid parent when mixed with it, one would have expected the proportion of haploids in the experimental culture to increase very much more steeply than in the control. If in fact the difference in growth rates between strains 44–14 and RA were the same, as shown in Fig. 4, one would predict a curve like that given by the upper dashed line. As seen in Fig. 5, the experimental points do fall very near the latter curve.

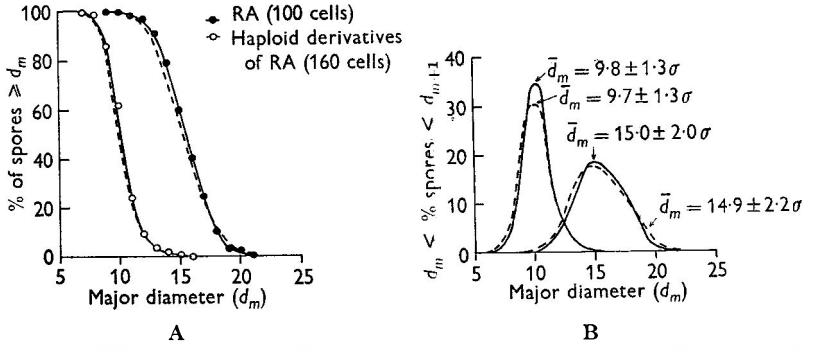


Fig. 3. Clones from Expt. I of Fig. 2 were scored for ploidy by chromosome strains. The major diameters of randomly chosen spores from these clones were then measured. The integral curves in A show the percentage of spores whose major diameters were equal to or greater than the values given in the abscissa. The differential curves in B were derived from the upper and show the percentage of spores falling between unit increments in major diameters. The dotted lines are curves previously obtained for RA, a diploid stock and NC–4, a stable haploid stock (Sussman & Sussman, 1962).

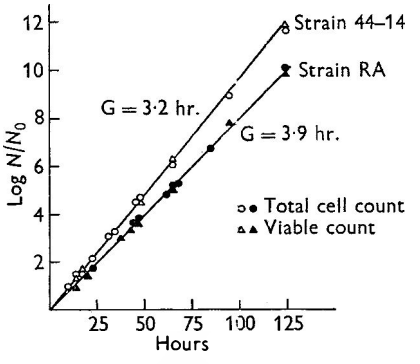


Fig. 4. Growth curves of *Dictyostelium discoideum*, strain RA and its haploid derivative, 44–14.

Haploidization and genetic segregation in a heterozygous diploid

Strain H–1 is a stable diploid which arose as a single clone from a mixed culture of two metastable mutant strains. The first, I–47, has fruiting bodies with yellow (wild-type) pigment plus a second reddish brown soluble pigment which stains both

the fruiting bodies and underlying agar. The second, I–262, remains pure white. H–1 is diploid and displays only the yellow pigment but a small (0·1–1) percentage of its clones are haploid and display pigmentation patterns corresponding to the four segregant classes (i.e. yellow-brown, white, white-brown, and yellow (Sussman, 1961*b*)). The haploid segregants breed true both for pigmentation and ploidy whereas the diploid clones continue to segregate at low frequency.

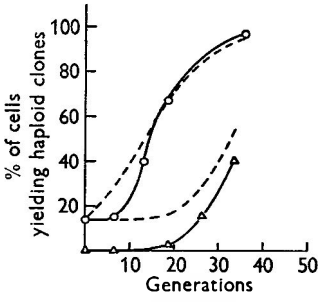


Fig. 5. Haploidization in *Dictyostelium discoideum*, strain RA (△) and a mixture of RA and 44–14 (○). The lower dashed curve would be expected for the mixture if RA and 44–14 grew at the same rate. The upper dashed curve would be expected if they grew at the rates deduced from Fig. 4.

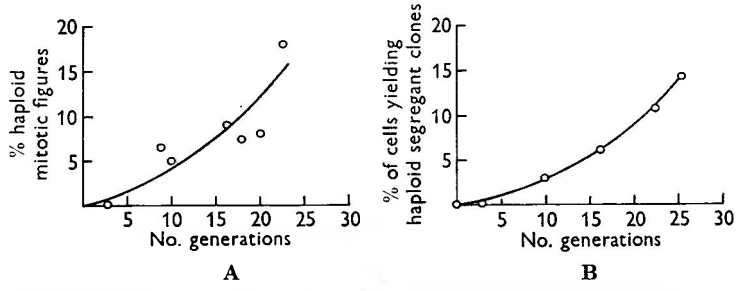


Fig. 6. A. the increase in haploid mitotic figures in samples taken from a growing culture of H–1. B. the increase in cells yielding haploid, segregant clones.

Table 1. *Haploid segregants from Strain* H–1 *during sustained exponential growth*

No. of generations	No. of clones examined	No. haploid	Pigment phenotypes				% of total clones
			White	Yellow-brown	White-brown	Yellow	
2·5	72	0	0	0	0	0	0
10	64	2	1	1	0	0	3
16	72	4	2	1	0	1	5·7
22·5	65	7	2	3	1	1	10·8
26	83	12	4	5	1	2	14·4

A liquid nutrient culture of H–1 was maintained in the exponential phase for 30 generations. Samples were withdrawn at intervals for staining. The incidence of haploid mitotic figures rose from an undetectable level to 18 % during 23 generations (Fig. 6A). Samples were also plated and randomly chosen clones scored for ploidy and incubated further to determine pigmentation. The incidence of haploid clones rose from a negligible level to 14 % after 26 generations (Fig. 6B). All clones deter-

mined to be diploid retained the wild-type pigmentation. All those first determined to be haploid, on subsequent incubation fell into one or another of the four segregant classes described above (Table 1).

Cytological observation also revealed, in the later samples, an appreciable incidence of tripolar and tetrapolar mitotic figures. An example of the former in telophase is shown in Pl. 1. A considerable number of binucleate cells was also detected. Strain RA also displayed these anomalies.

Haploidization of metastable strains

Table 2 summarizes the results of an experiment with strain I–2A. After 23 generations of sustained exponential growth, the incidence of haploid mitotic figures rose slightly and the incidence of cells that yielded haploid clones increased from a negligible initial level to 6 %. Another metastable strain, I–47, in which the majority of cells are haploid, yielded a large proportion of haploid clones after only 10 generations.

Table 2. *Haploidization of strain* I–2A

A. Incidence of haploid and diploid mitotic figures

No. of generations	No. diploid	No. haploid	% haploid
11	18	3	14
17	21	2	9
23	34	9	21

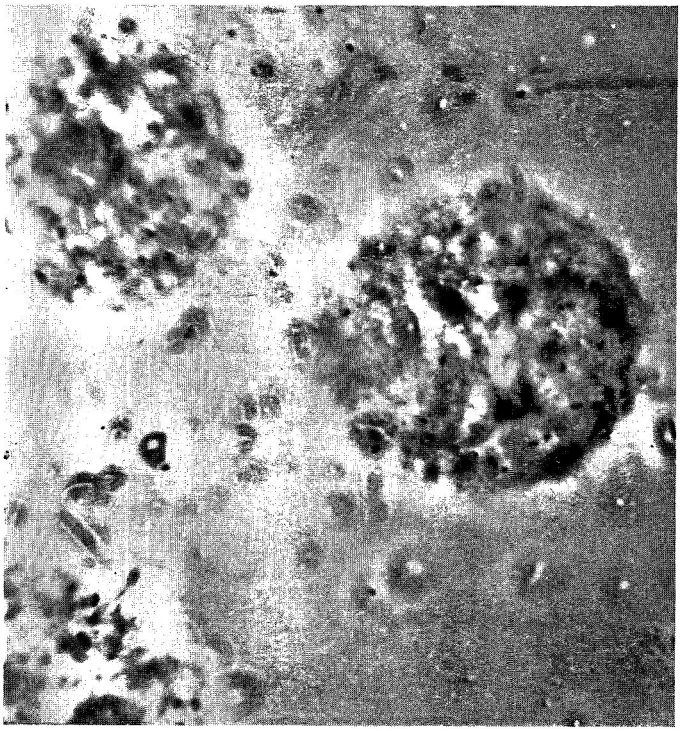
B. Incidence of stable haploid clones

No. of generations	No. clones examined	No. haploid	% haploid
3·8	50	0	0
17	51	0	0
23	86	5	6

DISCUSSION

The data indicate that conversion of diploid and metastable stocks of *Dictyostelium discoideum* to the stable haplophase occurs during growth and, in the absence of clonal re-isolation, there is a progressive increase in the proportion of stable haploids. Under certain cultural conditions this process is facilitated by selective advantages enjoyed by the haploid segregants, possibly because of the uncovering of recessive alleles affecting growth rate.

The mechanisms of diploidization and haploidization in *Dictyostelium discoideum* are unknown at present. The existence of heterozygotic strains like H–1 forces the conclusion that at least some diploids arise by cell fusion and karyogamy. Several mechanisms of haploidization can be envisaged. An orthodox meiotic reduction may occur such as proposed by Wilson (1953) and Wilson & Ross (1955). Cytological support for this process is suggestive but equivocal. Vegetative reduction division by means of tripolar and tetrapolar mitoses is a second possibility. Experimental support for this includes the tri- and tetrapolar mitotic figures encountered in haploidizing cultures (see Pl. 1) and observations of ternary and quaternary fissions by time-lapse cinematography (Sussman, Sussman & Ennis, 1960). A third



possibility is a piecemeal loss of chromosomes from the diploid by non-disjunction or other means. In this case the haplophase would be preceded by transitory aneuploidy lasting over several generations. The latter has been observed in at least one fungal group, Aspergillus (Käfer, 1960). At present it is impossible to choose among these hypotheses.

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EXPLANATION OF PLATE

Photomicrograph of tripolar mitotic (telophase) figure (× 2000). Aceto-orcein stain after Carnoy fixation and acid hydrolysis.

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Control of Internal Induction of Galactose Pathway Enzymes in an *Escherichia coli* Mutant

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SUMMARY

Galactokinaseless mutants of *Escherichia coli* are, in general, phenotypically constitutive for the remaining enzymes of the galactose pathway, a phenomenon which has been attributed to internal induction (Jordan, Yarmolinsky & Kalckar, 1962). One exceptional, kinaseless, mutant, though constitutive during stationary phase, is inducible during logarithmic growth. This mutant, w3092A, also produces a constitutive concentration of enzyme after confluent growth on solid medium and continues to do so even after 3–5 generations of subsequent logarithmic growth in liquid medium. This effect of growth on solid medium is peculiar to w3092A. It does not occur in other inducible strains, including galactose-fermenting revertants of w3092A, nor in w3092A itself grown as isolated colonies. Even in confluently grown organisms it is lost after storage at 4° for several days. In each case high degrees of enzyme synthesis can be prevented by an inhibitor of induction of the galactose enzymes, methyl-β-D-thiogalactoside, and are therefore attributed to internal induction. Though the enzyme concentrations decrease rapidly when the cultures resume logarithmic growth after stationary phase, high concentrations may persist up to the fifth generation after confluent growth on solid medium and then decline to reach the basal level only after the 12th generation. The inducibility of w3092A (A character) was shown to be independent of the mutation to kinaseless, since other kinaseless mutants derived from a galactose-fermenting revertant of w3092A still carried the A character.

INTRODUCTION

Galactose is converted to glucose in *Escherichia coli* by a series of inducible enzymes determined by genes located in the 'gal region' of the chromosome. Three enzymes and their corresponding cistrons have been identified (Morse, Lederberg & Lederberg, 1956*a*, *b*; Kurahashi, 1957; Kalckar, Kurahashi & Jordan, 1959; Lederberg, 1960; Soffer, 1961). These catalyse the reactions described by the equations given below:

$$\text{Gal} + \text{ATP} \xrightarrow[\text{cistron A}]{\text{galactokinase}} \text{Gal-1-P} + \text{ADP}$$

$$\text{Gal-1-P} + \text{UDPG} \underset{\text{cistron B}}{\overset{\text{transferase}}{\rightleftharpoons}} \text{UDPGal} + \text{G-1-P}$$

$$\text{UDPGal} \underset{\text{cistron D}}{\overset{\text{epimerase}}{\rightleftharpoons}} \text{UDPG}$$

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The following abbreviations are used: gal, D-galactose; gal-1-P, D-galactose-1-phosphate; G-1-P, α-D-glucose-1-phosphate; UDPG, uridine diphosphoglucose; UDPGal, uridine diphosphogalactose; NAD, nicotinamide-adenine dinucleotide; NADH, reduced NAD; ADP, adenosine diphosphate; ATP, adenosine triphosphate; galactokinase or kinase, ATP:D-galactose-1-phosphotransferase (E.C. 2.7.1.6); transferase, UDPglucose:α-D-galactose-1-phosphate uridylyltransferase (E.C. 2.7.7.12); epimerase, UDPglucose 4-epimerase (E.C. 5.1.3.2); UDPG pyrophosphorylase, UTP:α-D-glucose-1-phosphate uridylyltransferase (E.C. 2.7.7.9).

It was recently shown also that lack of the enzyme UDPG pyrophosphorylase results in an inability to metabolize galactose (Fukasawa, Jokura & Kurahashi, 1962). The gene controlling this enzyme is not located in the 'gal region'—nor is the enzyme inducible by galactose. An operator locus within the 'gal region' (Kalckar *et al.* 1959; Lederberg, 1960; Buttin, 1961) and a repressor locus outside this region responsible for the control of the galactose enzymes (Buttin, 1961) have also been identified.

It has been shown that the majority of kinaseless mutants are constitutive for the remaining enzymes of the galactose sequence and that revertants to galactose fermentation are again inducible. In this case constitutive synthesis can be inhibited by methyl-β-D-thiogalactoside (MTG), an inhibitor of induction for the galactose enzymes (Buttin, 1961). Constitutivity associated with lack of kinase was therefore attributed to internal induction by a substance which in the wild type is inactivated by a functional kinase (Jordan *et al.* 1962). The induction achieved by the addition of D-galactose or D-fucose corresponds to an increase in enzyme concentration of only a few fold; internal induction may be responsible for the high basal enzyme concentration even in the wild type.

One exceptional, inducible, kinaseless strain was found in our laboratory (Yarmolinsky & Wiesmeyer, 1959). This strain, w3092A, though inducible during logarithmic growth, is phenotypically constitutive in stationary phase. We previously reported (Yarmolinsky, Jordan & Wiesmeyer, 1961) a phage-produced alteration of enzyme inducibility in this strain. It was found that treatment of w3092A with phage, to effect prophage substitution, resulted in a change to constitutive enzyme production in logarithmic growth phase. Subsequent work has shown, however, that our original interpretation was erroneous. In these experiments one uncontrolled variable was systematically introduced: the type of colonial growth from which inocula were taken for the growth of assay cultures. It appears that a physiological modification, which may last for many generations, takes place in w3092A during confluent growth on solid medium. The nature of this modification is the subject of this paper.

METHODS

Materials and methods, including strains of *Escherichia coli* K-12, were essentially as described in Jordan *et al.* (1962).

Growth media. Tryptone broth: 1% Difco Bacto-Tryptone with 0·5% NaCl. 1·5% agar was added for tryptone agar.

Minimal agar: medium A (Hartman, 1956). A salts medium with NH_4^+ as nitrogen source and 1% glucose as carbon source—with 1·5% agar.

EMB agar: eosin-methylene blue agar (Campbell, 1957). 1 % galactose was added to make EMB gal agar.

Enzyme assay. The assay for epimerase, a modification of the method of Kalckar *et al.* (1959) was performed on lysozyme-disrupted organisms as follows. About 10^7 organisms were mixed in the cuvette with an equal volume of a lysis mixture (K. Paigen, personal communication) 0·3 mg lysozyme/ml. in 0·1 M-tris(hydroxymethyl)aminomethane-acetate (pH 8·0), containing $7{\cdot}5 \times 10^{-3}$ M-ethylenediaminetetraacetate. The reaction mixture, consisting of 100 μmoles glycine (pH 8·7) 0·05 μmole UDPGal (Wiesmeyer & Jordan, 1961), 2·5 μmole NAD and 100 units (Strominger, Maxell, Axelrod & Kalckar, 1957) of UDPG dehydrogenase, was added to the lysed organisms immediately. It was found that cells prepared in this way had negligible NADH oxidase activity.

Most of the assays reported in this paper were performed by the above method. In these cases, specific activity was calculated using optical density of the suspension of organisms at 650 mμ as a measure of protein. To obtain units comparable to those previously published, it was estimated that an optical density at 650 mμ of 5·0 corresponded to 1·0 mg. protein/ml. Thus the unit used here, μmole UDPG/hr/O.D.650 = 5, is roughly equivalent to μmole UDPG/hr./mg. protein, the unit used previously.

RESULTS AND DISCUSSION

For some time we were puzzled that the conversion of *Escherichia coli*, w3092A to the constitutive state occurred only during prophage substitution experiments. A close scrutiny of the protocols for such experiments finally revealed only one variable which correlated with conversion to constitutivity—the type of growth from which the inoculum for the assay cultures was taken. It seemed highly unlikely that differences in the origin of the inoculum could account for our findings, but a compilation of all relevant data seemed to bear out the hypothesis. This is presented in Table 1. It is seen that, under the conditions described, in about four

Table 1. *Effect of origin of inoculum on epimerase inducibility in strain* w3092A

	Confluent colonies		Isolated colonies	
Solid medium	No. constitutive	No. inducible	No. constitutive	No. inducible
EMB	48	12	0	28
Tryptone	16	5	0	5
Minimal glucose	2	0	0	6

Epimerase assays were performed on liquid cultures after about three generations of growth in the presence or absence of 10^{-2} M-galactose as inducer. The optical density at 650 mμ of the freshly inoculated cultures was between 0·01 and 0·02. Constitutive and inducible are defined as a specific activity ratio of + gal/− gal grown cultures of $< 2{\cdot}5$ and $\geqslant 2{\cdot}5$ respectively.

out of five cases, cultures derived from confluent colonies behave as if they were constitutive, while cultures derived from isolated colonies are inducible. While results for epimerase only are reported here, experiments in which transferase was assayed indicate that this enzyme behaves similarly. Furthermore, the method of cell disruption appears to be immaterial. Initial experiments (Yarmolinsky *et al*

1961) employed sonication and yielded results comparable to those obtained later with lysozyme.

With this encouragement, the experiment shown in Table 2 was performed. A culture of w3092A was streaked on EMB agar to obtain isolated colonies. Some of these (column A) were used directly as inocula for cultures to be assayed, others were first restreaked on EMB plates to obtain confluent growth (column B). Clearly all strains under column A, derived from isolated colonies, are inducible, while all except one under column B, derived from confluent colonies, are constitutive.

Table 2. *Effect of origin of inoculum on epimerase levels in strain* w3092A *and its reversibility on solid medium*

	A	B	C	D	E
Solid medium...	EMB	EMB	EMB	Min glu	Min glu*
Type of growth...	Isolated	Confluent	Isolated	Isolated	Confluent
Specific activity of epimerase in broth culture	4·2/16	16/11	3·2/14 1·7/10	3·4/15 2·8/13	11/6·7 —
	3·4/15	13/14	— —	2·3/11 2·8/9·5	6·1/5·7 —
	—	17/13	— —	1·3/6·5 2·1/8·4	— —
	4·0/17	13/19	4·0/15 2·7/15	—	—
	4·0/16	17/16	—	−gal/+gal	
	3·2/17	4·6/15	—		

The numbers are specific activities of epimerase after three generations growth in tryptone broth (expressed as μmole UDPG/hr./O.D.650 = 5). The numerator (denominator) of each ratio are the values obtained when 10^{-3}M-galactose was absent (present) in the broth culture. The source of the inoculum for each broth culture is indicated by the column headings. The cultures described in column B were derived from those described in column A by picking from the same plate and streaking out on EMB agar to obtain isolated colonies. Similarly, the cultures of columns C, D, E were derived by picking from the confluently grown column B cultures on to the indicated media.

* It appears from the data in this column that there is no inhibition of enzyme synthesis by glucose in this strain, W3092A. A glucose effect is observed in liquid cultures (Jordan *et al.* 1962). Its absence here may be due to depletion of glucose in the medium.

It was of interest to find out whether the phenomenon was reversible and whether it was medium-dependent. Conceivably the medium could contain a repressor of enzyme synthesis to which strain w3092A is sensitive and which is locally depleted by confluently growing cells. Such a repressor might not be present in minimal salts medium. Loopfuls were therefore picked from the streaks of column B and restreaked to obtain isolated or confluent colonies on EMB and minimal glucose agars. Column C indicates that the phenomenon is fully reversed by growth as isolated colonies. Columns D and E show that minimal solid medium gives essentially the same results as enriched medium. It is therefore unlikely that a repressor from the medium is involved.

Galactose might be formed endogenously in strain w3092A from UDPG by reversal of the reactions catalysed by epimerase and transferase and cleavage of the gal-1-P. Under conditions of confluent growth it might accumulate in this way, causing induction, while in the case of isolated colonies it could diffuse into the medium. As pointed out above, w3092A increases its level of epimerase when grown

into stationary phase in liquid medium as it does in confluent growth on a solid medium. This may also represent internal induction operating in w3092A during post-logarithmic growth.

Internal induction has been shown to occur in other kinaseless mutants previously studied (Jordan *et al.* 1962), in both post-logarithmic and logarithmic growth phases by demonstrating that MTG could prevent constitutive enzyme synthesis. The results of a comparable experiment with strain w3092A are shown in Table 3. Suspensions of organisms from confluent growths on EMB or tryptone agar have high concentrations of enzyme even though no galactose is added. However, when MTG is present in the agar, or in liquid medium during subsequent growth of the culture, the enzyme level becomes low. Similarly, the increase in enzyme concentration on going from logarithmic to stationary phase is prevented by MTG. The results therefore bear out the hypothesis that constitutivity in w3092A is the result of internal induction in each case.

Table 3. *Reversal of constitutivity in strain* w3092A *by the inhibitor of induction, methyl-β-*D*-thiogalactoside (MTG)*

		Specific activity of epimerase (μmole UDPG/hr./O.D.650 = 5)		
			$+10^{-2}$M-gal*	$+10^{-2}$M-MTG*
Confluent colonies	On EMB agar	14	—	—
		11	—	—
	On Tryptone agar	14	—	—
		13	—	—
	On Tryptone agar + MTG	1·0	—	—
		1·0	—	—
Logarithmic cultures derived from confluent colonies	On EMB agar	8·8	7·2	0·8
		7·4	9·7	0·4
		9·1	8·4	1·0
		6·1	9·1	1·0
	On Tryptone agar	14	19	2·3
		5·7	18	1·9
Cultures derived from a slant	log. phase	1·7	6·3	1·6
		—	17	1·7
	post-log phase	25	17	1·7

For assay of confluent colonies, cell suspensions were made in tryptone broth. Logarithmic cultures refers to cells grown about three generations in tryptone broth.

* Galactose or MTG were present during growth in broth.

It should be noted that cultures derived from confluent growth on a stock slant are *not* constitutive. This appears to be related to the age of such slants. It has been found that after several days storage at 4°, confluently growing organisms no longer are constitutive after growth in broth. Previous to experiments involving prophage substitution (Yarmolinsky *et al.* 1961) the inocula for the growth of w3092A cultures had been taken from slants stored several days in the cold. Since freshly made solid cultures of w3092A were first used routinely in the course of the prophage substitution experiments, we observed the dependence of enzyme concentration on the growth-history of the inoculum at this time, and mistakenly attributed the alteration in enzyme concentration to the treatment with prophage.

The high enzyme concentrations observed after internal induction on solid medium may be retained through many generations of logarithmic growth, as illustrated in Fig. 1. A suspension of confluently grown organisms from EMB agar was inoculated into tryptone broth with or without MTG and maintained in logarithmic growth for 15 generations by successive dilution every third generation. It is seen that in the absence of MTG the specific activity of epimerase remained high for about 5 generations and then decreased slowly to reach a stable, low level after 12 generations. In the presence of MTG the enzyme activity decreased more rapidly reaching after only three generations a level equal to the lowest level attained in the

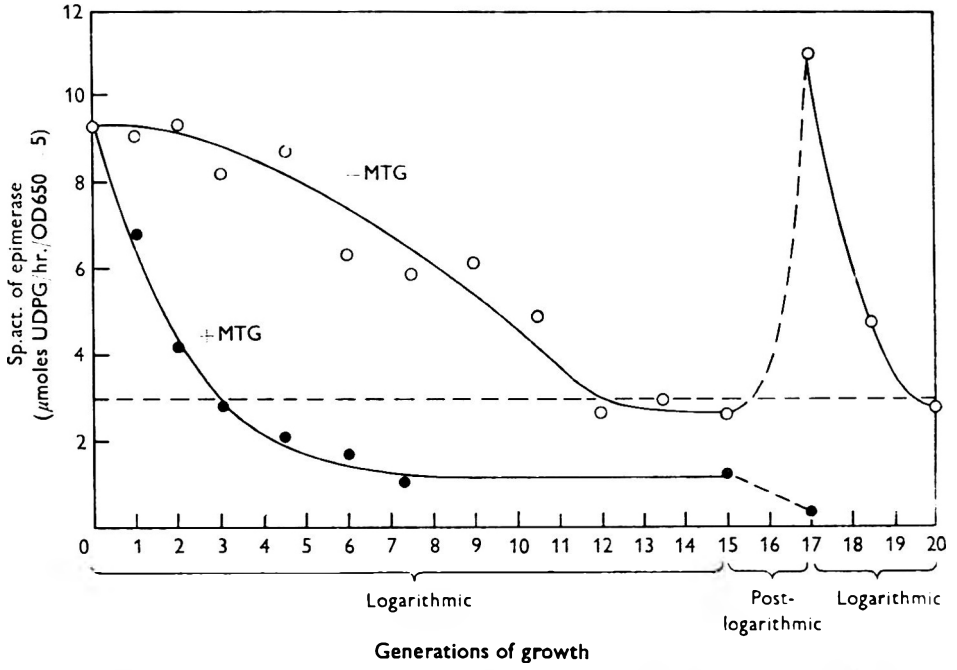

Fig. 1. Kinetics of decrease of epimerase activity after internal induction. A suspension of cells was made from confluent colonies on EMB agar and inoculated into broth $\pm 10^{-2}$ M-MTG. At the end of the 3rd, 6th, 9th and 12th generations the cultures were diluted tenfold to maintain them in logarithmic growth. After the 15th generation the cultures were allowed to go into post-logarithmic phase (until an O.D. 650 of 1·0 was attained) and then diluted to resume logarithmic growth. ----, dashed horizontal line represents the average basal enzyme level in this strain during logarithmic growth in tryptone broth.

absence of MTG and finally levelling off at about one-half this value. This indicates that in this particular strain there is a slight degree of internal induction even in logarithmic phase.

After the fifteenth generation the cultures were allowed to overgrow until an optical density at 650 mμ of 1·0 was reached. At this point they were again diluted and permitted to resume logarithmic growth. As seen in the figure, in the absence of MTG the enzyme concentration rises sharply during post-logarithmic growth. If the cells are allowed to continue post-logarithmic growth for longer times than in the experiment illustrated in Fig. 1, concentrations of epimerase two- to three-fold higher than those attained on solid medium may be obtained.

Upon suspension of cells in fresh medium after growth into stationary phase, the specific activity of epimerase falls exponentially to the basal level at the rate expected if internal induction ceases immediately upon resumption of logarithmic growth.

Upon suspension of organisms in fresh medium after confluent growth on solid medium, however, the specific activity of epimerase remains high for about five generations and then decreases at a non-exponential rate much slower than that observed after dilution of stationary phase liquid cultures. This result suggests that during confluent growth on solid medium internal inducer accumulates to a concentration requiring several generations of growth to be diluted out below the threshold concentration required for induction.

The observation, that in stationary phase cells epimerase concentrations are initially higher and yet decrease more rapidly upon suspension in fresh medium than in cells grown confluently on solid medium, seems paradoxical. It may be that growth conditions affect the synthetic capacity of the culture or the rate of elimination of the presumed internal inducer.

Table 4. *Independence of the* A *character and lack of galactokinase*

Strain		Specific activity of epimerase (μmole UDPG/hr./o.d.650 = 5)		
		−gal	$+10^{-2}$M-gal	+gal/−gal
w3092A		4·2	16	3·8
K⁻ from w 3092AR	*a*	2·8	10	3·6
	b	4·6	15	3·3
Average of other K⁻ strains		—	—	1·0*
Average of gal⁺ strains		—	—	7·2*

Assays were performed on logarithmic phase cultures derived from overnight cultures.
* Calculated from data of Jordan *et al.* 1962.

If the organisms are grown in broth containing MTG after growth on solid medium, their epimerase level decreases more rapidly than if they are grown in the absence of MTG, but not as rapidly as would be expected if MTG immediately inhibited the induction of epimerase completely. This suggests that MTG acts competitively with the presumed internal inducer and cannot inhibit enzyme synthesis completely until this inducer has been diluted out by growth.

Strain w3092A arose spontaneously in our laboratory from another kinaseless strain, w3092, which appears constitutive in all growth phases. This suggests that expression of inducibility in w3092A is the result of a mutation other than that leading to loss of kinase activity. One would therefore expect that other kinaseless strains derived from a galactose-fermenting revertant of w3092A should be inducible. This was found to be the case. The revertant w3092AR (described in Jordan *et al.* 1962) was treated with 0·02 % 2-amino purine according to Demerec (1960). Of the galactose-negative mutants obtained, two were kinaseless by the following criteria. They were transducible to galactose fermentation at high frequency by wild-type transducing phage, but only at low frequency by transducing phage from w3092A. The gal + colonies obtained at low frequency with w3092A are presumably the result of crossing over within the cistron. Furthermore, phage lysates from the

presumptive kinaseless mutants yielded many more gal + colonies on epimeraseless or transferaseless indicator strains than on the kinaseless w3092A. The appearance of some gal + colonies in crosses of the new kinaseless strains with w3092A indicates that neither of the new strains carries a mutation identical with that in w3092A.

Epimerase assays were performed on the new kinaseless strains derived from w3092R. Table 4 shows that epimerase in both of these strains is inducible, indicating that the mutation permitting inducibility to be expressed is a character independent of the mutation to kinaseless.

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The Effect of Lipids on Citric Acid Production by an *Aspergillus niger* Mutant

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SUMMARY

A mutant strain, 72-44, of *Aspergillus niger* was selected after ultraviolet irradiation of the spores of *A. niger* strain 72-4. This mutant was capable of giving high yields of citric acid in shaken flask fermentations. A study was made of the effect of various lipids on the yield of citric acid produced by *A. niger* strain 72-44. Fatty acids with less than 15 carbon atoms inhibited growth and no citric acid was produced. Natural oils with a high content of unsaturated fatty acids and oleic acid itself, when added at 2% (v/v) to suitable fermentation media, increased the yield of citric acid by about 20%. Lipids which improved the yield of citric acid had no effect on the dry weight of mycelium. The possible mode of action of effective lipids is discussed. It is suggested that unsaturated lipids act as alternative hydrogen acceptors to oxygen during the fermentation and thus improve the yield of citric acid.

INTRODUCTION

The problems associated with citric acid production by fermentation have been reviewed by Foster (1949), Johnson (1954) and Perlman & Sih (1960). In fermentations with *Aspergillus niger*, the amount of growth must be small and the medium must contain only very small concentrations of trace metals. These conditions present a serious problem when attempting to carry out this fermentation on an industrial scale. A major difficulty experienced by all workers is associated with inconsistencies in yield. In attempts to solve these problems, strains tolerant of trace metals have been selected (Bernhauer, 1929; Doelger & Prescott, 1934; Bernhauer, Knobloch & Iglauer, 1941), or substances to inhibit growth have been added to the medium. Gardner, James & Rubbo (1956) used X-rays and ultraviolet irradiation to produce mutants with a higher tolerance of trace metals. Perlman (1943), Gerhardt, Dorrell & Baldwin (1946), Bernhauer, Rauch & Gross (1949) and Martin & Waters (1952) used potassium ferrocyanide to decrease the trace metal content of beet molasses media to inhibit growth and increase the yield of citric acid. Moyer (1953) found that methanol, ethanol and isopropanol decreased growth and increased citric acid production from cane and beet molasses media. The present paper describes the selection of a potentially high-yielding mutant of *A. niger* strain 72-4 and the effect on the yield of citric acid of adding various lipids to the fermentation medium.

METHODS

Organisms. The parent culture was *Aspergillus niger* Wisconsin strain 72-4 ATCC 11414 (Perlman, Kita & Peterson, 1946). The mutant used in the present work, strain 72-44, was selected after ultraviolet (u.v.) irradiation of the parent

culture. Stock cultures were maintained as freeze-dried suspensions of spores in ampoules.

Media for fermentations (*Table* 1). Four different media were used for shaken flask fermentations, two for the screening mutants after u.v.-irradiation and two for testing the effect of lipids on yield of citric acid. The media used for testing lipids were modified from those reported by Shu & Johnson (1947, 1948*a*, *b*). All media were initially at pH 3; medium was dispensed in 50 ml. portions in 500 ml. conical flasks and autoclaved at 116° for 10 min.

Table 1. *Media used in shaken-flask citric acid fermentations by* Aspergillus niger *mutant* 72-44

Media constituents	Mutant screening		Lipid testing	
	Low trace metals	High trace metals	Medium A	Medium B
	(g./l.)		(g./l.)	
Sucrose	140·0	140·0	140·0	140·0
KH_2PO_4	1·00	1·00	1·00	0·30
$MgSO_4.7H_2O$	0·25	0·25	0·25	0·10
NH_4NO_3	1·87	1·87	1·87	1·40
	(mg./l.)		(mg./l.)	
Fe^{++}	3·0	16·0	2·0	2·0
Cu^{++}	0·3	10·0	0·3	0·3
Zn^{++}	0·1	2·0	0·1	0·1

The sucrose was A1 grade commercial cane sugar (Colonial Sugar Refineries, Australia). A 14% (w/v) solution was passed through a cation exchange resin Zeocarb 215 (Permutit Co.) in the H^+ form, and the effluent was tested for residual trace metals. It was found to contain (mg./l.): Fe^{++}, 0·1; Cu^{++}, 0·05; Zn^{++}, 0·05. The final concentrations of trace metals are listed in Table 1. A.R. grade chemicals were used for all media and no attempt was made to purify them further. Pyrex glassware was used to handle media and solutions of defined trace metal content and was cleaned in the usual way, washed in 10% (v/v) solution of redistilled concentrated HNO_3, and then rinsed three times with glass-distilled water.

In most cases lipids were added to media before autoclaving. To avoid oxidation, some of the highly unsaturated lipids (e.g. linseed oil, tung oil, linolenic and linoleic acids) were added to the sterile medium just before inoculation with spores.

Conditions of cultivation. All cultures were grown at 30°. All fermentations were carried out in shaken flasks. The shaker was housed in a warm room at 30° and was rotated at 210 rev./min. with a 2 in. amplitude throw. The table of the shaker had clips to hold 500 ml. conical flasks which were tilted at 15° to the horizontal to improve mixing. The sulphite value of these flasks, containing 50 ml. of solution, was 0·6 as determined by the method of Cooper, Fernstrom & Miller (1944).

Inoculum preparation. A stock freeze-dried culture was transferred to a beer wort agar slope. When sporing vigorously, the organism was subcultured to 20 similar slopes. When these were sporing, they were stored at 4° until required. The spores were then suspended in a 1/10,000 (w/v) sterile solution of Soaxit (sodium dioctyl-

sulphosuccinate; W. Hermon Slade & Co., Homebush, New South Wales, Australia) and spread over the surface of beer wort agar in an 8 in. Petri dish. To achieve good sporulation it was necessary to raise the lid of the Petri dish about 0·5 cm. The spores from the plate were harvested in sterile Soaxit, counted in a haemocytometer and, routinely, between 7×10^7 and 2×10^8 spores were used to inoculate 50 ml. of medium in shaken flasks.

Estimation of citric acid. Routinely, the yield of citric acid was measured by titrating a 2 ml. sample of culture fluid against 0·25 N-NaOH (phenolphthalein as indicator), and expressed as g. anhydrous citric acid/100 ml. of culture fluid. To determine whether the titratable acidity was due to citric acid alone, spot tests for oxalic acid were made according to Feigl (1960), citric acid was determined colorimetrically according to Marier & Boulet (1958) and chromatograms of the culture fluids were run with two different solvent systems, butanol + acetic + water (Smith, 1961) and butanol + formic + sodium formate + water (R. S. D. Read & N. F. Millis, personal communication.)

Estimation of sugar. Sugar was estimated before and after fermentation using Schaffer & Somogyi's reagent 50 (1933). For the initial sugar determination, the sucrose was inverted by heating a 1 ml. sample at 100° in 5 ml. of 0·1 N-H_2SO_4 for 10 min. Acid inversion was not necessary for residual sugar determinations.

Estimation of mould growth. The amount of mould growth was estimated by determining the dry weight of mycelium formed. The contents of a shaken flask were transferred to a weighed Whatman paper No. 541, washed three times with water and the mycelium dried at 60° for 24 hr. before weighing. When the fermentation was carried out in the presence of lipid, free lipid was removed by thoroughly washing the water-washed mycelium with ethanol before drying.

Estimation of oil utilization. To extract the oil after fermentation the mycelium was removed by filtration, washed with water and the filtrate and washings combined. The volume was reduced to one-tenth by vacuum distillation. The concentrate was mixed with 10 vol. ethanol and held overnight at 4°. The polysaccharide precipitate which formed was removed by centrifugation and washed with ethanol. The supernatant fluid and the ethanol washings were combined and the ethanol distilled off. The lipid was then extracted from the aqueous residue with ether. The removal of the polysaccharide was necessary to avoid the formation of emulsions at the ether water interface which occurred when ether extraction was attempted directly on the culture fluid.

The mycelium was dried at 60° for 24 hr., ground to a fine powder and lipid extracted with ether in a Soxhlet. The ether was distilled from both extracts and the residue weighed after drying over phosphorus pentoxide.

Estimation of iodine value. The iodine value of peanut oil samples was measured by Hanus's method as described in *Official Methods* (1955).

Estimation of peroxide value. The peroxide value of peanut oil samples was measured by the iodometric method of Jacobs (1958).

Saponification. Maize oil was separated into a saponifiable and a non-saponifiable fraction by refluxing with ethanolic KOH as described by Hilditch (1956).

RESULTS

The results reported here are the averages of duplicate fermentations. The divergence between the yields of citric acid in duplicate fermentations was not more than 0·7 g./100 ml.

Selection of mutant 72-44 *of* Aspergillus niger

The spores of the parent strain *Aspergillus niger* 72-4 were harvested from beer wort, filtered to remove clumps, counted in a haemocytometer and standardized at $1–2 \times 10^6$ spores/ml. One ml. of spore suspension was placed in a 4 in. Petri dish on a rotating table 12 cm. below a u.v. lamp (Sterilamp, 2537 Å, Westinghouse) for 10 min. Single spores were then spotted out on paper as described by James, Rubbo & Gardner (1956), except that the paper was soaked for 30 min. in a medium containing (g./l.): 140·0, cane sugar (A1 grade commercial); 1·00, K_2HPO_4; 0·25, $MgSO_4.7H_2O$; 1·87, NH_4NO_3; (mg./l.) 2·0, Fe^{++}; 2·0, Cu^{++}; 1·0, Zn^{++}; (ml./l.) 100, of 0·5% (w/v) bromcresol green in ethanol. This medium was selected in place of molasses used by James *et al.*, since the zones of acid production were more clearly defined and the concentrations of trace metals more accurately known. About 40,000 colonies which developed on paper from irradiated spores were inspected; of these 120 were selected as potentially better producers of citric acid than the parent. These selected colonies were grown on beer wort agar slopes and the spores tested for the yield of citric acid in shaken-flask fermentations in two media, one with a high-trace metal content and the other with a lower-trace metal content (Table 1). Mutants which yielded well in both media were tested twice more and when they behaved consistently they were retained as stock cultures. Finally, mutant 72-44 was selected as the most promising and used for all work reported in this paper.

Table 2. *Yield of citric acid by two strains of* Aspergillus niger *at 7 days*

Growth medium	Strain 72-4	Strain 72-44
	Citric acid (g./100 ml.)	
Medium A	1·0–1·3	6·2–7·2
Medium B	1·7–2·0	7·3–8·0

A comparison of the yields obtained with strains 72-44 and 72-4 when grown in media A and B are shown in Table 2. The mutant 72-44 gave about 5 g. more citric acid/100 ml. than did the parent strain. The superiority in yield with the mutant was not as marked when both strains were grown in the presence of peanut oil. Details of the conditions of fermentation which lead to high yields of citric acid with mutant 72-44 will be the subject of another paper.

Number of spores needed in the inoculum

Early in the study of conditions necessary for the accumulation of citric acid in shaken flasks, considerable variability was experienced under conditions which were apparently identical. Some of this variability was traced to the number of spores used in the inoculum. Fig. 1 shows the yield of citric acid from Medium A when the spores in the inoculum were varied, in two separate experiments, from 1×10^6 to 8×10^8/50 ml. of medium. Fig. 1 shows that the yield was best when the inoculum

size was between 7×10^7 and 2×10^8 spores/50 ml. medium. Subsequently, it was found that the yield of acid was always poor when inadequate numbers of spores were used or when the germination of adequate numbers of spores was inhibited.

Effect of the addition of lipids on the citric acid yield

Following an observation that peanut oil increased the yield of citric acid in aerated and stirred fermentations, a systematic investigation was made in shaken flasks of the effect of various lipids on the yield of citric acid. In any series of fermentations containing test lipids, controls were included with no lipid added and with 2% (v/v) peanut oil present. Generally the test lipid was added to medium A, but medium B was used for some tests, and the yields of citric acid were determined at 7 days.

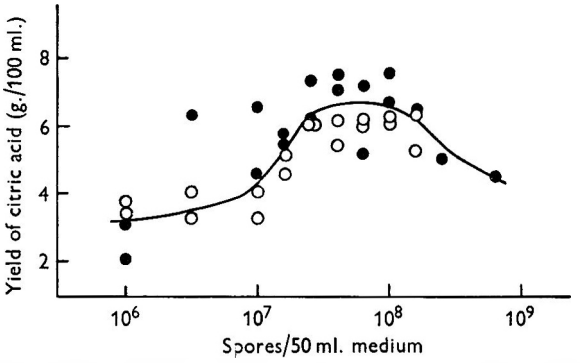

Fig. 1. The effect of the number of spores of *Aspergillus niger* 72-44 in the inoculum on the yield of citric acid in shaken culture. ○ = experiment 1; ● = experiment 2.

Table 3. *Lipids which inhibited growth of* Aspergillus niger *mutant* 72-44 *at 7 days*

Lipid added	Amount (%, v/v)	No addition	Lipid	Peanut oil 2%, v/v
		Citric acid (g./100 ml.)		
Natural animal oil: mutton bird	2·0		No growth	
Saturated fatty acids: acetic (2)*, propionic (3), butyric (4), valeric, isovaleric, levulenic (5), phenyl acetic (8), capric (10), undecylic (11), phenyl undecanoic (17)	0·05–1·0	5·0–8·4	No growth	8·4–10·4
Unsaturated fatty acids: 2,3-nonenoic (9), 2,3-decenoic (10), 10-undecenoic (11), 2,3-dodecenoic (12), 2,3-tridecenoic (13)	0·05–1·0	4·5–8·4	No growth	6·5–10·4
Glyceride: tributyrin	2·0	5·1	No growth	8·0

* Figures in parentheses refer to the number of carbon atoms in the preceding fatty acid.

It was found that some lipids either inhibited spore germination completely or allowed very little growth of the mould, and no citric acid was formed at 7 days. These lipids are listed in Table 3. The figures in parentheses indicate the number of carbon atoms in the preceding fatty acid. Saturated and unsaturated straight-chain

fatty acids containing from 2 to 13 carbon atoms were inhibitory to growth. Some lipids allowed normal growth of the mould but gave no improvement in the yield of citric acid. These are listed in Table 4. The figures in parentheses indicate the number of carbon atoms in the preceding fatty acid. Other lipids, listed in Table 5, allowed growth of the mould with increased yields of citric acid.

Table 4. *Lipids allowing growth but not increasing citric acid yield by* Aspergillus niger *mutant* 72-44 *at 7 days*

Lipid added	Amount (%, v/v)	No addition	Lipid	Peanut oil (2%, v/v)
		Citric acid (g./100 ml.)		
Natural vegetable oils:				
High % saturated fatty acid: coconut	2·0–10·0	5·0	5·0	8·1
High % unsaturated fatty acids: safflower, tobacco	0·5–1·0	5·0–6·5	6·0–6·5	8·2
Natural animal oil: cod liver	2·0–10·0	5·2	2·6–5·2	.
Natural mineral oils: liquid paraffin, light lubricating, heavy lubricating	2·0–10·0	6·0	2·0–6·5	9·5
Saturated fatty acids: tridecylic (13)*, myristic (14), pentadecylic (15), palmitic (16), chaulmoogric (18), arachidic (20)	(0·05–0·5 %, w/v)	4·4–8·4	3·0–6·2	6·5–10·4
Stearic, 12 OH-stearic, 9,10-di-OH-stearic (18)	0·2–1·0 (%, v/v)	.	.	.
Unsaturated fatty acids: 14 pentadecenoic (15), linoleic, linolenic, ricinoleic (18), erucic (22)	0·2–2·0 (%, w/v)	5·0–8·8	2·8–7·4	7·0–10·7
Vaccenic (18)	0·2 (%, v/v)	.	.	.
Esters of fatty acids: ethyl stearate, ethyl oleate, methyl linoleate	0·25–2·0	5·4–7·3	4·9–8·0	7·4–10·5
Glyceride: monoricinoleate	1·0 (%, w/v)	7·3	5·8	10·3
Sorbitan ester: polyoxyethylene oleate (Tween 80)	0·05–4·0	7·0	4·0–7·0	9·2

* Figures in parentheses refer to the number of carbon atoms in the preceding fatty acid.

Tables 4 and 5 show that the lipids which increased the yield of citric acid were natural oils high in unsaturated fatty acid, glycerides with at least one C18 unsaturated fatty acid and oleic acid itself. Natural oils with a high content of saturated fatty acids, saturated fatty acids and fatty acid esters were not effective. Of the C18 unsaturated fatty acids tested, only oleic acid (one double bond) was active; linoleic and linolenic acids (two and three double bonds, respectively) were not able to improve the yield of citric acid. In later experiments comparing oleic acid and peanut oil, oleic sometimes increased the citric acid yield as effectively as peanut oil, but more commonly peanut oil was superior.

In further experiments, natural oils which improved the yield of citric acid were treated in different ways and the treated oils tested. The yields of citric acid were estimated after 7 days. Peanut oil was dialysed for 4 days against medium A and then used in a fermentation. Maize oil was fractionated (Hilditch, 1956) and the

unsaponifiable fraction, glycerol and the mixed fatty acids from the saponifiable fraction were tested. Peanut oil samples were hydrogenated to give iodine numbers of 75 and 52 and these oils were compared in fermentations with the original peanut oil, iodine number 95. Peanut oil with iodine number 52 was added 24 hr. after inoculation, since germination of the spores was inhibited when this oil was added with the inoculum. The results of these fermentations are shown in Table 6, which shows that the activity of the oil lay in the saponifiable fraction, that no activity was lost with dialysis and that there was little loss in the ability of peanut oil to increase the citric acid yield until the iodine number had been decreased to 52.

Table 5. *Lipids increasing citric acid yield by* Aspergillus niger *mutant* 72-44 *at 7 days*

		Lipid (%, v/v)			
	No addition	2·0	4·0	10·0	Peanut oil (2 %, v/v)
Lipid added	Citric acid g./100 ml.				
Natural oils					
Vegetable					
Oleic + linoleic 70 % (w/v)					
Almond (in medium B)	7·9	11·4	11·2	.	
Linseed	6·6	7·8	.	5·6	
Maize	4·6	7·8	7·8	.	
Olive	6·5	9·5	.	9·8	
Peanut	6·2	9·1	9·1	.	
Soya bean	7·0	9·5	.	9·5	
Sunflower	7·0	9·0	.	9·5	
Ricinoleic 50 % (w/v)					
Castor	7·0	9·4	8·0	9·0	
Elaeostearic 50 % (w/v)					
Tung (in medium B)	8·7	10·8	.	.	
Animal					
Oleic 30 % (w/v) + C_{20} to C_{24} polyethenoid 40 % (w/v)					
Sperm	7·0	8·4	.	.	
Oleic 50 % (w/v)					
Neatsfoot	5·0	6·5	.	7·0	
Lard	5·0	7·0	.	8·0	
		Lipid (%, v/v)			
		0·5	1·0	2·0	
Glycerides					
Distearin	7·3	8·4	8·4	.	10·3
Triricinoleate	7·3	.	8·5	.	10·3
Monooleate	7·5	.	6·5	8·7	10·8
Trioleate	6·8	.	.	8·7	10·8
2 Oleate di-stearin	6·8	.	8·0	.	10·8
Monoglyceride of peanut oil (at 9 days)	7·0	.	.	11·1	11·5
Fatty acid					
Unsaturated					
Oleic (18)	6·0–7·9	6·6–7·2	7·8–9·9	8·1–9·9	9·4–10·9

Effect of 2,4-dinitrophenol

Several fatty acids including oleic acid are known to uncouple oxidative phosphorylation and to increase the respiration of isolated mammalian mitochondria (Pressman & Lardy, 1956). Since oleic acid and natural oils high in oleic acid were the only lipids effective in increasing the citric acid yield, it seemed possible that these substances might be uncoupling the rate-controlling effect of phosphorylation in this fermentation. This would allow more rapid respiration and oxidation of reduced pyridine nucleotides and so increase citric acid production. The effect on citric acid yield of 2,4-dinitrophenol, a typical uncoupling reagent of oxidative phosphorylation, was therefore tested. 2,4-Dinitrophenol was added to a series of flasks at two concentrations on different days of the fermentation. The yields obtained after 7 days are shown in Table 7, which shows that 2,4-dinitrophenol at 10^{-5}M did not increase the yield of citric acid; at 10^{-4}M, the yield was decreased and growth inhibited.

Table 6. *Effect of different treatments of maize and peanut oils on their ability to increase the yield of citric acid from* Aspergillus niger *mutant* 72-44 *at* 7 *days*

Lipid added	Amount of lipid (%, v/v)	Yield of citric acid (g./100 ml.)
No addition		6·5
Peanut oil dialysed 4 days	2·0	8·3
	4·0	8·3
	10·0	7·2
Peanut oil not dialysed	2·0	8·2
No addition		5·8
Maize oil	1·0	7·5
	2·0	8·0
	(%, w/v)	
Unsaponifiable fraction of maize oil	0·02	6·0
	0·1	5·8
	0·2	5·7
	(%, v/v)	
Saponifiable fraction of maize oil	1·0	8·3
	2·0	8·2
	4·0	8·5
Glycerol	1·0	6·2
No addition		8·2
Peanut oil, iodine number 95	2·0	11·5
Peanut oil, iodine number 75	2·0	10·7
Peanut oil, iodine number 52 (added 24 hr. after inoculation)	2·0	8·6

Effect of biotin. Since biotin is able to substitute as a growth factor for unsaturated fatty acids in some micro-organisms (Nieman, 1954) it was tested for its effect on citric acid accumulation. Over the range 0·2 to 40·0 μg./l. it had no effect on yield.

Amount of oil required and time of its addition

Peanut, maize and olive oils each gave good yields of citric acid when added at the 2% (v/v) in medium A. Table 8 shows the results at 7 days following the addition of various amounts of oil. These results show that above 2% (v/v) there was no further

marked improvement in yield. For subsequent work 2 % (v/v) peanut oil was selected since peanut was the cheapest effective oil. In another experiment, 2 % (v/v) peanut oil was added on different days after inoculation and the citric acid determined after 7 days. It was shown that the oil could be added and give an increase in the citric acid yield either on the day of inoculation or 48 hr. after inoculation; added later than this it was ineffective.

Table 7. *Effect of* 2,4-*dinitrophenol on citric acid yield by* Aspergillus niger *mutant* 72-44 *at* 7 *days*

Addition	Day of addition	Yield of citric acid (g./100 ml.)
Nil		4·5
Peanut oil 2 %, v/v	0	8·1
2,4-Dinitrophenol 10^{-5} M	0	4·8
10^{-5} M	1	4·3
10^{-5} M	2	4·6
10^{-4} M	2	2·3
10^{-5} M	3	4·8
10^{-5} M	4	4·6

Table 8. *Effect of different amounts of vegetable oils on the yield of citric acid with* Aspergillus niger *mutant* 72-44 *at* 7 *days*

Amount of oil added (%, v/v)	Oil added: Maize	Peanut	Olive
	Citric acid (g./100 ml.)		
0	4·6	7·0	5·2
0·1	6·7	—	5·7
0·2	5·7	—	5·2
0·5	6·8	7·3	7·0
1·0	7·5	9·0	7·2
2·0	8·1	9·9	8·5
3·0	8·0	9·2	8·5
4·0	8·5	9·0	8·4
5·0	9·4	10·0	—

The mycelial growth form was affected by the concentration of oil. In the absence of oil, the best yields of citric acid (6–7 g./100 ml.) were associated with small fine pellets 1–2 mm. in diameter, while larger pellets 3–4 mm. or porridge-like growth were associated with poor yields of 4 g. citric acid/100 ml. In the presence of 2 % (v/v) oil the pellets were about 1·0 mm. in diameter, with the highest yields associated with pellets less than 1 mm. in diameter in a fermentation mash of very low viscosity. In addition, at the end of fermentations which yielded $>9{\cdot}5$ g. citric acid/100 ml. some of the oil remained free in the medium as firm oval pellets of about 4×3 mm. with the consistency of margarine.

Utilization of oils

Microscopic examination of the mycelium showed the oil to enter the hyphae within 24 hr. of its addition, where it was clearly defined in droplets. The amount of oil used by the mould was determined by adding a weighed amount of peanut oil to

medium B in each of six flasks. The flasks were harvested after 7 days, pooled and the fermentation liquid, mycelium and solid oil pellets extracted with ether as outlined in Methods. The results are set out below:

	g.
Oil added	6·98
Oil recovered from:	
(1) mycelium	0·68
(2) fermentation liquid	0·07
(3) solid oil pellets	0·98
Total recovery	1·73
Recovery (%)	24·8

In this experiment, the addition of 2 g. oil/100 ml. resulted in an increase in citric acid yield of 2 g./100 ml., with 25 % of the oil recovered. Other experiments showed that oil caused no significant increase in dry weight of mycelium.

Properties of the recovered oil

As already indicated, the free oil which remained after fermentation was markedly changed in physical properties when the yield of citric acid had been increased by 1 to 3 g./100 ml. In experiments where the yield of citric acid was unaffected by added oil, the oil remained fluid to the end of the fermentation, indicating that the change in the physical properties was associated with its effect on citric acid yield. Changes in the melting point of oils can be caused by various changes in the fatty acids of the oil, for example, a change in chain length, the number, position or configuration of double bonds (Deuel, 1957). In addition, oxidation of the double bond to form epoxy acids can also decrease the iodine number. In an attempt to determine the changes which had occurred during fermentation, the iodine number of the peanut oil was determined by Hanus's method, before and after fermentation; it was found to be decreased from 95 to 72 in the recovered oil. This change might be due to saturation of double bonds or to other changes indicated above. The possibility that oxidation reactions were responsible for the decreased iodine number was tested by determining the peroxide values of the original and recovered oils as outlined in Methods.

The natural and recovered oils had similar peroxide values, indicating that the decreased iodine number was not due to oxidation reactions. Preliminary investigations by gas chromatography of the original and recovered oil indicated that the proportion of linoleic acid was decreased from 42 to 22 mole % during fermentation, while stearic acid was increased from 3 to 9 mole % and the oleic acid increased from 42 to 52 mole %. It is clear, therefore, that the recovered peanut oil was considerably more saturated than the original oil. When the recovered oil was tested in a new fermentation, it no longer increased the yield of citric acid.

Lipid as sole carbon source for citric acid production

To determine whether peanut and maize oils could act as the sole source of carbon for acid production 2 ml. of oil were added to 50 ml. medium containing inorganic salts at the concentrations listed for medium A in Methods. These media were inoculated and incubated statically or shaken for 7 days. The dry weight of mycelium

and the citric acid yield were then determined. The results are given in Table 9, which shows that while these oils were adequate for growth, they did not replace sucrose for citric acid production.

Table 9. *Oil as sole carbon source for citric acid production by* Aspergillus niger *mutant* 72-44 *at* 7 *days*

Oil added (4 %, v/v)	Growth condition	Dry wt. mycelium (g./100 ml.)	Yield of citric acid (g./100 ml.)
Peanut	Static	0·5	0·1
	Shaken	1·2	0·1
Maize	Static	0·5	0·1
	Shaken	1·5	0·1

Purity of fermentation products

Tests for the presence of organic acids other than citric were made as outlined in Methods. No acid other than citric was detected in any fermentation in the presence of the effective lipids peanut, maize, almond and tung oils, and oleic acid; nor in their absence.

DISCUSSION

Previous reports of substances which improved the yield of citric acid when added to fermentation media have shown these substances to be inhibitory to mould growth. In the present work on the effects of lipids on the yield of citric acid by *Aspergillus niger* mutant 72-44, the lipids which improved the yield were not inhibitory to growth; they could, in fact, act as the sole source of carbon for growth. There seem to be four possible ways in which lipids might act to increase the citric acid yield.

(1) *Physical effect*

The surface tension of liquids is altered by the addition of lipids, and it was formerly thought that, in fermentation media, lipids improved aeration efficiency. Deindoerfer & Gaden (1955) showed, however, that lipids decrease oxygen transfer in media. In the present work it seems unlikely that the lipids which increased the yield of citric acid were doing so by virtue of any physical effect, since only a few of the lipids tested improved the yield of citric acid; and yet most of the lipids have similar physical effects.

(2) *Source of acetyl co-enzyme A*

It seemed possible that β-oxidation of the lipids could provide additional acetyl co-enzyme A (acetyl coA) for citric acid formation and so increase the yield. Peanut oil entered the mycelium of *Aspergillus niger* mutant 72-44 readily and only 25 % of the added oil was recovered after fermentation, but the addition of lipid gave no increase in the dry weight of mycelium. It was possible then that the lipid was providing acetyl coA units for citric acid formation. However, saturated fatty acids like palmitic and stearic acids, oils with a high content of saturated fatty acids and the most highly hydrogenated peanut oil did not improve the yield of citric acid, and these lipids all provide acetyl coA units on β-oxidation. Although maize and peanut oils could act as the sole source of carbon for growth, they did not replace

sucrose for citric acid production. Improved citric acid yields in the presence of lipid could be accounted for by improved sugar utilization. These findings do not support the hypothesis that the lipids acted as additional sources of acetyl coA in improving citric acid yields.

(3) *Hydrogen acceptor*

The only lipids active in this citric acid fermentation were oils with a high content of unsaturated fatty acids or the unsaturated oleic acid. The changes observed in the oils as a result of the fermentation indicated that hydrogenation of constituent unsaturated fatty acids occurred. This suggested that the unsaturated fatty acids were acting as alternative hydrogen acceptors to oxygen during fermentation, thus allowing the mould to metabolize actively for longer and so improving the yield of citric acid. The importance of oxygen in all mould submerged culture fermentations has been stressed repeatedly (Finn, 1954; Arnold & Steel, 1958). When Shu (1953) used pure oxygen instead of air in a citric acid fermentation he obtained an increased citric acid yield.

Several observations in the present work support the theory that the effective lipids act as hydrogen acceptors. No saturated fatty acid or oils high in saturated fatty acids were effective. In fermentations with partially hydrogenated peanut oils, peanut oil with an iodine number of 52 gave no increase in yield, but oil with an iodine number of 75 was almost as active as the natural peanut oil (see Table 6). Hilditch (1947) showed that when peanut oil was hydrogenated, the component fatty acids were hydrogenated in a definite order, governed by the number and arrangement of the double bonds in the fatty acids. He found that in decreasing the iodine number from 93·3 to 71·6, the linoleate content was decreased from 26 to 3% and that the oleate content rose from 56 to 77%. In the present work, then, it seems probable that the peanut oil with an iodine number of 75 had a high content of oleic acid and gave a large increase in yield, and that it was not until the iodine number was decreased to 52 that the oleic acid content was so diminished that the oil was no longer active.

The hydrogen acceptor theory explains why saturated fatty acids and oils high in saturated fatty acids did not improve the citric acid yield and why oleic acid was effective. On this theory, however, it might be expected that linoleic and linolenic acids would also improve yield, but this was not found to be so. Their failure may have been due to steric differences caused by the larger number of double bonds in these two acids.

Trumpy & Millis (1963) found that when NH_4NO_3 was present at 2·7 g./l. then peanut oil did not improve the yield of citric acid, but at concentrations less than 2·7 g./l. addition of oil increased the yield. The dry weight of mycelium remained unchanged at all concentrations of NH_4NO_3 in the presence or absence of oil. This observation is difficult to reconcile with the hydrogen acceptor theory unless it is postulated that at higher concentrations of NH_4NO_3, the nitrate also acts as an alternative hydrogen acceptor, oil then being ineffective.

(4) *Uncoupling of oxidative phosphorylation*

In the citric acid fermentation one of the factors which improves the yield of acid is a low phosphate content in the medium. This leads to low concentrations of

adenosine diphosphate (ADP) and inorganic phosphate in the mycelium. Although growth in a high-yielding citric acid fermentation is limited, the conversion of sugar to citric acid proceeds rapidly. The rate of respiration would be increased if the rate-controlling effect of phosphorylation were uncoupled from respiration. In many cells the concentrations of inorganic phosphate and ADP govern the rate of respiration and thus the rate of oxidation of the reduced pyridine nucleotides and citric acid formation. Scholefield (1956) found that octanoic and decanoic acids were active as uncoupling reagents. Pressman & Lardy (1956) found that myristic acid and the unsaturated fatty acids with *cis* bonds, oleic, linoleic and linolenic acids, were also active as uncoupling reagents in mitochondrial preparations. It seemed possible therefore that in the citric fermentation by *Aspergillus niger*, the lipids might act by uncoupling respiration from phosphorylation. This would result in a faster rate of respiration and an improvement in citric acid accumulation. While this theory explains the activity of oleic acid, it cannot explain why linoleic, linolenic, myristic, octanoic and decanoic acids, which are also active uncoupling reagents, did not increase citric acid yield by *A. niger*. Similarly 2,4-dinitrophenol did not improve citric acid yield though it too is an active uncoupling reagent. It is always possible that the intact mycelium was impermeable to the ineffective reagents. This does not seem probable since 2,4-dinitrophenol at 10^{-4} M and saturated fatty acids with less than 13 carbon atoms all inhibited growth and must therefore have entered the mycelium.

Of the possible explanations of the mode of action of lipids in the citric acid fermentation by *Aspergillus niger*, it seems that the hydrogen acceptor theory is the one to which fewest objections can be raised. More definitive experiments need to be done with cell-free systems to determine whether or not unsaturated lipids can act as hydrogen acceptors and, if so, how specific the reaction is with respect to the structure of the acceptor fatty acid. The addition of appropriate lipids to the fermentation medium offers a considerable advantage for citric acid production on an industrial scale. This study, and others to be published, have shown that lipids improve the citric acid yield by 20–50 % in a wide variety of media. Lipids do not eliminate variability in yield, but the lowest yields in the presence of lipid are increased considerably compared with the lowest yields in the absence of lipid. In addition, in aerated and stirred fermentations lipids act as effective antifoam agents. The addition of lipids to fermentation media for citric acid production is the subject of a patent application by the University of Melbourne (1960).

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Nutritional Requirements of an *Aspergillus niger* Mutant for Citric Acid Production

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SUMMARY

The nutritional requirements for high conversion of sugar to citric acid in shaken flask fermentations were investigated for the mutant strain 72-44 of *Aspergillus niger*. Single variable and factorial experiments were used to determine the effects of nitrogen, phosphorus, magnesium, manganese, copper, zinc and iron on the yield of citric acid. When 2 % (v/v) peanut oil was added to the fermentation media it markedly improved the yield of citric acid. Two media were devised which yielded on average 10–10·5 g. anhydrous citric acid from 14 g. sugar, with occasional yields as high as 13 g. Both media contained 2 % (v/v) peanut oil and the following trace metals (mg./l.): Fe^{2+} 2·0; Cu^{2+} 0·3; Zn^{2+} 0·1. In medium A the major nutrients were (g./l.): ion-exchange purified cane sugar, 140; KH_2PO_4, 1·0; $MgSO_4.7H_2O$, 0·25; NH_4NO_3, 1·87; and in medium B (g./l.): ion-exchange purified cane sugar, 140; KH_2PO_4, 0·3; $MgSO_4.7H_2O$, 0·15; NH_4NO_3, 1·4. Interactions were found between: zinc and iron, nitrogen and iron, nitrogen and phosphorus. The implications of these findings for the fermentation of crude sugar substrates in citric acid production are discussed.

INTRODUCTION

Conditions which control citric acid accumulation in fermentation with *Aspergillus niger* have been reviewed by Foster (1949), Johnson (1954) and Perlman & Sih (1960). Published work shows that there are considerable variations in the conditions which different workers have found to be optimal for citric acid accumulation, that the mould strain is of great importance and that the yield of citric acid from carbohydrate metabolized by *A. niger* is extremely sensitive to trace metals, particularly under submerged culture conditions. Even under supposedly identical, ideal conditions, the yield of citric acid is variable. Previously, characterization of the cultural conditions which lead to high yields of citric acid has been based on experiments where single components of the medium have been varied and the best concentrations determined. The difficulty with this experimental design is that frequently the best concentration of the component under test is dependent on the concentrations of other components in the medium. An example of this was found by Shu & Johnson (1948) who showed with strain 72-4 of *A. niger* that the optimal concentration of KH_2PO_4 was twice as great when trace metals were limiting as when trace metals were not limiting.

Our laboratory has been attempting to define the conditions which will allow the production of citric acid by *Aspergillus niger* in submerged culture fermentation of Australian raw materials. This paper reports an investigation of the conditions

which lead to high yields of citric acid by the mutant strain 72-44 of *A. niger*. The media constituents were investigated in some experiments with single variables and in others with a factorial design with multiple variables. Millis, Trumpy & Palmer (1963) showed that certain unsaturated lipids increased the yield of citric acid from cane sugar with mutant 72-44. Peanut oil, which is an active lipid in this system, was included in fermentation media to see whether this affected the optimal concentrations of other media constituents and to determine the concentrations of media constituents which give maximal increases in citric acid yield in the presence of oil.

METHODS

Organism. The mutant 72-44 of *Aspergillus niger* was used for all fermentations. The mutant was selected after ultraviolet irradiation of the spores of strain 72-4 of *A. niger* (ATCC 11414) as described by Millis *et al.* (1963). Stock cultures were kept as spore suspensions freeze-dried in ampoules and working cultures on beer wort agar media.

Inoculum, fermentation techniques and determinations of fermentation products. The preparation of the spore inoculum, the conditions for shaken flask fermentations, the ion-exchange treatment of cane sugar used for media, the methods for the determination of sugar, citric acid and dry weight of mycelium, respectively, were as described by Millis *et al.* (1963).

Design of factorial experiments. For most experiments, three nutrients were investigated at three concentrations, making twenty-seven fermentations/experiment. This allowed the effect of each concentration of a nutrient to be estimated in nine fermentations containing three different concentrations of the other two nutrients.

Medium for single variable experiments with trace metals. The major nutrients of the fermentation medium in which the trace metals were varied were at the following concentrations (g./l.): 140, ion-exchange purified cane sugar; 1·0, KH_2PO_4; 0·25, $MgSO_4.7H_2O$; 1·87, NH_4NO_3. Trace metals, when not under test, were at the following concentrations (mg./l.): Fe^{2+}, 2·0; Cu^{2+}, 0·3; Zn^{2+}, 0·1. The medium was at pH 3·0. This medium will be referred to as medium A.

Peanut oil. Refined and deodorized peanut oil (Kristal Brand, Marrickville Margarine Co., N.S.W., Australia), was added to appropriate media at 2 % (v/v) before the medium was autoclaved.

RESULTS

Yield of citric acid

If all the cane sugar in 100 ml. of medium were converted to citric acid, 14 g. sucrose would yield 15·7 g. anhydrous citric acid. In high-yielding fermentations, however, between 1·0 and 1·5 g. dry wt. mycelium/100 ml. is formed and this would use between 1·8 and 2·7 g. sugar. Assuming all the sugar in the medium is utilized, between 11·3 and 12·2 g. sugar is, therefore, available for conversion to citric acid. This could theoretically yield 12·7–13·7 g. anhydrous citric acid/100 ml. medium. In the presence of oil, *Aspergillus niger* mutant 72-44 has on ten occasions yielded as high as 13 g. citric acid/100 ml. and on numerous occasions has yielded 12–12·5 g./100 ml., but the average yield in shaken flask fermentations was 10–10·5 g./100 ml. under conditions which were favourable for citric acid accumulation. A yield

of 10 g. citric acid/100 ml. represents 71 % conversion to citric acid of the sugar added to the medium but, of the sugar available after the growth requirements have been satisfied, 83 % has been converted to acid. This is, however, a low estimate, since between 1·0 and 1·5 g. sugar is unused at the end of a fermentation which yields 10 g. citric acid/100 ml. In this paper, yields of citric acid above 9·5 g./100 ml. (that is, if more than 68 % of sugar added to the medium is converted to citric acid) are considered high yields.

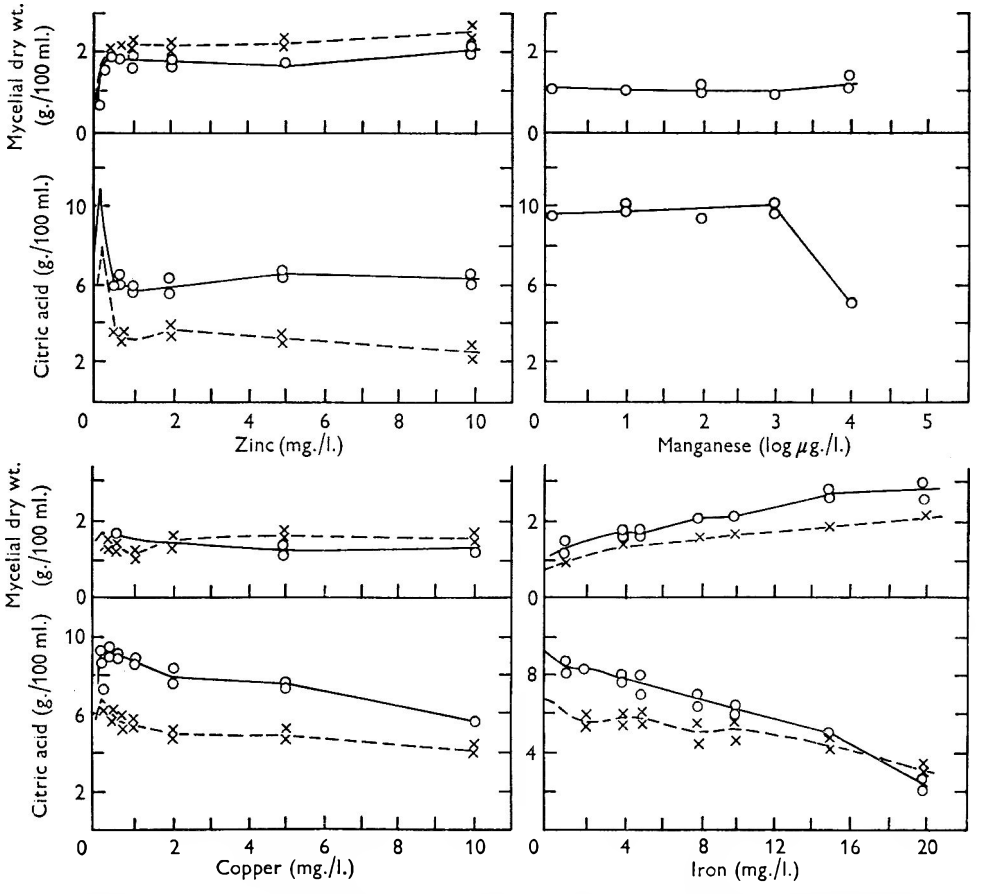

Fig. 1. Effect of trace metals on the yield of citric acid and the dry wt. of mycelium with *Aspergillus niger* mutant 72-44 grown in shaken culture: ○—○ results in the presence of 2 % (v/v) peanut oil; ×– – –× results in the absence of peanut oil.

Single variable experiments with trace metals and peanut oil

The effect of each of the trace metals iron, copper and zinc was tested in medium A by changing the concentration of one of these metals while the other trace metals were held at the concentration of medium A. Media were dispensed in quadruplicate and peanut oil added to one half of these flasks. The effect of manganese was tested in the presence of oil. After 7 days, the yield of citric acid and the dry weight of mycelium were determined; the results are shown in Fig. 1. The most conspicuous feature of these fermentations was the high yield of citric acid at very low

concentrations of zinc and the sensitivity of this yield to small additions of zinc. The fermentations also showed that *Aspergillus niger* mutant 72-44 gave considerably higher yields of citric acid at high concentrations of zinc, copper, iron and manganese as compared with the yields reported by Shu & Johnson (1948) for the parent strain *A. niger* 72-4. Like the parent strain, however, mutant 72-44 yielded citric acid best at low trace metal concentrations.

Figure 1 also shows that peanut oil greatly increased the yield of citric acid at all concentrations of metals, except at the highest concentrations of iron, but the concentrations of trace metals which gave the best yields of citric acid were the same in presence or in absence of oil. The oil had little effect on the dry-weight yield of mycelium, and at these concentrations of major nutrients, the dry-weight yield was little affected by trace metals, although there was a tendency for increased growth with high concentrations of iron.

Factorial experiments with major nutrients

To determine the best concentrations for citric acid accumulation, of the major nutrients, nitrogen, phosphate and magnesium, a factorial experiment involving twenty-seven media was designed to test three concentrations of each of the three nutrients. The concentrations chosen lay close to those already in use in medium A, since these were known to give good yields of citric acid. The sugar and trace metal concentrations were as in medium A. A similar set of twenty-seven media was

Table 1. *Effect of major nutrients and of peanut oil on yield of citric acid from* Aspergillus niger *mutant* 72-44 *in* 9 *days*

		NH_4NO_3 concentration (g./l.)								
		0·94			1·87			2·70		
		KH_2PO_4 concentration (g./l.)								
		0·25	0·5	1·0	0·25	0·5	1·0	0·25	0·5	1·0
		Yield of citric acid (g./100 ml.)								
$MgSO_4.7H_2O$ g./l.	0·15	*10·1**	*8·6*	*5·2*	*12·3*	*12·6*	*11·8*	*10·3*	*10·1*	*9·4*
		8·1	6·1	3·1	9·8	10·1	6·9	9·3	10·0	9·3
	0·25	*9·7*	*8·8*	*5·6*	*11·3*	*10·7*	*9·4*	*8·3*	*9·6*	*8·9*
		5·9	6·6	4·1	7·6	7·1	6·5	6·6	9·6	8·5
	0·35	*10·0*	*9·4*	*5·8*	*11·8*	*11·8*	*9·4*	*8·5*	*9·7*	*9·4*
		5·8	4·6	3·3	7·2	7·9	7·2	7·5	7·8	7·3

* *Italic numbers* = results in the presence of 2 % (v/v) peanut oil.

prepared and peanut oil added to each medium. The yield of citric acid from these fermentations at 9 days is shown in Table 1. As in the trace metal experiments, there was an improvement in citric acid yield in the presence of peanut oil at all nutrient concentrations tested. The mean citric acid yield in the presence of oil was 10·4 g./100 ml. compared with 7·9 g./100 ml. in its absence. The means of the yields from the three concentrations of each nutrient showed that the best yields in the presence of oil were obtained with (g./l.): $MgSO_4.7H_2O$, 0·15; KH_2PO_4, either 0·5 or 0·25; NH_4NO_3, 1·87. There was also a definite interaction between nitrogen and

phosphate; see Table 2. Clearly, increasing the phosphate depressed the yield of citric acid much more at the lowest nitrogen concentration than at high nitrogen concentrations, irrespective of the presence of peanut oil.

Table 2. *Interaction between nitrogen and phosphorus affecting the yield of citric acid by* Aspergillus niger *mutant* 72–44

	NH_4NO_3 concentration (g./l.)					
	0·94		1·87		2·70	
	+Oil	−Oil	+Oil	−Oil	+Oil	−Oil
KH_2PO_4 (g./l.)	Mean yield of citric acid (g./100 ml.)					
0·25	9·8	6·6	11·8	8·2	9·1	7·8
0·50	8·9	5·8	11·3	8·3	9·8	9·1
1·00	5·5	3·5	10·2	6·9	9·2	8·1

Table 3. *Effects of major nutrients on yield of citric acid by* Aspergillus niger, *mutant* 72-44 *in the presence of peanut oil at* 9 *days*

		NH_4NO_3 concentration (g./l.)								
		1·40			1·87			2·34		
		KH_2PO_4 concentration (g./l.)								
		0·30	0·50	0·70	0·30	0·50	0·70	0·30	0·50	0·70
$MgSO_4 \cdot 7H_2O$ g./l.		Yield of citric acid (g./100 ml.)								
0·10		9·1*	7·9	6·4	7·4	6·8	7·5	7·4	6·8	6·7
		10·2	9·3	9·1	8·7	8·2	7·9	7·6	7·8	7·2
0·15		10·8	9·8	8·3	9·6	9·4	6·8	9·0	9·7	7·8
		11·1	10·3	9·9	10·7	10·4	10·0	Lost	8·0	8·3
0·20		10·1	7·6	9·2	9·4	9·7	9·1	7·9	7·3	6·9
		10·2	8·9	8·1	11·1	9·6	8·5	9·2	8·9	8·8

* Top figure of each pair represents value in one replicate experiment.

The mean dry wt. yield of mycelium of the twenty-seven fermentations in the presence of oil was 1·30 g./100 ml. and was very similar in its absence, 1·26 g./100 ml. The dry wt. yield of mycelium increased with increasing phosphate (1·04–1·54 g./100 ml. in the presence of oil, and 1·12–1·35 g./100 ml. in its absence), but otherwise the different concentrations of nutrients had little effect on mycelial dry weight.

In further factorial experiments, the three concentrations of nutrients were spaced equally apart and close to the concentrations which gave best yields in the first factorial experiment. All the fermentations were carried out in the presence of peanut oil, and to estimate the variability between factorial experiments at different times, the whole experiment was repeated with a newly-prepared inoculum and medium. The yields of citric acid from these two replicates after 9 days are shown in Table 3. Here, the upper figure shown in each treatment belongs to one experiment. These results were analysed statistically. As the concentrations of each nutrient were equally spaced on a linear scale, estimates were made of the linear and

the quadratic trends associated with the variations in yield at these concentrations. The mean square due to error of the experiment was 0·589, and the interactions between pairs of nutrients and between the three nutrients proved to be not significant. The difference between replicates was significant at the 0·1 % level, as were the linear effects of each of the major nutrients. Magnesium showed a quadratic effect significant at the 0·1 % level. The main effects can be seen in Table 4 where the means of each level of each nutrient are shown together with the standard error (S.E.) and the S.E. of difference. The problem of variation of yield under supposedly similar conditions will be referred to in a later section.

Table 4. *Means of the effects of major nutrients on yield of citric acid from* Aspergillus niger *mutant* 72-44 *in the presence of peanut oil*

$MgSO_4.7H_2O$ (g./l.)	0·10	0·15	0·20
Citric acid (g./100 ml.)	7·94	9·42	8·92
KH_2PO_4 (g./l.)	0·30	0·50	0·70
Citric acid (g./100 ml.)	9·46	8·68	8·14
NH_4NO_3 (g./l.)	1·40	1·87	2·34
Citric acid (g./100 ml.)	9·29	8·93	8·06

Mean of replicate 1 8·31
Mean of replicate 2 9·22 } S.E.* = 0·15

S.E. = 0·18
S.E. (difference) = 0·25

S.E. = standard error.

The factorial experiments indicated that the yield of citric acid would be improved in the presence of peanut oil by using the following major nutrient concentrations (g./l.): $MgSO_4.7H_2O$, 0·15; KH_2PO_4, 0·3; NH_4NO_3, 1·4. These nutrients, plus 14 % (w/v) ion exchange purified cane sugar and trace metals at the following concentrations (mg./l.): Fe^{2+} 2·0; Cu^{2+} 0·3; and Zn^{2+} 0·1, will be referred to as medium B subsequently.

Factorial experiments with trace metals and oil

In Fig. 1, the yield of citric acid in medium A was reported for various concentrations of each of four trace metals. Factorial experiments were designed in which the major nutrients were as shown in medium B and the concentrations of three trace metals (iron, zinc, copper) were varied simultaneously in the presence and absence of peanut oil. The results after 9 days of fermentation are summarized in Table 5.

An analysis of variance was done on the results of fermentations in the presence of peanut oil. The error was 0·205, and the effect of different concentrations of copper and the interactions of zinc and copper and iron and copper were shown to be not significant. The effect of different concentrations of both zinc and iron were significant at the 0·1 % level and the interaction of iron and zinc was significant at the 5 % level. In the iron and zinc interaction, increasing zinc depressed the citric acid yield and increasing iron improved it but the improvement with increased iron was greatest when zinc was higher. These effects can be seen in the table of means listed in Table 6.

The results of fermentations in the absence of peanut oil were not analysed statistically, but inspection of the means of the citric acid yield at the different trace metal concentrations shows similar trends to those just discussed for fermentations in the presence of peanut oil, excepting that there is no evidence of interaction between iron and zinc (Table 7).

Table 5. *Effect of trace metals and peanut oil on the yield of citric acid from* Aspergillus niger *mutant* 72-44 *at* 9 *days*

	Zn^{2+} concentration (mg./l.)								
	0·05			0·1			0·2		
	Fe^{2+} concentration (mg./l.)								
	0·1	2·0	4·0	0·1	2·0	4·0	0·1	2·0	4·0
Cu^{2+} (mg./l.)	Yield of citric acid (g./100 ml.)								
0·1	*9·1*	*10·2*	*10·9*	*6·6*	*10·4*	*10·7*	*4·9*	*8·6*	*8·6*
	7·0	8·2	8·1	5·9	7·1	5·9	4·5	5·6	4·8
0·3	*8·6*	*10·7*	*11·6*	*7·0*	*9·9*	*10·0*	*4·6*	*7·7*	*8·1*
	7·1	8 1	8·0	5·9	7·3	6·3	4·5	5·2	5·3
0·9	*8·7*	*9·5*	*10·5*	*7·3*	*9·3*	*9·0*	*4·7*	*8·3*	*8·5*
	7·1	8·4	8·2	6·7	6·7	5·8	4·7	4·7	5·0

* *Italic* numbers = results in the presence of 2 % (v/v) peanut oil.

Table 6. *Means of the effects of trace metals in the presence of peanut oil on yield of citric acid by* Aspergillus niger *mutant* 72-44

	Fe^{2+} (mg./l.)			Means
	0·1	2·0	4·0	of Zn^{2+}
Zn^{2+} (mg./l.)	Yield of citric acid (g./100 ml.)			
0·05	8·80	10·13	11·00	9·97
0·1	6·97	9·87	9·90	8·91
0·2	4·73	8·23	8·40	7·12
Means of Fe^{2+}	6·83	9·41	9·77	8·67

Cu^{2+} (mg./l.)	Means of Cu^{2+}
0·1	8·90
0·3	8·69
0·9	8·43

Standard error (marginal means):	0·15
Standard error (means within table):	0·26
Standard error (different marginal means):	0·21
Standard error (different means within table):	0·38

A comparison of Table 6 with Table 7 shows that peanut oil was most effective in increasing citric acid yield when the iron concentrations were highest (between 2 and 4 mg./l.). It was then decided to test the effect of even higher concentrations of iron in the presence and absence of oil. The highest concentration of copper in the

factorial experiment was less than 1 mg./l. and this had no effect on yield. In the second factorial experiment, it was decided to increase the concentration of copper as well as that of iron to determine whether oil increased the citric acid yield at higher copper concentrations. Since the yield was so greatly depressed by zinc, fermentations were carried out with only two concentrations of zinc (0·05, 0·10 mg./l.). The major nutrients were as in medium B and the citric acid yields were estimated after fermentation for 9 days. These results are shown in Table 8.

Table 7. *Means of the effect of trace metals in the absence of peanut oil on the yield of citric acid by* Aspergillus niger *mutant* 72-44

	Fe^{2+} (mg./l.)			
	0·1	2·0	4·0	Means of Zn^{2+}
Zn^{2+} (mg./l.)	Yield of citric acid (g./100 ml.)			
0·05	7·1	8·2	8·1	7·9
0·1	6·2	7·0	6·0	6·4
0·2	4·6	5·2	5·0	4·8
Means of Fe^{2+}	6·0	6·8	6·4	6·4

Cu^{2+} (mg./l.)	Means of Cu^{2+}
0·1	6·3
0·3	6·4
0·9	6·4

Table 8. *Effect of trace metal concentrations and of peanut oil on the yield of citric acid from* Aspergillus niger *mutant* 72-44 *after* 9 *days*

	Zn^{2+} (mg./l.)					
	0·05			0·1		
	Fe^{2+} (mg./l.)					
	4·0	8·0	12·0	4·0	8·0	12·0
Cu^{2+} (mg./l.)	Yield of citric acid (g./100 ml.)					
1·0	*8·1**	*10·7*	*8·9*	*11·7*	*10·6*	*9·3*
	8·2	7·8	7·0	8·4	7·3	5·3
4·0	*9·0*	*9·9*	*9·0*	*11·0*	*9·8*	*8·6*
	7·2	7·9	7·1	7·3	5·6	7·7
8·0	*8·8*	*8·8*	*8·9*	*10·1*	*10·3*	*7·4*
	7·4	6·7	5·3	5·8	4·3	3·8

* Italic numbers = results in presence of 2% (v/v) peanut oil.

An analysis of variance of the citric acid yields in the presence of oil, given in Table 8, indicated that at these higher concentrations of trace metals there was an interaction between zinc and iron significant at the 1 % level. Neither of the other interactions, nor the effect of copper, was significant. The analysis of the means of the effect of iron and of zinc in the presence of peanut oil is shown in Table 9. The influence of peanut oil on the yield at various trace-metal concentrations can be seen

in Table 10 which shows the mean of the effect of each metal concentration in the presence and absence of oil.

Oil improved the citric acid yield in all treatments. The yield was unaffected by 8 mg. copper/l. in the presence of oil but was depressed by copper above 4 mg./l. in the absence of oil. As in the presence of oil, an interaction between zinc and iron occurred in the absence of oil; the upper concentration of zinc depressed the citric

Table 9. *Means of the effect of zinc and iron on the yield of citric acid by* Aspergillus niger *mutant* 72-44 *in the presence of peanut oil at* 9 *days*

	Fe^{2+} (mg./l.)			
	4·0	8·0	12·0	Means of Zn^{2+}
Zn^{2+} (mg./l.)	Yield of citric acid (g./100 ml.)			
0·05	8·63	9·80	8·93	9·12
0·1	10·93	10·23	8·43	9·86
Means of Fe^{2+}	9·78	10·02	8·68	9·49

S.E.* 0·35
S.E. of difference 0·49

* S.E. = standard error.

Table 10. *Means of effects of trace metals and of peanut oil on the yield of citric acid from* Aspergillus niger *mutant* 72-44 *at* 9 *days*

	+Oil	−Oil		+Oil	−Oil		+Oil	−Oil
	Yield of citric acid (g./100 ml.)			Yield of citric acid (g./100 ml.)			Yield of citric acid (g./100 ml.)	
Cu^{2+} (mg./l.)			Zn^{2+} (mg./l.)			Fe^{2+} (mg./l.)		
1·0	9·80	7·33	0·05	9·12	7·18	4·0	9·78	7·38
4·0	9·55	7·13	0·10	9·86	6·17	8·0	10·02	6·60
8·0	9·05	5·55	.	.	.	12·0	8·68	6·03

Mean + oil = 9·5; mean − oil = 6·7.

acid yield markedly when iron was greater than 4 mg./l.; at the lower concentrations of zinc higher amounts of iron could be tolerated. At the higher zinc concentration (0·10 mg./l.), oil improved the citric acid yield at all concentrations of iron to a greater extent than when zinc was at the lower concentration (0·05 mg./l.). At 8–12 mg. iron/l., 2–3 mg. more citric acid /100 ml. were formed in the presence of oil than in its absence, but the actual citric acid yield decreased when iron was increased from 8 to 12 mg./l., even in the presence of oil.

Factorial experiment to test for interaction between major nutrients and trace metals

The previous experiments indicate that there were interactions between major nutrients and between trace metals influencing the yield of citric acid. It is also clear that zinc is a particularly critical metal for *Aspergillus niger* mutant 72-44.

It was thought possible that major nutrients and trace metals may also interact, and if so, that this suggests changes in major nutrients which would improve the tolerance of the mutant to trace metals, and in particular to zinc. This possibility was investigated in a factorial experiment with six media components each at two concentrations. The experiment was designed as a half replicate of the full factorial experiment, and was arranged in two randomized blocks, each of sixteen media. The concentration chosen lay on either side of those found optimal in the earlier factorial experiments. Peanut oil was added to all media and the yields of citric acid estimated after fermentation for 9 days. Statistical analysis of the citric acid yields indicated that the block effect and all interactions except that of NH_4NO_3 with iron were not significant. With the nitrogen and iron interaction, the higher concentration of nitrogen combined with the higher concentration of iron decreased the citric acid yield, whereas with the lower concentration of nitrogen, increasing the amount of iron did not depress the citric acid yield. The effect of KH_2PO_4 was significant at the

Table 11. *Effect of six components of the growth medium on the yield of citric acid by* Aspergillus niger *mutant* 72-44 *in the presence of peanut oil at* 9 *days*

Medium component	Mean yield citric acid (g./100 ml.)	Standard error of difference	Significance
$MgSO_4.7H_2O$ (g./l.)			
0·1	9·44	0·263	None
0·2	9·93		
KH_2PO_4 (g./l.)			
0·25	9·94	0·215	5 %
0·5	9·42		
Zn^{2+} (mg./l.)			
0·05	10·10	0·263	1 %
0·15	9·27		
Cu^{2+} (mg./l.)			
1·0	9·88	0·263	None
4·0	9·48		

Table 12. *Interaction of* NH_4NO_3 *and iron on yield of citric acid by* Aspergillus niger *mutant* 72-44

	NH_4NO_3 (g./l.)		
	1·2	2·0	Means of Fe^{2+}
Fe^{2+} (mg./l.)	Yield of citric acid (g./100 ml.)		
6·0	9·81	9·99	9·90
10·0	10·06	8·87	9·47
Means of NH_4NO_3	9·93	9·43	9·68

S.E. of difference = 0·322; significance = 5 %.

5 % level, while the effect of zinc was significant at the 1 % level. Both of these nutrients gave better citric acid yields at the lower concentrations. These results are summarized in Tables 11 and 12.

Variation in citric acid yield

Within any one factorial experiment, there was no doubt that the major nutrients significantly affected the yield of citric acid. The concentrations of nutrients used in medium B were always superior to those used in medium A in the factorial experiments. Later, on several different occasions, medium A and medium B were compared in the same experiment and there was no consistent difference in citric acid yield; sometimes medium A was even superior to medium B. This can only mean that unrecognized and uncontrolled factors can so affect citric acid yield that, if these factors are favourable to high yields, then the nutrient differences in the two media are not significant. If the uncontrolled factors are unfavourable to high citric acid yields then medium B is superior to medium A.

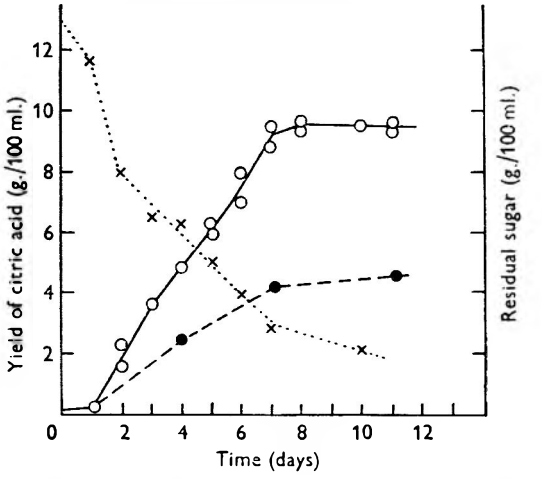

Fig. 2. Rate of citric acid production and sugar utilization by *Aspergillus niger* mutant 72-44 in medium A. ○—○ citric acid in the presence of 2 % (v/v) peanut oil; ●– – –● citric acid in the absence of peanut oil; ×– – –× residual sugar in presence of 2 % (v/v) peanut oil.

During the investigation, three different dried cultures were used to prepare inocula and different batches of sugar were used. It was possible that these factors were responsible for some of the variation; fermentations were therefore made in medium A and medium B to test this possibility. No significant differences were found between the citric acid yields from the two media when tested for all combinations of sugar batch and inoculum source. It should be pointed out, however, that this variability was from 9·5 to 13 g. citric acid/100 ml., that is, variability between good yields and yields very close to what is, theoretically, possible.

Sugar utilization

With a yield of citric acid of 10·0–10·5 g./100 ml., there was residual sugar of 1·0 and 1·5 g./100 ml., which was decreased to less than 0·2 g./100 ml. as the citric acid

yield increased to 13 g./100 ml. Fermentations in the presence of oil had a faster rate of sugar utilization and a lower residual sugar than those without oil. In Fig. 2, the rate of citric acid accumulation and sugar utilization is shown in the presence of oil, as compared with the rate of citric acid accumulation in the absence of oil.

Purity of fermentation products

Although all citric acid yields reported here are calculated from total titratable acidity, frequent tests were made to ensure that only citric acid was being produced. Spot tests were made for oxalic acid (Feigl, 1960), chromatograms were run on culture fluids (Millis *et al.* 1963) and citric acid was estimated colorimetrically by the method of Marier & Boulet (1958). In these tests, no acid other than citric was detected, and the agreement between the colorimetric and titratable-acidity estimations varied between 98 and 103%. These tests were made on culture fluids containing peanut oil, oleic acid and a high content of trace metals as well as with media with low trace metal content and no lipid present.

DISCUSSION

Accumulation of large amounts of citric acid is the result of metabolic malfunction of *Aspergillus niger* in a medium of low pH value with a high sugar content and carefully controlled concentrations of other nutrients; and the amount of growth must be limited. This has been clearly shown in these investigations where the best yields of citric acid were always associated with mycelial pellets about 0·5 mm. in diameter suspended in a culture medium of very low viscosity, virtually free from mycelial fragments and with less than 1 g. dry wt. mycelium/100 ml.

Multiple nutritional deficiencies can cause limited growth; the nitrogen, phosphorus, zinc and iron concentrations are particularly important. At very low concentrations of zinc, growth was severely limited, but once the zinc content was increased above 0·1 mg./l., further increases have no effect. At higher zinc concentrations, iron, nitrogen or phosphorus then limited growth. Medium B had a low nitrogen and phosphorus content and this contributed to the growth-limiting effect of this medium.

Although growth must be limited, this of itself is not sufficient to give high yields of citric acid. Low concentrations of certain trace metals are also required. With this mutant 72-44 of *Aspergillus niger*, citric acid yield is particularly sensitive to zinc but less so to iron, copper and manganese. The interaction between iron and zinc offers some opportunity of improving tolerance of zinc, since higher concentrations of zinc do not depress citric acid yield as greatly when combined with higher concentrations of iron. In addition, higher concentrations of iron do not depress the citric acid yield when the concentration of zinc is very low. The interaction of iron and nitrogen also offers a means of overcoming the depressing effect of high iron concentration. A high iron concentration does not decrease the citric acid yield if the nitrogen concentration is low.

The addition of peanut oil to the fermentation medium has shown an improvement of 2–3 g. citric acid/100 ml. with a wide variety of media. Oil improves the yield most markedly when trace metal concentrations are high, particularly iron and copper. Low phosphorus and high magnesium media also respond well to oil.

At 0·05 mg. zinc/l., yields of 10–11 g. citric acid/100 ml. were obtained at 20 mg. iron/l. When iron was at 0·01 mg./l. and zinc greater than 0·05 mg./l., oil gave no improvement. Oil was not effective with 2·7 g. or more NH_4NO_3/l. (yields in these media were high in presence and in absence of oil).

The variability of citric acid yield shown in this study when every care was taken to standardize procedures indicates that all factors which affect this yield are not yet known. The diversity of known individual factors and interrelated factors which affect citric acid yield make it scarcely surprising that no one medium emerges clearly as being optimal for citric acid production. This work does, however, allow some generalizations to be made about the behaviour of *Aspergillus niger* mutant 72-44. This mutant is more tolerant of higher concentrations of trace metals than is the parent, but it yields citric acid best at low concentrations of trace metals. Zinc is the most critical single medium component. Mutant 72-44 will yield citric acid well in the presence of oil despite a relatively high content of iron and copper, as long as a major nutrient like nitrogen or phosphorus is low. It seems then, that several different nutrient deficiencies can lead to the accumulation of citric acid. If a natural crude sugar is to be used as a substrate for this fermentation, then it would be profitable to add peanut oil to all fermentations and to select samples of crude sugar as low as possible in nitrogen and phosphorus where trace metal deficiencies are not so critical, and to see that there is sufficient iron present to minimize the depressing effect of zinc on citric acid yield. The proper explanation of the highly complex interactions shown in this study must await a full biochemical investigation.

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Damage and Survival of Bacteria during Freeze-Drying and during Storage over a Ten-Year Period

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SUMMARY

Extrapolation from 10-year survival curves indicates that many lyophilized bacterial suspensions may be expected to yield viable organisms after centuries of storage, but that suspensions of Pseudomonadaceae may become sterile within one to several decades. The characters of Lactobacteriaceae and other bacteria appear unaltered as a result of lyophilization and storage for 5 years, but some Bacteroides isolates manifested apparently altered fermentation reactions under the same circumstances. Freezing bacteria in pellets is suggested as an alternative method of storage for those species which are particularly sensitive to lyophilization.

INTRODUCTION

For storage over long periods freeze-drying is a method often used for the maintenance of bacterial cultures. Although there are numerous reports about technique and conditions for freeze-drying, there is little quantitative information about viability and the stability of traits over a long period. Changes in interests and techniques within a given laboratory, as well as the personnel turnover through the years, distract from and render difficult long-term study. The present paper summarizes viability data on 12 species which have been stored in the dried state for 10 years.

METHODS

Methods of preparation of the desiccates. Escherichia coli 69L–15, *Aerobacter aerogenes* and *Proteus vulgaris* were cultivated at 35° on a shaking apparatus for 18–24 hr. in 1 % (w/v) yeast-extract broth. *Acetobacter melanogenum* American Type Culture Collection (ATCC) 9844 was cultivated at 27° on the shaker for 2 days in the same medium containing 2 % (w/v) glucose. *Micrococcus albus* and a psychrophilic *Pseudomonas* sp. (isolated as a contaminant from a refrigerated room) were cultivated at 27° on the shaker for 1 day in nutrient broth (Baltimore Biological Laboratories). *Pseudomonas chlororaphis* (from W. C. Haynes, North Regional Research Lab., U.S. Department of Agriculture) was cultivated at 27° on the shaker for 1 day in a defined liquid medium developed by Kögl & Postowsky (1930); *Pseudomonas aeruginosa* was cultivated under the same conditions except the temperature was 35°. *Serratia marcescens* ATCC 274 was cultivated at 27° on the shaker for 1 day in a defined liquid medium devised by Bunting (1946). *Vibrio costicolus* (from N. E.

Gibbons, National Research Council, Ottawa) was cultivated at 27° on the shaker for 1 day in 1 % (w/v) Trypticase (Baltimore Biological Laboratories) containing 2 % (w/v) NaCl (these ingredients were autoclaved separately). *Lactobacillus bifidus* 20–32 and *L. fermenti* 69L–3 were grown at 37° in static culture for 1 day in yeast-extract + Trypticase + glucose + calcium carbonate broth.

The media used for counting viable organisms were of the same composition as the corresponding liquid media which had been used to grow the organisms initially but contained 1·5 % agar. Plates were incubated at the appropriate temperatures until development of good colonies (12 hr. to several days, depending upon the particular organism).

Preparation of organisms for storage. Forty ml. of a liquid culture were centrifuged, the supernatant fluid decanted, and the pellet of organisms taken up in 8 ml. of double-strength skim milk (prepared from the Difco dehydrated product). The suspension was well mixed and dispensed in 0·2 ml. portions into sterile cotton-plugged ampoules (10 mm. diam. × 35 mm.). All but two of these ampoules were placed at −60° in ethanol + solid CO_2. Viable counts were made on the two unfrozen suspensions as follows. Each ampoule was carefully wiped with 95 % ethanol, air dried, and aseptically placed into a sterile milk-dilution bottle containing 20 ml. 0·85 % NaCl and several marbles. The two bottles were vigorously shaken until the ampoules were well pulverized. From these 1/100 dilutions further serial dilutions were prepared and samples plated on appropriate nutrient agars.

Two of the frozen ampoules were thawed (at room temperature) and assayed as described above. The other ampoules were removed from the −60° bath and quickly placed in a jar (at room temperature) connected to a vacuum pump assembly. Drying from the frozen state was at 70–100 μ Hg and continued overnight. The ampoules were then removed from the jar, placed into phials (14 mm. diam. × 60 mm.), the ends packed with asbestos fibre, and the phials sealed under a pressure of 70–100 μ Hg. Two phials were opened immediately and the viable counts made. The other phials were stored at 8° for various periods up to 10 years. The results will be expressed as total number of viable organisms/ampoule.

Several strains of a large (1–2·5 μ × 5–20 μ) Gram-negative obligate anaerobe, which had been isolated from faeces of turkeys (Harrison, 1949) and which will be referred to as *Bacteroides* sp. were handled somewhat differently. Growth was washed from agar slopes with double-strength skim milk and several tenths ml. dispensed into ampoules (7 mm. diam. × 80 mm. tubes with 11 mm. diam. bulbs at the distal end). The suspensions were frozen at −60°, dried directly from the freezing bath overnight at 70–100 μ Hg (Model 103 PM, F. J. Stokes Corp., Phila, Pa., U.S.A.), and the ampoules sealed without being disconnected from the manifold. Storage was at room temperature. Species of Lactobacteriaceae were lyophilized in the same way. Viable counts of these organisms were not made; it was noted simply whether the contents of an ampoule, when decanted into suitable broth, produced a growing culture. A detailed physiological study before and after (Stevens, 1956) storage of the lyophilized Bacteroides organisms was undertaken by the methods already outlined (Harrison & Hansen, 1954).

RESULTS AND DISCUSSION

Table 1 summarizes the viability data for 12 bacterial species. Freezing (with subsequent thawing) caused little, if any, decrease in count. On the other hand, subsequent manipulation (either drying or exposure to oxygen of the air after drying; Lion & Bergmann, 1961) caused decrease in count, the response differing greatly among the species. Semilog-time plots of the data are useful to predict the longevity of the dried suspensions and to demonstrate the relative sensitivity of the different species. The death rate may decrease with time, thus a survival curve may

Table 1. *Viability of bacteria after freezing, drying, and storage in the dried state**

(All assays represent the total number of viable organisms by colony count in the ampoule.)

Species	Initial count	After freezing	After drying	After storage for 1 week	4 weeks	8 weeks	12 weeks	10 years
Escherichia coli	$8{\cdot}2\times10^{9}$	$9{\cdot}2\times10^{9}$	$5{\cdot}6\times10^{9}$	$9{\cdot}4\times10^{7}$	$8{\cdot}2\times10^{8}$	$4{\cdot}2\times10^{8}$	$3{\cdot}4\times10^{8}$	$2{\cdot}2\times10^{8}$
	$9{\cdot}4\times10^{9}$	$8{\cdot}6\times10^{9}$	$9{\cdot}8\times10^{9}$	$5{\cdot}4\times10^{8}$	$1{\cdot}8\times10^{9}$	$8{\cdot}2\times10^{7}$	$1{\cdot}2\times10^{8}$	$1{\cdot}5\times10^{8}$
Vibrio costicolus	$4{\cdot}0\times10^{8}$	$3{\cdot}8\times10^{8}$	—	$7{\cdot}4\times10^{4}$	$1{\cdot}4\times10^{4}$	$2{\cdot}2\times10^{4}$	$7{\cdot}6\times10^{4}$	$<2{\cdot}0\times10^{1}$
	$2{\cdot}2\times10^{8}$	$3{\cdot}8\times10^{8}$	—	$2{\cdot}4\times10^{4}$	$8{\cdot}6\times10^{3}$	$7{\cdot}8\times10^{4}$	$5{\cdot}2\times10^{3}$	$<2{\cdot}0\times10^{1}$
Lactobacillus bifidus	$2{\cdot}0\times10^{8}$	$3{\cdot}4\times10^{8}$	$3{\cdot}6\times10^{8}$	$1{\cdot}4\times10^{9}$	$8{\cdot}8\times10^{8}$	$3{\cdot}4\times10^{8}$	$3{\cdot}6\times10^{8}$	$2{\cdot}2\times10^{7}$
	$4{\cdot}8\times10^{8}$	$2{\cdot}8\times10^{8}$	$2{\cdot}6\times10^{8}$	$1{\cdot}3\times10^{9}$	$8{\cdot}8\times10^{8}$	$5{\cdot}0\times10^{8}$	$2{\cdot}8\times10^{8}$	$2{\cdot}6\times10^{7}$
L. fermenti	$5{\cdot}6\times10^{8}$	$6{\cdot}0\times10^{8}$	$2{\cdot}4\times10^{8}$	$2{\cdot}0\times10^{8}$	$1{\cdot}0\times10^{8}$	$1{\cdot}4\times10^{8}$	$1{\cdot}8\times10^{8}$	$1{\cdot}2\times10^{8}$
	$8{\cdot}0\times10^{8}$	$5{\cdot}2\times10^{8}$	$3{\cdot}0\times10^{8}$	$2{\cdot}4\times10^{8}$	$1{\cdot}5\times10^{8}$	$1{\cdot}1\times10^{8}$	$1{\cdot}4\times10^{8}$	$9{\cdot}0\times10^{7}$
Acetobacter melanogenum	$8{\cdot}2\times10^{8}$	$7{\cdot}6\times10^{8}$	$5{\cdot}4\times10^{7}$	$3{\cdot}2\times10^{7}$	$5{\cdot}6\times10^{7}$	$9{\cdot}6\times10^{7}$	—	$<2{\cdot}0\times10^{1}$
	$5{\cdot}8\times10^{8}$	$7{\cdot}2\times10^{8}$	$5{\cdot}4\times10^{7}$	$2{\cdot}8\times10^{7}$	$4{\cdot}2\times10^{7}$	$7{\cdot}0\times10^{7}$	—	$<2{\cdot}0\times10^{1}$
Aerobacter aerogenes	$4{\cdot}4\times10^{9}$	$4{\cdot}4\times10^{9}$	$2{\cdot}0\times10^{9}$	$2{\cdot}2\times10^{9}$	$1{\cdot}0\times10^{9}$	$1{\cdot}9\times10^{9}$	$8{\cdot}0\times10^{8}$	$3{\cdot}6\times10^{9}$
	$4{\cdot}8\times10^{9}$	$5{\cdot}0\times10^{9}$	$2{\cdot}6\times10^{9}$	$5{\cdot}8\times10^{8}$	$9{\cdot}6\times10^{8}$	$1{\cdot}4\times10^{9}$	$1{\cdot}2\times10^{9}$	$8{\cdot}8\times10^{8}$
Pseudomonas sp.	$5{\cdot}6\times10^{9}$	$7{\cdot}6\times10^{9}$	$1{\cdot}1\times10^{9}$	$1{\cdot}3\times10^{9}$	$1{\cdot}2\times10^{9}$	$4{\cdot}2\times10^{8}$	$9{\cdot}8\times10^{8}$	$3{\cdot}6\times10^{8}$
	$7{\cdot}8\times10^{9}$	$1{\cdot}1\times10^{10}$	$1{\cdot}3\times10^{9}$	$3{\cdot}2\times10^{8}$	$5{\cdot}2\times10^{8}$	$4{\cdot}0\times10^{8}$	$9{\cdot}0\times10^{8}$	$4{\cdot}2\times10^{8}$
P. aeruginosa	—	$3{\cdot}0\times10^{9}$	$3{\cdot}2\times10^{8}$	$1{\cdot}0\times10^{8}$	$1{\cdot}5\times10^{8}$	$8{\cdot}6\times10^{7}$	—	$1{\cdot}5\times10^{6}$
	$4{\cdot}4\times10^{9}$	$2{\cdot}2\times10^{9}$	$6{\cdot}8\times10^{7}$	$1{\cdot}0\times10^{7}$	$1{\cdot}1\times10^{8}$	$3{\cdot}0\times10^{7}$	—	$2{\cdot}0\times10^{5}$
P. chlororaphis	$8{\cdot}8\times10^{9}$	$8{\cdot}2\times10^{9}$	$2{\cdot}0\times10^{6}$	—	$1{\cdot}9\times10^{6}$	$1{\cdot}0\times10^{5}$	$6{\cdot}6\times10^{5}$	$2{\cdot}0\times10^{5}$
	$1{\cdot}0\times10^{10}$	$1{\cdot}0\times10^{10}$	$3{\cdot}2\times10^{7}$	—	$1{\cdot}3\times10^{5}$	$1{\cdot}9\times10^{5}$	$6{\cdot}0\times10^{5}$	$4{\cdot}6\times10^{5}$
Proteus vulgaris	$3{\cdot}2\times10^{9}$	$2{\cdot}8\times10^{9}$	$6{\cdot}8\times10^{7}$	$5{\cdot}4\times10^{7}$	$9{\cdot}2\times10^{6}$	$9{\cdot}8\times10^{6}$	—	$4{\cdot}6\times10^{6}$
	$3{\cdot}0\times10^{9}$	$3{\cdot}2\times10^{9}$	$6{\cdot}0\times10^{7}$	$5{\cdot}0\times10^{7}$	$3{\cdot}6\times10^{5}$	$1{\cdot}0\times10^{7}$	—	$5{\cdot}2\times10^{6}$
Serratia marcescens	$9{\cdot}4\times10^{8}$	$8{\cdot}8\times10^{8}$	$1{\cdot}8\times10^{8}$	$8{\cdot}8\times10^{7}$	$3{\cdot}8\times10^{8}$	$1{\cdot}4\times10^{8}$	$2{\cdot}2\times10^{7}$	$2{\cdot}2\times10^{7}$
	$1{\cdot}0\times10^{9}$	$8{\cdot}4\times10^{8}$	$1{\cdot}6\times10^{8}$	$3{\cdot}4\times10^{8}$	$1{\cdot}2\times10^{8}$	$9{\cdot}8\times10^{7}$	$7{\cdot}4\times10^{6}$	$7{\cdot}4\times10^{7}$
Micrococcus albus	$4{\cdot}8\times10^{9}$	$4{\cdot}2\times10^{9}$	$2{\cdot}6\times10^{9}$	$2{\cdot}8\times10^{9}$	$2{\cdot}8\times10^{9}$	$4{\cdot}4\times10^{8}$	—	$1{\cdot}3\times10^{9}$
	$4{\cdot}2\times10^{9}$	$4{\cdot}8\times10^{9}$	$2{\cdot}6\times10^{9}$	$1{\cdot}8\times10^{9}$	$2{\cdot}0\times10^{9}$	$1{\cdot}4\times10^{9}$	—	$7{\cdot}8\times10^{8}$

* Suspended in double-strength skim milk, frozen at −60°, dried at 70–100 μ Hg and stored at 8°.

have a continuously decreasing slope. Upon extrapolation from the 12-week (or 8-week) and 10-year points we find that the ampoules containing the Vibrios and Acetobacters can be expected to become sterile within several decades, whereas (assuming constant death rate) most ampoules can be expected to contain some viable organisms after centuries of storage. (Of course, the longevity will depend upon the number of organisms initially present.) We can compare sensitivities by means of death rates. Thus, the numbers of years required to cause a mere ten-fold

decrease in count are, respectively, 100 (for *Escherichia coli*), 3, 9, 40, 1, 40, 25, 5, 25, 35, 17, and many 100's (for *Micrococcus altus*). There appears little correlation with phylogeny, although members of Pseudomonadaceae (Acetobacter, Vibrio, Pseudomonas) are among the most sensitive. (*Vibrio cholera* also does not tolerate lyophilization well according to Rhodes & Fisher, 1950; Stamp, 1947.) Appreciable variation in sensitivity occurs among different genera of Enterobacteriaceae.

Although it may be possible to retain viability of a suspension in the dried state for very long periods, it might be unwise to allow too great a decrease in count to take place. Unequal selection of different genotypes within the population might occur, thus the mass characters of the culture might be altered. Indeed, the reason

Table 2. *Final* pH *values of broth cultures of two Bacteroides strains before lyophilization and after lyophilization and storage at room temperature for* 5 *years**

Substrate	Strain 21–28		Strain 28–30	
	Before†	After‡	Before†	After‡
Glycerol	6·9	6·6	7·0	*4·7*
Xylose	*4·8*	*5·1*	*4·7*	6·4
Arabinose	*4·8*	6·5	*4·7*	6·4
Rhamnose	6·8	6·6	6·8	6·4
Sorbitol	*5·3*	6·7	*5·1*	6·4
Mannitol	*5·5*	6·6	*5·3*	6·5
Inositol	7·0	6·6	7·0	6·6
Fructose	*5·3*	*4·8*	*5·0*	*5·0*
Glucose	*4·9*	*5·1*	*4·9*	*4·8*
Mannose	*5·1*	*5·9*	*4·9*	*4·8*
Galactose	*5·0*	*4·8*	*4·8*	6·2
Sucrose	*5·9*	*5·0*	6·6	6·8
Trehalose	*4·8*	6·6	*4·8*	6·5
Maltose	*4·8*	*5·0*	*4·9*	6·6
Cellobiose	6·8	6·6	*4·8*	6·4
Melibiose	*4·8*	6·5	*4·7*	6·5
Lactose	*4·9*	6·5	*4·7*	6·2
Melezitose	6·9	6·6	6·9	6·5
Raffinose	*4·8*	6·6	*4·8*	6·8
Dextrin	6·5	6·5	6·5	6·7
Starch	7·0	6·6	7·0	6·5
Salicin	*5·0*	6·6	6·7	6·7
No substrate	7·0	6·7	7·0	6·6

* Incubation was in anaerobic jars at 37° for 2 weeks in a basal medium of 1 % trypticase, 0·2 % K_2HPO_4, 0·01 % $MgSO_4.7H_2O$, and with 1 % substrate. All substrates were sterilized by autoclaving in the basal medium except xylose, arabinose, maltose, cellobiose and melibiose which were sterilized by filtration. Significant fermentation is indicated by italic figures.

† Harrison (unpublished data).

‡ Stevens (1956).

why lyophilized material appears to maintain its characters so well may be because the usual holding period (a few years) is relatively short as compared with the potential longevity of the suspension; thus the decreases in count may not have been great enough to bring about much selection. Storage at higher temperatures increases the death rate (Weiser & Hennum, 1947; Proom & Hemmons, 1949; Heckly, Anderson & Rockenmacher, 1958; Heckly, Faunce & Elberg, 1960), thus selection should occur more rapidly. However, changes in physiological traits of

lactobacilli, including the anaerobic *L. bifidus*, streptococci, betacocci, and pediococci stored for 5 years at room temperature have not been encountered. Sharpe & Wheater (1955) found little alteration of characters of lactobacilli lyophilized and stored for 6 months. On the other hand, with some unusuallylarge Bacteriodes spp., lyophilized and stored in an identical manner, pronounced alterations were observed.

Table 2 presents the fermentation spectrum of two strains of Bacteroides before lyophilization and after lyophilization and storage. Losses in the ability to ferment the polyhydric alcohols and di- and tri-saccharides have been sustained. Strain 28–30, in addition, had lost the ability to ferment galactose and, more surprising, had gained the ability to ferment glycerol. The end-products from glucose fermentation were not altered: for example, lactic acid remained the principal end-product and the zinc lactate remained the laevorotatory isomer. Morphology, temperature requirements and gross growth characteristics also were unaltered. Quantitative viability studies were not made on these ampoules, but it is likely that great decreases in count had occurred, because after three additional years storage all ampoules had become sterile. A pleomorphic Bacteroides strain 29–9, also lyophilized, did not survive even the 5-year period. Thus, some Bacteroides appear much more sensitive to lyophilization than the lactic acid bacteria.

Table 3. *Viability of bacteria after freezing as pellets*

Species	Temp. (°)	Initial count	After storage for (weeks) 1	2	4	8	12
Escherichia coli	−25	$1{\cdot}2\times10^{9}$	$1{\cdot}1\times10^{9}$	$1{\cdot}1\times10^{9}$	$8{\cdot}7\times10^{8}$	—	$9{\cdot}5\times10^{8}$
	−60	$1{\cdot}2\times10^{9}$	$8{\cdot}5\times10^{8}$	$1{\cdot}0\times10^{9}$	$9{\cdot}5\times10^{8}$	—	$9{\cdot}4\times10^{8}$
Vibrio costicolus	−25	$8{\cdot}0\times10^{8}$	$1{\cdot}1\times10^{9}$	$1{\cdot}3\times10^{9}$	$1{\cdot}3\times10^{9}$	$5{\cdot}0\times10^{8}$	$3{\cdot}6\times10^{8}$
	−60	$8{\cdot}0\times10^{8}$	$9{\cdot}3\times10^{8}$	$5{\cdot}6\times10^{8}$	$4{\cdot}0\times10^{8}$	$4{\cdot}0\times10^{7}$	$9{\cdot}5\times10^{6}$
Lactobacillus bifidus	−25	$1{\cdot}1\times10^{9}$	$3{\cdot}2\times10^{8}$	$1{\cdot}6\times10^{8}$	$3{\cdot}8\times10^{7}$	$5{\cdot}6\times10^{7}$	$8{\cdot}1\times10^{7}$
	−60	$1{\cdot}1\times10^{9}$	$9{\cdot}5\times10^{8}$	$9{\cdot}8\times10^{8}$	$7{\cdot}7\times10^{9}$	$9{\cdot}1\times10^{8}$	$1{\cdot}1\times10^{9}$
L. fermenti	−25	$4{\cdot}3\times10^{8}$	$4{\cdot}9\times10^{8}$	$2{\cdot}1\times10^{8}$	$1{\cdot}5\times10^{8}$	$1{\cdot}2\times10^{8}$	$8{\cdot}5\times10^{7}$
	−60	$4{\cdot}3\times10^{8}$	$7{\cdot}8\times10^{8}$	$4{\cdot}7\times10^{8}$	$4{\cdot}4\times10^{8}$	$3{\cdot}4\times10^{9}$	$4{\cdot}5\times10^{8}$
Serratia marcescens	−25	$5{\cdot}9\times10^{9}$	$5{\cdot}7\times10^{9}$	$4{\cdot}9\times10^{9}$	$6{\cdot}1\times10^{9}$	$3{\cdot}7\times10^{9}$	$4{\cdot}4\times10^{9}$
	−60	$5{\cdot}9\times10^{9}$	$4{\cdot}8\times10^{9}$	$3{\cdot}6\times10^{9}$	$6{\cdot}3\times10^{9}$	$3{\cdot}7\times10^{9}$	$4{\cdot}9\times10^{9}$

Frozen storage as pellets is suitable for some bacteria that are sensitive to lyophilization. Broth cultures are centrifuged, the broth decanted, and the centrifuge tubes containing the pellets placed directly in the deep-freeze unit. Results are presented in Table 3. Quantitative experiments have been undertaken with this method only over a 12-week period, but it is noteworthy that survivals may be better than with lyophilized material during this interval (Table 1). Storage as thick water suspensions may also be satisfactory, since many bacteria are not affected adversely thereby. For example, it has been observed repeatedly that *Escherichia coli* titres are not diminished as a result of freezing and thawing in distilled water (Harrison, 1956; Clement, 1961; Lion & Bergmann, 1961).

Freezing agar slope and butt cultures at −22° gives good results and is convenient, but a quantitative viability study of this method has not been carried out. It is important that toxic end-products such as acid are minimized by using glucose concentrations in the medium of 0·25% or less. The following bacteria have been

maintained frozen for 2–3 years with no detected changes in morphology, cultural characteristics, or physiological traits: 10 species of Streptococcus, including 14 strains of *Streptococcus faecalis*, 7 species of Bacillus, 6 species of Lactobacillus, 3 species of Acetobacter, 2 species of Microbacterium, 2 species of Propionibacterium, 2 species of Sarcina, 3 species of Betacoccus, 2 species of Micrococcus, Chromobacterium, Pediococcus, Proteus, Escherichia, Aerobacter, Alkaligenes, Pseudomonas and Saccharomyces. Anaerobic Bacteroides and Lactobacillus have been stored under aerobic conditions successfully for a year with no change in characters noted.

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Fine Structure of Vegetative Hyphae of Rhizopus

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SUMMARY

Ultrathin sections of vegetative hyphae of *Rhizopus sexualis* and *R. homothallicus* examined by electron microscopy revealed structure essentially similar to that reported for some other filamentous fungi and yeasts. The cell wall in section consists of elongated elements tangentially orientated. The nucleus is surrounded by a double membrane interrupted by pores and contains a denser body identified as the nucleolus. The cytoplasm contains well-defined mitochondria, vesicles (cisternae), unidentified spherical bodies, oil drops and vacuoles. There is a complex system of cytoplasmic membranes or membrane-like layers including the outer layer or plasmalemma, membranes (tonoplasts) surrounding the vacuoles and, in the zone of extension growth behind the hyphal tip, a more or less continuous, convoluted 'cortical membrane' separating a central core of cytoplasm from a peripheral zone. The possible significance of such a membrane in relation to cytoplasmic streaming is discussed.

INTRODUCTION

The physiology of zygospore formation in *Rhizopus sexualis* (Smith) Callen and some other members of the Mucorales is being studied in this laboratory (Hawker, Hepden & Perkins, 1957; Hepden & Folkes, 1960; Hepden & Hawker, 1961; Hawker & Hepden, 1963). Unpublished observations with the light microscope suggest that chemical changes occur in the hyphae before conjugation. A study of the fine structure of the hyphae, as revealed by the electron microscope, was therefore made to assist interpreting experimental results. Additional observations have been made with another homothallic species, *Rhizopus homothallicus* Hesseltine & Ellis (Hesseltine & Ellis, 1961) and with a single strain of *Rhizopus nigricans* Ehrenb.

METHODS

Rhizopus sexualis was grown on 3 % malt agar for 24 hr. at 20°; *R. homothallicus* for 30 hr. at 25°. Tufts of young hyphae were picked off from the edge of a colony and fixed immediately. A single tuft of hyphae from an older culture of *R. homothallicus* was also fixed.

The methods of fixation, dehydration and embedding outlined below were based on information supplied by Dr E. H. Mercer of the Chester Beatty Institute, and on a recent publication (Mercer & Birbeck, 1961).

Fixation. The hyphae were either (*a*) fixed in Luft's permanganate, 2 % (pH 7) (Luft, 1956) in a refrigerator for 20 min., centrifuged for 10 min. and then washed in cold distilled water, or (*b*) fixed by a dual fixation method described by Thyagarajan,

Conti & Naylor (1961) as follows; Luft's permanganate diluted to 1·5 % with buffer (pH adjusted to 7·0), for 10 min. at room temperature, washed with cold distilled water, followed by fixation in osmium tetroxide, 1·0 % (pH 7·2) for 70 min. in a refrigerator, centrifuged for 10 min. while still in the osmic fixative and washed with veronal buffer.

Dehydration. After fixation the hyphae were left for 15 min. in 30, 50 and 80 % alcohol successively and were finally left for 1 hr. in absolute alcohol.

Embedding. Surplus alcohol was drained off and the tuft of hyphae gently dried with filter paper and placed in a 50/50 mixture (v/v) of alcohol and 'Araldite' (a proprietary epoxide embedding medium) for 1 hr. The hyphae were then passed through two changes of araldite mixture (without accelerator) and left in the second of these overnight. They were finally placed in gelatine capsules containing araldite to which accelerator had been added, and were left at 62° for 2–3 days until the araldite had hardened.

Sectioning. Sectioning proved to be unusually difficult owing to the exceptionally tough walls of the hyphae. Sections of these fungi were cut on a Huxley ultramicrotome (Cambridge Instrument Co.). A diamond knife proved unsatisfactory and a new glass knife was used for every set of sections. The microtome was usually set to cut sections 0·05 μ thick, but the actual thickness probably varied from 0·05 to 0·1 μ. A few thicker sections (0·15 μ) of *Rhizopus homothallicus* were cut.

The sections were floated on 10 % acetone and caught on small copper grids of the usual type. Owing probably to the tough nature of the cell wall it was difficult to get properly stretched thin sections as the araldite tended to tear away from the sections of hyphae.

Electron microscopy. The sections were examined and photographed with a Phillips E.M. 200 or occasionally a Phillips E.M. 100 electron microscope in the Department of Physics, University of Bristol.

RESULTS

The main object of the investigation was to determine the structure of the cytoplasm of vegetative hyphae in young cultures before the onset of reproduction. Observations were also made of the structure of the nucleus and cell wall.

The cell wall

The cell wall was seen only in section. It consists of elongated elements laid down tangentially, giving it a striate appearance (Pl. 2, fig. 8). The wall varies in thickness from 0·05 to 0·5 μ. It is assumed that thickness is correlated with distance from the hyphal tip, so affording a means of estimating the relative physiological age of the hyphae. There is no visible difference in structure between the outer older portions and the inner newly formed layers of the thick wall of a mature hypha. Wall structure was similar in both species of Rhizopus studied.

Cell contents

Nuclei. The nuclei are relatively large bodies (*c.* 1·25–2·2 μ diam.), approximately globose or ellipsoidal but showing minor irregularities in outline (Pl. 1, figs. 1, 4; Pl. 2, fig. 5).

The internal mass of the nuclei shows no clearly defined structure. There is usually a denser patch which, by comparison with suitably stained preparations viewed under the light microscope, is assumed to be the nucleolus (Pl. 1, fig. 1; Pl. 2, fig. 5).

The nuclei are surrounded by a clearly defined double membrane interrupted by pores (Pl. 2, fig. 5). Occasionally the nuclear membrane is seen to be in direct continuity with membranous inclusions in the cytoplasm. The nuclear substance was in direct contact with the cytoplasm through pores in the nuclear membrane.

Although the presence of paired nuclei suggests that nuclear division occurs fairly frequently, there is no indication that discrete chromosomes are formed. Robinow (1957), from a study of stained material of *Mucor hiemalis*, *M. fragilis* and *Phycomyces blakesleeanus* by the light microscope, concluded that 'division of the chromatinic elements is direct and involves neither spindle nor metaphase plate.'

Mitochondria. Numerous clearly defined mitochondria are present in hyphae of all ages. In transverse sections they are usually more or less circular or broadly elliptical (Pl. 1, figs. 1, 2; Pl. 2, figs. 5, 7) but in longitudinal sections of young hyphae many of them are elongated (Pl. 1, fig. 4; Pl. 2, fig. 6). They are surrounded by a double membrane, the inner layer of which is infolded to form a varying number of cristae. In relatively thick sections these are seen to be lamellae and are frequently arranged in groups of two or more roughly parallel plates. In young hyphae there are relatively few cristae and they extend less deeply into the central matrix of the mitochondria than do those of the mitochondria of older hyphae. The range of size of the mitochondria is similar in all parts of the hyphae (i.e. 0·4–0·7 $\mu \times 0{\cdot}5$–0·8 μ).

Intact hyphae stained with toluidine blue and examined under the light microscope show numerous mitochondria (Pl. 2, figs. 11, 12) and confirm the electron microscope findings relating to size, shape and distribution.

Vesicles, tubules or cisternae; possible Golgi bodies. The cytoplasm also contains sparsely distributed irregular vesicles or tubules (Pl. 1, figs. 2, 3; Pl. 2, figs. 6, 7) resembling the cisternae seen by Blondel & Turian (1960) in Allomyces. Occasionally these are grouped together but are not so regular in arrangement as the typical Golgi apparatus seen in plant cells by Buvat (1958) and others.

Unidentified spherical bodies. In some sections there are groups of irregularly arranged spherical bodies with homogeneous contents. These did not stain black with osmium fixation and their nature was not determined.

Oil drops and food vacuoles. Numerous homogeneous globose bodies, stained black in material fixed with osmic acid, are thought to be oil drops. They occur as clear white patches of regular outline in material fixed with permanganate only (Pl. 2, fig. 7). Material stained with Sudan III and examined under the light microscope contains oil drops of similar size and distribution.

Stellate vacuoles (Manton, 1961) containing amorphous material are also present (Pl. 2, fig. 7). They resemble the storage vacuoles in conidiophores of *Stilbum zacoloxanthum* McAlear & Edwards (1959). The nature of the stored material is not known, but suitably stained hyphae under the light microscope show the presence of considerable quantities of glycogen.

Cytoplasmic 'membranes'. A thin dark layer, the plasmalemma, appearing as a membrane consisting of one or more layers, can be seen enclosing the cytoplasm. Similar layers (tonoplasts) surround the vacuoles. The plasmalemma is not always

in close contact with the wall and may show a crinkled edge (Pl. 2, fig. 8). The curious structures termed lomasomes by Moore & McAlear (1961) and thought by them to be characteristic of fungi, were not seen, although occasionally vacuoles could be seen in contact with the plasmalemma and apparently about to break through it (Fig. 1). Large vacuoles, present only in mature hyphae, have a regular outline (Fig. 2) but smaller ones in younger hyphae have irregular or stellate shape (Pl. 2, fig. 7). All are surrounded by a distinct 'membrane'. Often one vacuole is invaginated by another, suggesting that the surrounding layer is a definite membrane.

In addition to the plasmalemma and the membranes surrounding the vacuoles there is a complex system of membrane-like layers in the endoplasm. These are most clearly marked in sections which, from the extreme thinness of the cell wall, must have been taken at or near the tip of the hypha, probably from the zone of extension growth just behind it. In many such sections (both transverse and longitudinal) a complete or almost complete sinuous 'cortical membrane' could be traced, separating an irregular central core from a peripheral zone (Pl. 1, figs. 1–4; Fig. 3).

The core is always less dense than the peripheral layer and contains all or most of the nuclei and few mitochondria. The peripheral zone is often crowded with mitochondria (Fig. 3). The presence of such zoning is confirmed by observation of living hyphae by phase contrast (Pl. 2, figs. 9, 10).

In slightly older hyphae, as judged by the rather thicker cell walls, the 'cortical membrane' is no longer continuous but is broken and tends to envelop small stellate vacuoles (Pl. 2, fig. 7). In still older hyphae, with relatively thick walls, the small vacuoles coalesce and may extend across the central part of the hyphae, leaving only a shallow peripheral zone of cytoplasm containing nuclei and numerous mitochondria, neither of which show signs of deterioration (Fig. 2). The vacuoles of hyphae at this stage still show contents thought to be food reserves, probably glycogen. In very old hyphae the vacuole occupies a still greater volume and no longer contains regularly spaced material but is apparently empty or contains irregular masses of unidentifiable substances. The peripheral cytoplasmic residues in such hyphae are irregular and mitochondria and nuclei are distorted. They remain recognizable, however, even after the endoplasm shows signs of disintegration.

DISCUSSION

No complete study of fine structure in a member of the Mucorales has yet been published, although the structure of the wall of *Phycomyces blakesleeanus* has been investigated (Roelofsen, 1959) and a single electron micrograph of a section of a hypha of a species of Syncephalastrum has been published by Moore & McAlear (1961). A few fungi from other groups have been studied, notably *Allomyces macrogynus* (Blondel & Turian, 1960), *Neurospora crassa* (Shatkin & Tatum, 1959) and various yeasts (Thyagarajan *et al.* 1961; Kawakami & Nehira, 1959). The structure of the hyphae of *Rhizopus sexualis* differs from that of other fungi studied, chiefly in the greater development of 'membranes' in the cytoplasm of the young hyphae. This may not be a real difference, since other studies may not have included such young hyphae.

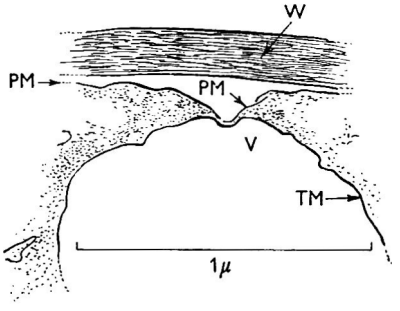

Fig. 1

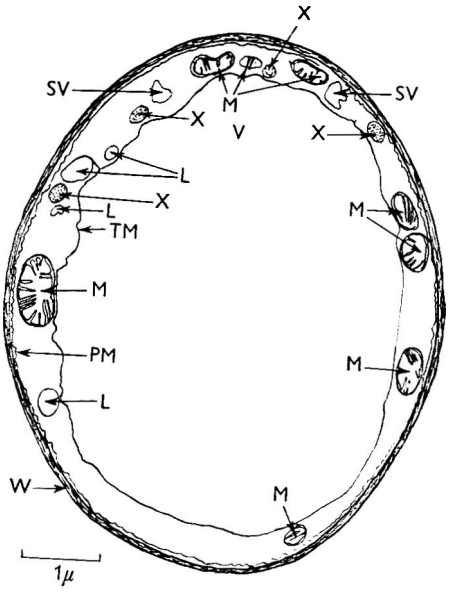

Fig. 2

Fig. 1. *Rhizopus homothallicus*, tracing from electron micrograph showing vacuole (V) in contact with plasmalemma (PM) and apparently about to break through it. W = cell wall, TM = tonoplast.

Fig. 2. *Rhizopus homothallicus*, tracing of electron micrograph of T.S. of mature hypha. Note large central vacuole (V) and peripheral cytoplasm. M = mitochondria, PM = plasmalemma, TM = tonoplast, W = cell wall, L = lipid body, SV = small stellate vacuole, X = spherical body of unknown nature.

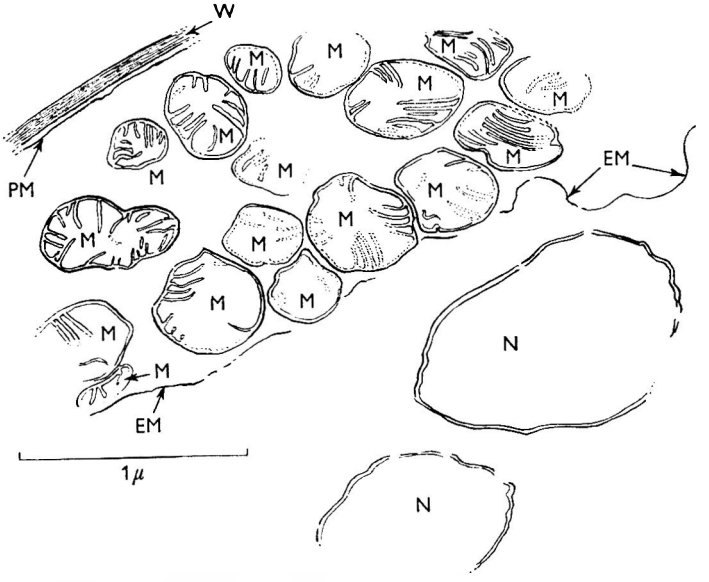

Fig. 3. *Rhizopus sexualis*, tracing of electron micrograph showing endoplasmic cortical membrane separating outer peripheral zone containing numerous mitochondria from inner central cylinder containing nuclei. M = mitochondria, N = nucleus, PM = plasmalemma, EM = endoplasmic membrane, W = cell wall.

The general structure of the nuclei, mitochondria and other cytoplasmic inclusions resembles that described for other fungi.

The most interesting feature in the anatomy of Rhizopus is the separation of an outer zone containing numerous mitochondria from an inner core containing nuclei and a few mitochondria in the region of extension growth of the hypha. These zones appear to be separated by a membrane, often of two or even three layers. It is not certain whether this is a true membrane or merely the effect of two surfaces in contact. Such a division is of particular interest in view of the observation of Arthur (1897) with *Rhizopus nigricans*, confirmed by Schröter (1905) and Buller (1933), of the occasional presence of particles moving in a direction opposite to that of the main stream within the hyphae. These authors describe a broad vacuolated axial stream of protoplasm and a sheath-like peripheral return current of non-vacuolated protoplasm moving basipetally. This observation has been doubted by some later workers and it has been suggested that such a streaming of protoplasm in opposite directions in one and the same hypha at the same time presupposes the existence of a cytoplasmic membrane separating the two streams of cytoplasm. We examined living hyphae under the ordinary light microscope and by phase contrast. In mature parts of the living hypha streaming was rapid, but at any particular time was in one direction only. In the region of extension growth near the apex of the hyphae, however, streaming was usually slower but some particles could be seen moving towards the tip while others were moving away from it. The particles were of a size comparable with that of mitochondria. The peripheral particles moved in jerks as if they were encountering obstacles. Plate 1, figs. 1–4, indicate that such obstacles may well exist in the form of folds of the 'cortical membrane'. The great concentration of mitochondria in the peripheral zone suggests that these may be actively engaged in the linear extension of the cell wall. The internal arrangement of this part of the hyphae and the observations on particle movement are consistent with the hypothesis that food material and nuclei move up the centre of the young hypha and that mitochondria may accumulate at the tip and then move slowly down the peripheral layer. Their progress is impeded by folds of the membrane and it may be that during this slow backward progress they are concerned in the addition of new material to the growing wall. Buller (1933) reported the presence of small moving particles in the peripheral stream. In older parts of the hypha, where the membranes are not continuous, the cytoplasmic streaming is unidirectional at any particular time. Particles flowing in this stream together with small vacuoles are apparently unimpeded by any resistant structure, in contrast to those moving nearer the tip. This is again consistent with the observed structure of older hyphae in which continuous cortical membranes are lacking.

We wish to thank Dr J. Fraymouth of this Department for advice on methods of fixing, embedding and sectioning the material and Mr G. A. Bassett and Mrs J. Dyer of the Physics Department, University of Bristol, for taking many of the electron micrographs. We also wish to thank Dr C. W. Hesseltine of the U.S. Department of Agriculture, Peoria, Illinois for the culture of *Rhizopus homothallicus*.

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EXPLANATION OF PLATES

Rhizopus—electron micrographs

W = cell wall, N = nucleus, NU = nucleolus, NM = nuclear membrane, NP = pores in nuclear membrane, M = mitochondria, MC = cristae, C = cisternae, PM = plasmalemma, TM = tonoplast, EM = endoplasmic or 'cortical' membranes, SV = stellate vacuole, L = lipid or oil drop, X = unidentified spherical bodies. Figs. 1–4, fixed permanganate and osmium tetroxide; figs. 5–8, fixed permanganate only.

PLATE 1. *Rhizopus sexualis*

Fig. 1. T.S. hypha through zone of extension growth behind hyphal tip, as indicated by extremely thin cell wall. Note complex endoplasmic membrane enclosing a central 'core' containing a nucleus; peripheral zone is denser and contains numerous mitochondria and cisternae.

Figs. 2, 3. Parts of same section (outlined by dotted lines in fig. 1) photographed at higher magnification. Fig. 2 shows thin wall of tangentially arranged elements, thin plasmalemma indicated by arrow, mitochondria and lobed endoplasmic 'membranes'. Fig. 3 shows similar endoplasmic membranes of complex nature, small endoplasmic vesicles or cisternae and an unidentified spherical body.

Fig. 4. A longitudinal tangential section of a similar hypha, showing central zone containing a nucleus and small vacuoles and a peripheral zone containing mitochondria and vacuoles. Mitochondria, vacuoles and nucleus are longitudinally elongated and orientated in direction of cytoplasmic streaming.

PLATE 2. *Rhizopus homothallicus*, *R. nigricans* and *R. sexualis*

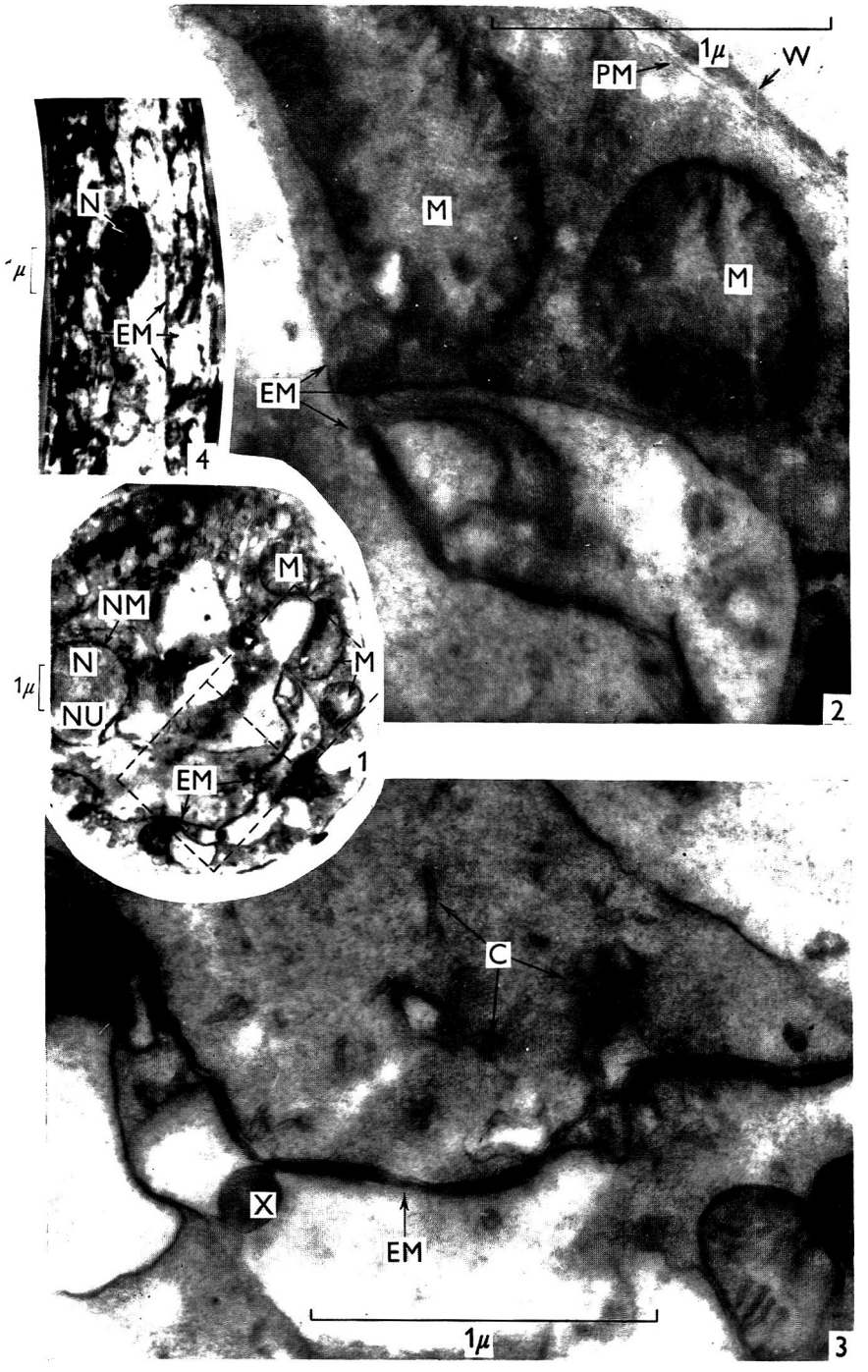
Figs. 5, 6. T.S. and L.S. respectively of young hypha of *R. homothallicus*, showing nucleus with nucleolus, 3-layer nuclear membrane interrupted by pores; mitochondria with complex membrane, inner layer of which is infolded to form plate-like cristae arranged parallel to one another in groups and extending well into the interior; endoplasm with sparsely distributed endoplasmic reticulum, which in fig. 5 shows continuity with nuclear membrane; small vacuoles; unidentified spherical bodies; plasmalemma and thin cell wall. Note elongated mitochondria in fig. 6.

Fig. 7. T.S. older hypha of *R. homothallicus* showing mitochondria, vacuoles, lipid bodies, sparse endoplasmic reticulum with occasional cisternae, plasmalemma and cell wall.

Fig. 8. Section through cell wall of old hypha of *R. homothallicus* showing tangentially striate structure.

Figs. 9. 10. Hypha of *R. nigricans* vitally stained with Janus green, photographed at two different focusings under phase contrast. Note roughly cylindrical central column in zone of extension growth, indicating presence of 'cortical' membrane or the juxtaposition of masses of cytoplasm of different texture.

Figs. 11, 12. Young hyphae of *R. sexualis*, stained with toluidine blue, photographed under oil immersion lens, showing mitochondria and nuclei (clear bodies with stained nucleolus, one nucleus outlined with dots). Note elongated shape of mitochondria in older hypha.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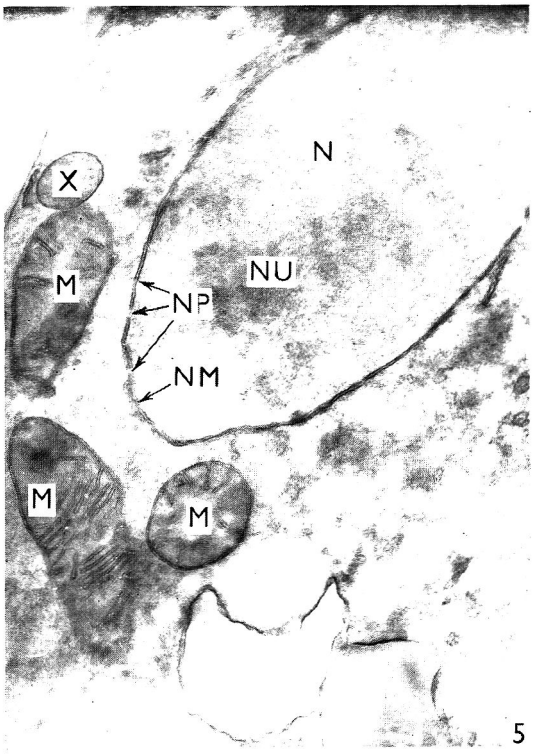

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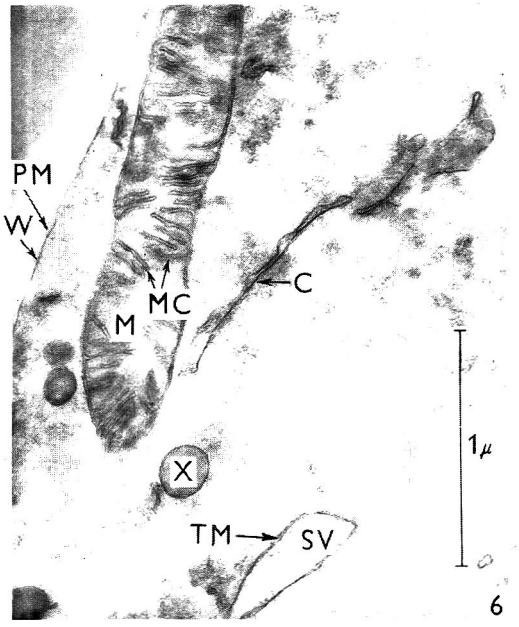

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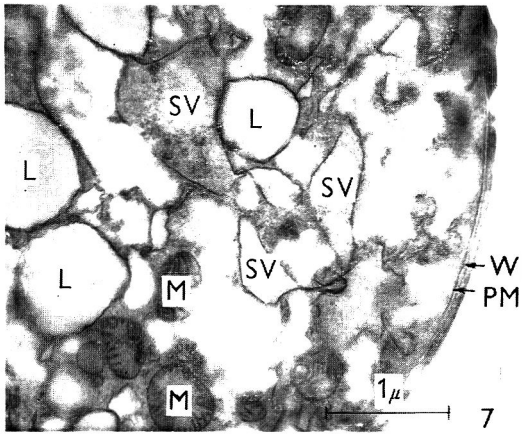

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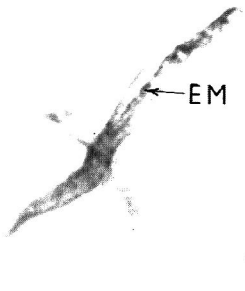

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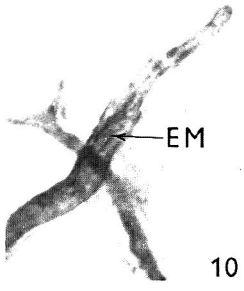

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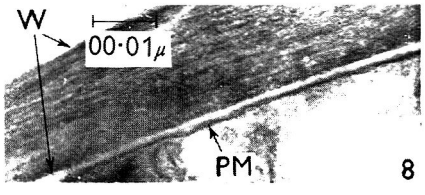

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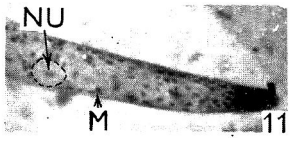

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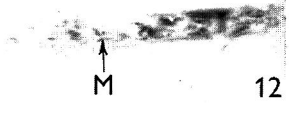

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A Classification of Micrococci and Staphylococci Based on Physiological and Biochemical Tests

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SUMMARY

1250 Gram-positive and catalase-positive cocci were isolated from bacon, pig and human skin and dust, and their morphology, physiology and biochemical characters examined. The genera *Staphylococcus*, *Micrococcus* and *Sarcina* were recognized. Staphylococci were distinguished by their ability to form acid from glucose anaerobically and sarcinas by the formation of cubical packets. 570 isolates could be placed in the genus *Staphylococcus*, 677 in the genus *Micrococcus* and 3 in the genus *Sarcina*. Six subgroups were recognized within the genus *Staphylococcus* and seven within the genus *Micrococcus*. The relationship of these subgroups to previously defined genera and species is discussed.

INTRODUCTION

Many classifications of the Gram-positive and catalase-positive cocci have been suggested. Some authors place all organisms in a single group or genus, others into two or more genera. The classification of Shaw, Stitt & Cowan (1951) proposed combining all organisms in the genus *Staphylococcus*. Evidence for the more traditional approach of recognizing several genera was obtained by Evans, Bradford & Niven (1955) and Hill (1959). The latter concluded from computer data that members of Shaw, Stitt & Cowan's genus *Staphylococcus* were better placed into two genera. The genus *Staphylococcus* was retained for those organisms which formed acid from glucose aerobically but did not possess a pink pigment. The genus *Micrococcus* was re-introduced to contain the pink-pigmented organisms and those types unable to form acid from glucose. Evans *et al.* (1955) suggested a different classification based on the observation of Evans (1947, 1948) that coagulase-positive staphylococci, together with some coagulase-negative, Gram-positive and catalase-positive cocci, could grow and form acid from glucose anaerobically. They proposed that the two genera *Staphylococcus* and *Micrococcus* should be recognized and separated on the ability of the former genus to grow and to produce acid anaerobically. This classification forms the basis of that followed in the most recent edition of *Bergey's Manual* (1957). It was criticized by Thatcher & Simon (1957) who found that all their isolates formed similar amounts of acid both aerobically and anaerobically, and more recently by Garvie, Higgs & Neave (1961) who reported finding coagulase-positive staphylococci which were unable to attack glucose anaerobically. I have been unable to confirm either of these observations and my results based on a study of 1250 isolates agree with those of Evans *et al.* (1955). The object of the present work was to classify the aerobic, Gram-positive and catalase-positive cocci found on bacon, human and pig surfaces, and dust.

METHODS

1250 isolates of Gram-positive and catalase-positive cocci were freshly isolated; 920 of these isolates were from bacon. The bacon was macerated aseptically and plated on tryptone yeast glucose salt agar (TYGSA) containing 6% (w/v) sodium chloride (Cavett, 1962). Colonies from plates incubated at 30° for 3 days were subcultured on to nutrient agar plates and tested for catalase by using 3% (w/v) hydrogen peroxide (Society of American Bacteriologists, 1957). Single colonies of catalase-positive organisms were picked on to slopes of yeast glucose agar (YGA) of the following composition (%, w/v): glucose, 0·5; Evans peptone, 0·5; Na_2HPO_4, 0·5; Difco yeast extract, 0·5; Lab-Lemco, 0·5; agar 1·5. These cultures were incubated for 24 hr. at 30° and then stored at 5° until required. Coagulase-positive staphylococci were isolated on the selective and diagnostic medium of Baird-Parker (1962). 152 isolates were from armpits and hands of 24 volunteers. The skin was subjected to a standardized washing procedure to remove transient organisms (Hurst, Stuttard & Woodroffe, 1960). Organisms recovered in the wash-water after this procedure were cultivated on blood agar. 70 isolates were from 24 pig trotters. Isolations were made from ground-up skin, previously washed by the same procedure as used for making isolations from human skin. 108 isolates were from household dust. Plates of TYGSA were exposed to air for 30 to 60 min.; after incubation for 3 days at 30°, catalase-positive colonies were picked on to nutrient agar plates.

Morphology. Smears were made from nutrient agar plates incubated for 24 hr. at 30° and stained by Gram's method. The Gram reaction, size and arrangement of organisms was noted. Hanging drop preparations of 24 hr. nutrient broth cultures were used for motility tests and for further observations on morphology. The appearance of growth in nutrient broth was noted. Features of 3-day colonies (30°) on nutrient agar were determined by using a ×50 binocular plate microscope. Pigmentation of colonies was noted at the same time and compared where possible with the pigment of similarly aged colonies on milk agar.

Physiological test methods. Experiments were so arranged that the isolates were subcultured as little as possible before testing. Organisms stored at 5° were revived by plating on YGA. Single colonies were further plated on to nutrient agar followed by picking into nutrient broth. These cultures were incubated overnight at 30° and used to inoculate the test media outlined in the following sections. Analar or Difco products were used unless otherwise stated, and bacteriological grade 'sugars' were used throughout; also unless otherwise stated the pH value of media was adjusted to between pH 7·2 and 7·4. Media were sterilized at 120° for 15 min. Liquid media were dispensed (5 ml.) in 1 oz. screw-capped McCartney bottles. Tests on solid media were made in Petri dishes, 6 isolates being radially streaked per plate. After inoculation, liquid media were incubated for 14 days at 30° before testing. Solid media were also incubated at 30° and read daily up to a maximum of 7 days.

Phosphatase. Ability to produce phosphatase was tested in the following medium (%, w/v); Evans peptone, 0·5; Lab-Lemco, 0·5; NaCl, 0·5; agar, 1·5. To 100 ml. the molten basal medium (45°) was added 1 ml. of a 1·0% Seitz-filtered solution of phenolphthalein diphosphate (sodium salt; L. Light, Colnbrook, England; Barber & Kuper, 1951). Inoculated plates were incubated for 3–5 days at 30°. It should be noted that this procedure differs from the original one used by Barber & Kuper for

detecting coagulase-positive staphylococci; they incubated for only 18 hr. at 37°. The release of free phenolphthalein was detected using a drop of 0·880 sp. gr. ammonia placed in the lid of each Petri dish. Phosphatase-producing colonies turned deep pink immediately on exposure to ammonia vapours.

Coagulase. The production of free coagulase (Duthie, 1954) was tested by the method of Fisk (Mackie & McCartney, 1960). Difco dried rabbit plasma was reconstituted and 0·3 ml. added to 0·1 ml. of an overnight culture in heart infusion broth. Tubes were incubated at 37° and read at 1 and 3 hr. and after standing overnight at room temperature. Any degree of coagulation of the plasma was recorded positive.

Acid production from carbohydrates. Ability to produce acid from glucose aerobically and/or anaerobically was tested by Hugh & Leifson's (1953) method with the following medium (%, w/v): $NH_4H_2PO_4$, 0·1; KCl, 0·02; $MgSO_4.7H_2O$, 0·02; yeast extract, 0·1; bromocresol purple, 0·004; agar 0·2; pH 7·0. The medium was sterilized in 90 ml. amounts and 10 ml. of a 10 % (w/v) Seitz-filtered solution of glucose added to the molten base (about 45°) immediately before use. It was dispensed aseptically into sterile 6 in. × ½ in. test tubes which were two-thirds full. The medium was steamed for 15 min. to drive off dissolved oxygen and kept at 5° until set. Duplicate tubes were stab inoculated throughout their length with a heavy inoculum from nutrient agar plates. After inoculations the surface of one tube of each duplicate was covered with a 1–2 in. layer of sterile liquid paraffin. After incubation for 5–10 days the tubes were examined for acid production. The production of acid from carbohydrates other than glucose was detected on plates of the same mineral salt yeast extraction medium as used for glucose, except that the agar concentration was increased from 0·2 to 1·5 % (w/v). Seitz filtered 5 to 10 % solutions of the carbohydrates under test were added to the molten base to give final concentrations of 0·25–0·5 %. After a preliminary trial with 28 carbohydrates the following 14 were selected for use: L-arabinose, cellobiose, dextrin, galactose, α-methyl-D-glucoside, glycerol, inositol, lactose, maltose, mannitol, raffinose, rhamnose, salicin, xylose.

Acetoin production and final pH *value in glucose broth.* Medium (%, w/v): Tryptone, 1·0; Lab-Lemco, 0·3; yeast extract, 0·1; glucose, 2·0. The pH value of the inoculated medium was determined electrometrically after incubation for 14 days. The presence of acetoin was detected by the method of Barritt (1936) and in some cultures by Batty-Smith's (1941) method.

Aesculin hydrolysis. Medium (%, w/v): Tryptone, 1·0; Lab-Lemco, 0·3; yeast extract, 0·1; Na_2HPO_4, 0·5; aesculin, 0·5. The release of the phenolic aglycone was detected by adding 0·5 ml. of a 1 % solution of ferric ammonium citrate, allowing to stand at room temperature for 1–2 min., and then examining for blackening of the medium and loss of fluorescence (Barnett, Ingram & Swain, 1956).

Starch hydrolysis. Medium (%, w/v): Tryptone, 1·0; yeast extract, 0·1; Lab-Lemco, 0·3; starch, 0·02; agar, 1·5 (modified from Eckford's, 1927, medium). Starch hydrolysis was detected by flooding with Lugol's iodine.

Milk agar. The following basal medium was made up to four fifths of the finally required volume and dispensed in 80 ml. amounts (%, w/v): Tryptone, 1·0; Lab-Lemco, 0·3; yeast extract, 0·1; Na_2HPO_4, 0·5; agar, 1·5. Low fat milk was obtained by standing milk for 3 hr. in a 45° incubator and skimming off the free fat. The milk

was adjusted to pH 7·0 and dispensed in 20 ml. amounts. It was sterilized by autoclaving at 115° for 10 min.; 20 ml. of warmed milk was added to 80 ml. of the basal medium before use. Inoculated plates were examined daily for clearing around the colonies. After 5 days, plates were flooded with acid mercuric chloride (Frazier, 1926) to distinguish between true proteolysis and clearing of the milk due to solubilization of the milk protein by alkaline end products of metabolism (Hastings, 1904).

Egg yolk agar. The following basal medium was prepared (%, w/v): Evans peptone, 0·5; Lab-Lemco, 0·5; NaCl, 0·5; agar, 1·5. Oxoid concentrated egg yolk emulsion was added to the molten base to give a final concentration of 5% (v/v). Plates were read as for the milk agar plates.

Gelatin agar (%, w/v): Tryptone, 1·0; yeast extract, 0·1; Lab-Lemco, 0·3; gelatin, 0·4; agar, 1·5. Gelatin hydrolysis was detected by the method of Frazier (1926).

Ammonia production in arginine broth (%, w/v): Tryptone, 1·0; yeast extract, 0·1; Lab-Lemco, 0·3; Na_2HPO_4, 0·5; L-arginine HCl, 0·3. Ammonia was detected by removing 1 ml. of culture supernatant fluid and adding to it 0·5 ml. Nessler's reagent.

Breakdown of other amino acids. Washed suspensions of organisms were used for detecting ammonia production from arginine, asparagine, cysteine, glycine, methionine, and serine. Decarboxylation of glutamic acid, lysine and arginine was tested by the methods of Moeller (1955) and Carlquist (1956). Anaerobic breakdown of arginine was determined by the method of Thornley (1960).

Hydrogen sulphide production. The decomposition of specific organic and inorganic compounds with H_2S formation (Olitzki, 1954) was investigated by using washed suspensions of organisms and the following substrates: cysteine, methionine, thioglycollate, thiosulphate, sulphite.

Hippurate hydrolysis. Medium (%, w/v): Tryptone, 1·0; Lab-Lemco, 0·3; yeast extract, 0·1; glucose, 0·1; Na_2HPO_4, 0·5; sodium hippurate, 1·0. It was sterilized by autoclaving at 115° for 10 min. The release of benzoate was tested by adding 1·5 ml. of 50% (v/v) conc. sulphuric acid to 1 ml. of culture supernatant fluid and standing at room temperature for 4 hr. Free benzoic acid was precipitated as fine crystals (Ayers & Rupp, 1922).

Nitrate reduction. Medium (%, w/v): Tryptone, 1·0; Lab-Lemco, 0·3; yeast extract, 0·1; glucose, 0·1; Na_2HPO_4, 0·5; sodium nitrate, 0·02. Reduction of nitrate was determined by adding 1 ml. each of Griess–Ilosvay's reagents to the culture fluid (Recommended method of Society of American Bacteriologists, 1957). Results were read after standing for about 5 min. Reduction of nitrate beyond nitrite was tested by adding a knife tip of zinc dust. Production of ammonia from nitrite was determined by using washed suspensions of organisms incubated for 6 hr. at 30° in 2×10^{-5}M-sodium nitrite buffered at pH 7·5 with M/15 sodium cacodylate.

Lipolysis. Ability to hydrolyse butter and lard with the release of free fatty acids was tested by using the Jones & Richards' (1952) medium. Care was taken to emulsify the fats by vigorous shaking before pouring the plates. When the medium was almost set it was poured into cooled plates (5°) in order to prevent separation of the fats. The hydrolysis of Tweens with the release of free fatty acids was tested in Sierra's (1957) medium; Tweens 20, 40, 60 and 80 (Honeywill-Atlas Ltd.) were used as substrates.

Ammonium phosphate utilization. The medium used was that published by Hucker (1924). Difco Nobel agar was used to solidify the medium. Slopes were inoculated with organisms from mineral-salt yeast-extract agar in order to minimize the transfer of organic material. Inoculated slopes were examined weekly for growth and acid production; they were incubated for 1 month.

Growth in media containing sodium chloride. Ability to grow in a peptone medium containing no added sodium chloride, or 5 or 15 % (w/v) sodium chloride was tested by using the media and methods suggested by Pohja (1960).

Growth at 10° *and* 45°. Tests were carried out in stirred water baths with an accuracy of ±0·25°. Yeast-extract glucose broth was brought to the temperature of incubation and inoculated with about 0·5 ml. of an overnight nutrient broth culture. Inoculated bottles were incubated submerged to their necks.

Recording of results. The results obtained were recorded on Paramount brand punch cards in order to compare characters of the isolates. All positive characters, weak and strong, were punched out; the strength of the reaction was noted on the cards. The isolates were sorted into groups, taking into account all their characters.

Experiments on variation of physiological characters. To avoid defining groups of isolates on unstable characters, attempts were made to cause variation in a collection of 6 isolates when frequently subcultured. Two strains each of Shaw *et al.* (1951) subgroups 1, 2 and 3 were resuscitated from 12–14 months old, freeze-dried cultures and plated on YGA. Single colonies were picked and replated. The process of picking and replating was repeated five times, and from the final platings 6–8 colonies of each organism were inoculated into nutrient broth. The broths were incubated overnight and used to inoculate the tests outlined in the previous sections of this paper. All tests were read after incubation for 7 days. This procedure was repeated four times, the first culture of the preceding series being used as the mother culture for the next series.

RESULTS

Morphology

The 1250 isolates were Gram-positive and catalase-positive cocci which divided in more than one plane, either regularly to form tetrads or cubical packets, or irregularly to form different sizes of loose or compact clusters. The ability to form cubical packets appeared to be a stable character produced both in broth and on solid media. The formation of loose or compact clusters showed considerable variation on different media, and tetrads were formed by many strains which otherwise grew as irregular clusters. Although the organisms were Gram-positive in young culture this character was frequently lost in cultures older than 48 hr. The organisms were often slightly elongated, and divided by septa formed at right angles to their long axis; short chains of 3–4 cocci were commonly present. Motile strains were not found among these isolates.

Colonies were mainly convex (0·5–2·0 mm. diam.) with an entire edge and a smooth or slightly granular surface sometimes with radial striations. Some organisms when first isolated formed hard, irregular colonies clinging to the surface of the agar, but this property was unstable and lost by most isolates on subculture. Organisms undergoing regular division and forming cubical packets usually possessed

characteristic large granular matt colonies. Colonies were white, yellow or orange pigmented. Yellow- or orange-pigmented isolates often gave rise to white variants which when tested were physiologically identical to their parent types.

The appearance of the growth in broth was variable. The anaerobic glucose utilizers (staphylococci) grew usually as an easily suspendable fine sediment. Sometimes the culture fluid became turbid with a tendency to form a ring of growth at the surface of the medium in cultures older than 48 hr. In contrast, the growth in broth of those organisms which utilized glucose only aerobically (micrococci) mostly gave a mucoid sediment which was difficult to disperse; the culture fluid above these sediments remained clear. Some organisms grew as a granular sediment, often with small colonies developing on the sides of the bottles.

Physiology

Phosphatase. After incubation at 30° for 3 days all coagulase-positive isolates were strongly phosphatase-positive together with 378 coagulase-negative isolates which utilized glucose anaerobically and 4 isolates which utilized glucose only aerobically. Weak activity was recorded from 4 isolates which did not utilize glucose and from 2 which attacked glucose only aerobically.

Coagulase. Twenty-two isolates were positive after incubation in the presence of rabbit plasma for 3 hr.; 2 isolates were only positive after leaving for a further 18 hr. at room temperature.

Acid from carbohydrates. Isolates showed three types of activity in the breakdown of glucose in the mineral-salt yeast-extract medium used for the Hugh & Leifson test: (1) acid throughout the open and sealed tubes; (2) acid at the surface and upper part of the open tube, no acid in depth of sealed tube; (3) no acid, i.e. no change in the bromocresol purple indicator, in either the open or the sealed tube. All coagulase-positive isolates together with 546 other organisms grew and formed acid throughout the open and sealed tubes; of the remainder, 669 isolates formed acid at the surface and upper part of the open tubes and sometimes slight acid at the surface of the sealed tubes. The location of acid production coincided with growth. Growth was most rapid in the open tubes and when acid production occurred in the depth of the agar it was accompanied by growth. Acid production in the depth of the sealed tube was considered to take place under anaerobic conditions and organisms which showed this activity will be referred to in this paper as anaerobic glucose utilizers. Pohja (1960) found that many of his Gram-positive and catalase-positive cocci formed acid weakly anaerobically, i.e. pH $> 5{\cdot}7$. These would not be detected with bromocresol purple as an indicator of acid production.

Many isolates utilized a wide range of carbohydrates besides glucose. These carbohydrates were incorporated in an agar medium since it was found, in agreement with Hill (1959), that a plate test gave clear-cut results. The carbohydrates most frequently attacked by the anaerobic glucose utilizers were: galactose, glycerol, lactose, maltose, mannitol. Those isolates which utilized glucose aerobically but not anaerobically also frequently formed acid from L-arabinose, cellobiose, inositol, rhamnose and xylose. Some organisms also attacked α-methyl D-glucoside, dextrin, raffinose, salicin and starch; the breakdown of starch was usually weak. Daily readings of plates was necessary since acid from some carbohydrates, particularly L-arabinose, was often present only transiently, with subsequent reversion to alkaline conditions.

Acid from mannitol was produced both aerobically and anaerobically by those coagulase-positive staphylococci able to attack this sugar alcohol. In contrast, only 2 out of 40 coagulase-negative anaerobic glucose utilizers which formed acid from mannitol aerobically were able to do so anaerobically. These results agree with those of Evans (1948) and Dr D. A. A. Mossel (1962).

Acetoin and final pH value in glucose broth. Acetoin was produced mainly by the anaerobic glucose utilizers; its production usually was accompanied by a terminal pH value in 2 % glucose broth below pH 5·5. Identical results were obtained for 100 isolates when the presence of acetoin was tested for by the method of Barritt (1936) and the more sensitive method of Batty-Smith (1941). A larger number of organisms was positive in the phosphate-free 2 % glucose medium used in the present work than in the glucose phosphate medium commonly used for the Voges–Proskauer (VP) test (Mackie & McCartney, 1960). Similar results were reported by Abd-El-Malek & Gibson (1948) and Shaw *et al.* (1951). It was necessary to incubate for at least 14 days to avoid missing late acetoin producers.

Aesculin. Aesculin was mainly hydrolysed by those isolates which were acetoin-negative and utilized glucose only aerobically.

Effects in milk agar, egg-yolk agar and gelatin agar. Most isolates which utilized glucose anaerobically caused clearing of milk in milk agar plates; many of these also cleared egg yolk and hydrolysed gelatin. These activities were generally weak or absent from the other isolates, although some packet formers were actively gelatinolytic. Clearing of milk and egg yolk was due mainly to solubilization of the proteins by alkaline end products of metabolism since treatment with acid mercuric chloride (Frazier, 1926) resulted in a decrease or disappearance of the clearing.

Decomposition of amino acids and hydrogen sulphide production. Most isolates produced ammonia when grown in arginine broth. All coagulase-positive isolates formed ammonia from arginine anaerobically in Thornley's (1960) medium, whereas only 5 out of 24 coagulase-negative isolates which formed ammonia aerobically were also positive anaerobically. Washed suspensions of 12 of 20 isolates formed ammonia from arginine, 15 of these also formed small amounts of ammonia from serine; no ammonia was detected from asparagine, cysteine, glycine or methionine. Glutamic acid, lysine and threonine were not deaminated. Traces of hydrogen sulphide were formed from thiosulphate (7 of 20 isolates) and from cysteine (9 of 20 isolates) but not from methionine, thio-glycollate nor sulphite.

Hippurate was hydrolysed by most isolates.

Nitrate reduction. Nitrate was reduced by most isolates; gas was not detected. Many isolates which utilized glucose anaerobically reduced nitrate to ammonia, whereas most of those isolates which utilized glucose only aerobically or did not attack glucose, reduced nitrate to nitrite.

Lipolysis, and hydrolysis of Tweens. Lard, butter and Tweens were hydrolysed by most of the isolates which utilized glucose anaerobically. Tweens 20, 40 and 60 were more frequently attacked than Tween 80. The remaining isolates generally did not attack these substrates except for some phosphatase and acetoin producers, and some of the organisms which did not form acid from carbohydrates.

Ammonium phosphate utilization. Eleven of the isolates which utilized glucose anaerobically grew weakly on the ammonium phosphate medium and formed small amounts of acid after incubation for 21–28 days. Forty isolates which formed acid

only aerobically from glucose, grew and produced acid between 7 and 21 days. Several isolates grew weakly on the medium but failed to form acid.

Growth in the presence of 5 % *and* 15 % *sodium chloride.* All isolates grew in Pohja's (1960) medium without added sodium chloride with exception of 2 coagulase-negative phosphatase-positive isolates. However, growth of many of the anaerobic glucose utilizers was weak. All isolates grew in the presence of 5 % sodium chloride and most in the presence of 15 %.

Growth at 10 *and* 45°. With the exception of 5 isolates all the coagulase-negative organisms which formed acid from glucose anaerobically did not grow at 10°. Growth at 45° was a more variable character but most of the isolates grew at this temperature. In contrast, all but 21 of the isolates which formed acid only aerobically grew at 10°, and with the exception of 38 isolates, did not grow at 45°. Most of the coagulase-positive isolates grew at 10 and 45° as also did some of the packet formers and isolates which did not attack glucose.

Examination of instability of some physiological characters

Six cultures of Shaw *et al.* (1951) subgroups 1, 2 and 3, which were freeze-dried and examined about 1 year later, showed only minor changes in physiological reactions on retesting. None of the strongly positive characters was lost although one subgroup 3 isolate had lost the ability to attack xylose and hydrolyse casein. However, both of these characters were weak when the isolate was tested before freeze-drying. One isolate apparently gained the character to form acid weakly from galactose. Further apparent changes were noted when the strains were subcultured frequently and tested over a period of 1 month. Series of colonies picked from the subgroup 1 and 2 strains reduced nitrate to nitrite or a more reduced end product, and showed change in ability to form detectable ammonia when grown in arginine broth. Some of these colonies also apparently became able to produce acetoin. This was associated with a terminal pH greater than 5·5 in 2 % glucose broth; cultures having a final pH of 4·5 to 5·0 contained acetoin. The acetoin-negative cultures became positive when retested with the incubation time extended from 7 to 14 days. Similarly, variations in end-products of nitrate reduction and the production of ammonia from arginine were eliminated by longer incubation. Thus tests on end products of glucose, arginine and nitrate metabolism should be performed after 14 days incubation to avoid negative results due to weak activities

Grouping of isolates

The isolates examined could be divided into 3 main groups. Group 1 contains 570 isolates which utilize glucose anaerobically; it corresponds to the genus *Staphylococcus* as defined by Evans in *Bergey's Manual* (1957). Members of group 2 (677 isolates) only utilized glucose aerobically or not at all; this group corresponds to the genus *Micrococcus* as defined by *Bergey's Manual* (1957). Group 3 contains 3 packet-forming isolates corresponding to the genus *Sarcina* Goodsir.

Group 1 (*Staphylococcus* Rosenbach emend. Evans). It was possible to recognize 6 subgroups within this group. The main characters of these are shown in Table 1; complete characters together with those of groups 2 and 3 are tabulated in Table 3.

Group 2 (*Micrococcus* Cohn emend. Evans). Members of this group could be divided

into 7 subgroups on their physiological characters. Their main characters are shown in Table 2, complete characters in Table 3.

Group 3 (*Sarcina* Goodsir). Too few isolates were obtained to recognize subgroups. Complete characters of the 3 isolates are listed in Table 3.

Table 1. *Main characters of subgroups within Group* 1 (Staphylococcus *Rosenbach emend. Evans*)

	Subgroups					
Characteristic	I	II	III	IV	V	VI
Coagulase	+	−	−	−	−	−
Phosphatase	+	+	+	−	−	−
Mannitol (aerobic)	+ (usually)	−	−	−	−	+
Mannitol (anaerobic)	+ (usually)	−	−	−	−	− (usually)
Acetoin	+	+	−	+	+	+
Lactose	+ (usually)	+ (usually)	v	−	+	v
Maltose	+	+	−	v	+	v
Growth at 10°	+ (usually)	−	−	−	−	− (usually)
No. of isolates	24	354	24	53	19	96

v = variable

Table 2. *Main characters of subgroups within Group* 2 (Micrococcus *Cohn emend. Evans*)

	Subgroups						
Characteristic	1	2	3	4	5	6	7
Acid from glucose (aerobic)	+	+	+	+	+	+	−
Phosphatase	−	−	−	−	−	+	−
Acetoin	+	+	+	+	−	−	−
Average end pH value in 2% w/v glucose broth	4·6	5·1	5·0	5·2	5·5	5·3	6·5
Arabinose	−	−	−	+	v	+	−
Lactose	−	+	v	+	+ (usually)	+	−
Maltose	v	+	+ (usually)	+	+ (usually)	+	−
Mannitol	−	−	+	+	+ (usually)	+	−
Lipolysis	v	+	+	−	− (usually)	+ (usually)	v
Tween hydrolysis	− (usually)	−	v	−	−	+	v
Growth at 10°	− (usually)	+ (usually)	+ (usually)	+	+ (usually)	+	+ (usually)
No. of isolates	17	8	75	19	544	4	10

v = variable

Table 3. *Complete characters of subgroups*

Characteristics	Group 1. *Staphylococcus* Rosenbach emend. Evans — Subgroups I	II	III	IV	V	VI	Group 2. *Micrococcus* Cohn emend. Evans — Subgroups 1	2	3	4	5	6	7	Group 3 *Sarcina* Goodsir
Colony														
(1) Smooth, convex regular edge	+	340*	+	52	+	94	14	+	71	18	475	+	2	–
(2) Nodular irregular edge	–	–	–	1	–	–	–	–	3	1	22	–	–	–
(3) Granular surface regular edge	–	14	–	–	–	2	3	–	1	–	47	–	8	+
Pigment														
(1) White	1	341	11	27	17	36	13	+	72	+	516	–	5	–
(2) Yellow	1	2	1	10	1	37	1	–	1	–	20	–	5	+
(3) Orange	22	11	12	16	1	23	3	–	2	–	8	4	–	–
Appearance in broth														
(1) Fine deposit	23	288	20	44	16	73	14	5	45	4	71	4	9	1
(2) Granular deposit	–	32	–	5	–	15	1	2	25	1	189	–	–	2
(3) Mucoid deposit	1	34	4	4	3	8	2	1	5	14	282	–	1	2
Size														
$< 1\mu$	23	300	28	37	12	72	8	2	18	–	30	4	2	–
$1–1{\cdot}5\mu$	1	54	1	13	4	22	9	6	56	16	487	–	5	–
$> 1{\cdot}5\mu$	–	–	–	3	3	2	–	–	1	3	27	–	3	+
Glucose Aerobic acid production	+	+	+	+	+	+	+	+	+	+	+	+	–	(2)
Glucose Anaerobic acid production	+	+	+	+	+	+	–	–	–	–	–	–	–	–
Coagulase production	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Phosphatase production	+	+	+	–	–	–	–	–	–	–	– (2)†	+	(3)	(1)
Terminal pH value in glucose	4·3–4·6 av. 4·4	4·2–5·3 av. 4·4	5·0–5·3 av. 5·0	4·1–4·8 av. 4·5	4·2–4·9 av. 4·4	4·3–5·4 av. 4·6	4·2–4·8 av. 4·6	4·2–5·3 av. 5·1	4·3–5·5 av. 5·0	4·3–5·5 av. 5·2	5·0–5·9 av. 5·5	5·3 av. 5·3	6·0–7·3 av. 6·5	6·1–7·3 av. 6·6
VP test	+	+	–	+	+	95	+	+	74 (1)	11 (8)	–	–	(4)	(1)
Anaerobic acid production from: Mannitol	+ 18/18‡	NT	NT	NT	NT	+ 2/40	NT	NT	NT	NT	NT	NT	NT	NT
Aerobic acid production from:														
Arabinose	–	–	–	–	–	–	–	–	–	16(2)	336(24)	+	–	–
Cellobiose	–	–	–	–	–	–	–	–	–	3	22(3)	1	–	–
Dextrin	(2)	2	–	–	–	–	–	–	–	–	1(1)	–	–	–
Galactose	7 (2)	177 (51)	14 (1)	2 (2)	6	3 (4)	–	5 (3)	19 (5)	7 (1)/18	239 (84)/543	+	–	–
α-Methyl-D-glucoside	–	–	–	1	–	–	–	–	–	–	–	–	–	–
Glycerol	+	353	22 (2)	+	+	+	16	+	+	+	541/541	+	–	–
Inositol	–	–	–	–	–	–	–	–	–	2	51 (2)	–	–	–
Lactose	21 (1)	331 (2)	19 (2)	–	+	12	(1)	6 (2)	35 (1)	15/18	478 (3)/543	+	–	–
Maltose	23/23	353 (1)	–	41 (9)	+	79	8 (2)	7 (1)	74	17/18	512 (11)/536	+	–	(1)
Mannitol	17/17	–	–	(1)	–	+	(1)	–	74	+	532/543	+	–	–
Raffinose	–	–	–	–	–	–	–	–	–	–	–	1	–	–
Rhamnose	–	–	–	–	–	–	–	–	–	–	64 (32)	+	–	–
Salicin	1	–	–	–	–	–	–	–	–	–	2 (2)/543	+	–	–
Xylose	–	–	–	–	–	–	(2)	–	7	16	433 (31)/543	+	–	–

Table 3 (*cont.*)

Characteristics	Group 1. *Staphylococcus* Rosenbach emend. Evans — Subgroups						Group 2. *Micrococcus* Cohn emend. Evans — Subgroups							Group 3 *Sarcina* Goodsir
	I	II	III	IV	V	VI	1	2	3	4	5	6	7	
Hydrolysis of														
Aesculin	1	(2)	–	(11)	–	4 (14)	3 (1)	(1)	7 (15)	15	393 (27)/539	+	–	–
Starch	–	–	–	–	–	–	–	–	–	–	2 (8)	–	–	(2)
Hippurate	20/21	329/334	23	49/49	16/17	81/82	12/14	+	37/37	11	473 (3)/527	3	6	–
Gelatin	23	237 (12)	+	5	5	11 (1)	(1)	(1)/7	6 (1)	3	12 (47)/543	1	8 (1)	+
Clearing of														
Casein	23 (1)	302 (40)	+	15 (11)	12 (2)	48 (24)	5	2 (4)	11 (17)	3 (7)	51 (225)/542	3	2	1 (1)
Egg yolk	+	124 (11)	+	19 (2)	5	54	3	(1)	17	1	14 (11)	–	–	1
Ammonia from arginine broth	+	244 (19)	23	45 (1)	10 (1)	75 (1)	9 (1)	6 (1)	59	11 (1)	244 (70)	–	(1)	(1)
Nitrate														
No reduction	–	12	–	–	1	2	1	5	46	1	10/540	–	4/9	2
Reduced to nitrite	–	39	–	19	4	58	6	2	21	15	489/540	3	(1)/9	(1)
Reduced beyond nitrite	+	303	+	34	14	36	10	1	7	3	41/540	1	4/9	–
Lard hydrolysis	23	353	11/11	22 (2)	+	80 (8)	6 (2)	7 (1)	53 (1)/74	–	10 (4)/542	3	2/9	2
Butter hydrolysis	23	343	21/23	28 (15)	+	89 (1)	7 (1)	+	53	–	11 (2)	3	3 (1)	+
Tween 20 hydrolysis	22/23	307 (10)/353	11 (1)	17 (4)	14 (2)	58 (6)	–	–	23 (2)	–	–	3	4/8	2
Tween 40 hydrolysis	23	259 (10)/337	22	15 (3)	11	61 (5)/95	– (1)	–	22 (1)/74	–	–	3	5/8	2
Tween 60 hydrolysis	23	290 (16)/349	23	15 (4)	10 (2)	74 (4)/95	–	–	23	–	–	3	4 (1)/9	2
Tween 80 hydrolysis	18	86 (24)	–	14 (2)	6 (1)	32 (2)	–	–	21	–	–	3	3	–
Ammonium phosphate used	–	(10)	–	–	–	(1)	–	(2)	29 (4)	2	41 (33)	2 (2)	1 (1)	2
Growth in the presence of no added NaCl	17 (1)/18	111 (239)	22 (2)	50 (3)	11 (8)	91 (5)	16 (1)	+	68 (7)	17 (2)	540 (4)	+	7 (3)	+
Growth in presence of 5 % NaCl	17 (1)/18	352 (2)	23 (1)	51 (2)	18 (1)	95 (1)	15 (2)	+	71 (4)	+	+	+	+	2/1
Growth in the presence of 15 % NaCl	15 (3)/18	259 (52)	5 (16)/21	20 (7)	9	25 (28)	8	4 (3)	54 (16)	17 (2)	521 (14)	+	1	–
Growth at 10°	6 (10)/20	(2)/227	–	1/37	–	2/69	2/13	5/6	62/67	+	238/243	+	7/9	2/2
Growth at 45°	+	216/227	23	35/37	12/14	55/69	12/13	1/6	2/67	4	17/243	2	7/9	–
Total number of isolates:	24	354	24	53	19	96	17	8	75	19	544	4	10	3

\+ All isolates positive.
* Numbers = no. of isolates positive.
† Numbers in brackets = no. of isolates weakly positive.
‡ Fractions = $\frac{\text{no. isolates positive (no. isolates weakly+ve)}}{\text{no. isolates tested}}$.
NT = Not tested.

DISCUSSION

The Gram-positive and catalase-positive cocci isolated from bacon, human and pig skin, and dust can be divided into 3 main groups similar to the genera *Staphylococcus*, *Micrococcus* and *Sarcina* as defined in *Bergey's Manual* (1957). The genera are closely related and form a series of morphological and physiological types ranging from the biochemically active small staphylococci to the relatively inactive large sarcinas. Evidence was obtained, in agreement with the proposals of Evans *et al.* (1955), that two genera, *Staphylococcus* and *Micrococcus*, could be separated by the ability of *Staphylococcus* to grow and form acid from glucose anaerobically. The 3 isolates which formed packets were morphologically distinct from these two genera although physiologically related to *Micrococcus*. There appeared to be no reasonable grounds for ignoring the genus *Sarcina*, as suggested by Shaw *et al.* (1951), since the formation of packets appeared to be stable. However, it could be possible to confuse the true packet formers with some of the tetrad-forming micrococci unless hanging drop preparations were examined under phase contrast. Following these observations those organisms which grew as tetrads were placed in the genus *Micrococcus*. They were mostly members of subgroup 7.

Hill (1959) suggested that all non-pink pigmented Gram-positive and catalase-positive cocci which produced acid from glucose should be placed in the genus *Staphylococcus*. This suggestion was made from a study of only 49 strains analysed according to the Adansonian classification as suggested by Sneath (1957). It sets broad limits to the genus *Staphylococcus* and results in classifying the unrelated saccharolytic sarcinas in the same genus as the coagulase-positive staphylococci; the non-saccharolytic sarcinas would be placed in the genus *Micrococcus*. Pohja (1960), who also used the Adansonian classification, proposed to limit the genus *Staphylococcus* to Shaw, Stitt & Cowan's two species, *Staphylococcus aureus* and *S. saprophyticus*, the latter being defined to contain Gram-positive and catalase-positive cocci which formed acetoin from glucose. If this classification were accepted my subgroup III staphylococci, which are acetoin-negative but ferment glucose anaerobically (Table 3), would be placed in a *Micrococcus* subgroup. This classification would disregard the main distinguishing character between staphylococci and micrococci, which is the fermentative or oxidative utilization of glucose.

Separation of staphylococci from micrococci on anaerobic glucose utilization, i.e. ability to grow and produce detectable acid in the depth of sealed tubes with bromocresol purple as an indicator, results in the delimitation of a group of organisms which are morphologically and physiologically similar to *Staphylococcus aureus*. The latter is accepted as type species of the genus *Staphylococcus* (Judicial Commission of the International Committee of Bacteriological Nomenclature and Taxonomy; Stockholm, 1958). It appears reasonable to suggest, therefore, that since anaerobic glucose utilization, under the conditions mentioned, separates a group of organisms which are closely related to the type species of the genus *Staphylococcus*, this is a better method of separating staphylococci from micrococci than the divisions proposed by Hill or Pohja. Two strains of *S. aureus* (NCDO 1236, 1238) were described by Garvie *et al.* (1961) as not forming acid from glucose anaerobically; but both organisms did so when tested by the Hugh & Leifson test as described in the present paper. Some convergence between the proposed genera can be seen from the results listed in

Table 3. For example, some *Staphylococcus* subgroup IV isolates were distinguished from those of *Micrococcus* subgroup 1 solely on anaerobic glucose utilization. The presence of intermediate types between genera and species must be expected if evolutionary concepts are to be accepted (Gibson, 1955; Lysenko, 1961); convergencies can be found between almost every genus in bacteriology.

Proposed classification

Six subgroups were recognized within the genus *Staphylococcus*. Members of subgroup I produce coagulase and belong to the species *Staphylococcus aureus* as defined by Shaw *et al.* (1951). They also produce phosphatase and are able usually to form acid from mannitol, aerobically and anaerobically. Members of subgroup II although phosphatase-positive are well separated from those of subgroup I by not forming coagulase or acid from mannitol. These organisms are found frequently on human and pig surfaces and in household dust. They are also found in large numbers in bacon stored at 30° (Cavett, 1962). Closely related to subgroups II are members of subgroups V which are phosphatase-negative. They are found in similar habitats to subgroup II members. Subgroup III organisms are found frequently on pig skin but were not isolated from human skin or household dust. This subgroup although phosphatase-positive and mannitol-negative is distinguished from subgroup II by not forming acetoin from glucose or acid from maltose. Subgroup IV members are unable to produce phosphatase and although acetoin-positive, attack only a limited range of carbohydrates. They were isolated from skin surfaces and from bacon. In subgroup VI are placed those phosphatase-negative staphylococci which form acid from mannitol, usually only aerobically; within this subgroup 3 types can be distinguished on ability to attack maltose and lactose (see definitions).

Seven subgroups were recognized within the genus *Micrococcus*. As previously mentioned subgroup 1 members are similar to *Staphylococcus* subgroup IV but are unable to form acid from glucose anaerobically when tested by the described method. Members of this subgroup were isolated mainly from dust and bacon. Subgroup 2 is separated from subgroup 1 by forming acid from lactose. They usually have a terminal pH value $> 5{\cdot}0$ in glucose broth and usually grow at 10°; they were mostly isolated from bacon. Subgroup 3 organisms occur mainly in dust and vacuum-packed bacon. They are closely related to members of subgroup 2 but are mannitol-positive; 3 types can be separated on ability to hydrolyse lard and Tweens, and to grow and form acid in a medium containing glucose and ammonium dihydrogen phosphate as sole carbon and nitrogen sources (see definitions). Subgroups 4 and 5 are closely related, being distinguished only by the formation of acetoin. Both of these subgroups are found frequently in bacon and subgroup 5 is the predominant *Micrococcus* subgroup found in this product. Subgroup 6 is a small subgroup found on pig skin. The ability of this subgroup to produce phosphatase together with its other characters clearly separates it from the other subgroups (see Table 3). Subgroup 7 contains a heterogeneous collection of organisms which produce little or no acid from glucose and no detectable acid from other carbohydrates. Members of this subgroup were isolated from all sources studied.

Relationship of proposed classification to previous classifications

Staphylococcus aureus constitutes a well-defined species when defined on ability to form coagulase (Evans & Niven, 1950; Shaw *et al.* 1951); it corresponds to my subgroup I. The recognition of other species of staphylococci and micrococci is controversial. Shaw *et al.* (1951) said that their classification 'like all other classifications of cocci is arbitrary and artificial'. Hill (1959) concluded that *S. saprophyticus* of Shaw *et al.* contained a natural group of organisms corresponding to *S. epidermidis* (*Bergey's Manual*, 1957). My results do not support this conclusion. First, *S. epidermidis*, as defined, is always mannitol-negative whilst *S. saprophyticus*, to quote Hill, is often positive. Secondly, it does not appear that organisms classified in *S. saprophyticus* constitute a natural group. According to my grouping the following subgroups would belong to *S. saprophyticus*: *Staphylococcus* subgroups II, IV, V and VI and *Micrococcus* subgroups 1, 2, 3 and 4. The two most dissimilar subgroups, namely *Staphylococcus* subgroup II and *Micrococcus* subgroup 4, show a greater diversity of characters than between many members of Shaw, Stitt & Cowan's *S. lactis* and *S. saprophyticus*. For example, *Micrococcus* subgroup 4 (*S. saprophyticus*, Shaw *et al.*) is more closely related to *Micrococcus* subgroup 5 (*S. lactis*, Shaw *et al.*) than either *Staphylococcus* subgroup II and *Micrococcus* subgroup 4 are related to each other. In agreement with Hill (1959) my results show that *S. lactis* is not a natural group; this species contains my *Staphylococcus* subgroup III and *Micrococcus* subgroups 5 and 6, organisms which are very different (Table 3).

Organisms corresponding to *Staphylococcus roseus* of Shaw *et al.* were not isolated from the habitats studied. However, examination of NCTC strains of this species indicated that it would be better named *Micrococcus roseus* as suggested by Hill (1959).

Staphylococcus afermentans of Shaw *et al.* (1951) was not recognized as a species name by Hill (1959) and he did not regard it as a valid group. This conclusion agrees with the present work. Members of the *Micrococcus* subgroup 7 are most closely related to *S. afermentans*. However, this subgroup contains a miscellaneous collection of morphological and physiological types which possess the common character of forming little or no detectable acid from carbohydrates. Too few isolates have been studied to suggest better division of this subgroup.

Definitions of species of staphylococci and micrococci in editions of *Bergey's Manual* have been criticized by Abd-El-Malek & Gibson (1948) and by Pohja (1960). I agree with many of their criticisms, particularly regarding the difficulty in trying to type isolates according to the somewhat contradictory characters given in the *Bergey's Manual*. It was impossible to relate with any degree of certainty my *Micrococcus* subgroups to species of micrococci recognized in the *Bergey's Manual*, although the *Staphylococcus* subgroups could be mostly placed in one or the other of the two species recognized in the 7th edition. *Staphylococcus aureus* is similar to my subgroup I and subgroups II, III, IV and V belong to *S. epidermidis* (Winslow and Winslow) Evans. The characters of subgroup VI fit none of the species recognized in *Bergey's Manual* although the two members of this subgroup which utilize mannitol anaerobically would apparently be placed with *S. aureus*. The physiological characters of the tetrad formers fitted none of the species of *Gaffkya* recognized in *Bergey's Manual*. Two of the *Sarcina* isolates corresponded to the species *Sarcina lutea*.

NOMENCLATURE

Staphylococcus aureus appears to be the only species about which there is general agreement as to its validity; most of the other so-called species of staphylococci and micrococci are not true species. It is suggested, therefore, that at present species names other than *S. aureus* should not be used and that non-pathogenic staphylococci and micrococci should be classified into subgroups along the lines proposed in the present paper. To define more clearly the subgroups recognized in this work I have followed the procedure used by Shaw *et al.* (1951) namely, to separate essential characters of the proposed subgroups from the usual or variable characters.

Definitions

Group 1. Staphylococcus *Rosenbach emend. Evans*

General characters. Regular, small celled (0·5–1·5 μ) Gram-positive and catalase-positive cocci which form acid anaerobically from glucose and grow as compact or loose clusters. Usually acetoin is produced as an end product of glucose metabolism with a terminal pH value less than 5·0 in 2 % glucose broth. Acid is formed usually from glycerol, lactose and maltose. Mannitol is utilized aerobically and anaerobically by coagulase-producing strains and usually only aerobically by other organisms which attack mannitol. Acid is not usually formed from α-methyl-D-glucoside, arabinose, dextrin, inositol, raffinose, rhamnose, salicin or xylose. Aesculin and starch are not usually hydrolysed. Many organisms are phosphatase-positive. Proteins, hippurate, fats and Tweens are usually actively hydrolysed and ammonia produced in arginine broth. Nitrate is usually reduced beyond nitrite. Ammonium phosphate is not utilized as sole nitrogen source. Growth can usually occur in the presence of 15 % sodium chloride and at 45°. Usually no growth at 10° with the exception of some coagulase producers.

Subgroup I: (Staphylococcus aureus) *essential characters.* Coagulase-positive and phosphatase-positive; usually form acid from mannitol, aerobically and anaerobically.

Other characters. Acid from glycerol and maltose, usually from galactose and lactose, and sometimes from dextrin and salicin; acetoin produced in glucose broth. Nitrate reduced beyond nitrite. Ammonia from arginine produced both aerobically and anaerobically. Egg yolk always cleared and usually casein and gelatin hydrolysed; usually lipolytic to lard and butter. Tweens are hydrolysed by most strains. Growth occurs in the presence of 15 % sodium chloride and usually at 10° and 45°.

Subgroup II: *essential characters.* Phosphatase-positive and acetoin-positive, coagulase-negative and mannitol-negative.

Other characters. Acid from glycerol and maltose, usually from galactose and lactose, and sometimes from dextrin. Nitrate usually reduced beyond nitrite. Arginine usually only attacked aerobically with the release of ammonia. Usually caseinolytic, hydrolyse gelatin, and may also clear egg yolk. Lipolytic and usually hydrolyse Tweens. Generally poor growth in media with no added sodium chloride; no growth at 10° but usually growth at 45°.

Subgroup III: *essential characters.* Phosphatase-positive, coagulase-negative; no acid from maltose or mannitol; acetoin-negative.

Other characters. Acid from glycerol and usually from lactose and galactose.

Nitrate reduced beyond nitrite; ammonia from arginine. Casein and egg yolk are cleared and gelatin hydrolysed. Most strains are lipolytic and hydrolyse the lower Tweens. Poor growth in the presence of 15 % sodium chloride; no growth at 10° but usually growth at 45°.

Subgroup IV: *essential characters.* Acetoin-positive, phosphatase-, coagulase-, lactose- and mannitol-negative.

Other characters. Acid from glycerol and sometimes galactose. Some strains may form acid from maltose and hydrolyse fats and Tweens.

Subgroup V: *essential characters.* Acetoin-positive, acid from lactose and maltose phosphatase- and coagulase-negative; no acid from mannitol.

Other characters. Acid from glycerol and sometimes from galactose. Nitrate usually reduced beyond nitrite. Casein usually cleared but egg yolk and gelatin not usually attacked. Butter and lard are hydrolysed, and usually Tweens. Many strains do not grow in the presence of 15 % sodium chloride.

Subgroup VI: *essential characters.* Acetoin-positive; acid from mannitol (usually aerobic only), phosphatase- and coagulase-negative.

Other characters. Acid from glycerol and sometimes from galactose; aesculin may be hydrolysed. Strains differ as to ability to utilize lactose and maltose and by the use of these characters 3 types can be separated:

Type	No. of isolates	Lactose	Maltose
1	12	+	+
2	68	−	+
3	16	−	−

Most strains form ammonia in arginine broth and reduce nitrate to nitrite or a more reduced product. Many strains are inhibited by 15 % sodium chloride; most grow at 45° but not usually at 10°.

Group 2. Micrococcus *Cohn emend. Evans*

General characters. Variable sized (0·5–3·0 μ) Gram-positive and catalase-positive, cocci forming irregular clusters or tetrads. Acid from glucose, if at all, only aerobically.

Acetoin is produced by some strains; terminal pH value usually greater than 5 in 2 % glucose broth. Most strains form acid from arabinose, glycerol, lactose, maltose, mannitol and xylose. Some form acid from cellobiose, dextrin, galactose, inositol, raffinose, rhamnose, salicin and starch. Aesculin is usually hydrolysed. Few strains are phosphatase-positive. Casein, egg yolk and gelatin are hydrolysed by some strains but generally only weakly. Fats and Tweens are hydrolysed by some strains. Nitrite is usually the end product of nitrate reduction. Some strains utilize ammonium phosphate as sole nitrogen source. Most grow in the presence of 15 % sodium chloride and at 10°; usually no growth at 45°.

Subgroup 1: *essential characters.* Acetoin-positive. No acid from mannitol or lactose.

Other characters. Terminal pH value usually less than 5·0 in glucose broth. Acid usually from glycerol, maltose and sometimes from xylose; aesculin hydrolysed by some strains. Casein and egg yolk may be cleared. Usually nitrate reduced to nitrite or a more reduced product. Some strains are lipolytic; Tweens are not usually

hydrolysed. Most strains grow at 45° but usually not at 10°; many are inhibited by 15% sodium chloride.

Subgroup 2: *essential characters.* Acetoin-positive, acid from lactose and maltose.

Other characters. Acid from glycerol and galactose. Nitrate usually not reduced. Lipolytic but generally proteins only weakly attacked. Usually growth at 10° but not at 45°.

Subgroup 3: *essential characters.* Acetoin-positive, acid from maltose and mannitol.

Other characters. Acid from glycerol, usually from lactose and sometimes from galactose and xylose; aesculin hydrolysed by some strains. Three types can be recognized on the hydrolysis of lard and Tweens and ability to grow and form acid on a medium containing ammonium dihydrogen phosphate as sole nitrogen source.

Type	No. of isolates examined	Hydrolysis of		Ammonium phosphate utilization
		Lard	Tweens	
1	28	+	+	−
2	30	+	−	+
3	17	−	−	−

Subgroup 4: *essential characters.* Acetoin-positive; acid from arabinose.

Other characters. Acid from glycerol and mannitol and usually from lactose, maltose and xylose; sometimes from cellobiose, galactose and inositol. Aesculin usually hydrolysed. Nitrate reduced by most strains to nitrite. Proteins usually not attacked. Lard and Tweens not hydrolysed. Grows at 10° but not usually at 45°. Some strains utilize ammonium phosphate.

Subgroup 5: *essential characters.* Acid aerobically from glucose; acetoin-negative.

Other characters. Acid from glycerol and usually from galactose, lactose, maltose, mannitol and xylose; sometimes from dextrin and cellobiose. Aesculin hydrolysed by most strains. Nitrate usually reduced to nitrite. Usually no hydrolysis of casein, egg yolk or fat; Tweens not hydrolysed. Usually growth at 10° but not at 45°. Some strains grow and form acid in Hucker's ammonium phosphate medium.

Subgroup 6: *essential characters.* Phosphatase-positive; acid formed aerobically from glucose, arabinose and mannitol.

Other characters. Acetoin-negative; acid from galactose, glycerol, lactose, maltose, salicin and xylose and sometimes from cellobiose, raffinose and rhamnose. Tweens and fat hydrolysed. Growth at 10° and sometimes at 45°.

Subgroup 7: *essential characters.* Unable to form detectable amounts of acid from glucose in the described Hugh & Leifson test (above).

Other characters. Usually large-celled irregular cocci or tetrad formers. Gelatin is usually hydrolysed and casein sometimes cleared. Some strains are actively lipolytic and hydrolyse Tweens. Most grow at 10° and 45° but are unable to grow in the presence of 15% sodium chloride.

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Morphogenesis and Nutrition in the Memnionella–Stachybotrys Group of Fungi

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SUMMARY

A regular pattern of morphogenetic responses to factorially arranged variations in concentrations of NH_4Cl, K_2HPO_4 and glucose was found common to ten strains of Memnionella and Stachybotrys. Three phases of morphogenesis were observed: sterile swollen mycelium, sterile filamentous hyphae, and hyphae with conidiophores. The morphological status of the mature colony was found to be related both to the absolute concentrations of NH_4Cl and K_2HPO_4, and to the ratio of these concentrations. At high values of the ratio the mycelium remained in the sterile swollen phase; at intermediate values the mycelium was mainly filamentous and sporing occurred; at low values the hyphae were filamentous but less fertile. Increase in concentration of K_2HPO_4 favoured filamentation whereas increase in the concentration of NH_4Cl favoured swelling of the cells and suppressed sporulation. Glucose concentration determined mainly the extent of growth and intensity of sporulation, although some interaction between the effects of glucose and NH_4Cl on sporulation were observed. A general set of conditions is proposed for the propagation of Memnionella and Stachybotrys in the sporing phase. It is suggested that the factorial design of nutritional experiment is appropriate for cultural studies fundamental to the taxonomy of fungi.

INTRODUCTION

Interest in the Memnionella–Stachybotrys group of fungi centres around its capacity to utilize cellulose as a major carbon source and hence to damage fabrics made of plant fibres (White, Yeager & Shotts, 1949). The physiology of these organisms is known only in sketchy outline but the biochemistry of some glucosidases of a strain of *Stachybotrys atra* (1, Defence Standard Laboratories (DSL)) was studied by Jermyn (1962). The group is characterized by the morphology of imperfect forms though a perfect form in the Pyrenoomycetes was reported for *Stachybotrys socia* (Booth, 1957). Two genera have been constructed, differentiated on the basis of the shape and arrangement of the conidia. The single species of *Memnionella*, *M. echinata*, produces predominantly spheroidal spores in chains, whereas the type species of *Stachybotrys*, *S. atra*, forms mainly ellipsoidal conidia which may be borne in slime. However, Zuck (1946) established that some monospore cultures produced conidia of both types, indicating that the genera are very closely related. None the less, cultures of the Memnionella type examined in the course of the present work were readily distinguishable by the more granular appearance of their colonies and the predominance of spheroidal catenulate spores.

The taxonomy of the group remains very indefinite since isolates belonging to

both genera have not been subjected to rigorous comparative examination. Bisby (1943, 1945) reduced some species of Stachybotrys to synonymy, and later Bisby & Ellis (1949) defined a new species, but these authors, though recognizing the morphological affinities of Stachybotrys and Memnionella, did not reach any conclusion concerning their taxonomic relationships. One of the factors which has hindered taxonomic studies is the great variability in colonial morphology which occurs with changes in cultural conditions, and the present investigation was designed to provide a sounder basis for such studies.

Stock cultures are usually propagated on potato glucose media but it has been found in this laboratory that these media do not favour sporulation, especially among Stachybotrys isolates. The further finding that addition of ammonium, nitrate or phosphate salts to potato extract media modified morphogenesis in a strain of *Stachybotrys atra* led to the adaptation of the defined media of Marsh & Bollenbacher (1946), Perlman (1948) and Jermyn (1953) for surface culture. The trace element concentrations common to these media were retained but the concentrations of ammonium, phosphate and glucose were varied in the experimental media, on which the microscopic and colonial morphologies of cultures of standard isolates were investigated.

METHODS

Media. All defined media contained the following components (g./l.): agar (Difco Bacto-agar), 10; $MgSO_4.7H_2O$, 1·0; $CaCl_2$, 0·02; $FeSO_4.7H_2O$, 0·01; $ZnSO_4.7H_2O$, 0·002; $MnSO_4.4H_2O$, 0·001; biotin, 2×10^{-5}. Glucose, NH_4Cl and K_2HPO_4 were added at concentrations indicated in Results.

Inocula. Cultures were propagated on a defined medium (optimal for sporulation) where possible; it comprised the basal components (g./l.): NH_4Cl, 0·1; K_2HPO_4, 1·0; and a strip of cellulose paper (Whatman no. 1). When the fungus did not grow on this medium yeast extract (0·5 g./l.) was added as a supplement. Test slopes were inoculated with spores; hyphal transfer was used only for non-sporing cultures.

Test cultures. Cultures were prepared on slopes (7 ml. medium in 19×150 mm. test tubes plugged with cotton wool) which were incubated in closed glass containers. During most of the period of incubation they were kept in the dark. They were examined macroscopically and microscopically on maturity (usually about 10 days).

RESULTS

Morphogenetic responses to variations in concentrations of NH_4Cl, K_2HPO_4 and glucose were examined in the following isolates: *Memnionella echinata*, 24003; *Stachybotrys atra*, 49534 (Commonwealth Mycological Institute; IMI); *M. echinata*, 11973 (American Type Culture Collection; ATCC); *S. subsimplex*, 42309; *Stachybotrys* sp., 39834; *S. atra*, 42311; *M. echinata*, 15377 (IMI); *S. atra*, 9182 (ATCC); *S. parvispora*, 62338; *S. lobulata*, 11716 (IMI). The isolates are listed according to the intensity of sporulation shown in these tests; macroscopically visible sporulation graded from very intense in isolate 24003 to barely perceptible in isolate 9182; in isolates 62338 and 11716 sporulation was so sparse that it could be detected only by microscope.

A single experimental design was used; the three nutrients were introduced at three concentrations each, (0·1, 1·0, 10 g./l.), in factorial combination. Each

isolate was tested once. In response to this grid of treatments all ten isolates showed the three following morphological phases: (i) sterile swollen hyphae, (ii) sterile filamentous hyphae, (iii) filamentous hyphae bearing sporophores. Cultures consisting chiefly of swollen cells were compact, pale and showed no macroscopically visible sporulation, whereas those containing filaments showed aerial hyphae and deeper colour. The compact colonies varied from off-white to salmon pink and the filamentous colonies from pale buff to deep reddish brown. The colour of the reverse and the medium was most intense in those treatment combinations where sporulation seemed to be incipient in less fertile isolates; those more fertile isolates which showed sporulation in identical treatments were less highly coloured. The most intensely sporing cultures showed sparse mycelium and a lightly coloured medium; they were superficially jet black owing to aggregates of conidia supported on upright conidiophores.

The least fertile isolates formed glabrous colonies with no aerial mycelium, but with increasing fertility the colonies became increasingly matt and showed more aerial mycelium. In sterile filamentous colonies of isolates of intermediate fertility there occurred occasionally aerial strands of aggregated hyphae, giving the colonies a spidery appearance. Floccose aerial mycelium was not profuse but formed a fine layer based on a leathery pellicle. In most colonies there were streams of filaments projecting deeply from the pellicle into the medium.

Beneath minor variations among the ten tests a regular pattern of morphogenetic responses was discernible. Since the concentration of glucose determined mainly the quantity of growth, but not its type, the presentation of the data may be simplified by considering the responses at one concentration of glucose. Growth was virtually absent at the lowest concentration of glucose and was most profuse at the highest. The effects of the other factors will therefore be considered first at the latter concentration.

In general the cultures passed from the sterile swollen phase at high values (100, 10) of ammonium chloride: potassium phosphate ratio, through a filamentous phase at unity and a sporulation phase at 0·1, to a filamentous variably sporing phase at 0·01. There were also observable effects of the absolute values of the concentrations of nutrients. The tendency to swollen cell formation varied directly with ammonium chloride and inversely with phosphate concentrations. Sporulation, when it occurred, was always most intense at the lowest ammonium chloride and medium phosphate concentrations. A minor interaction between the effects of ammonium chloride and glucose on sporulation was observed. At the lowest concentration of ammonium the high glucose + median phosphate combination was optimal for sporulation; at the median level, median glucose + median phosphate was optimal. Sporulation in this last treatment combination was very light, and occurred in four of the more fertile isolates.

As a concrete model the detailed characters of the colonies of *Memnionella echinata* 24003 are set out in Table 1 and some microscopic forms in Pl. 1. This isolate proved the most prolific sporer in the series of ten isolates tested, and showed aggregates of spores typical for both Memnionella and Stachybotrys (approximately 10 % of the spores were Stachybotrys type). Sporophores of Memnionella, Stachybotrys and mixed types can be seen in Pl. 2. Less fertile isolates (7) did not spore in the low ammonium + high phosphate treatment, and the least fertile

(5 isolates) spored only in the low ammonium + median phosphate combination, which may therefore be taken as the nearest to optimal (termed optimal for convenience) combination for sporulation.

The ammonium + phosphate treatment combination apparently optimal for sporing was tested for its suitability in propagating the following isolates on cellulose: *Stachybotrys atra*, 42309 (IMI); *S. atra*, 1 (DSL); *S. atra* f. *lobulata* (Central Bureau voor Schimmelcultures Baarn, CBS); *Memnionella echinata*, 16201 (IMI); *M. echinata*, 11973 (ATCC); *S. atra*, 42311 (IMI); *S. atra*, 9182 (ATCC); *S. atra*, v. *brevicaule* (CBS); *M. echinata*, 24003; *M. echinata*, 48570; *Stachybotrys* sp., 39834; *M. echinata*, 15377; *S. atra*, 72155; *M. echinata*, 45547; (IMI); *S. atra* f. *genuina*; *S. atra* v. *angustispora*; (CBS); *S. atra*, 11695 (ATCC); *M. echinata*,

Table 1. *Colony form and micromorphology of* Memnionella echinata *strain* 24003 *in relation to concentrations of* NH_4Cl and K_2HPO_4

All cultures showed a dense suspension of submerged vertical hyphal filaments.

	K_2HPO_4 (g./l)		
NH_4Cl (g./l.)	0·1	1·0	10
0·1	Sterile, swollen cells, compact, matt, pale colour	Fertile (maximal), filamentous, sparse superficial hyphae, pale colour	Fertile (lightly), filamentous, pellicle, mod. colour
1·0	Sterile, swollen cells, compact, matt, pale colour	Sterile, filamentous, matt, pale colour	Fertile (highly), filamentous. Superficially floccose, pellicle with some swollen cells, very deep in colour
10	Sterile, swollen cells, compact, glistening, pale colour	Sterile, swollen cells, compact, matt, pale colour	Sterile,* filamentous, pigmented hyphae, deep colour

* One isolate (11973) spored in this treatment combination

24287; *S. atra*, 32542; *S. atra*, 49534; *S. parvispora*, 62338 (IMI); *S. lobulata*, 11716 (ATCC); *Stachybotrys* sp. 24004; *S. atra*, 42310; *S. dichroa*, 17506 (IMI). The isolates are listed in descending order of their intensity of sporulation. Twenty-one of the cultures could be propagated on this medium and, except for *S. parvispora*, spored freely. The last five isolates would not grow on this medium unless it was supplemented with yeast extract or when the cellulose was replaced by glucose, and they showed no macroscopic evidence of sporulation.

Some of these standard isolates which had been in cultivation for decades spored very poorly when first transferred to the cellulose + minerals medium but steadily improved in fertility on serial subculture with spores as inocula. Since it was suspected that selection was the basis for this induced drift in fertility, the possibility of genetic heterogeneity was checked. Spores from isolate 49534, which is moderately fertile after twenty years of laboratory cultivation, were spread on the optimal medium, and five colonies showing fertility varying from high to zero were selected for serial culture. These resulting five sublines retained their fertility characteristics through several subcultures, indicating that genetic heterogeneity existed in the parent culture.

DISCUSSION

The literature indicates that, among other factors, initial concentrations of nutrients can determine the extent of sporulation of fungi. High concentration of nitrogen compounds has been found to inhibit sexual reproduction in *Allescheria boydii* (Benham & Georg, 1948; Gordon, 1957) and in *Coprinus* spp. (Bille-Hansen, 1953; Madelin, 1956); but where the effects of carbohydrates have also been examined both groups of nutrients governed reproduction in a wide variety of fungi (Westergaard & Mitchell, 1947; Buston & Basu, 1948; Basu & Bose, 1950; Hirsch, 1954; Miller & Halpern, 1956; Das Gupta & Nandi, 1957; Morton, 1961). The present study suggests however that these effects may depend on the concentration of phosphate, and in consequence there is some uncertainty in assessing the relative importance of nitrogen and carbohydrate nutrition in morphogenesis where the effects of phosphate are not known. Furthermore, in unpublished studies on *Stachybotrys atra* (1, DSL), we found that the effects of potassium phosphate on mycelial growth interacted heavily with those of magnesium sulphate (ammonium + glucose medium), suggesting that for comparative studies the possibility of relationships between the effects of phosphate and magnesium should not be neglected, especially where ammonium is the nitrogen source.

Those variations in the composition of the medium used here would be expected to result in large variations (during growth) of physico-chemical factors such as ionic strength, water availability, solubility of gases, course of pH changes. Although the interpretation of the responses in such terms is not possible in the present study, it should be noted that Sörgel (1953) found analogous phases in the morphogenesis of *Mycosphaerella pinodes* to be clearly correlated with the pH of the medium.

Many factors other than the concentrations of the major nutrients have been found to govern morphogenesis in fungi; among these are growth factors, trace elements, humidity and light. These factors were not strictly controlled in the present investigation. Some isolates appeared to require some undetermined growth factors when cultured with cellulose. Contamination on glassware (washed in tap water) and in chemicals was relied on to supply trace elements required in microquantities. Furthermore, temperature was fixed at a value set by previous workers (Marsh & Bollenbacher, 1946) without further investigation, humidity was controlled merely sufficiently to prevent desiccation, and lighting was irregular. Nevertheless, the isolates examined grew fairly regularly in serial culture under the better defined conditions, and it can be claimed that the Memnionella–Stachybotrys group may now more certainly be grown as surface cultures in particular morphological phases, so facilitating studies on their physiology, genetics and taxonomy.

The data indicate that the application of systematically arranged grids of nutritional treatments might prove more informative in taxonomy than the use of single media selected by trial and error. The factorial design permitted both rapid pin-pointing of the balance of major constituents suitable for sporulation, and detection of the fundamental control over morphogenesis exerted by the balance between ammonium chloride and potassium phosphate. However, observations on *Stachybotrys atra* 49534 suggest that the lines studied here may be genetically heterogeneous with regard to fertility, and the morphogenetic responses within strains may therefore be partly a reflexion of such heterogeneity. Furthermore,

since the histories of these lines were not known, they were regarded as isolates rather than strains. Gordon (1957) nevertheless showed that strains (monospore derivatives) of *Allescheria boydii* showed an analogous gradation of fertility, and Jinks (1954) induced a fading of fertility through serial culture in a line of *Aspergillus nidulans*, by selecting infertile cells. Such observations stress the need for a clear understanding of the interaction between nutrition and genetic constitution of serially cultured fungi.

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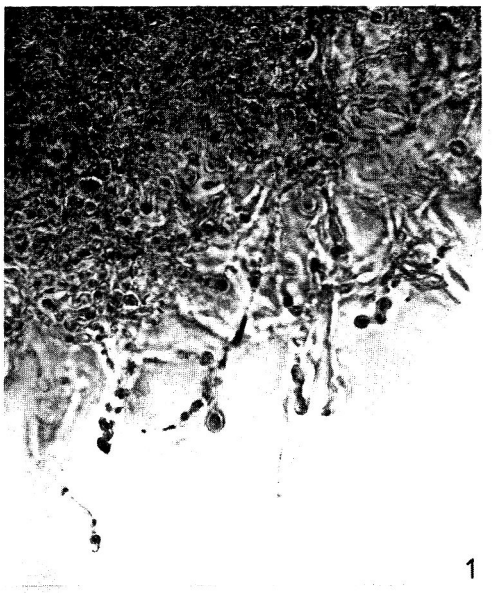

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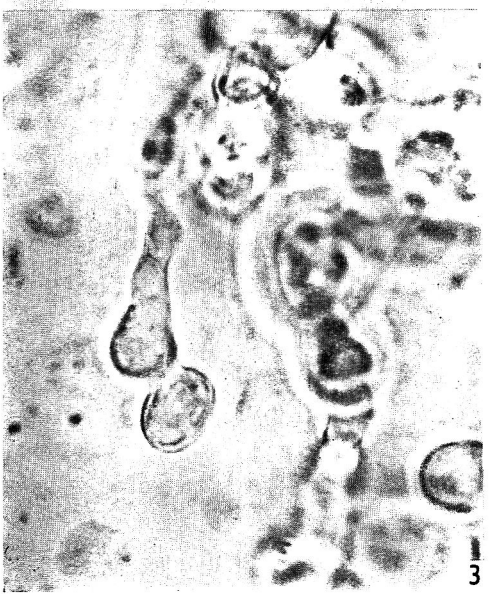

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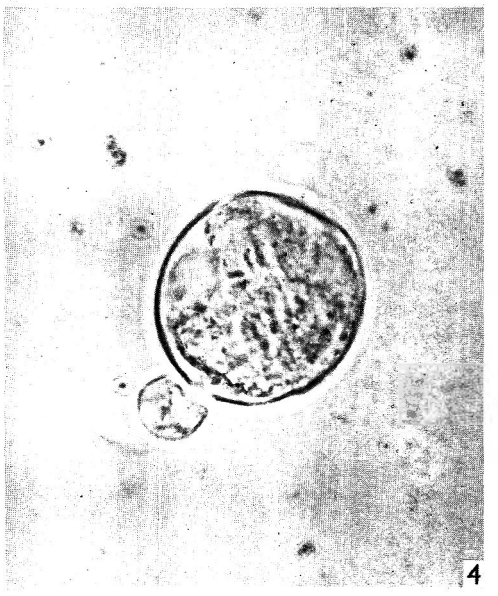

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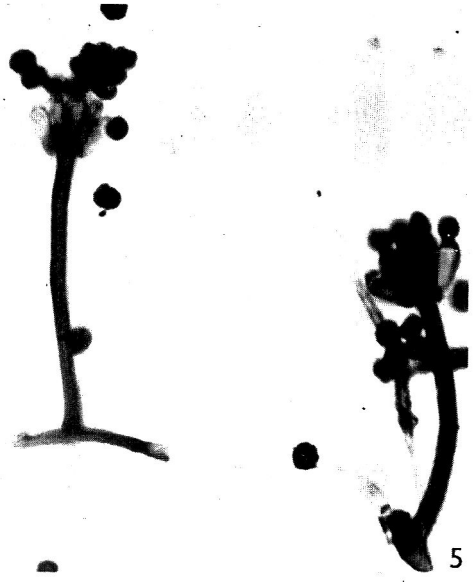

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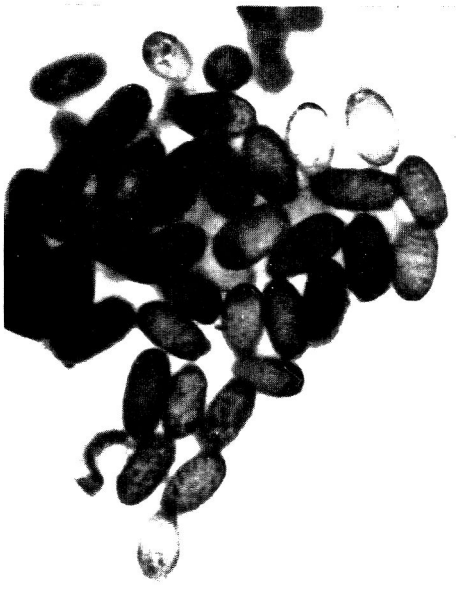

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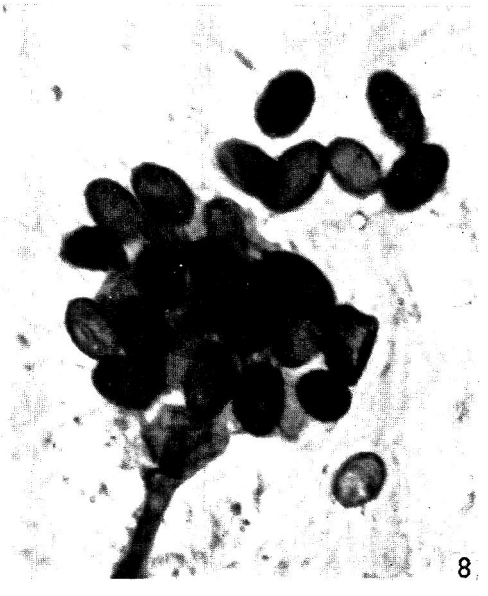

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EXPLANATION OF PLATES

All figures are phase-contrast photomicrographs. For Figs. 1 and 5, ×220; for 2–4 and 6–8, ×990.

PLATE 1. Sterile phases of *Memnionella echinata*.

Fig. 1. Compact mycelium with swollen cells, *M. echinata* 11973.

Fig. 2. Unswollen mycelium from culture with swollen cells, *M. echinata* 24003.

Fig. 3. Swollen cells, *M. echinata* 24003.

Fig. 4. Separated swollen cells, *M. echinata* 24003.

PLATE 2. Fruiting bodies of *Memnionella echinata* and *Stachybotrys atra*.

Fig. 5. Mycelium with sporophores, *M. echinata* 24003.

Fig. 6. Spores in chains on sporophore and typical for *M. echinata* (24003) with some spores adjacent to the sporophore but typical for Stachybotrys.

Fig. 7. Cluster of spores produced in *M. echinata* 24003.

Fig. 8. Spores of *S. atra* 42311 with sporophore, and with background of filamentous hyphae.

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The Examination of Brucella Cultures for Lysis by Phage

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SUMMARY

A total of 3919 brucella cultures was examined for lysis by five brucella phages, using a routine test dilution (R.T.D.) and 10,000 × R.T.D. All cultures of *Brucella abortus* examined were lysed by all five phages at both dilutions. In addition, all cultures with the oxidative metabolic pattern characteristic of *B. abortus*, irrespective of their properties as determined by conventional typing methods, were lysed by both phage dilutions. Cultures of *B. suis* were not lysed by phages at R.T.D. but all showed lysis by phages at 10,000 × R.T.D. Cultures of *B. melitensis* and those with the oxidative metabolic pattern characteristic of *B. melitensis*, irrespective of their properties as determined by conventional typing methods, were not lysed by phages at either dilution. All five phages used (Tb, 10/I, 24/II, 212/XV and 371/XXIX) displayed an identical host range; this was confirmed by neutralization tests with antiphage sera.

INTRODUCTION

In a previous publication (Morgan, Kay & Bradley, 1960) it was shown that a brucella phage obtained from the U.S.S.R. (Tbilisi or Tb phage) when used at a routine test dilution was able to lyse cultures of *Brucella abortus* but not of *B. suis*. Some of the *B. melitensis* cultures examined were lysed but others were not. Cultures of *B. melitensis* isolated abroad (U.S.A., Malta) were not lysed whilst cultures with the biochemical and serological properties of *B. melitensis* isolated from cattle in Great Britain were lysed. It was therefore possible to distinguish between these British cultures and the Mediterranean strains. Four new phages isolated in Poland (Parnas, 1961) have now become available to us. The purpose of the present paper is to report on the examination of these phages when tested with a larger collection of brucella cultures.

METHODS

Phages. In addition to the Soviet phage Tb, four other phages received from Professor J. Parnas (Lublin, Poland), were used; phages 10/I, 24/II and 371/XXIX had been isolated from carrier cultures of *Brucella abortus* and phage 212/XV from a culture of *B. suis*. Phage stocks were prepared by the double agar layer technique (Adams, 1959) with a suspension of organisms from a 24 hr. culture of *B. abortus* 544 (FAO/WHO reference strain) as propagating culture. The phages were suspended in phosphate buffer (pH 7·2) containing albumin (Fildes & Kay, 1955), the suspension centrifuged and filtered through Oxoid millipore (0·8 μ diam.) filters and stored

at 4°. All the phages were used at a routine test dilution (R.T.D.) prepared in phosphate buffer containing albumin with *B. abortus* 544 as the indicating culture. The number of plaque-forming particles (pfp)/ml. in these preparations was assayed by the method of Adams (1959) with *B. abortus* 544. Counts were made after 48 hr. of incubation at 37° in air containing 10 % (v/v) added CO_2.

Partial purification of phage Tb was made by alternate low- and high-speed centrifugation. Phage was centrifuged at 100,000 *g* in a Spinco centrifuge and resuspended, after two such centrifugations, in albumin buffer.

An antiserum against phage Tb was prepared in rabbits by using as antigen a crude preparation containing $2{\cdot}5 \times 10^{10}$ pfp/ml. Four injections were given every other day, and a week after the last injection two more were given after an interval of 2 days. Blood was collected 10 days after the last injection and the sera Seitz-filtered and stored at 4°. Antisera were also prepared in rabbits against the partially purified phage Tb. Neutralization tests for assessing the K values were made by the method of Adams (1959).

Brucella cultures. Cultures recently isolated at this laboratory or sent here from various parts of Great Britain for routine typing constituted the majority of the cultures studied. Tests were made as soon as possible after isolation. Cultures received from other parts of the world were also studied. The type and countries of origin of these cultures are given later in Tables 1 and 4. All cultures were typed by conventional methods (as recommended by the World Health Organization Expert Committee on Brucellosis; Report, 1953); details of the technique and media have already been published (Morgan, 1961).

Media. Serum glucose agar (Morgan, 1960) was used for the growth of cultures and as the basal medium for the propagation and plaque-counting of phage. For the double agar layer technique, the top layer consisted of nutrient agar + 0·5 % (w/v) glucose and 0·7 % (w/v) agar.

For routine typing, growth from 48 hr. slopes was suspended in buffered saline (pH 6·4) and a loopful streaked across well-dried serum glucose agar plates. When the five phages were used, three cultures were streaked; plates were dried with the lid partly open for 1 hr. at 37°. One drop (0·02 ml.) of each phage suspension at its routine test dilution (R.T.D.) was spotted on the cultures, allowed to dry and the plates incubated for 48 hr. in air + 10 % (v/v) CO_2.

RESULTS

Phage preparations containing between 2 and 6×10^{11} pfp/ml. were obtained. R.T.D. preparations contained approximately 1×10^7 pfp/ml. Plaques were not observed when *Brucella suis* 1330 or *B. melitensis* 16 M were used as lawns; but lysis of *B. suis* though not of *B. melitensis* occurred at the highest dilutions of phage (10^{-1} and 10^{-2} with high titre stocks).

In Table 1 are given the results of testing with phage, at a routine test dilution, 3919 brucella cultures. Of 3775 cultures isolated in Britain, representing *Brucella abortus* type I, *B. abortus* type II (dye-sensitive), the variety which is *B. abortus* biochemically and culturally but *melitensis* serologically and the variety isolated in Great Britain from cattle which has the biochemical, cultural and serological properties of *B. melitensis*, 3736 cultures (98·8 %) were lysed by all the phages used.

Many of the cultures that were not lysed were rough; the remainder had been disposed of before tests for roughness were made.

Of 144 cultures received from abroad, all 23 strains of *Brucella abortus* III (thionin-resistant) were lysed and none of 22 cultures of *B. suis*. Fifteen of 37 cultures of *B. melitensis* were lysed by phage, thus resembling these cultures with

Table 1. *The lytic effect of brucella phage at its routine test dilution* (R.T.D.) *on* 3919 *brucella cultures*

Species or type	No. of cultures Examined	Lysed	Remarks
B. abortus type I and type II	3454	3423	Britain
B. abortus type III (thionin-resistant)	23	23	Germany, Uganda and Turkey
B. abortus type III, but agglutinating in both monospecific sera	4	4	Turkey
Biochemically *B. abortus*/serologically *B. melitensis*	65	65	Includes 3 cultures that were dyesensitive/serologically *B. melitensis* (Britain)
Biochemically *B. melitensis*/serologically *B. abortus*	13	7	4 (Turkey), 1 (Germany) and 1 (Khartoum) lysed. 7 (Turkey) not lysed
B. melitensis isolated from cattle in Britain	256	248	Britain. The 8 not lysed were rough
B. melitensis (foreign)	37	15	14 (Germany), 1 (Uganda) lysed. 3 (Africa), 1 (Mexico), 1 (U.S.A.), 15 (Germany), 2 (Malta) not lysed
B. melitensis (foreign), but agglutinating in both monospecific sera	45	6	5 (Uganda), 1 (Turkey) lysed. 22 (Germany), 9 (Italy), and 8 (Turkey) not lysed
B. suis type I (4 cultures), *B. suis* type II (16 cultures), *B. suis* type III (2 cultures)	22	0	Denmark, U.S.A., Switzerland, Germany and Singapore

the biochemical and serological properties of *B. melitensis* isolated from cattle in this country. Six cultures that were culturally and biochemically *B. melitensis* but were *B. abortus* serologically were lysed by phage, as also were six cultures that were *B. melitensis* but which agglutinated in both 'abortus' and 'melitensis' monospecific sera.

In every case a culture was either lysed by all five phage preparations or by none; in no case was a culture lysed by one or two phages and not by the others.

The effect of using phage at R.T.D. and at 10,000 × R.T.D. was examined on a small number of cultures of each of the three main species; the results are in Table 2. Strains of *Brucella abortus* were lysed by both dilutions, *B. melitensis* by neither; the strains of *B. suis* showed complete lysis with 10,000 × R.T.D. but no lysis with R.T.D. With these *B. abortus* strains, resistant colonies developed in the area of lysis on incubation for four days but resistant colonies did not develop with *B. suis* strains.

To determine whether this apparent lysis of *Brucella suis* was caused by phage or by some toxic factor(s) present in the lysate, phage Tb was partially purified by differential centrifugation. The results (Table 3) show that the partially purified

phage containing $6{\cdot}5 \times 10^{11}$ pfp/ml. caused lysis of *B. abortus* and *B. suis* but not of *B. melitensis*. At the R.T.D., only *B. abortus* cultures were lysed. The supernatant fluid obtained after high-speed centrifugation caused no lysis of *B. suis* nor *B. melitensis* but caused lysis of *B. abortus* because of the presence of undeposited phage (45×10^8 pfp/ml.). Attempts to propagate phage Tb with *B. suis* as propagating culture (in solid and liquid media) were unsuccessful, the filtrate either gave no lysis of *B. abortus* or *B. suis* or only a very weak effect on *B. abortus* with the undiluted preparation.

Table 2. *The effect of using phage at routine test dilution and at* 10,000 × *routine test dilution*

	Lysis by phage*									
	Tb		10/I		24/II		212/XV		371/XXIX	
Strain	R.T.D.	10,000 × R.T.D.	R.T.D.	10,000 × R.T.D.	R.T.D.	10,000 × R.T.D.	R.T.D.	10,000 × R.T.D.	R.T.D.	10,000 × R.T.D.
B. abortus 544 (FAO/WHO reference strain)	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.
B. abortus S19	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C L	C.L.	C.L.	C.L.
B. abortus 61/1	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.
B. melitensis 16 M (FAO/WHO reference strain)	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.
B. melitensis 177	N.L.	N.L.	N.L.	N.L.	N.L.	N L.	N.L.	N.L.	N.L.	N.L.
B. suis 1330 (FAO/WHO reference strain)	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.
B suis 61/59 (type II)	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.
B. suis 61/178 (type II)	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.

* C.L. = Complete lysis; N.L. = no lysis.

Table 3. *The lytic effect of partially purified phage Tb*

	Species*			
Phage preparation	*B. abortus* 544	*B. melitensis* 16 M	*B. suis* 1330	*B. suis* 20 other cultures
Phage Tb undiluted stock preparation ($2{\cdot}5 \times 10^{11}$ pfp/ml.)	C.L.	N.L.	C.L.	C.L.
Phage Tb at routine test dilution (20×10^6 pfp/ml.)	C.L.	N.L.	N.L.	N.L.
Phage Tb partially purified by 2 centrifugations at 100,000*g* for 1 hour ($6{\cdot}5 \times 10^{11}$ pfp/ml.)	C.L.	N.L.	C.L.	C.L.
Phage Tb partially purified R.T.D. (25×10^6 pfp/ml.)	C.L.	N.L.	N.L.	N.L.
Supernatant of phage Tb collected after centrifugation at 100,000*g* for 1 hr. (45×10^8 pfp/ml.)	C.L.	N.L.	N.L.	—

* C.L. = complete lysis; N.L. = no lysis; — = test not done.

The results obtained by using phage at the two concentrations on further cultures are presented in Table 4. Cultures that were lysed by phage at R.T.D. were also lysed by phage at 10,000 × R.T.D. All the cultures of *Brucella suis* showed lysis by phage at 10,000 × R.T.D. but not at R.T.D. On the other hand, of 31 cultures of *B. melitensis* which were not lysed at R.T.D. none was lysed at 10,000 × R.T.D. These included *B. melitensis*, cultures with the biochemical and cultural characters of *B. melitensis* but which agglutinated in both monospecific *abortus* and *melitensis* sera as well as cultures that were biochemically *B. melitensis* but *abortus* serologically.

Table 4. *Comparison of the lytic effect of Brucella phage at routine test dilution and at* 10,000 × R.T.D.

Particulars of lysis	Particulars of cultures
Lysed by phage at R.T.D. and at 10,000 × R.T.D.	20 cultures of *B. abortus* (type I) 30 cultures of 'British' *B. melitensis* including strains from Germany, Italy and Uganda 18 cultures of *B. abortus* type III 4 cultures of *B. abortus* type III but agglutinating in both monospecific sera 3 cultures biochemically *B. abortus* but serologically *melitensis* 1 culture biochemically *B. melitensis* but agglutinating in both monospecific sera
Lysed by phage at 10,000 × R.T.D. but not lysed at R.T.D.	22 cultures of *B. suis*
Not lysed by phage at either concentration	13 cultures of *B. melitensis* (including WHO/FAO reference culture 16M) and others from Malta, Mexico and Germany 15 cultures of *B. melitensis* but agglutinating in both monospecific sera (7 from Italy and 8 from Germany) 3 cultures of *B. melitensis* biochemically but *abortus* serologically (1 from Germany and 2 from Italy)

Table 5. *The neutralization of* 5 *Brucella phages by phage antiserum*

	K value using phage antiserum produced against	
Phage	Tb phage crude preparation	Tb phage, partially purified
Tb	492	165
10	485	163
24	502	144
212	640	157
371	587	145

The results of neutralization tests of the five phages by a phage antiserum produced in rabbits against crude and partially purified phage Tb are given in Table 5. The results clearly show that the five phages are very closely related antigenically.

DISCUSSION

By using the conventional methods of species differentiation in the genus *Brucella*, most cultures can be placed into one of the three species—*B. abortus*, *B. melitensis* or *B. suis*; but over the years, an increasing number of cultures has been found which show some properties of two species, e.g. those which are *B. abortus* biochemically but *B. melitensis* serologically; cultures which are *B. melitensis* biochemically but *B. abortus* serologically; cultures which are *B. melitensis* except that they produce H_2S or require added CO_2 for growth (Stableforth, 1959).

By studies of oxidative metabolism Meyer & Cameron (1961*a*, *b*) and Meyer (1961) showed that each of the species of *Brucella* showed a characteristic metabolic pattern and that biotypes within the species also gave the metabolic pattern characteristic of the species. Many of the cultures used in the present paper (*Brucella melitensis* from Britain as well as practically all the cultures received from abroad) have been examined for their oxidative metabolic patterns (Meyer, 1962; Meyer & Morgan, 1962). These studies showed that, without exception, all cultures with the oxidative metabolic pattern characteristic of *B. abortus*, irrespective of their biochemical and serological properties as determined by conventional methods, were lysed by phage at R.T.D. Cultures which showed the oxidative metabolic pattern of *B. melitensis*, irrespective of their serological properties, were not lysed by phage at R.T.D. There is, therefore, no correlation between susceptibility to lysis by phage and serological and biochemical properties as determined by conventional methods.

By using two dilutions of phages it was possible to divide the cultures into species corresponding with oxidative metabolic patterns. The apparent lysis of *Brucella suis* by concentrated phage has not been reported previously; that such lysis was not due to some toxic factors present in the lysate was shown by the results obtained by partially purifying the phage by centrifugation. Replication of phage in *B. suis* has not been observed and the apparent lytic effect of 10,000 × R.T.D. may be due to lysis from without. The number of cultures of *B. suis* examined was, however, small and the observations need to be extended.

From the host range tested on a large number of cultures, all five phages studied were identical. This close similarity was confirmed by neutralization tests with phage antiserum.

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The Swelling of Bacterial Spores during Germination and Outgrowth

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SUMMARY

The swelling of spores of *Bacillus cereus* and *B. subtilis* during germination and outgrowth was followed by measuring changes in packed cell volume (pcv) and by photomicrographic measurements of single organisms. Three stages of swelling were recognized: (1) germination swelling involving a rapid increase of about 20% in pcv as the spore germinated; (2) pre-emergence swelling of up to 100% increase in pcv before emergence from the spore coat; (3) elongation. Germination swelling was due mainly to an increase in the breadth of the spore. It occurred in the absence of oxygen and was not inhibited by nisin whereas pre-emergence swelling was oxygen-dependent and was inhibited by nisin. Post-emergence swelling occurred during elongation of the new vegetative form. The significance of these findings is discussed.

INTRODUCTION

The transition from a dormant bacterial spore to a dividing vegetative form used to be termed germination. However, recent research has shown that this transition may be divided into several distinct stages. The term germination is thus now reserved for the first stage, and the transformation of the germinated spore into the vegetative form is called outgrowth (Campbell, 1957) or post-germinative development (Levinson & Hyatt, 1956). Germination involves loss of resistance to heat and other injurious agents, loss of refractivity when examined by the phase-contrast microscope (Pulvertaft & Haynes, 1951), decrease in optical density of suspensions and increase in stainability of cells (Powell, 1950), decrease in dry weight due to loss of dipicolinic acid, calcium and a non-dialysable mucopeptide (Powell & Strange, 1953) and the initiation of respiration (Murrell & Scott, 1958). Germination is induced by simple nutrients and may be differentiated from outgrowth by certain inhibitors (Murty & Halvorson, 1957; Halvorson, 1959). Temperature requirements for germination are often different from those for outgrowth (Mol, 1957; Wolf & Mahmoud, 1957). Outgrowth includes four stages occurring in the following order: swelling, emergence from the spore coat, elongation of the emergent organism, and finally division of the elongated organism. Each stage is characterized by different requirements. For example oxygen uptake becomes detectable during germination, increases in rate during swelling and increases still further after emergence (Mandels, Levinson & Hyatt, 1956). Phosphates are required early in the outgrowth process (Hyatt & Levinson, 1959), but sulphur only becomes essential later on (Hyatt & Levinson, 1957); different metal ions are toxic to different stages of outgrowth

(Hyatt & Levinson, 1957). The changes occurring during and after germination can thus be grouped into one or other of two major processes, which are: (i) the breakdown of spore structure; (ii) the synthesis of new vegetative cell material. These two processes have been intensively studied, but the swelling of spores has received little attention. We have therefore studied changes in the volume of spores during germination and outgrowth and related these changes to the stages of development.

METHODS

Cultures. The organisms used were *Bacillus cereus* NCTC 945 and *B. subtilis* M3, a laboratory isolate. Both conformed to the descriptions of these species given by Smith, Gordon & Clark (1946).

Production and cleaning of spores. Organisms were grown at 30° on Oxoid potato extract agar reinforced with 1 % (w/v) yeast extract (Difco) and adjusted to pH 7. When sporulation was complete and lysis of the sporangia had occurred, the freed spores were washed off the agar with cold water and washed a further three times with cold water by centrifuging. Suspensions were cleaned by repeatedly centrifuging and discarding the uppermost layers of the pellets (Long & Williams, 1958). Efficient cleaning was necessary because any cell debris contaminating the spore suspensions interfered with measurements of volumes of packed spores. Furthermore, the packed volume of cell debris in suspensions which had not been cleaned was variable; for instance it decreased when suspensions were heated, probably because of disruption or coagulation of cytoplasmic debris. This did not occur with cleaned spores. Spore suspensions were stored at 4° in water and activated by heating at 70° for 30 min. before use, to ensure rapid germination.

Experimental technique. Spore suspensions (70 ml.) were warmed to 37° and rapidly mixed with an equal volume of yeast glucose broth (YGB) also at 37°. At the same time a stop-watch was started. Samples (25 ml.) were removed at intervals and rapidly pipetted into bottles precooled in ethanol and solid CO_2 mixture and then stored in ice water until required. This cooling procedure effectively arrested the development of spores and enabled optical measurements to be made.

A drop of the same suspension was spread on a prewarmed microscope slide so that it quickly dried. This was stained by a modified Ziehl-Neelsen stain (Powell, 1950), and viewed with a light microscope. Another drop of the suspension was viewed with a phase-contrast microscope. The two microscopic methods and measurements of the optical density of suspensions at 580 mμ were used to determine the extent of germination and outgrowth.

Measurement of packed cell volume (pcv). Samples were centrifuged at 2000*g* for 20 min. at 4° and the pellets drained. Each pellet was then emulsified in 0·5 or 1·0 ml. of saline at 4° containing 2 drops of capryl alcohol/100 ml. to prevent foaming. These concentrated samples were drawn into M.S.E. capillary haematocrit tubes and centrifuged at 2700*g* for 15 min. at 4° in the M.S.E. microhaematocrit centrifuge head (Measuring and Scientific Equipment Ltd., London, S.W. 1). The length of the pellet and the total liquid length in each capillary tube were measured with a travelling microscope with vernier scale. The packed cell volume (pcv) was expressed as a % by using the formula: pcv = length of pellet (mm.) × 100/ length of pellet + length supernatant fluid (mm.)

Photomicrographic measurements. Suspensions of spores (about 10^7/ml.) were dried on 1 in. square no. 0 cover glasses. These were placed on flat pads of yeast glucose agar (about 1 mm. in thickness) on 1 × 3 in. microscope slides in a microscope stage incubator at 37°. A group of spores was immediately brought into focus and photographed, first under phase-contrast illumination then under light field illumination. At intervals photography of the same group of spores was repeated so that the successive stages of development were recorded. The micrographs were enlarged and printed at final magnifications of ×3840 (*Bacillus subtilis*) or ×2400 (*B. cereus*). Lengths and breadths of phase and light field photographs of individual organisms were measured. In addition to measuring cell size, an estimate of the stage of germination reached by each spore was made from its appearance under phase-contrast illumination. These were recorded as: (1) phase bright ungerminated spore; (2) first sign of phase darkening; (3) germinating but not fully phase-dark spore; (4) germinated fully phase-dark spore. Measurements of each spore were plotted against time, and the stage of germination was related to change in size.

RESULTS

The effect of spore concentration on swelling

Figure 1 shows the effect of spore concentration on the increase in packed cell volume (pcv) of *Bacillus subtilis* spores in medium which was not agitated (i.e. aeration was solely by diffusion through the liquid surface). Spore concentration was expressed as the initial pcv of the suspensions. The concentrations used were roughly doubling. The curves show that the rate of swelling was inversely dependent on the spore concentration. The rate of germination was such that 98 % of the spores had germinated at all three concentrations within the first 25 min. The initial increase of about 20 % pcv, which occurred within the first 25 min., was not decreased by increasing the spore concentration and swelling began at the same time as germination, without a lag phase. These results suggested that there might be two phases of swelling, the first occurring synchronously with germination and not dependent on spore concentration, and the second a post-germinative swelling which was dependent on spore concentration. Since the suspensions used were concentrated and not well aerated, oxygen was the obvious choice for the role of rate-limiting nutrient.

The effect of aeration on swelling

The increase in pcv of *Bacillus subtilis* spores under conditions of good and poor aeration is shown in Fig. 2. With a constant spore concentration (4·5 % initial pcv) the increase in pcv depended on the aeration rate. Germination was not affected by poor aeration, 98 % germination occurred in 25 min. in each case. However, with poor aeration (static culture with a small surface area), although germination occurred rapidly there was only a small increase in pcv of about 20 % and no post-germinative swelling; after 80 min. no outgrowth was observed. Figure 2 therefore shows that the effect of spore concentration (Fig. 1) was due to a deficiency of oxygen rather than of any other nutrient. Germination swelling still occurred when the oxygen tension was further lowered by bubbling nitrogen through the medium. Germination was therefore always accompanied by an increase in pcv, and this increase could be differentiated from the post-germinative increase in pcv.

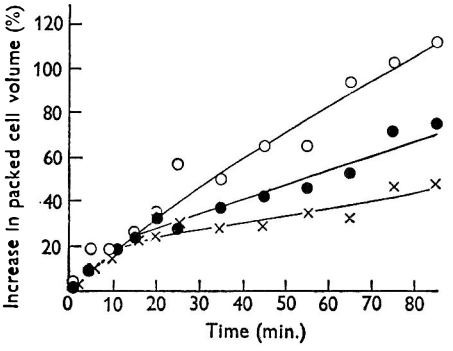

Fig. 1

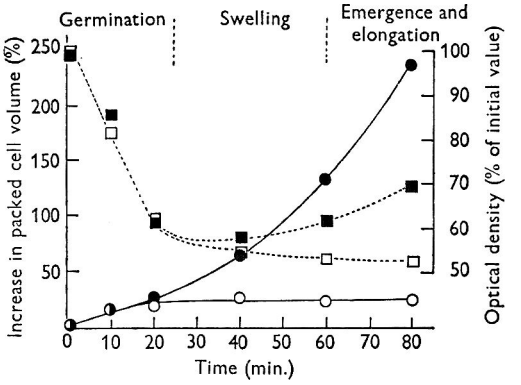

Fig. 2

Fig. 1. Effect of spore concentration on the increase in pcv of *Bacillus subtilis* spores. Spores incubated in yeast glucose both at 37 °. Germination was 98 % complete in 25 min. and no emergence occurred in 80 min. Spore concentration is expressed as the initial packed cell volume: ○—○, 2·2 % pcv; ●—●, 4·1 % pcv; ×—× 7·8 % pcv.

Fig. 2. Effect of aeration on the increase in pcv of *Bacillus subtilis* spores. The initial packed cell volume was 4·5 % for both cultures. ———, Packed cell volumes; – – – –, optical density; ●, and ■, well-aerated culture; ○ and □, poorly aerated culture.

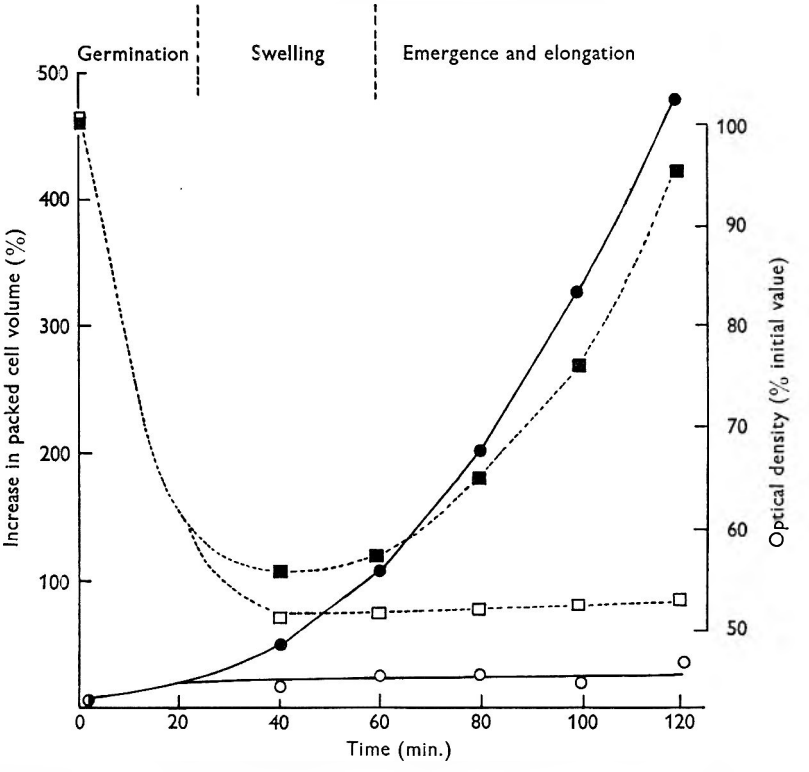

Fig. 3. Effect of nisin (0·5 μg./ml.) on the increase in pcv of *Bacillus subtilis* spores. The initial pcv were 3·1 % and both cultures were well aerated. ———, pcv; – – – – –, optical density; ● and ■, in absence of nisin; ○ and □, in presence of nisin.

Increase in spore volume also occurred during the germination of *Bacillus cereus* NCTC 945 spores and could be measured photomicrographically. However, the presence of exosporia around these spores made the increase in pcv that occurred during germination swelling appear relatively smaller. This fact coupled with the error of the pcv technique resulted in variable measurements. Post-germination and pre-emergence increase in pcv was readily detected.

The effect of nisin on swelling

Nisin is known to allow spores of sensitive organisms to germinate but to prevent their outgrowth (Campbell & Sniff, 1959). The actual stage of outgrowth inhibited is coat rupture, and since *Bacillus subtilis* is sensitive to nisin it was of interest

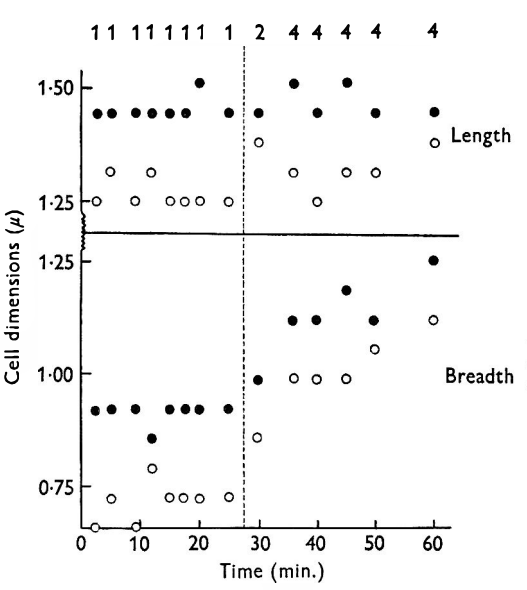

Fig. 4

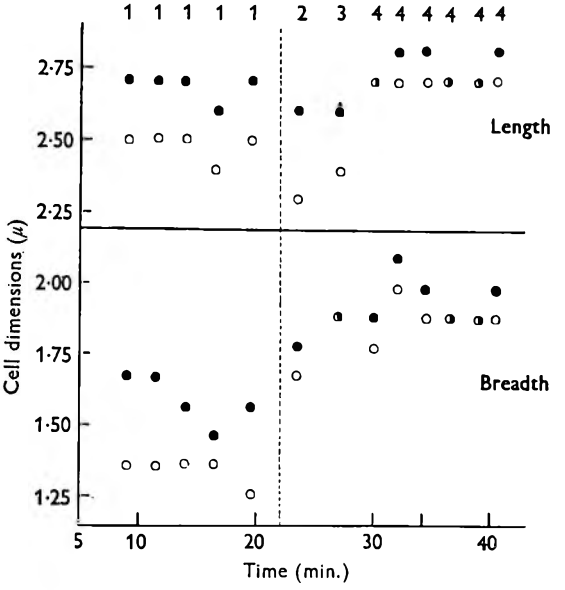

Fig. 5

Fig. 4. The length and breadth of a typical *Bacillus subtilis* spore during germination. Measurements from phase-contrast photomicrographs (●) and light-field photomicrographs (○). Key to stages of germination: 1, phase-bright ungerminated spore; 2, first sign of germination and phase-darkening; 3, germinating but not fully phase-dark spore; 4, germinated, fully phase-dark spore. Stage 3 was not observed with this particular spore.

Fig. 5. The length and breadth of a typical *Bacillus cereus* spore during germination. Measurements from phase-contrast photomicrographs (●) and light-field photomicrographs (○). Identical phase and light-field measurements are shown thus: ◑. Key to stages of germination as in Fig. 4.

to find what effect nisin had on spore swelling. Figure 3 shows the result of incubating spores in a well-aerated medium containing an inhibitory concentration of nisin. The initial swelling occurred as before, accompanying germination of the spores, but nisin was effective immediately after germination, for no post-germinative swelling occurred. This again suggests that the small constant germination-swelling

is distinct from the swelling of the germinated spore, which occurs before emergence of the vegetative forms.

Germination swelling observed microscopically

Figures 4 and 5 show length and breath measurements of typical *Bacillus subtilis* and *B. cereus* spores before, during and after germination. Photographs taken with phase contrast and light-field illumination both revealed that during the change from the refractile ungerminated spore to the non-refractile germinated spore, cell breadth increased by about 40 % whilst length increased only slightly (about 5 %). The change in shape can be seen in Pl. 1 and 2, and corresponds to the increase in pcv measured during germination. It is also noticeable that the increase in breadth of individual spores occurred rapidly and at the first sign of germination; i.e. germination-swelling takes place concurrently with germination and does not closely follow it.

DISCUSSION

Powell (1957) reported that swelling occurs after phase darkening and therefore after germination. Rode & Foster (1960) stated that the germination process itself is characterized by slight swelling. Black & Gerhardt (1962) also referred to several other authors who have reported swelling during germination. In the present work we have attempted to correlate these observations by describing swelling quantitatively and by relating it to the germination process. In what follows the results are discussed in relation to the facts known about germination.

The sudden increase in size and change in shape of spores during the very first phase of germination indicates that the spore coat must become elastic very early in the process, i.e. during the loss of dipicolinic acid, calcium and spore mucopeptide, since these events all follow closely the fall in optical density of the suspension and other changes that collectively constitute germination. Is weakening of the spore coat, for instance by partial lysis with enzymes, the event that starts the process or is weakening of the coat a subsequent event? The synchrony of germination-swelling with other changes that define germination suggests that the former is more probable. Strange & Dark (1957*a*, *b*) described enzymes which attacked spore coats. If the mucopeptide released early in the germination process is derived from the spore coat, then early enzymic weakening of this structure seems likely. Furthermore, nisin prevents the rupture or lysis of spore coats during emergence but does not inhibit germination or germination swelling. Of course changes in spore shape and volume do not necessarily involve weakening of the spore coat, but might reflect changes in the properties of some underlying structure, such as the cortex. This is a structure inside the spore coat and surrounding the central core. Lewis, Snell & Burr (1960) suggested that the core might have a low free-water content because it is squeezed dry by a contractile cortex. This presupposes that an early event in germination would be loss of strength of this structure, perhaps by lysis with release of hexosamine-containing peptides. Takagi, Kawata & Yamamoto (1960) have shown that the cortex is disrupted early in the germination process, so this structure could equally well be enzymically weakened rather than the coat, and allow the core to swell. In any case, hydration and swelling of the core could then be reflected in swelling of the spore and a change to more spheri-

cal shape as it became turgid. Some internal structure becomes visible in light-field photographs of *Bacillus subtilis* spores early in the germination process (Pl. 1) and is probably the core and cortex (Powell, 1957; Robinow, 1960). The core does not obviously swell in the first few minutes of germination; however, the structure surrounding it, which may be the cortex, does increase in thickness (Pl. 1).

In contrast to germination-swelling, pre-emergence (post-germination) swelling requires good aeration and an external energy source. Pre-emergence swelling is therefore probably due to synthesis coupled with uptake of water and is similar to the elongation process. Since Bacillus spores can germinate anaerobically (Roth & Lively, 1956) and many species can outgrow in the absence of oxygen to give viable vegetative forms, pre-emergence swelling must be able to occur anaerobically but after a lag. Nevertheless, pre-emergence swelling needs external energy sources under anaerobiosis.

The prime event in germination is usually assumed to be something that causes a change in the permeability of the dormant spore. Murrell & Scott (1958) demonstrated the permeability of dormant spores to water. Black & Gerhardt (1962) showed that dormant spores are permeable to water, germination stimulants and non-germination stimulants. They listed the evidence for an increase in permeability during germination and demonstrated it for glucose and water in *Bacillus cereus*. Thus germination swelling is probably due to water uptake for rehydration of the spore, which is widely assumed to contain little free water, and is therefore an inseparable part of germination. As germination stimulants are utilized in almost undetectable amounts (Harrell & Halvorson, 1955) and respiration is only just detectable during germination (Murrell, 1955) there may be an endogenous energy source and an electron acceptor taking the place of oxygen e.g. dipicolinic acid (Doi & Halvorson, 1961). Thus although the breaking of the impermeability barrier may be metabolic, germination swelling is probably solely a consequence of physical rehydration.

We are grateful to Mr A. W. Knight for technical assistance.

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EXPLANATION OF PLATES

The scale marks represent 5μ.

PLATE 1. Germinating spores of *Bacillus subtilis*

Figs. 1–4. Phase-contrast photographs.

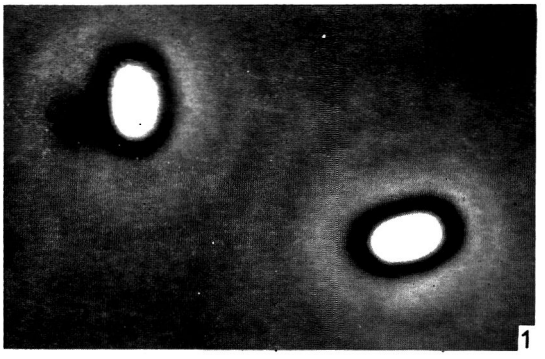
Figs. 5–8. Light-field photographs.

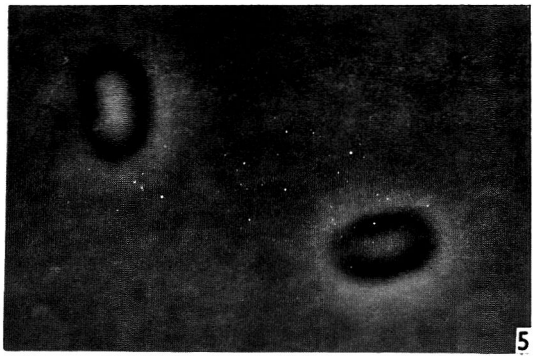
Figs. 1, 5. Ungerminated spores.

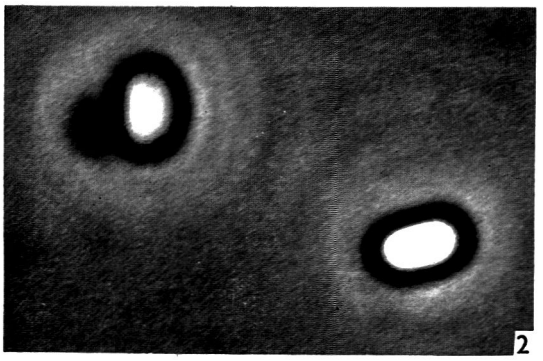
Figs. 2, 6. First signs of germination and swelling after 3 min.

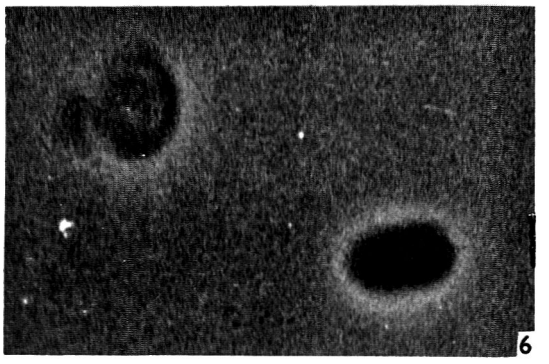
Figs. 3, 7. Core and cortex visible after 6 min.

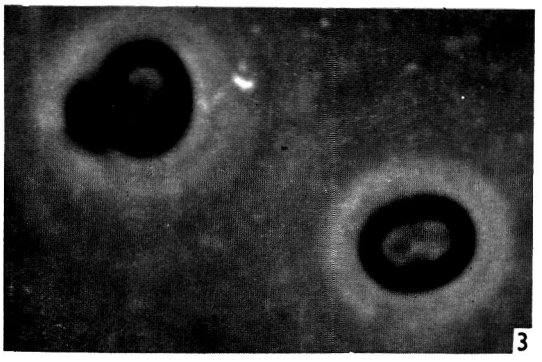
Figs. 4, 8. Spores fully germinated and swollen with core and cortex clearly visible after 10 min.

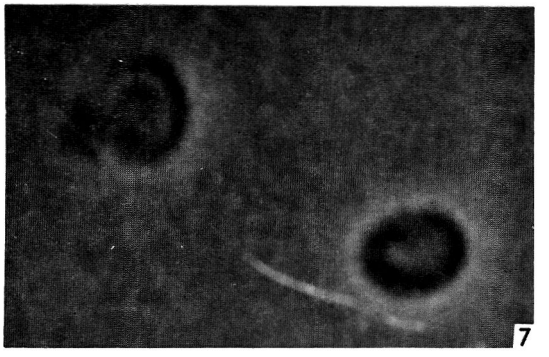


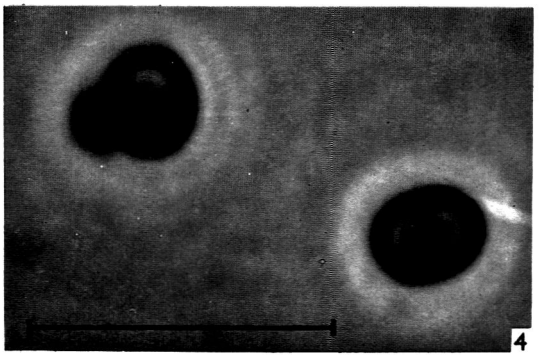


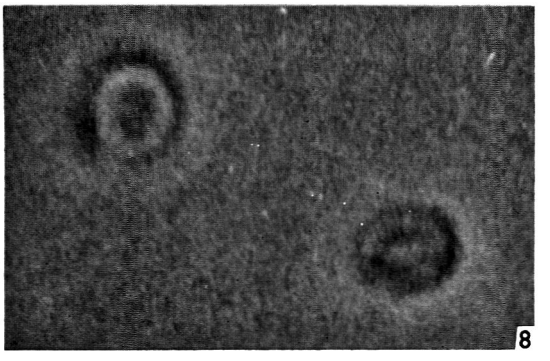












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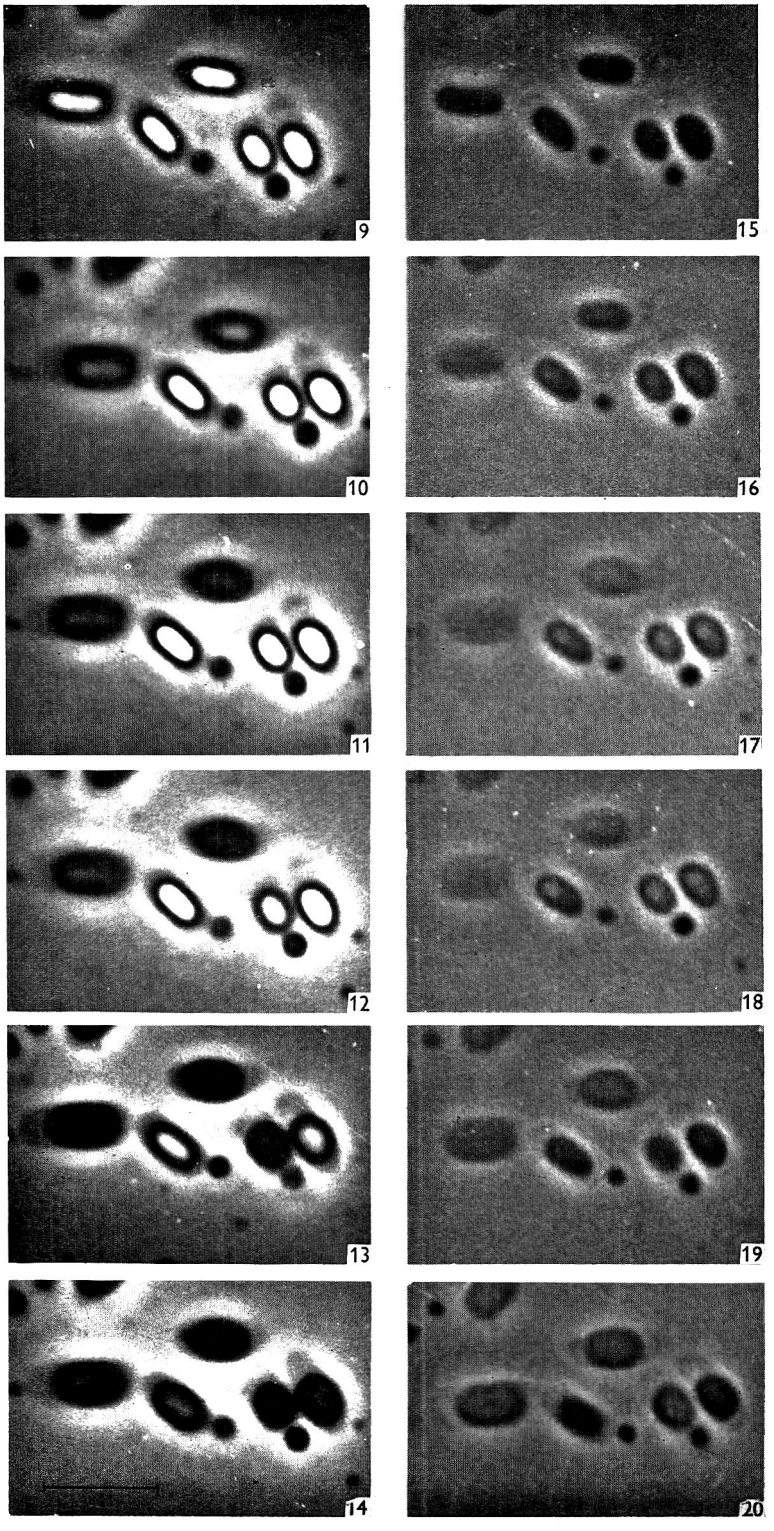

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PLATE 2. Germinating spores of *Bacillus cereus*

Figs. 9–14. Phase-contrast photographs.

Figs. 15–20. Light-field photographs.

Figs. 9, 15. Group of five ungerminated spores and two parasporal bodies. One spore is beginning to germinate and shows peripheral darkening by phase contrast. Exosporia are visible as pale sheaths interrupting the light haloes.

Figs. 10, 16. After 3 min. germinating spores are swelling.

Figs. 11, 17. After 5·5 min.

Figs. 12, 18. After 8 min.

Figs. 13, 19. After 13 min.

Figs. 14, 20. After 18 min. all spores have germinated. Core and cortex are not detectable.

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Isolation and Composition of Staphylococcal Alpha Toxin

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SUMMARY

A method is described for the preparation, on a laboratory scale, of staphylococcal alpha toxin having a purity of about 70% and in a yield of 40%. The method entails ammonium sulphate fractionation followed by curtain electrophoresis. The toxin so obtained contains two impurities, one of which can be removed electrophoretically, and the other ultracentrifugally. The toxin itself is a protein of molecular weight 44,000. It contains most of the usual amino acids which vary in amount from 4 residues for histidine to 44 for aspartic acid. Although electrophoretically heterogeneous its electrophoretic components produce the biological effects classically associated with alpha toxin. As by-products of purification, two other proteins were isolated in crystalline form. Their significance for pathogenicity, if any, remains to be determined.

INTRODUCTION

Alpha toxin is a substance, presumably protein, found extracellularly in cultures of *Staphylococcus aureus*. Capacity to produce it is closely associated with pathogenicity, and 'it is probably the most important and certainly the best known of the staphylococcal toxins' (Van Heyningen, 1950). Preparations of alpha toxin (*a*) are haemolytic *in vitro*, (*b*) are lethal for small laboratory animals, (*c*) produce necrotizing lesions following injection into the skin of rabbits, and (*d*) cause smooth muscle to undergo contraction and loss of function. The effects are commonly attributed to a single product of growth, but rigorous proof for this belief appears to be lacking.

Purification of alpha toxin has been carried out by Wittler & Pillemer (1948), Turpin, Relyveld, Pillet & Raynaud (1954), Butler (1959), Robinson, Thatcher & Montford (1960), and Goshi & Cluff (1960), but in no instance have the products satisfied the usual tests of homogeneity. In order to define more clearly the nature and properties of the toxin, its purification and characterization were undertaken once more. In the course of the work, two other proteins unrelated to alpha toxin were isolated in crystalline form. Their properties, so far as they are known, will also be described.

METHODS

Strain. The Wood 46 strain of *Staphylococcus aureus*, kindly supplied by Dr A. Thal, was used almost exclusively.

Measurement of haemolytic activity. Preparations of toxin were diluted in saline solution (M/13 NaCl) buffered at pH 7·0 with phosphate (M/15) containing 0·1% (w/v) bovine serum albumin as stabilizing agent. To 1 ml. of each of a series of

dilutions increasing in steps of about 30 % was added 1 ml. of a twice-washed suspension of rabbit red blood cells. The concentration of the red cell suspension used (about 0·7 %, v/v) was adjusted so that a sample, after haemolysis with saponin and adding an equal volume of diluent, gave a Beckman absorbance at 545 mμ of 0·80. The mixtures of toxin dilutions and red cells were placed in a 37° water bath for 30 min., and then centrifuged briefly. The colour of the haemoglobin in the supernatant fluids was compared visually with that of standard haemoglobin solutions, and the dilution haemolysing 50 % of the red cells in the suspension was determined by interpolation. A unit of haemolysin is defined as that amount which liberates half the haemoglobin in the test red cell suspension under the conditions stated.

Suspensions of red cells prepared from different rabbits show wide variation in sensitivity to lysis by alpha toxin. For example, a toxin preparation tested against red cells from three different rabbits titrated at 335,000, 274,000 and 117,000 haemolytic units/ml., and a second toxin preparation tested against the same three cell suspensions gave 18,700, 14,700 and 6,500 haemolytic units/ml. An indication of the variability of red cells from different rabbits can be found in the observations of Timmerman (1937). To make all titrations comparable, a standard toxin preparation, stable when stored under ammonium sulphate, was titrated with each unknown, and the results corrected according to the deviation from the fixed value of the standard.

Protein. Protein was estimated by reading absorbance at 280 mμ in a Beckman DU spectrophotometer, with optical cells of 10 mm. light path. Because none but the crudest preparations contained a significant amount of material absorbing strongly at 260 mμ, no correction for absorbance at this wavelength was applied, and protein concentration was assumed to be directly proportional to 280 mμ absorbance.

Specific haemolytic activity. On the assumption that alpha toxin is a protein, specific activity was expressed as haemolytic activity/unit absorption at 280 mμ, the latter being called for convenience an 'optical unit'.

Lethal activity. Twofold dilutions of toxin were injected intravenously into Swiss mice (20 g.). Not less than three mice were used for each dilution. The toxin was diluted in 0·85 % (w/v) sodium chloride solution containing 1 mg. gelatin/ml., and the injection volume was 0·25 ml. Mice surviving for less than 24 hr. were scored as deaths.

Starch gel electrophoresis. The method of electrophoresis and protein staining with Amido-Black as described by Smithies (1955) was used.

Ouchterlony plates. The technique described by Ouchterlony (1958) was used.

Analytical procedures. Nitrogen was estimated by a modified micro-Kjeldahl procedure; phosphorus by the method of Lohmann & Jendrassick (1926); carbohydrate by means of the anthrone reagent (Carroll, Longley & Roe, 1956); amino acids by the Piez & Morris (1960) modification of the Stein & Moore method.

RESULTS

Preparation of partially purified alpha toxin

Cultivation of organism. The medium and conditions of cultivation were, with slight modification, those devised by Dr A. Pinsky and shown by him to yield consistently satisfactory titres of alpha toxin (in 1960–61 when he was a postdoctoral fellow in this laboratory). The importance of yeast dialysis diffusate for toxin production had been demonstrated by Bramann & Norlin (1951). Medium of the following composition was used: dialysate diffusate of yeast extract 2600 ml., acid hydrolysed casein, 64 g.; glucose, 8 g.; thiamine, 0·4 mg.; nicotinic acid, 3·7 mg.; water to make volume 3200 ml. This medium was adjusted to pH 7·1, and distributed among six 2 l. Erlenmeyer flasks, and sterilization carried out for 20 min. at 123° (17 lb. steam pressure). The diffusate of yeast extract was prepared by dialysing in the cold and with occasional mixing, 1 l. of a 40 % (w/v) solution of yeast extract (Difco Laboratories, Detroit, Michigan) against 4 l. of distilled water. After 6 days the contents of the dialysis sac were discarded, and the outside solution (diffusate) now having a volume of approximately 2·6 l. was used as indicated above.

Table 1. *Purification and recovery of Staphylococcal alpha toxin*

		Volume (ml.)	Total haemolytic activity (units of haemolysin)	Specific activity (units of haemolysin per optical unit)	Recovery of activity (%)
Stage 1.	Culture supernatant fluid	3000	$2{\cdot}75 \times 10^6$	11	100
Stage 2.	Extract of crude ammonium sulphate precipitate	113	$1{\cdot}70 \times 10^6$	235	62
Stage 3.	0·6 saturated ammonium sulphate precipitate extracted with 0·4 saturated ammonium sulphate	102	$1{\cdot}68 \times 10^6$	440	61
Stage 4.	Extract of stage 3 precipitated, dialysed and centrifuged	23·5	$1{\cdot}13 \times 10^6$	2,300	41
Stage 5.	Continuous-flow electrophoresis; pooled toxic fractions	67	$1{\cdot}05 \times 10^6$	11,000	38
Product.	First ammonium sulphate precipitate	5	$0{\cdot}08 \times 10^6$	13,700	39
	Second ammonium sulphate precipitate	5	$0{\cdot}31 \times 10^6$	9,600	

The inoculum was prepared by washing the cocci of an overnight broth culture of *Staphylococcus aureus* strain Wood 46, and suspending them in 0·5 vol. of sterile saline. The flasks were inoculated with 0·3 ml. each, and incubated 15–18 hr. at 37° on a rotary shaker operating at 220 cycles/min.

Purification of toxin. The cultures were chilled, pooled, centrifuged 30 min. at 8000 rev./min. and the cocci discarded. The supernatant fluid (stage 1, Table 1)

was saturated by addition of solid ammonium sulphate, and allowed to stand overnight at 4°. All subsequent steps were carried out in the cold.

The precipitate was recovered by stirring into the saturated culture supernatant fluid 15 g. powdered cellulose and filtering through a large coarse sintered-glass filter. The filtrate was discarded, the precipitate emulsified in 40 ml. 0·03 M-borate buffer (pH 8·6) and stirred for a few minutes. Following centrifugation for 10 min. at 10,000 rev./min. the supernatant fluid was decanted, and the precipitate extracted three more times with 40 ml. portions of the same buffer. The four extracts were combined (stage 2) and the residue discarded.

The pooled extract was made approximately 0·6 saturated with ammonium sulphate by addition of 4·2 g. solid ammonium sulphate for each 10 ml., and stirred for 25 min. Following centrifugation for 10 min. at 10,000 rev./min., the supernatant fluid was discarded and the precipitate stored overnight. The precipitate was extracted five times with 20 ml. portions of 0·4 saturated ammonium sulphate, allowing for each extraction 30 min. with intermittent stirring. The extracts were pooled (stage 3).

The extract was brought to 0·6 saturation by dissolving 1·4 g. ammonium sulphate for each 10 ml. After standing for 25 min. with occasional stirring, the mixture was centrifuged, and the clear supernatant fluid discarded. The precipitate was dissolved in 3 ml. 0·03 M-borate buffer (pH 8·3) and with the aid of additional small amounts of buffer totalling 2·5 ml., was quantitatively transferred to a cellophan sac. The solution was dialysed overnight against 6 l. distilled water. When, after dialysis, the volume exceeded 25 ml., it was reduced by pervaporation to 15–25 ml. The small amount of precipitate which formed during dialysis was removed by centrifugation and discarded (stage 4).

After adding 0·3 ml. of 0·04% (w/v) phenol red, as a marker, the solution was fractionated by means of a Spinco Model CF curtain electrophoresis apparatus (Beckman Instruments, Inc., Belmont, California) according to the following conditions: buffer: 0·06 M-borate (pH 8·3) containing M/5000 ethylenediaminetetraacetic acid (EDTA); paper: that supplied by Spinco; buffer level at feeder curtain: 6·5 cm.; Feed-tab cut above drip-point no. 21; sample feed rate: about 1 ml./hr.; drip-point collection rate: about 0·5 ml./tube/hr.; temperature 3 mm. from curtain 8°; potential difference: 400 V. between electrodes; current: 13 mA.; duration of run: 20–30 hr.

The distribution of protein as measured by light absorption at 280 mμ, and of toxic (haemolytic) activity of the 32 fractions resulting from continuous flow electrophoresis are shown in Fig. 1. It can be seen that the alpha toxin activity coincides with the major protein peak. As a routine, the alpha toxin was recovered in concentrated form by pooling appropriate fractions and dialysing against 75% saturated neutral ammonium sulphate. In the present instance, the five fractions obtained from drip-points no. 17 to no. 21 were pooled (stage 5) and then dialysed against two changes of 60% saturated neutral ammonium sulphate, 200 ml. each. After 7 days, the silky precipitate which had formed was separated by centrifugation and suspended in 5 ml. of 60% saturated neutral ammonium sulphate solution (first precipitate). The supernatant fluid was dialysed overnight against 200 ml. of 80% saturated neutral ammonium sulphate. The precipitate which formed was separated and suspended in 5 ml. of 80% saturated neutral ammo-

nium sulphate (second precipitate). The two precipitates are designated 'partially purified alpha toxin'. The recovery and degree of purification at each stage are shown in Table 1.

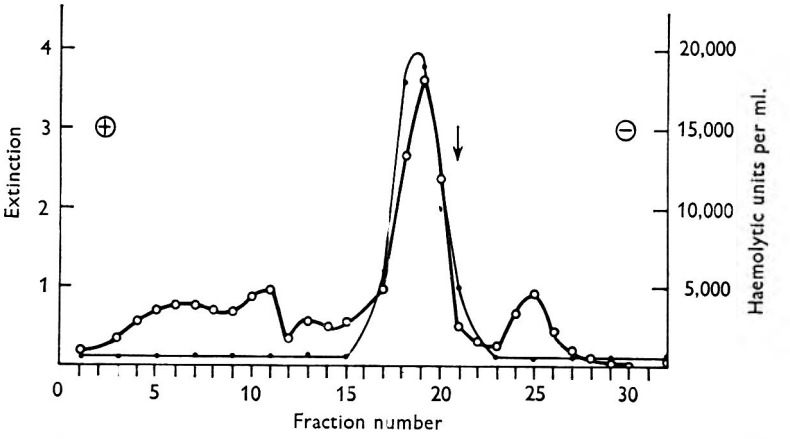

Fig. 1. Continuous-flow electrophoretic pattern of stage 4 material showing distribution of alpha toxin as haemolytic activity (solid circles) and of protein as extinction at 280 mμ (open circles).

Analysis of partially purified alpha toxin spectrophotometrically, ultracentrifugally, by starch-gel electrophoresis, and immunologically

The product obtained by the foregoing procedure, dialysed free from ammonium sulphate, yielded a faintly yellowish solution whose ultra violet (u.v.)-absorption curve resembled those usually given by proteins. It showed a maximum at 277 mμ, a minimum at 247 mμ, and a slight but definite shoulder at 290 mμ.

Examination in an analytical ultracentrifuge of a solution containing 7·5 optical units of partially purified alpha toxin/ml. 0·03 M-borate buffer (pH 8·3) revealed: (*a*) a major component accounting for 85 % of the area of the pattern and having an S_{20} value of 3 Svedberg units; (*b*) a minor component accounting for 15 % and having an S_{20} value of 12 Svedberg units (Fig. 2). By means of a separation cell, a portion of the major component was isolated free from the minor component. Its specific haemolytic activity was, within experimental error, the same as that of the starting material. These observations indicate that the major component was toxin.

Starch-gel electrophoresis of 0·5 optical unit of partially purified toxin, followed by protein staining with Amido-Black revealed two bands on the cathode side of the origin (Fig. 2*b*). The heavy-staining band was estimated to account for roughly 90 % of the total protein; the light-staining band for 10 %. The position of the heavy-staining band coincided exactly with one previously identified as alpha toxin from among many bands present in electrophoretic patterns of unfractionated crude mixtures of staphylococcal extracellular proteins (Bernheimer & Schwartz, 1961). The 12S component, which was subsequently isolated, yielded no stainable band when subjected to starch gel electrophoresis.

An Ouchterlony plate (Fig. 4*b*) in which one optical unit of partially purified alpha toxin was diffused against commercial antitoxin known to contain a variety of antibodies against staphylococcal proteins, revealed only two antigens: one

expressed by a heavy line of precipitate and identifiable with a pure alpha toxin line (Fig. 4*c*); a second which was expressed as a faint line and presumably the same material as the 10 % component demonstrable by starch gel electrophoresis.

Fractionation and analysis of partially purified alpha toxin by means of zone electrophoresis in a density gradient

The foregoing observations are interpreted as indicating that the toxin preparations obtained by the procedure described have a purity of 70–85 %. They were studied further by means of zone electrophoresis in a sucrose density gradient, a technique shown not only to be capable of considerable resolving power but one which is also useful preparatively (Bernheimer, 1962).

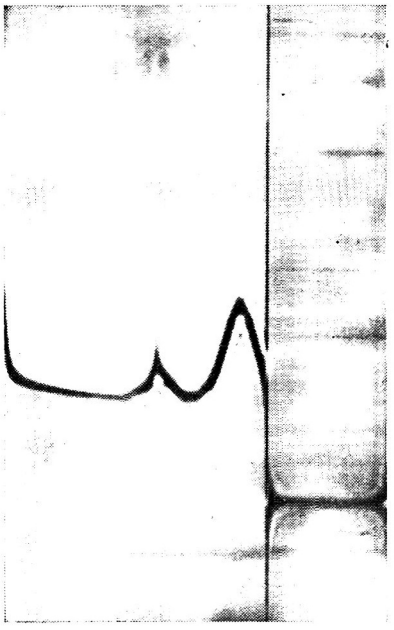

Fig. 2

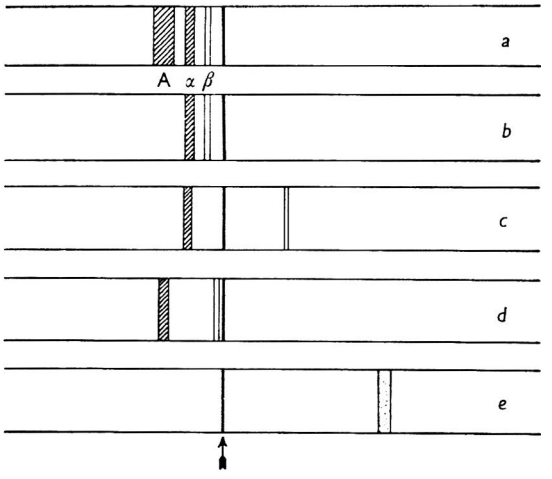

Fig. 3

Fig. 2. Sedimentation pattern of a 0·5 % (w/v) solution of partially purified alpha toxin in 0·03 M-borate buffer (pH 8·3) recorded after 20 min. at 59,780 rev./min. in the model E Spinco centrifuge at 20°.

Fig. 3. Representation of starch gel electrophoretic patterns. Arrow indicates origin; anode is to the right of origin. *a*, Crude extracellular proteins prepared as described by Bernheimer & Schwartz (1961). The complex of bands on the anode side of the origin is not shown but can be seen in Fig. 1. of the paper cited. *b*, 0·5 optical unit of partially purified alpha toxin. *c*, 0·5 optical unit of alpha$_n$ toxin. *d*, 0·5 optical unit of A-protein. *e*, 0·5 optical unit of X-protein.

Ninety-six optical units of partially purified alpha toxin were fractionated in the apparatus designed by Svensson (1960), using the general conditions described earlier (Bernheimer, 1962). The distribution among the fractions, of toxin as haemolytic activity, and of protein as optical units, is shown in Fig. 5. Seventy-seven % of the input haemolytic activity was recovered. Aside from the small protein peak at

Fraction 260 and that at Fraction 110 which appears to represent a small amount of toxin adsorbed to a finely divided precipitate, the protein between Fractions 160 and 240 is divisible into four zones, namely, from right to left in Fig. 5, $alpha_a$, $alpha_b$, $alpha_c$ and $alpha_d$. The mean specific haemolytic activities of the fractions of these zones were 15000, 14,700, 9500 and 250, respectively. (Dialysis of the fractions of $alpha_d$ against 0·03 M-borate containing M/5000 EDTA followed by ammonium sulphate precipitation was accompanied by an 18-fold increase in activity; from this it is concluded that an appreciable part of the protein of the $alpha_d$ zone consisted of inactive toxin capable of activation).

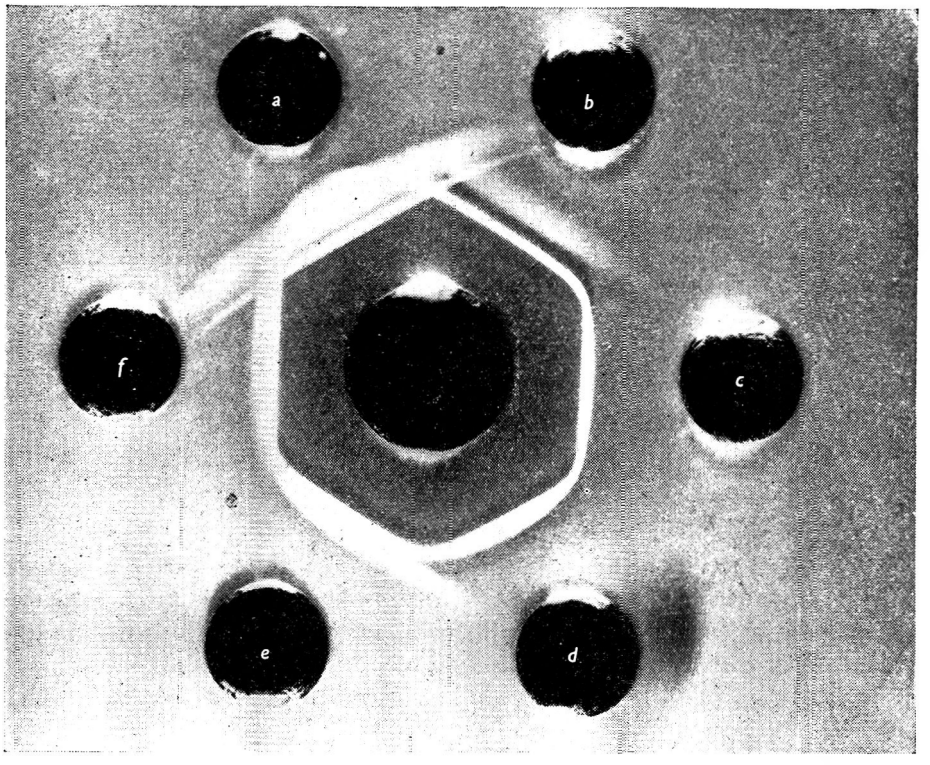

Fig. 4. Precipitin reactions in Ouchterlony plate after 5 days. Centre well: 0·1 ml. antitoxic horse serum. *a*, 0·5 mg. crude staphylococcal extracellular proteins prepared as described by Bernheimer & Schwartz (1961); *b*, one optical unit of partially purified alpha toxin (stage 5 material); *c*, one optical unit of $alpha_a$ toxin; *d*, 0·5 optical unit of $alpha_b$ toxin; *e*, 0·5 optical unit of $alpha_c$ toxin; *f*, 0·5 optical unit of $alpha_d$ toxin.

Starch gel electrophoretic patterns done on samples containing 0·5 optical unit revealed a single heavily-stained alpha toxin band at the same locus for all four varieties of toxin. In addition, $alpha_a$ and $alpha_b$ showed a very faint band on the anode side of the origin. The latter was not consistently demonstrable and its origin is obscure. $Alpha_b$, $alpha_c$ and $alpha_d$ showed a faint but distinct band (beta of Fig. 3*b*) slightly to the right of the heavy band; the pattern for $alpha_a$ can be seen in fig. 3*c*.

The results of agar diffusion analysis (Fig. 4) were in the main consistent with

those obtained from starch gel electrophoresis. Alpha$_a$ showed a single heavy line of precipitate. Alpha$_b$, alpha$_c$ and alpha$_d$ produced a similar line giving a reaction of identity with that of alpha$_a$, but alpha$_c$ showed a second, fainter line which in time tended to merge with the heavy line.

Besides having similar specific haemolytic activities, alpha$_a$, alpha$_b$ and alpha$_c$ are potent lethal agents (Table 2). The ratio of haemolytic to lethal potency of culture supernatant fluid, alpha$_a$, alpha$_b$ and alpha$_d$ are, within experimental error, identical, but that of alpha$_c$ is somewhat out of line with the others. The fractions of alpha toxin, when injected into the skin of rabbits, induced the formation of necrotic lesions identical in appearance with the classical description (Elek, 1959) of dermonecrosis associated with alpha toxin. About 1 μg. of partially purified alpha toxin contained in 0·1 ml. gelatin saline produced in 72 hr. a lesion 20 mm. in diameter, as did also the same weight of alpha$_b$. Alpha$_a$ in the same amount, produced a lesion of about half this diameter.

Table 2. *Comparison of haemolytic and lethal activities of staphylococcal toxin fractions*

Toxin preparation	Mouse LD 50 dose/ml.	Units of haemolysin / Mouse LD 50 dose
Alpha$_a$	5700	22
Alpha$_b$	2600	22
Alpha$_c$	1620	41
Alpha$_d$	2400	19
Culture supernate (strain Wood 46)	108	15
Culture supernate (strain 4423)	20	17

The foregoing results are interpreted as meaning that four species of molecules are demonstrable in partially purified alpha toxin when it is subjected to analysis by density gradient electrophoresis. They are alpha toxins, which exhibit different electrophoretic mobilities, but they are antigenically identical, and they are identical, or nearly so, in their biological (lethal, haemolytic, dermonecrotic) activity. However, a fifth species of molecule was detectable by starch gel electrophoresis and was antigenically distinct from toxin. Its relationship, if any, to alpha toxin is unknown.

Further examination of alpha$_a$. Because the component designated alpha$_a$ appeared to be a homogeneous substance, except for a trace of contaminating protein, and because it had properties identical with those classically associated with alpha toxin, it was examined further. It was prepared by electrophoretically fractionating partially purified toxin in a sucrose density gradient, combining appropriate fractions, dialysing the pool against 0·03 M-borate buffer to remove sucrose, and precipitating with 75 % saturated neutral ammonium sulphate. It was stored under ammonium sulphate, and portions were dialysed free from salt as needed. A solution having an absorbance at 280 mμ of 1·00 contained 109 μg. N/ml., 13,000 haemolytic units/ml., and 600 mouse LD 50 per ml.

One of the features characteristic of alpha toxin as distinct from other staphylococcal haemolysins is the sensitivity of rabbit, and to a less extent of sheep

erythrocytes to its action, and the relative resistance of red cells of certain other animal species (Elek, 1959). In Table 3 are shown the results obtained when the same preparation of alpha$_a$ toxin was tested against suspensions of diverse types of red cell made up to contain the same concentration of haemoglobin. It can be seen that the variation in the sensitivity of the red cells to toxin was very great indeed, and in general was in agreement with the experience of earlier workers.

Table 3. *Sensitivity to alpha toxin of red blood cells from different animals*

Animal	Specific haemolytic activity using species of cells indicated	μg. toxin required to cause 50 % haemolysis
Rabbit	14,900	0·048
Mouse	2,400	0·29
Sheep	580	1·2
Human	99	7·2
Horse	62	11·5
Guinea pig	62	11·5

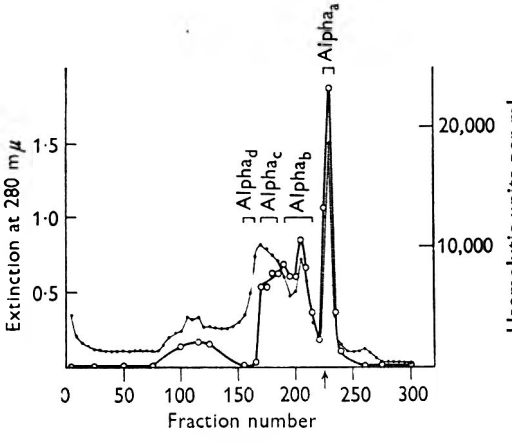

Fig. 5

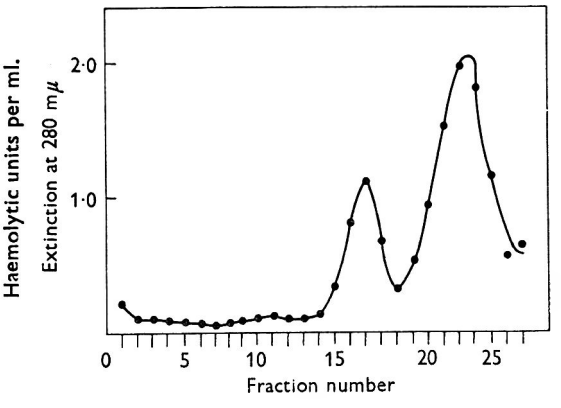

Fig. 6

Fig. 5. Density gradient electrophoretic pattern of partially purified alpha toxin. Solid circles: protein as extinction at 280 mμ; open circles. alpha toxin as haemolytic activity. Arrow indicates starting zone. Anode to left of starting zone. Buffer: 0·03 M-borate (pH 8·3) containing M/5000 EDTA. Sucrose gradient: 0–50 % (w/v); 800 V and 21–18 mA.; 21 hr. Sample consisted of 2·24 ml. borate buffer containing 96 optical units of partially purified toxin and 170 mg. glycine.

Fig. 6. Separation of 12 S component (small peak) from staphylococcal alpha toxin (large peak) by ultracentrifugation in sucrose density gradient.

Examination of 0·6 % (w/v) solution of alpha$_a$ in a Spinco Model E ultracentrifuge (Beckman Instruments, Inc., Belmont, California) revealed a pattern essentially identical with that of Fig. 2. It was evident, therefore, that alpha$_a$ was contaminated with the 12S component that had been detected earlier in partially purified toxin.

Separation of the 12S component from alpha toxin. Zonal ultracentrifugation in a sucrose density gradient was carried out according to conditions similar to those

described by Martin & Ames (1961). One ml. of a 1 % (w/v) solution of partially purified toxin was layered on a 27 ml. linear gradient of 5–40 % sucrose contained in a 25 by 75 mm. polyethylene tube. After centrifugation for 20 hr. at 25,000 rev./min, a hole was punched in the bottom of the tube and 27 fractions of approximately one ml. each were collected and estimated for protein. As shown in Fig. 6 the 12S component separated from the alpha toxin. The fractions comprising the 12S peak possessed no haemolytic activity while those of the alpha toxin peak had the expected activity of 12,000 to 13,000 haemolytic units/optical unit. One hundred μg. of the 12S component, or roughly 100 times as much as the LD 50 of alpha toxin, did not kill when injected intravenously into mice. Electrophoresis, in starch gel, of as much as 500 μg. of the isolated 12S component, followed by protein staining, revealed no band. A solution of 12S material containing 1 mg./ml. had a 280 mμ. absorbance of 0·60 but the u.v. absorption spectrum showed no well-defined peak at 280 mμ. nor elsewhere. It is concluded from these findings that the 12 S substance is probably not a simple protein and that it is unrelated to alpha toxin.

Characterization of 12S-free alpha toxin. For further characterization, partially purified alpha toxin free from 12S component rather than the alpha$_a$ component was used. This choice was made because alpha$_a$ represents only about one-fifth of the active material of partially purified toxin and it may not be representative, in all respects, of the whole. 12S-free toxin was prepared by ultracentrifugation in sucrose density gradients as described in the preceding paragraph. The fractions comprising the major peak were pooled and dialysed overnight in the cold against borate buffer to remove sucrose. The toxin was then salted out by dialysing the solution, in two steps, against 20 vol. of neutral 85 % saturated ammonium sulphate. The precipitate was stored under ammonium sulphate and dialysed free from ammonium sulphate as needed, against borate buffer.

Ammonium sulphate precipitated alpha toxin consisted microscopically of very small granules which were slightly birefringent but which exhibited no uniformity of shape. When the ammonium sulphate precipitated toxin was dissolved in borate buffer (pH 8·3) and dialysed in the cold against the same buffer to a final toxin concentration of 2 % (w/v), there developed a precipitate having a silky sheen. The particles comprising the precipitate were nearly invisible when examined by ordinary light microscopy, but were seen by dark field to consist of feathery lenticular units that were relatively uniform in size and shape. After washing in saturated ammonium sulphate solution and dissolving in buffer, the resulting solution had the same specific activity as had been found earlier for highly purified toxin preparations.

Solutions of toxin buffered at pH 8·3 retained full activity for several weeks when stored at $-15°$ but showed significant loss when stored for more than 2 weeks at 4°. The toxin could be kept for at least several months under ammonium sulphate at 4° without loss. Highly dilute solutions underwent rapid loss of activity but they could be effectively stabilized by gelatin or bovine serum albumin at 1 mg./ml. Dialysis of 0·1 % (w/v) solution against cold distilled water was accompanied by marked loss of activity and the loss was not prevented by M/5000 EDTA.

Ultracentrifugal analysis of a 0·6 % (w/v) solution of toxin revealed a single component having a sedimentation constant of 3·0 Svedberg units. The molecular weight, as determined by the Archibald method (Schachman, 1959) was 44,000;

this is an average of 42,930 at the meniscus and 45,150 at the bottom of the cell. The toxin contained less than 0·025% P and less than 1% carbohydrate (as glucose); its amino acid composition is shown in Table 4.

Absence of proteolytic activity. It has been suggested that alpha toxin is a proteolytic enzyme (Robinson *et al.* 1960). By the casein digestion method of Kunitz (1947) the amount of trichloracetic acid-soluble 280 mμ-absorbing material liberated from 10 mg. casein by 100 μg. of partially purified alpha toxin, or of the alpha$_a$ component, was negligible. Under the same conditions one-fiftieth this weight of crystalline trypsin liberated a measurable amount of acid soluble 280 mμ-absorbing material. Similarly, partially purified alpha toxin in a final concentration of 10 μg./ml. neither liquefied 1 % gelatin nor clotted milk. It is concluded that alpha toxin does not cause significant proteolysis of the substrates tested.

Table 4. *Amino acid composition of staphylococcal alpha toxin*

Constituent	mg./16 mg. N	Residues*
Cysteic acid	0	.
Taurine	0	.
Urea	0	.
Methionine sulphoxides	1·77	.
Hydroxyproline	0	.
Aspartic acid	13·2	44
Glutamine and asparagine	0	.
Threonine	6·15	23
Serine	5·22	22
Glutamic acid	7·15	21
Citrulline	0	.
Proline	1·91	7
Glycine	3·90	23
Alanine	2·52	12
Cystine	0	.
α-Amino-*n*-butyric acid	0	.
Valine	3·22	12
Methionine	1·92	10
Isoleucine	3·78	13
Leucine	4·34	15
Tyrosine	3·84	9
Phenylalanine	3·62	10
Ornithine	0	.
Ammonia	2·76	71
Lysine	7·68	23
Histidine	1·37	4
Arginine	3·89	10
Tryptophan	?	.
? Ethanolamine	0·21	.
Unidentified peak no. 1	0·176†	.
Unidentified peak no. 2	0·252†	.
Unidentified peak no. 3	0·292†	.

* Residues calculated for molecular weight 44,000; † Uncorrected leucine equivalents.

A- and X-proteins. As by-products of the purification of alpha toxin two proteins designated A and X were obtained in crystalline form. A was isolated on several occasions by dialysing against cold 60–70 % saturated neutral ammonium sulphate the fractions comprising the small protein peak to the right of alpha toxin (Fig. 1). It consisted of cottony aggregates of fine needles. Protein X was isolated in two

instances only, both times by dialysing the protein of or near Fraction 11 (Fig. 1) against cold 55 % saturated acid ammonium sulphate; it consisted of strongly birefringent needles. Each protein gave rise to a characteristic band on starch gel electrophoresis (Fig. 3*d* and 3*e*), protein A to a band on the cathode side of the origin, and protein X to a very faint band on the anode side. Both showed u.v.-absorption spectra characteristic of proteins. In an Ouchterlony plate with a commercial staphylococcal antitoxic serum, protein A yielded in 3 weeks a single faint band of precipitate. Intravenous injection into mice of about 100 μg. of either protein was followed by no obvious manifestation of illness. Neither protein has thus far been identified with any of the known extracellular products of staphylococcal growth.

DISCUSSION

The foregoing results indicate that staphylococcal alpha toxin is a protein and that its physical and chemical properties do not differ uniquely from those of many other proteins. Assuming a nitrogen content of 16 %, the amino acids and related compounds recovered from acid hydrolysed toxin accounted for 82 % of the weight of the toxin. The apparent absence of cystine is notable. Using the same figure for nitrogen content, 1 mg. of pure toxin is calculated to have 19,000 haemolytic units, and since cultures contain as much as 1600 haemolytic units/ml., (most or all of the activity appearing to be alpha) it follows that 1 l. of culture can contain as much as 84 mg. toxin, i.e. about 2 % of the dry weight of the cocci that produced it. The concentration of toxins in bacterial filtrates in general has been estimated to be from 5 to 20 mg./l. at most (van Heyningen, 1950). Many of the earlier reports on the purification of alpha toxin do not contain sufficient detail about methods to allow repetition of the work, and in most instances no indication has been given of the yields obtained relative to the amounts of crude toxin started with. The present paper describes methods which permit the preparation of toxin of about 70 % purity, in yields of about 40 %, and without the need for industrial facilities, and procedures are described whereby further purification can be achieved.

Three kinds of preparations were studied: (*a*) 'partially purified toxin', (*b*) an electrophoretic fraction of (*a*) designated alpha$_a$; (*c*) ultracentrifugally purified toxin derived from (*a*) designated 12S-free toxin. Although none of these preparations proved to be completely homogeneous when examined by a sufficient number of physical methods, their state of heterogeneity is operationally definable. The partially purified toxin contained two minor components which appear to be unrelated to toxin: a protein designated beta and demonstrable immunologically and by starch gel electrophoresis, and a substance designated 12S that was demonstrated ultracentrifugally. Together they account for most of the impurity. Aside from these substances, alpha toxin itself seems to be heterogeneous inasmuch as it is partially resolvable into several components by means of density gradient electrophoresis. So far as these have been studied, the electrophoretically separable components do not differ from each other in any very striking fashion as regards their biological properties, but further study of this point would be desirable. The results support the original and subsequently much discussed suggestion of Burnet (1929) that lethal, haemolytic and dermonecrotic effects are caused by a single toxin. The slowest moving electrophoretic fraction, alpha$_a$, was physically isolated and

although it was free from the beta impurity it still contained 12S material. Similarly, ultracentrifugally pure material can be obtained but contaminated with beta.

Aside from its gross biological effects, virtually nothing is known of the mode of action of staphylococcal alpha toxin. Contrary to an earlier suggestion, it does not appear to be a proteolytic enzyme. In view of the rapidity with which it brings about cell damage and in view of its remarkable lytic action on red blood cells, as distinct from diphtheria, tetanus and botulinum toxins which have neither of these properties, perhaps the best hypothesis is that it alters or disrupts cell membranes.

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Following completion of the manuscript of this paper, there appeared several publications concerned partly or wholly with the purification of staphylococcal alpha toxin. Madoff & Weinstein (1962) obtained by metal-ion precipitation, column chromatography and curtain electrophoresis, a highly active, unstable product which yielded a single line in Ouchterlony plates, and two bands when examined by means of starch gel electrophoresis. Kumar & Lindorfer (1962) and Kumar, Loken, Kenyon & Lindorfer (1962) obtained by means of curtain electrophoresis alone, alpha toxin in a purified state. There are, however, several discrepancies between the results of the latter authors and ours. Their product contained significant amounts of carbohydrate and had a sedimentation constant at 18·6° of 1·4, while our alpha toxin is free from carbohydrate and has a sedimentation constant at 20° of 3. Data on the kinetics of haemolysis induced by partially purified alpha toxin were reported by Lominski & Arbuthnott (1962).

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Cell-wall Constituents of Rickettsiae and Psittacosis-Lymphogranuloma Organisms

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SUMMARY

The taxonomic position of organisms belonging to the rickettsiae and psittacosis-lymphogranuloma groups is controversial. Although, like viruses, all pathogenic forms of these organisms are obligate intracellular parasites, in other respects they resemble bacteria. Since a cell wall, deriving its rigidity from mucopeptide, which contains the amino sugar muramic acid as a key constituent, has so far only been found in bacteria and the closely related blue-green algae, the presence of muramic acid in an organism may be used as a taxonomic criterion. The mucopeptides of bacterial cell walls are also often sensitive to lysozyme, so that dissolution by this enzyme serves as an indication of the presence of mucopeptide. Organisms of the rickettsiae and psittacosis-lymphogranuloma groups have been examined for the presence of muramic acid. Critical chemical tests have shown that this substance is present in organisms of both groups. Cell walls of *Rickettsia burnetii* were dissolved by lysozyme. In the light of these and other results the taxonomic position of these groups of organisms is discussed.

INTRODUCTION

The taxonomic position of organisms belonging to the rickettsia and psittacosis-lymphogranuloma groups is controversial (Andrewes, 1952; Bedson, 1959). All pathogenic forms of these organisms appear to be obligate intracellular parasites; no unequivocal evidence has been obtained that they multiply in cell-free media. In this respect these organisms resemble viruses, but in other important properties they are quite distinct from them. Viruses may be regarded as biological entities which contain protein and either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) but not both (Allison & Burke, 1962), and they do not multiply by binary fission; instead, the virus nucleic acid and protein constituents replicate more or less independently and are later reassembled (see Cohen, 1957; Schafer, 1959). Furthermore, viruses, unlike bacteria, are insensitive to penicillin, chloramphenicol and tetracyclines; in contrast, the rickettsia and psittacosis-lymphogranuloma organisms contain both DNA and RNA (Allison & Burke, 1962), and are sensitive to these antibiotics (Hurst, 1953). As discussed below, it seems highly probable that at least some typical rickettsia and psittacosis-lymphogranuloma organisms replicate by binary fission, although there is uncertainty about the exact mode of replication of other members of the psittacosis-lymphogranuloma group. In view of these difficulties there is a need for independent evidence to help clarify the

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affinities of rickettsiae and psittacosis-lymphogranuloma organisms. A contribution might be made by study of their cell walls, the composition of which has come to be recognized as characteristic of different groups of organisms. All the bacteria so far studied (Eubacteriales and Actinomycetales, excluding permanent L-forms) have been found to possess cell walls which contain relatively rigid mucopeptide. An essential component of mucopeptide is muramic acid (Cummins & Harris, 1956; Work, 1961). Some, but not all, mucopeptides also contain diaminopimelic acid. These two components, typical of bacterial cell walls, have not been found in protozoa or fungi, although both have been shown to occur in some blue-green algae (Work & Dewey, 1953; Frank, Lefort & Martin, 1962).

Evidence is presented in this paper that bacterium-like cell walls are present in rickettsiae and psittacosis-lymphogranuloma organisms. A new and highly sensitive technique for detection of muramic acid is also presented. A preliminary account of the results with rickettsiae has already been published (Allison & Perkins, 1960). Jenkin (1960) mentioned the presence of muramic acid in the feline pneumonitis organism (a member of the psittacosis-lymphogranuloma group), although no technical details were given. The results of these and other studies suggest that rickettsiae and psittacosis-lymphogranuloma organisms have evolved from bacteria by adaptation to intracellular replication; this conclusion is discussed.

METHODS

Rickettsiae organisms. Q fever (*R. (Coxiella) burnetii*); the Nine Mile strain isolated by Davis & Cox (1938) in Montana; number of passages is unknown. Scrub typhus *R. tsutsugamushi*, Karp strain, isolated in 1943 from the blood of an American soldier in New Guinea (Derrick & Brown, 1949); material from 101st yolk-sac passage was used. Rocky Mountain Spotted Fever, *R. rickettsii* (Maxcy, 1929) Bitterroot strain after an unknown number of passages in guinea pigs and eggs. Epidemic typhus, *R. prowazekii*, Madrid E attenuated strain, isolated in 1941 during an epidemic in Madrid (Clavero & Perez-Gallardo, 1943); material in the 267th yolk-sac passage was used.

Psittacosis-lymphogranuloma organisms. Psittacosis (Ornithosis) P-4 pigeon strain isolated in 1942 from a sick pigeon in New York (Smadel, Wall & Gregg, 1943), material from a 42nd yolk-sac passage was used. Mouse pneumonitis (Nigg) Hampstead strain, isolated by Andrewes (see Andrewes & Horstmann, 1949) and passaged in mouse lungs. Feline pneumonitis, strain isolated by Baker (1944), used after an unknown number of yolk-sac passages. Trachoma, strain TE-55, isolated by T'ang, Chang, Huang & Wang (1957) and passaged in the yolk sac.

Preparations of organisms. All organisms except mouse pneumonitis were propagated in yolk sacs of embryonated eggs, inoculated at 6 days and harvested after a further 6 days of incubation at 35°. Tests for bacterial contamination were negative. The yolk sacs were homogenized in Ten Broeck grinders, centrifuged at 2000*g* for 10 min. and the supernatant fluids centrifuged at 25,800*g* for 2 hr. at 2°. The deposits were resuspended in Gey's saline solution and used for preparation of cell walls or for chemical analysis. Mouse pneumonitis organisms were grown in mouse embryo cell cultures prepared as described by Allison & Armstrong (1960). They were harvested after 5 days of incubation at 36°, homogenized and centrifuged as

described above. Control material was prepared in the same way from uninfected yolk sacs.

Viruses. Concentrated suspensions of vaccinia and influenza viruses were also examined. Vaccinia virus (Lister egg-adapted strain) was grown in the chorio-allantoic membranes of embryonated eggs (Westwood, Phipps & Boulter (1957). The membranes were homogenized and centrifuged as already described. The titre of the final preparation was 8×10^9 pock-forming units/ml., from which it is likely that the number of virus particles was of the order of 10^{11}/ml. (Kaplan & Valentine, 1959). Influenza virus (strain Mel 1953) was grown in the chick embryo. Allantoic fluid was harvested 40 hr. after inoculation of 0·05 ml. of seed virus suspension into the allantoic cavity of 10-day chick embryos and incubated at 35°. Centrifugation was at 38,000*g* for 30 min. The deposit was resuspended in 0·05 M-tris saline (pH 7·0). The number of virus particles in the final suspension, estimated from the haemagglutination end-point (Donald & Isaacs, 1954) was 8×10^{11} particles/ml.

Preparation of cell walls. These were prepared by a technique like that described by Schaechter *et al.* (1957*b*). Suspensions of *Rickettsia burnetii* were incubated at 45° with 1 % (w/v) sodium deoxycholate in 0·1 M-tris buffer (pH 7·0) for 4 hr. with constant stirring. The cell-wall preparations were centrifuged at 50,000*g* for 30 min. and resuspended in 0·1 M-tris buffer (pH 8·0). In some experiments the purified cell walls were incubated with lysozyme in the presence of ethylenediaminetetra-acetate under the conditions described below.

Detection of muramic acid. After extraction of lipids, suspensions of organisms were precipitated with 5 % (w/v) trichloroacetic acid. Much protein was removed at this stage by incubation with pepsin (1 mg./ml. in 0·05 N-HCl) and then with trypsin (1 mg./ml. in 0·05 M-phosphate buffer, pH 7·6); these enzymes do not dissolve bacterial mucopeptides (Cummins & Harris, 1956). The residues were hydrolysed in sealed ampoules with 4N-hydrochloric acid at 105° for 4 hr. After removal of excess hydrochloric acid *in vacuo* hexosamines in the hydrolysates were concentrated by chromatography in a column of Dowex 50 (H^+). The hexosamine peak was transferred to a charcoal + Celite column, and glucosamine was eluted with water (Perkins & Rogers, 1959). Substances eluted by 10 % (v/v) ethanol in water were subjected to paper chromatography in butanol + pyridine + water (6 + 4 + 3 by vol.). A sample of the material eluted from the expected position of muramic acid was submitted to the Elson-Morgan test as described by Rondle & Morgan (1955) except that, to increase sensitivity, the final volume in the reaction mixture was 1·5 ml. Absorption curves were drawn from measurements with the spectrophotometer (Unicam S.P. 500) using small cuvettes with a 2 cm. light path. Muramic acid gives a maximum absorption at 510 mμ or, on standing overnight, 505 mμ (Crumpton, 1959).

Identification of microgram quantities of muramic acid. When the sample of material tested by the Elson–Morgan reaction gave an absorption curve with a maximum well below 530 mμ, suggesting that muramic acid might be present, the remainder of the material was treated as follows. To a sample dissolved in 20 μl. water was added 2·5 μl. pyridine followed by 0·1 μl. 1-^{14}C-acetic anhydride (specific activity 1·7 mc./mmole) from a graduated capillary tube (total capacity 0·7 μl.). The acetylation reaction was allowed to take place at room temperature for 1 hr. The sample was then dried *in vacuo* over soda-lime, and 0·2 ml. of 10 % aqueous

acetic anhydride (non-radioactive) was added and removed *in vacuo*; this step was repeated three times. At this stage 50 μg. *N*-acetylmuramic acid was added as a carrier, to the sample and also to a control tube which had been treated in the same way throughout. The samples were transferred to the origin of a paper chromatogram and run in butanol + acetic acid + water (63 + 10 + 27 by vol.). In this solvent *N*-acetyl-muramic acid runs twice as far as *N*-acetylglucosamine. The position of *N*-acetylmuramic acid was determined from a marker strip by using the spray system of Partridge (1948) except that the final heating after spraying with Ehrlich's reagent was omitted. Radioactivity in the corresponding region of the chromatogram was detected by radioautography. The radioactive region was eluted and counted on a planchette with an end-window counter.

Under these conditions samples of 0·2, 1 and 10 μg. (0·8, 4, and 40×10^{-3} μmole) of muramic acid originally present before the addition of carrier gave final counts of 61, 295, and 3022 counts/min., respectively. Since the counting efficiency was such that 1 μc. gave 2×10^5 counts/min., this corresponded to a 45% over-all yield of labelled *N*-acetylmuramic acid.

In some experiments the samples of radioactive *N*-acetylmuramic acid were converted to a Morgan–Elson chromogen, which retains the acetyl group, by heating in a sealed tube with aqueous triethylamine at 100° for 8 min. (Perkins, 1960*a*). The reagent was removed *in vacuo*, the sample transferred to a paper chromatogram (solvent *n*-butanol + pyridine + water; 6 + 4 + 3 by vol.) and run overnight. The site of radioactivity was found by radioautography; subsequently the position of the chromogen was determined by spraying with Ehrlich's reagent in butanol (Partridge, 1948). When the resulting purple spot coincided exactly with the area that contained radioactivity, it was concluded that the parent labelled-compound was indeed muramic acid.

Conversion of muramic acid to a substituted pentose. Samples of the material isolated from chromatograms and suspected to be muramic acid were heated at 100° for 30 min. in sealed ampoules with ninhydrin in aqueous pyridine (Stoffyn & Jeanloz, 1954). Authentic samples of muramic acid were treated in the same way. The reaction mixtures were dried and run on paper chromatograms in butanol + pyridine + water overnight, together with markers of the four pentoses. The dried paper was sprayed with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950).

Incubation of residues from cultures of Rickettsia burnetii *with lysozyme.* Samples (5 mg.) of the residue obtained from cultures by treatment with fat solvents, trichloroacetic acid and proteolytic enzymes, as described above, were incubated in tris buffer (pH 8·0, 0·02 M), sodium chloride (0·02 M) and ethylenediaminetetraacetate (0·002 M) for 2 hr. at 37°, with or without lysozyme of final concentration 50 μg./ml. The residue was removed by centrifugation at 11,000*g* and the samples concentrated *in vacuo* to 0·25 ml. The reaction for *N*-acetylhexosamines was then done as described by Aminoff, Morgan & Watkins (1952), except that the heating period was 12 min. since it is known that the lysozyme digestion products of mucopeptide give the highest yield of colour under these conditions (Perkins, 1960*b*). A purple colour with an absorption maximum at 585 mμ was considered to be indicative of the presence of liberated *N*-acetylhexosamine end-groups.

RESULTS

Incubation of Rickettsia burnetii *with lysozyme*

Purified cell-wall preparations of *Rickettsia burnetii* were digested with lysozyme in the presence of ethylenediaminetetra-acetate. This treatment led to the disappearance of cell walls recognizable as such in the electron microscope. The conditions used were like those under which lysozyme will digest the walls of some Gram-negative bacteria (Repaske, 1958). No precipitation was observed, so that it seems unlikely that aggregation in the presence of the highly basic protein lysozyme was responsible for the disappearance of the cell walls. Under suitable conditions the cell walls of some Gram-positive and some Gram-negative bacteria are attacked by egg-white lysozyme with liberation of material which gives a positive Morgan–Elson reaction for *N*-acetyl-hexosamines. This reaction is due to the liberation of reducing groups of the walls (Perkins, 1960*b*; Salton & Ghuysen, 1960). Since other evidence suggested that muramic acid was present in *R. burneti*, we attempted to determine whether preparations from this organism could undergo a similar reaction with lysozyme. When these preparations were treated with lysozyme (as described in Methods), material giving an absorption maximum at 585 mμ in the Morgan–Elson reaction for *N*-acetylhexosamines was obtained. Although bacterial cell-wall mucopeptides containing muramic acid are not the only polymers hydrolysed by lysozyme (for instance chitin, a β(1-4)-linked poly *N*-acetylglucosamine, is attacked to some extent as Berger & Weiser (1957) showed), the result with *R. burneti* is at least consistent with the other evidence for the presence of muramic acid described below.

Chemical evidence for the presence of muramic acid

So far as is known muramic acid is a compound which is specific to the mucopeptides found in bacterial cell walls. Thus, when an organism is shown to contain muramic acid it is reasonable to assume that it possesses a cell wall which contains a mucopeptide resembling that of bacteria. Preparations of rickettsiae and the psittacosis-lympho-granuloma group of organisms were therefore examined for the presence of muramic acid. Hydrolysed samples were fractionated as described, and the material likely to contain muramic acid was subjected to the Elson–Morgan reaction for amino sugars. After colour development the tubes were allowed to stand overnight, and the light absorption then measured over the range 490 to 540 mμ. Absorption curves typical of muramic acid were found in specimens derived from several of the organisms studied (see Table 1), as shown in the curves in Fig. 1; these results suggested that muramic acid was present in the preparations. The chemical identity of the isolated material was examined further.

The *N*-acetylated derivatives of hexosamines are compounds which yield the characteristic Morgan–Elson reaction; this reaction can be used to detect their presence on chromatograms. The material isolated from the rickettsiae and psittacosis-lymphogranuloma organisms and suspected of being muramic acid was therefore converted to its *N*-acetyl derivative. Because of the small quantities of substance available, the reaction was performed with 1-^{14}C-acetic anhydride of high specific activity and the identity of the labelled material with non-radioactive

N-acetylmuramic acid added as carrier was traced chromatographically. The radioactivity due to the labelled acetyl group ran in the same position as the *N*-acetylmuramic acid detected by the Morgan–Elson reaction (Table 1). Further confirmation that radioactivity present in the region corresponding to *N*-acetylmuramic

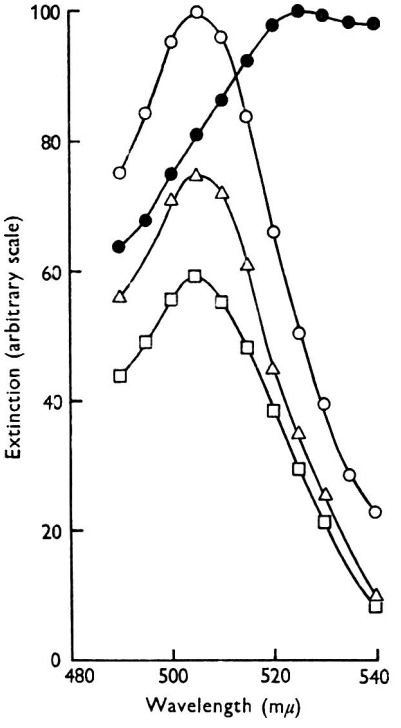
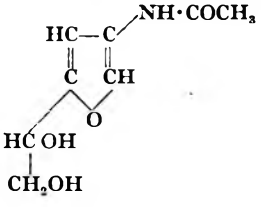


Fig. 1. Absorption curves of substances in the Elson–Morgan reaction. Samples were treated by the procedure of Rondle & Morgan (1955) and the final reaction mixtures were allowed to stand overnight before preparation of the absorption curves. ●—●, Glucosamine; △—△, muramic acid; ○—○, material from *R. burnetii*; □—□, material from mouse pneumonitis (Nigg) organism.

acid was indeed due to this compound was obtained as follows. *N*-acetylhexosamines heated with alkali at 100° yield the chromogens of the Morgan–Elson reaction (1934), the chief of these being the compound:



(Kuhn & Kruger, 1957), which retains its acetyl group. Heating *N*-acetylmuramic acid with alkali produces the same chromogen (Perkins, 1960*a*). If, therefore, material containing radioactivity due to an acetyl group can be converted by heating in alkali to a Morgan–Elson chromogen which is also radioactive, it is

probable that the original compound was an *N*-acetylhexosamine. If, also, this material came from chromatographically isolated *N*-acetylmuramic acid, then it is almost certain that the compound originally acetylated with radioactive acetic anhydride was muramic acid. The radioactive spots of *N*-acetylmuramic acid described above were eluted from the paper and heated with aqueous triethylamine to produce the chromogen, which was then run on another chromatogram. In all instances this procedure gave a radioactive spot which coincided with the chromogen, thus confirming that the radioactive acetyl group apparently belonging to *N*-acetylmuramic acid on the first chromatogram was indeed combined in an acetylhexosamine. No other type of acetyl compound would be converted to Morgan–Elson chromogen in this way. The results of this test are shown in Table 1.

Table 1. *Tests for muramic acid applied to organisms of the rickettsia and psittacosis-lymphogranuloma groups*

R. burneti showed conversion to substituted pentose by ninhydrin.

Organism	Chemical test as described in the text: Elson–Morgan reaction for muramic acid	Chemical test as described in the text: Conversion to radioactive *N*-acetylmuramic acid and chromogen
Rickettsia		
R. burnetii	+	+
R. tsutsugamushi	+	+
R. rickettsiii	+	.
R. typhi (*mooseri*)	+	.
R. prowazeki	±	.
Psittacosis-lymphogranuloma group organisms		
Psittacosis	+	+
Mouse pneumonitis (Nigg)	+	.
Feline pneumonitis	+	+
Trachoma	±	+

+ indicates a positive reaction. A point indicates that the test was not applied, usually because of lack of total material. ± indicates a weak reaction only.

Since the validity of this work largely depends upon the positive chemical identification of small quantities of substance isolated from the organisms in question, with authentic muramic acid, when sufficient material was available a further critical reaction was performed. When 2-amino-2-deoxyhexoses are heated with ninhydrin they lose their reducing groups; the $-CHNH_2-$ group at C_2 is converted to an aldehyde group, so that the corresponding pentose results (Stoffyn & Jeanloz, 1954). Similarly, muramic acid yields a product which is presumably 2-*O*-carboxyethylarabinose. Samples of the substance suspected to be muramic acid and samples of the authentic compound were treated in this way, and the final products run on a paper chromatogram. Spraying with *p*-anisidine hydrochloride revealed that muramic acid and the test material both yielded slow running spots of the same colour in the same position ($R_{\text{arabinose}} = 0{\cdot}46$). This

result gave additional chemical evidence for the identification of muramic acid isolated from rickettsiae. The results obtained in the chemical tests for muramic acid applied to the organisms examined are given in Table 1. They show that muramic acid is present in the rickettsiae and in the members of the psittacosis-lymphogranuloma group of organisms, as in bacteria. The same tests applied to yolk-sac preparations, uninfected tissue cultures and vaccinia and influenza viruses all gave negative results.

DISCUSSION

The observations presented show that a bacterium-like cell wall consisting of mucopeptide containing muramic acid is present in rickettsiae and psittacosis-lymphogranuloma organisms. Preparations of cell walls of *Rickettsia typhi* (*mooseri*) have been shown to contain amino acids, glucose, glucuronic acid and galactose (Schaechter *et al.* 1957*b*), although these components are not confined to the mucopeptides of bacterial cell walls. Jenkin (1960) reported the presence of muramic acid in cell-wall preparations of meningopneumonitis virus. The presence of a cell wall of bacterial type is also evident in electron micrographs (Plotz, Smadel, Anderson & Chambers, 1943; Wissig, Caro, Jackson & Smadel, 1956; Stoker, Smith & Fiset, 1956; Armstrong, Valentine & Fildes, 1963). In air-dried preparations of suspensions of psittacosis-lymphogranuloma organisms the cell wall has a characteristic appearance like the brim of a hat lying flattened around the shrivelled central mass of the organism. In ultra-thin sections the cell wall appears as a distinct layer outside the delicate cytoplasmic membrane of rickettsiae and psittacosis-lymphogranuloma organisms. These cell walls resemble closely those seen in ultra-thin sections of bacteria (Glauert, Brieger & Allen, 1961). The free acidic groups in the cell wall might account, at least in part, for the well-known basophilia of the organisms when studied by conventional staining techniques. The fate of the cell wall during intracellular replication of the organisms is not yet known. It is widely accepted that rickettsiae undergo binary fission in the cytoplasm of infected cells, as shown by the time-lapse cinematographic studies of Schaechter, Bozeman & Smadel (1957*a*). If this be so, it is reasonable to suppose that a typical and intact cell wall is present throughout the cycle of replication of rickettsiae.

The situation is less certain in the psittacosis-lymphogranuloma group. Bedson (1959) concluded that the organisms pass through a series of developmental forms larger than the 0·2μ elementary bodies when multiplying by binary fission. On the other hand, electron micrographs of thin sections of cells infected with high multiplicities of psittacosis organisms (Tajima, Nomura & Kubota, 1957) and trachoma organisms (Armstrong *et al.* 1963) indicate that an early stage of development is the formation of comparatively large bodies or plaques. In the first 24 hr. after infection no definite cell walls surrounding the small invading organisms are seen, and although the photographs are difficult to interpret unambiguously, the appearances suggest that there may be an intimate association of the organisms with one another and even with the host cell cytoplasm. Later newly formed small bodies with discrete cell walls appear in large numbers and seem to undergo typical binary fission. One interpretation of these findings is that the relatively rigid cell walls are temporarily lost during the early phases of intracellular replication, the organisms being analogous to the L-forms of bacteria, and correspondingly pleo-

morphic. With the re-formation of cell walls later would come the regular appearance of the 'elementary bodies' which are stable and highly infectious.

Another argument has been presented to support the view that the psittacosis organism undergoes a virus-like replication. Tanami, Pollard & Starr (1961) showed that multiplication of psittacosis organisms in tissue-culture cells was inhibited by 5-fluoro-2-deoxyuridine (FUDR) and FU (5-fluorouracil). From the effects of adding FUDR and FU at different times during the course of development they concluded that synthesis of psittacosis DNA preceded synthesis of psittacosis protein by about 7 hr. They therefore suggested that psittacosis DNA replicates independently and is later incorporated into newly formed organisms. However, several points about this investigation make these conclusions unconvincing. In the first place, the inhibitory effect of FUDR was annulled by uracil in only slightly higher concentrations than by thymidine. This is difficult to reconcile with a specific FUDR inhibition of thymidylate synthetase (see Rich, Saslaw & Eidinhoff, 1960). Furthermore, the conclusion of Tanami *et al.* (1961) that psittacosis DNA synthesis occurs relatively early is at variance with the observations of Becker, Mashiah & Bernkopf (1962) that most DNA synthesis (incorporation of labelled thymidine) occurred late in the intracellular developmental cycle of the related trachoma organism.

There is another quite different inhibitory effect of these drugs which may account for such results. FU powerfully inhibits not only RNA and protein synthesis (Tanami *et al.* 1961), but also the synthesis of the mucopeptide component of bacterial cell walls, which takes place through uridine intermediates (Rogers & Perkins, 1960). Thus the inhibition by FU of the formation of small infectious elementary bodies of the psittacosis organism, despite accumulation of large amounts of RNA (Starr, Pollard, Tanami & Moore, 1960; Pollard, Moore, Tanami & Starr, 1961), may well be because mucopeptide cell walls are required for this process.

Penicillin is another compound known to act on bacteria by inhibiting the synthesis of cell-wall mucopeptides (see Rogers, 1962). It has been suggested that this action is specifically due to a structural relationship between penicillin and *N*-acetylmuramic acid (Collins & Richmond, 1962). Thus the observation that penicillin also prevents the formation of infectious elementary bodies of the psittacosis organism (Starr *et al.* 1960; Pollard *et al.* 1961) may be due to its specific action on the synthesis of mucopeptide structures containing muramic acid like those of bacteria.

In view of these and all the other findings mentioned above it seems difficult to escape the conclusion that rickettsiae and psittacosis-lymphogranuloma organisms have closer biochemical affinities with Gram-negative bacteria than with the animal viruses. Perhaps they have evolved from bacteria by loss of certain enzymes necessary for independent replication, and so have become obligate intracellular parasites. Bacteria such as *Mycobacterium lepraemurium* are also restricted to intracellular replication. The apparent inability of suspensions of rickettsiae to utilize glucose, even though they metabolize tricarboxylic acid cycle intermediates (Bovarnick & Snyder, 1949; Price, 1953), supports this suggestion. There is a substantial body of opinion that all rickettsiae have evolved from insect parasites, and among the rickettsiae which infect insects there seems to be no sharp line of distinction between intracellular and free-living forms (Steinhaus, 1946).

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The Examination of Sulphur Auxotrophs: A Warning

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SUMMARY

The chemical instability and reactivity of certain biologically important sulphur derivatives are recalled and their relevance to the study of sulphur auxotrophs is pointed out.

In the early stages of the biological utilization of inorganic sulphur sources the compounds involved are often relatively unstable. In the conditions used for the examination of their specific biological effects the compounds themselves, even when initially pure, may undergo chemical change by reaction with other compounds in growth media or the oxygen of the air, for example. Many inorganic sulphur compounds, particularly those which involve intermediate oxidation states of the sulphur, are unstable (see for example, Pollard & Jones, 1958) and exchange reactions readily occur between these compounds and organic -SH or -SS-compounds. Such hazards are familiar to many workers in the field of microbial sulphur metabolism (e.g. Vishniac & Santer ,1957; Szczepkowski, 1958). Deductions about metabolic pathways which involve such sulphur compounds and are based on growth and metabolic experiments, particularly those of long duration, should be made circumspectly. Experiments of this kind should include controls to assess the stability of the compounds in the test conditions used. Some publications concerned with the metabolism of microbial mutants which require sulphur compounds (parathiotrophs) have not explicitly described such controls. A short remainder of some of the experimental hazards involved in studying the biological activity of sulphur compounds may not be unhelpful.

Sulphite. Analytical grade sodium sulphite contains sulphate which may reach as much as 75 mole% in old opened bottles (Postgate, 1952). Solutions aerated at physiological pH values oxidize spontaneously to sulphate; for example, a 0·1 M solution of sodium sulphite (Na_2SO_3) in physiological saline buffered with phosphate fell to 0·07 M after shaking in air for 1 hr. at 37° and to 0·022 M after $2\frac{1}{2}$ hr. The autoxidation of sulphite is catalysed by metals including some (Cu, Zn) that are normal 'trace' constituents of microbial media. Copper is known to be catalytically active at 10^{-9} g./l. (Travers, 1931); its effect is antagonized by certain anti-oxidants, some of which (e.g. sucrose) may be present in microbial media.

Metabisulphite. Salts of the ion S_2O_5'' exist in the solid state and in strong solutions (Golding, 1960) but become indistinguishable from sulphites in solution because of hydrolysis of the ion to sulphite and bisulphite. Buffered to neutral pH values, metabisulphite yields the same ionic species as does sulphite. Commercial metabisulphite preparations usually contain sulphate, though in smaller proportions than sodium sulphite preparations.

Thiosulphate. This ion is reasonably stable around pH 7. Below pH 5 it hydrolyses in a complex manner to yield sulphite, free sulphur dioxide and pentathionate; sulphur and H_2S may be present among its acid hydrolysis products (Pollard & Jones, 1958); in aerated alkaline conditions it oxidizes to sulphate. At neutral pH values thiosulphate reacts with thiol groups (such as will be present in many biological extracts and microbial inocula) to yield free H_2S, organic S-sulphonic acids (e.g. cysteine-S-sulphonate + H_2S is formed from thiosulphite and cysteine) and possibly polythio-homologues of these (Szczepkowski, 1958).

Tetrathionate. This undergoes reactions like thiosulphate with thiol groups, yielding sulphite in place of H_2S.

Dithionite ('*hydrosulphite*'). The dithionite ion S_2O_4'' is extremely unstable in solution; in air, autoxidation to sulphite takes place rapidly (at pH 7 a 0·1 M solution of commercial sodium dithionite was free from this ion after 10 min. shaking in air at room temperature). Even in the absence of oxygen dithionite solutions are unstable and hydrolyse to a mixture of thiosulphate, sulphite and free sulphur. Sodium dithionite is only stable in the anhydrous form; commercial preparations rarely claim to be pure.

Sulphoxylate. The ion SO_2'' exists out of solution only as the cobaltous salt. Sodium formaldehyde sulphoxylate is a half ester and yields no sulphoxylate ion in solution.

Sulphide. The sulphide ion at pH 7 is largely hydrolysed to free H_2S (van Niel, 1931), which as a gas evaporates rapidly from solution. For example, when a drop of 0·1 M-Na_2S solution was placed on the surface of agar buffered at pH 7·3, no sulphide was detectable with lead acetate at the site of the drop after 15 min. at 37° in air. Where evaporation is slow, sulphide solutions photo-oxidize to form sodium thiosulphate; trace metals catalyse formation of free sulphur. In the author's experience analytical grade Na_2S, even when stored in black bottles, has always contained sulphate. Many microbes which have no specialized inorganic sulphur metabolism (e.g. yeasts, *Sphaerotilus*) oxidize sulphide to free sulphur (Skerman, Demantjev & Skyring, 1957).

Cysteine. For completeness I recall the well-known fact that cysteine autoxidizes in air to cystine at values below pH 2; even at refrigerator temperature (4°) slow autoxidation to cystine takes place in acid solution over a week or two; this autoxidation is catalysed by metal ions (Cu, Hg).

Cysteine-S-sulphonic acid. This substance has recently assumed importance in suggested pathways of synthesis of sulphur-containing amino acids. In solution it undergoes a hydrogen ion-dependent dissociation of the general type:

$$2RSSO_3H \rightleftharpoons RSSR + 2HSO_3'$$

(Cecil & McPhee, 1955; Cecil & Wake, 1962).

Cysteinesulphinic and *Cysteinesulphenic acids.* These compounds are subject to slow spontaneous hydrolysis (rate depending on pH value) to yield disulphides and inorganic sulphur-containing anions such as sulphite (see Eldjarn, 1955). Such reactions are special cases of $RSSR + SXH \rightleftharpoons RSSX + RSH$ equilibria, which proceed rapidly and spontaneously at physiological pH values and temperatures (Kolthoff, Stricks & Kapoor, 1955). Eldjarn & Pihl (1956) recorded that cysteamine and glutathione established such an equilibrium in 1·25 to 3 min. at pH 7·3 and 37°.

Polythionates having 3, 5 or 6 sulphur atoms are difficult to obtain pure and are indifferently stable in aqueous solution. *Sulphane monosulphonates* (Schmidt, 1957) probably do not exist in aqueous environments.

Dithionate (S_2O''), *sulphate*, *cystine* and *cysteic acid* are stable and may be obtained pure.

DISCUSSION

The data quoted above, except those with specific citations, were obtained from text-books of chemistry (e.g. Sidgwick, 1950). Obviously, in experiments involving the use of these compounds with biological systems exposed to air in physiological conditions of pH value and temperature, rigorous controls are needed to evaluate their stability, and consequently the significance of any biological effects observed. Such controls have not always been mentioned in published work. For example, Clowes (1958) incubated sulphide, sulphite, cysteine or dithionite in air at 37° in media in Petri dishes, used substances which contained sulphate as impurity (sulphite, dithionite) for competition experiments involving labelled sulphate, and distinguished results with metabisulphite from sulphite in a buffered system. Nakamura (1962) incubated sulphite, thiosulphate, sulphide, cysteine or cysteine-S-sulphonic acid aerobically for 8 days with spores of *Aspergillus nidulans* in physiological conditions. Hockenhull (1949) accepted sodium formaldehyde sulphoxylate as a source of sulphoxylate; Shepherd (1958) examined some highly autoxidizable sulphur derivatives by a procedure involving incubation in air for 48 hr. at 37° with media in Petri plates. It would be invidious to discuss the relevance of these criticisms to the scientific value of the papers cited since in some cases the authors may have been so well aware of the hazards mentioned that they felt it unnecessary to mention that they included appropriate controls.

The purpose of this note is to remind workers in the field of sulphur auxotrophy that the hazards listed above are real, and that publication of explicit controls to take care of them is necessary if experimental work on sulphur auxotrophy is to be accepted as significant.

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Effects of Glucose on the Production by *Escherichia coli* of Hydrogen Sulphide from Cysteine

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SUMMARY

Glucose and certain other sugars accelerated hydrogen sulphide production from L-cysteine by washed cells of *Escherichia coli* (strain Crookes) which had been grown in the presence of L-cysteine. On the other hand, glucose or some other sugars in protein hydrolysate media containing L-cysteine suppressed the synthesis of an enzyme(s) which mediates the formation of hydrogen sulphide from L-cysteine. Glucose accelerated sulphide formation from L-cysteine by sonicated preinduced cells, although activity was unstable to such treatment. Both effects of glucose were influenced by the amino acid content of the medium. Sulphide production probably resulted through the action of cysteine desulphydrase; certain evidence suggested that a transaminase linked to β-mercaptopyruvate desulphurase also may have functioned. Apparently glucose repressed the induction of one or more enzymes concerned with cysteine degradation.

INTRODUCTION

The ability of glucose and other fermentable carbohydrates to suppress the formation of a variety of inducible enzymes in bacteria is well established, although the mechanism has not been elucidated fully (Cohn & Horibata, 1959; Magasanik, 1957; Magasanik, Magasanik & Neidhardt, 1959; Magasanik, 1962; Pardee, 1961; Pardee & Prestidge, 1961). During the course of an investigation of the production of hydrogen sulphide from L-cysteine it was noted that the presence of glucose in tryptone broth cultures of *Escherichia coli* (strain Crookes) presumably inhibited the induction of cysteine desulphydrase, an inducible enzyme (Fromageot, 1951), whereas glucose stimulated hydrogen sulphide production from cysteine by preinduced organisms (cf. Desnuelle & Fromageot 1939). This report will describe these effects of glucose and certain other fermentable carbohydrates and will present some evidence for nutritional regulation of the production of the enzyme(s) which liberate hydrogen sulphide from L-cysteine.

METHODS

Micro-organism. Escherichia coli, strain Crookes (ATCC 8739).

Culture media. Two basal media were used: (i) a mineral salts defined medium (Davis & Mingioli, 1950) to which glucose (generally 0·5 %, w/v) and/or monosodium glutamate (0·5 or 1·0 %, w/v) were added; and (ii) a crude complex medium containing (%, w/v) Bacto-tryptone (1·0) and K_2HPO_4 (0·12). The media were

dispensed in 250 ml. quantities in 500 ml. Erlenmeyer flasks which were stoppered with gauze-wrapped cotton and autoclaved for 20 min. at 121°. Glucose was autoclaved separately as an aqueous 10 % (w/v) solution and, when needed, added aseptically to the sterilized medium. L-cysteine.HCl was sterilized by filtration of a freshly prepared aqueous solution through a UF sintered glass filter and added aseptically (0·05 or 0·1 %, w/v) to the sterile medium prior to inoculation. Other modifications of the media will be described in Results. The medium was adjusted to the desired pH value with sterile NaOH or HCl, just before inoculation.

Production and preparation of organisms. Stock cultures of *Escherichia coli* were maintained by monthly transfer on nutrient agar slopes. The inoculum for 250 ml. of medium was 0·75 ml. of a 24 hr. culture grown at 37° in a medium of the same composition as that in the flask. The inoculated flasks of media were incubated at 37° under static conditions, generally for 12–16 hr. The organisms were harvested by centrifugation for 30 min. at 1800 *g* and resuspended in 5 ml. de-ionized water. After a second washing in de-ionized water the organisms were stored in the packed state at 4° for no more than 4 hr. prior to assay for cysteine desulphydrase activity. Further washing or prolonged storage, especially in the unpacked state, markedly decreased any desulphydrase activity.

Assay for hydrogen sulphide production. One volume of a freshly prepared concentrated buffered substrate solution (16 or 32 mM L-cysteine.HCl, 0·1 or 0·2 M-KH_2PO_4, 0·05 M-$MgSO_4$; adjusted to pH 6·4 with 0·1 N-NaOH) was mixed with four volumes of washed organisms resuspended from the packed state in de-ionized water to a density of 0·75–1·5 mg. dry weight. Replicate 5 ml. quantities of the substrate-organism suspension were placed in Pyrex tubes (12 × 100 mm.) with rubber stoppers to minimize entrance of air and incubated at 37° in a water bath. At intervals tubes were removed, 0·25 ml. of a 40 % (w/v) solution of NaOH was added to terminate the reaction and the stoppers were replaced. The tubes were gently agitated for 20 min. at room temperature, and the cell-free supernatant fluid recovered after centrifugation for 10 min. at 2800 *g*. The supernatant fluid was assayed for hydrogen sulphide within 2 hr. by a modification of the method of Delwiche (1951): 1–4 ml. of the supernatant fluid was mixed with 2 ml. of 2N-NaOH, 4 ml. lead acetate reagent (Pb acetate, 1·0 g.; glacial acetic acid, 2·5 ml.; gum arabic, 2·5 g.; distilled water to 1 l.) was added and the resulting brownish yellow colour was measured promptly in a Klett–Summerson photoelectric colorimeter (filter 54) previously adjusted to 100 % transmission with a reagent blank. The readings were compared with those of an alkaline sodium sulphide solution standardized by iodometric titration (Bethge, 1953). Over a limited range in concentration of sulphide, a plot of colorimeter readings against μM-sulphide yielded a straight line. The assay was simple and reproducible (replicates agreed within limits of ± 1 % two-thirds of the time), being sensitive to 0·1 μg. H_2S/ml. substrate solution.

Assay for ammonia production. Portions of the same washed suspensions as employed for the assay of hydrogen sulphide were tested on occasion for their ability to produce ammonia from L-cysteine; in such experiments the reaction was terminated with 0·25 ml. of 50 % (w/v) trichloroacetic acid. Ammonia was released by K_2CO_3 solution, absorbed in 1 % (w/v) boric acid contained in Pyrex microdiffusion dishes (64 mm. diameter, 15 mm. deep, 37 mm. diameter centre well),

and titrated with 0·002 N-HCl (Conway, 1950). The distilled water and reagents were checked for any traces of ammonia and a suitable correction applied when necessary.

Terminology. The term cysteine desulphydrase will connote the biological formation of hydrogen sulphide from L-cysteine, although other pathways of sulphide production from cysteine may have functioned under certain conditions (see Discussion). Cysteine desulphydrase activity was expressed as Q_{H_2S} ($\mu g.H_2S$ produced/mg. dry cells/hr.) estimated from the amount of product formed over a period of time during which not more than 20 % of the cysteine sulphur appeared as sulphide.

RESULTS

Optimal cultural conditions for cysteine desulphydrase activity. Although cystine and cysteine are present in Bacto-tryptone, supplementation of the tryptone broth with L-cysteine.HCl encouraged desulphydrase production (cf. Kallio & Porter, 1950). Preliminary experiments showed that organisms of highest specific activity were obtained from tryptone broth (*a*) devoid of glucose, (*b*) supplemented with 0·1 % (w/v) L-cysteine.HCl, (*c*) adjusted to pH 5·5 and (*d*) incubated at 37° for 10–16 hr. In the defined medium, cysteine—or possibly sulphide arising from degradation of this amino acid—was inhibitory to growth when serving as the sole energy source, although the meagre number of organisms which appeared after several days of incubation at 37° had good desulphydrase activity. Supplementation of the defined medium containing L-cysteine (0·05 %, w/v) with glucose (0·1 %, w/v) partially overcame the toxicity of cysteine. Monosodium glutamate or aspartic acid (0·1 %, w/v) supported excellent growth in the cysteine-supplemented defined medium, but did not produce organisms with good cysteine desulphydrase activity unless the medium was fortified also with glucose. The initial pH of the defined medium was generally adjusted to 6·0–6·4, being not as critical toward formation of cysteine desulphydrase as was noted for tryptone broth.

Effect of test conditions. In addition to the nature of the culture medium, the composition of the substrate solution in which washed cells were suspended for determination of desulphydrase activity was important. Since the enzymic desulphydration of cysteine is an anaerobic process, the presence of oxygen or oxidative processes may lead to non-enzymic loss of sulphide and cysteine, or influence the pathway of dissimilation (Kallio, 1951; Tamiya, 1954). Incubation in the small rubber-stoppered tubes, almost completely filled with substrate + organism suspension, apparently maintained conditions sufficiently anaerobic for good activity. Flushing with nitrogen before incubation resulted in no improvement of activity or stability of the desulphydrase system. Additions to the buffered substrate of mercaptoethanol or ascorbic acid had no detectable effect, while sodium thioglycolate increased, after a delay, the yield of sulphide, possibly by non-enzymic degradation of thioglycolate (Olitzki, 1954). On the other hand, reduced gluthathione lowered the output of sulphide. Although the cysteine codesulphydrase is a derivative of vitamin B_6, additions of pyridoxine, pyridoxal or pyridoxal phosphate to the substrate solution had no effect on the desulphydrase activity of induced cells. (Likewise, additions of B_6 to the culture medium had no effect on induction.) In agreement with the reports of a number of investigators, arsenate, cyanide, monoiodoacetate and azide, at

concentrations of 10^{-3}M in the substrate solution, were found to be inhibitory to cysteine desulphydrase. Fluoride also was highly inhibitory, although it has been reported to have no influence on this enzyme in cell-free liver preparations (Fromageot, Wookey & Chaix, 1940).

Table 1. *Effects of glucose in the medium and in the cysteine substrate solution on L-cysteine desulphydrase activity of* Escherichia coli *strain Crookes*

Organisms were grown in the media indicated and tested for desulphydrase activity as washed suspensions in the presence or absence of glucose (see Methods). Results from two separate experiments, one of which tested a graded series of glucose concentrations.

			Glucose present during test (M × 10^{-3})							
	pH*		0	0·0054	0·022	0·087	0·34	1·4	2·8	5·5
			Desulphydrase activity (Q_{H_2S})†							
Growth medium	Initial	Final								
Tryptone + cysteine (3·2 mM)	5·7	5·8	9·0	—	—	—	—	—	31·0	—
	5·5	5·7	9·0	13·7	16·7	18·0	—	25·6	—	51·0
Same + glucose (0·1 %, w/v)	5·7	5·3	0·6	—	—	—	—	—	1·4	—
Defined + cysteine (3·2 mM) + glutamate (1 %, w/v)	6·4	6·4	1·6	—	—	—	—	—	97·0	—
Same + glucose	6·4	5·0	30·0	—	—	—	—	—	60·0	—
(1·0 % and 0·5 %, w/v)	5·7	4·7	30·5	—	—	39·5	45·0	51·0	—	54·0

* Initial = pH at time of inoculation; final = pH at time of harvest.
† μg. H_2S/hr./mg. washed dry cells.

Table 2. *Effects of various sugars on activity of* L-*cysteine desulphydrase*

Organisms were grown in the defined glutamate-cysteine medium and tested for activity as washed suspensions in the presence of the indicated sugars (0·5 or 1·0 mg./ml. buffered L-cysteine solution; see Methods). The sugars were reagent grade and were not checked for purity.

Nature of sugar present during test	Glucose in medium (%, w/v)		
	0*	Q_{H_2S}†	0·1‡
Control	16·5		21·4
Glucose	106·0		148·0
Xylose	18·6		23·4
Fructose	82·4		118·0
Galactose	30·0		24·6
Sucrose	109·0		57·0

* Initial pH at time of inoculation = 5·5; final pH at time of harvest = 5·7.
† μg. H_2S/hr./mg. washed dry cells.
‡ Initial pH = 5·5; final pH = 5·2.

Influence of glucose and other sugars on the formation and activation of cysteine desulphydrase. Regardless of its presence in the growth medium, glucose ($5·4 \times 10^{-6}$M or more) increased the desulphydrase activity of washed *Escherichia coli* recovered from media supplemented with L-cysteine (Table 1). The stimulatory effect of glucose of desulphydrase activity was of the same order as that of sucrose or fructose while xylose and galactose were slightly stimulatory (Table 2). The presence of glucose

in the medium, however, either enhanced (defined medium) or inhibited (tryptone broth) the formation of the enzyme (Table 1). The latter effect was detectable at a glucose concentration of 0·01 % (w/v) (Table 3) and was essentially duplicated by five other sugars (Table 4).

Table 3. *Sensitivity of formation of cysteine desulphydrase to glucose in tryptone broth*

Organisms were grown in tryptone broth (initial pH of 6·0) containing various concentrations of glucose and tested as washed suspensions for activity in the buffered L-cysteine solution (with and without 0·5 mg. glucose/ ml.; see Methods). Activity expressed as Q_{H_2S} (μg. H_2S/hr./mg. washed dry organisms).

Glucose concentration in medium (%, w/v)	Final pH	Activity in L-cysteine solution containing glucose concentration of (mg./ml.)	
		0	1
0	6·0	10·0	30·0
0·01	6·1	9·2	21·0
0·05	5·8	3·0	16·5
0·10	5·3	1·5	9·0
0·20	4·7	0·5	4·0

Table 4. *Effect of various sugars in tryptone broth upon* L-*cysteine desulphydrase activity of* Escherichia coli

Organisms were grown in L-cysteine-fortified tryptone broth containing one of the indicated sugars and tested as washed suspensions for activity in the buffered L-cysteine solution (see Methods).

Sugar in medium	Concn. of sugar (%, w/v)	pH*		Q_{H_2S}†
		Initial	Final	
Control	—	5·6	5·8	46·2
Fructose	0·05	5·6	5·4	6·4
	0·30	5·6	4·7	0·4
Galactose	0·05	5·5	5·4	14·6
	0·30	5·5	4·9	0·3
Glucose	0·05	5·6	5·4	3·6
	0·30	5·6	4·7	0·5
Lactose	0·05	5·7	5·3	10·6
	0·30	5·7	4·9	2·6
Sucrose	0·05	5·5	5·8	20·4
	0·30	5·5	5·4	1·6
Xylose	0·30	5·5	4·8	0·5

* See Table 1.
† See Table 1.

The observed stimulatory action of glucose on the cysteine desulphydrase activity of intact pre-induced organisms could be interpreted as a need for an exogenously supplied source of energy for maximal absorption of substrate. *Escherichia coli* was grown in the defined cysteine + glutamate + glucose medium: the organisms were washed once with distilled water and exposed to vibrations of a Raytheon 9 KC Sonic Oscillator (Model S-102A, 50 W., frequency maximal, plate voltage of

78) for various measured times while being cooled with ice water. Plate counts in a nutrient agar were made of portions of the suspension removed at each time interval; the remainder of each sample was diluted with the buffered substrate and tested for cysteine desulphydrase activity. Activity declined rapidly in particulate as well as in the supernatant fractions; viability paralleled rather closely loss in enzyme activity as determined in the presence of glucose. In the absence of glucose, enzyme activity was found to decline more rapidly than loss in viability during the first 8 min. of sonication (Table 5). Nevertheless, at every interval of treatment, especially during the first 6 min., glucose enhanced the apparent activity of cysteine desulphydrase. Similar results were obtained with cells grown in tryptone-cysteine broth. These data suggest that glucose activation of cysteine desulphydrase operates within the cytoplasm.

Table 5. *Effects of glucose on desulphydrase activity of sonicated* Escherichia coli

Organisms were grown in the defined glutamate + glucose + cysteine medium, washed, sonicated and tested for activity in the presence and absence of glucose (see Methods). The initial pH of the medium was 5·8; final pH was 5·1. Viable organisms remaining after sonication were enumerated by customary plate count method. Activity expressed as Q_{H_2S} (μg. H_2S /hr./mg. washed dry organisms).

Period of exposure (min.)	Viable organisms (no./ml.)	Activity in L-cysteine solution containing glucose at conc. of (mg./ml.): 0	1	Degree of stimulation from glucose*
0	$6·9 \times 10^9$	9·3	45·3	4·9
2	$4·4 \times 10^9$ (64 %)†	3·3 (35 %)†	35·0 (77 %)†	10·6
4	$2·3 \times 10^9$ (33 %)	1·2 (13 %)	20·4 (45 %)	17·0
6	$1·9 \times 10^9$ (28 %)	0·7 (7 %)	12·7 (28 %)	19·5
8	$7·7 \times 10^8$ (11 %)	0·7 (7 %)	2·9 (6 %)	4·5
10	$3·0 \times 10^8$ (4·3 %)	0·5 (5·4 %)	1·6 (3·5 %)	3·2
12	$1·8 \times 10^8$ (2·6 %)	0·24 (2·6 %)	0·65 (1·4 %)	2·7
15	$6·0 \times 10^7$ (0·9 %)	—‡	—‡	—

* Ratio of activity in presence of glucose to that in absence of glucose.

† Figures in parentheses indicate percentile viability or activity remaining after indicated period of treatment.

‡ Negligible activity.

Since the cysteine desulphydrase reaction produces equimolar amounts of sulphide, ammonia and pyruvic acid (Fromageot, 1951; Meister, 1957), simultaneous analysis for more than one of these products might indicate whether cysteine desulphydrase was solely responsible for the production of hydrogen sulphide by L-cysteine-induced suspensions of *Escherichia coli*. Pre-induced washed organisms were therefore suspended in the buffered cysteine substrate solution at 37° and desulphydrase activity assayed by measuring both hydrogen sulphide and ammonia evolution. The ratio of H_2S:NH_3 was consistently very close to 1·0. Glucose (0·5 mg./ml. buffered cysteine substrate solution) or α-ketoglutaric acid (0·5 mg./ml.), however, upset this ratio and altered the rates of appearance of the two products: glucose accelerated the rate of production of both products and raised the ratio of H_2S:NH_3 (on occasion to as high as 1·5), while α-ketoglutarate slowed down the reaction rates and lowered the initial (20 min.) ratio. A mixture of glucose and α-ketoglutarate moderately accelerated production of sulphide and ammonia and raised the

H_2S-NH_3 ratio, especially during the first 20 min. of incubation (Table 6). These deviations might result from interposition of other enzyme(s) leading to release of sulphide and ammonia from transamination or degradation products of L-cysteine. One such mechanism could yield alanine (see Discussion) but alanine was not detected by paper chromatography of vacuum-distilled concentrates from active cell-substrate preparations exposed to glucose. Control strips clearly resolved L-alanine and L-cysteine. No attempts were made to detect glutamic acid in preparations exposed to α-ketoglutarate.

Table 6. *Stoicheiometry of hydrogen sulphide and ammonia formation from* L-*cysteine and the influence of glucose and α-ketoglutarate*

Organisms were grown in the defined glutamate + glucose + cysteine medium (initial pH of 6·5; final pH of 5·2) and tested as washed suspensions for desulphydrase activity by quantitating the NH_3 and H_2S formed after 20 and 60 min. exposure to buffered L-cysteine solution containing 0·5 mg. α-ketoglutaric acid and/or glucose per ml. (see Methods and Results). Activity expressed as μmole NH_3 or H_2S formed/mg. dry cells over indicated time.

Incubation period (min.)	Product formed	Activity in the presence of: Control	Ratio*	α-Ketoglutarate	Ratio	Glucose	Ratio	α-Ketoglutarate + glucose	Ratio
20	H_2S	0·74	1·00	0·41	0·53	2·17	1·24	1·61	1·48
	NH_3	0·74		0·78		1·75		1·09	
60	H_2S	1·63	1·08	0·95	1·09	3·02	1·31	1·71	1·12
	NH_3	1·51		0·87		2·30		1·53	

* Ratio H_2S:NH_3.

Efficiency of enzyme action. Tryptone-grown organisms were able to convert from 85 to 95 % of cysteine sulphur to sulphide, regardless of the presence of glucose in the substrate solution, while sulphide produced from cysteine by organisms recovered from the defined glucose + glutamate medium never exceeded, in the absence of glucose, an 80 % conversion. In the presence of glucose, the latter organisms converted less than 65 % of the cysteine sulphur to sulphide. Prolonged incubation of these preparations resulted in the gradual formation of a pronounced yellow colloid material, most intense in the depth of the tube, which disappeared within 30 min. after unstoppering the tube. No attempt was made to identify this chromogenic material, but it might have been polysulphide formed through degradation of β-mercaptopyruvic acid (Hylin & Wood, 1959).

Interaction of glucose and organic nitrogen in the medium on induction of cysteine desulphydrase. Because glucose exerted different effects in the two basal media on the hydrogen sulphide-producing competency of *Escherichia coli*, the influence of various relative amounts of amino nitrogen and glucose as nutrients on the induction of cysteine desulphydrase was examined. One such experiment (Table 7) indicated that glucose as a constituent of the medium enhanced the resultant desulphydrase activity of organisms from the defined cysteine-glutamate medium, but only if the organic nitrogen mixture (Casitone, Difco) was omitted or very limited during growth. Glucose activation of the system lessened as the amount of amino

nitrogen in the medium was raised. Highest activities were obtained with organisms grown in media containing Casitone, but not more than 0·01 % (w/v) glucose, provided activity was determined in the presence of glucose. The omission of glutamate from the medium had no apparent effect on these findings.

Table 7. *Inter-relationship between casein hydrolysate and glucose in medium on cysteine desulphydrase formation by* Escherichia coli *during growth*

The various indicated amounts and combinations of an enzymic casein hydrolysate (Casitone, Difco) and glucose were incorporated in the defined monosodium glutamate (0·8 %, w/v) + L-cysteine.HCl (0·05 %, w/v) basal medium. Organisms were tested for activity as washed suspensions in the buffered L-cysteine solution (with and without 1·0 mg. glucose/ml.; see Methods and Results). The initial pH of all media was 5·8. Activity expressed Q_{H_2S} (μg. H_2S/hr./mg. washed dry organisms).

Addition to medium				Activity in L-cysteine solution containing glucose concentration of (mg./ml.):	
Casitone (%, w/v)	Glucose (%, w/v)	Final pH	Relative* growth	0	1
0	0	5·9	36	—	230·0
	0·01	5·8	216	19·8	196·0
	0·10	5·1	254	54·7	163·0
	0·20	5·2	298	95·0	193·0
0·05	0	5·7	76	13·7	418·0
	0·01	5·8	147	30·1	318·0
	0·10	5·1	324	25·4	119·0
	0·20	5·1	320	40·5	98·7
0·50	0	5·9	180	4·9	347·0
	0·01	5·8	216	5·6	312·0
	0·10	5·3	404	8·1	43·6
	0·20	5·0	396	4·8	27·0

* Scale reading of Klett-Summerson photoelectric colorimeter with filter no. 42.

Effects of glucose on the cysteine desulphydrase activity of other strains of Escherichia coli. Three stock (ATCC 9637, 10795, 10586) and three wild strains of the colon bacillus were grown in tryptone broth, with and without glucose supplementation, and in the defined glucose + glutamate medium. L-cysteine.HCl (0·05%, w/v) was added to both media. In every case glucose enhanced cysteine desulphydrase activity, while being inhibitory to production of the enzyme if present in tryptone broth. Glucose enhancement of activity was greater in organisms recovered from tryptone broth (about 34– to > 100-fold) than in those grown in glucose-fortified tryptone broth (about 3- to 14-fold). Specific activities ranged from 1·0 to 187 μg. H_2S/hr./mg. washed dry organisms. Organisms grown in glucose-supplemented tryptone broth were less active than organisms from plain tryptone broth, such difference being particularly striking when desulphydrase activity was measured in the presence of glucose.

DISCUSSION

Two routes of cysteine desulphydration are known: (i) the classical pyridoxal phosphate-dependent inducible cysteine desulphydrase yielding hydrogen sulphide and the unstable α-aminoacrylic acid which hydrolyses to ammonia and pyruvic acid (Fromageot, 1951; Meister, 1957; Singer & Kearney, 1955); (ii) de- or

trans-amination reactions yielding an amino acid and an intermediate, β-mercaptopyruvic acid, which is decomposed by a desulphurase to pyruvic acid and sulphur (Hanson & Mantel, 1953; Meister, Fraser & Tice, 1954). Sulphur produced by the latter reaction is reduced to sulphide by thiosulphate, glutathione, cysteine, etc. (Hylin & Wood, 1959; Sörbo, 1957). The β-mercaptopyruvic acid desulphurase is constitutive, has no requirement for vitamin B_6 and has no effect on L-cysteine. Hydrogen sulphide may also be produced from cysteine through elimination of ammonia with the formation of an intermediate that is neither α-aminoacrylic or β-mercaptopyruvic acid (Suda, Saigo & Ichihara, 1954).

While it is tempting to claim that a 'glucose effect' on yet another inducible enzyme has been demonstrated, the diversity of the aforementioned paths by which *Escherichia coli* can produce hydrogen sulphide from cysteine obscures interpretation of the results. The inhibitory effects of glucose in amino acid-containing media on the formation of 'cysteine desulphydrase' might be a result of repression of either the latter enzyme or of cysteine-pyruvic acid transaminase. In either case, formation of sulphide from cysteine would be impaired and the assay employed would not distinguish which mechanism was operative. Moreover, a dilemma arises in distinguishing between 'basal' cysteine desulphydration and that which is seemingly activated by glucose. Conceivably, cysteine degradation, with an intermediate product (pyruvic acid) common to one from glucose dissimilation, might be repressed by glucose when a good supply of amino acids encourage more vigorous synthesis of enzymes concerned with glucose degradation than was possible in an amino acid-limited menstruum.

The concept of active transport of amino acids (Britten & McClure 1962; Christensen, 1955; Edelman, 1961; Gale & Folkes, 1953; Kleinzeller & Kotyk, 1961; Roberts *et al.* 1955) encourages speculation that an exogenously supplied energy source facilitates the assimilation of L-cysteine, thereby maximizing the activity of cysteine desulphydration. Kun, Bradin & Dechary (1956) reported that the degradation of cysteine to sulphide by *Endamoeba histolytica* was completely dependent upon concurrent glycolysis while producing sulphide from cysteine. To date, a cysteine permease has not been reported, although glucose is strongly inhibitory to the induction of β-galactoside permease (Cohn & Horibata, 1959) and indifferent in the induction of tryptophan permease (Freundlich & Lichstein, 1960). Since sonically disrupted cysteine-induced organisms were stimulated by glucose to produce sulphide from cysteine, glucose activation may operate at subcellular barriers, some of which are known to control enzymic function (Gross, 1959; Siekevitz, 1959). Delwiche (1951) noted that most of the cysteine desulphydrase activity resided in the particulate fraction of *Escherichia coli*.

Because the pathway by which hydrogen sulphide is produced from cysteine was obscure in this study, experiments in which glucose and α-ketoglutarate were shown to affect the rates of appearance and relative amounts of ammonia and hydrogen sulphide from washed cysteine-induced organisms suggest that more than one mechanism was operative. Both glucose and α-ketoglutarate might encourage transamination reactions with cysteine to result in the formation of β-mercaptopyruvate and alanine (transamination with pyruvate) or glutamic acid (transamination with α-ketoglutarate). While glucose raised appreciably the molar ratios of sulphide to ammonia, alanine could not be detected in the reaction mix-

tures. On the other hand, α-ketoglutarate lowered the ratio of these products and slowed down their rate of appearance, suggesting the intervention of some mechanism other than transamination. Metaxas & Delwiche (1955) reported α-ketoglutarate to discourage sulphide formation from L-cysteine in alumina-ground *Escherichia coli*, an effect attributed to competition for a limited supply of pyridoxal phosphate. Glucose activation of the sulphide-producing system studied probably was a result of cysteine-pyruvate transaminase activity since it raised the ratio of sulphide to ammonia and, in some cases, apparently caused the accumulation of sulphur and polysulphide, evidence of β-mercaptopyruvic desulphurase activity (Hylin & Wood, 1959). Further work is needed to unravel the apparent glucose activation and repression effects and to establish the nutritional role of amino nitrogen and glucose in cysteine catabolism.

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The Decomposition of Dichloropropionate by Soil Micro-organisms

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SUMMARY

When a column of air-dried soil was perfused with a mineral salt solution containing 'Dalapon' (Dow Chemical Company, Michigan), the concentration of dichloropropionate (Dalapon's herbicidal component) became minimal in 15–17 days. The soil was reperfused and the dichloropropionate became minimal in 4–5 days.

Three strains of the genus *Pseudomonas*, exhibiting a requirement for dichloropropionate, were isolated from the perfusate. Two strains attacked dichloropropionate when allowed to perfuse through sterilized soil.

A further seven micro-organisms, with a similar requirement, were isolated from pasture soils treated with Dalapon.

INTRODUCTION

Audus (1951), Walker (1954), Theigs (1955) and Jensen (1957) showed that many of the indigenous micro-organisms of soils could break down herbicides. In the absence of leaching, Theigs (1955) attributed the disappearance of Dalapon in the soil to the action of Dalapon-decomposing organisms, as the rate of decomposition was greater in soils of higher bacterial numbers than in infertile soils. He found similarities to the pattern of 2,4-dichlorophenoxyacetic acid (2,4-D) disappearance observed by Audus (1951).

Jensen (1957) isolated micro-organisms of the genus *Agrobacterium* from soil and found them able to attack Dalapon. Magee & Colmer (1959) isolated eight micro-organisms able to decompose Dalapon, six tentatively classified as species of Agrobacterium and two of Pseudomonas. Hirsch & Alexander (1960) isolated a number of bacteria and species of Streptomyces, Nocardia, Penicillium and Aspergillus that could attack Dalapon. The present investigation seeks further evidence for the degradation of dichloropropionate in the soil by micro-organisms.

METHODS

Soil. Soil for perfusion was taken from three adjacent plots of pastureland, each 2 sq.yd. in area; they had never been treated with Dalapon and had been under grass for several years. Soil from one plot was sampled and removed to the laboratory. The two remaining plots were sprayed with aqueous solutions of Dalapon for 8 consecutive months from summer to early spring, one at 10 lb./acre, the other at 5. Both treated plots were sampled at the end of the 8-month period.

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In sampling the soil, adjacent 6 in.-deep sods were dug and the top 1 in. of turf removed. After air-drying at room temperature between layers of newsprint, the 1·0–4·0 mm. crumb fraction was collected by sieving.

Perfusion fluid and apparatus. The perfusion fluid was as follows: $MgSO_4.7H_2O$ 1·0 g., $CaCl_2$ 1·0 g., NaCl 1·0 g., K_2HPO_4 5·0 g., $(NH_4)_2SO_4$ 5·0 g., $FeCl_3.6H_2O$ 10·0 mg., distilled water to 1 l. It was adjusted to pH 6·8 and when prepared in bulk steamed for 2 hr. daily for 3 days.

The perfusion apparatus was similar to that used by Audus (1946).

Dichloropropionate. The source of crude dichloropropionate was Dalapon (W. R. Mullison, private communication) and added so that the final concentration in the perfusion fluid was 0·2 %. Pure sodium 2,2-dichloropropionate was prepared as described by Macgregor (1960).

Dichloropropionate was assayed colorimetrically. Samples were assayed for pyruvate (Friedman & Haugen, 1943) following autoclaving for 40 min. at 120°.

Soil perfusion

Untreated soil. Soil crumbs from the untreated plot were perfused with the salt solution containing an estimated 2 mg. dichloropropionate/ml., until no dichloropropionate could be detected. The soil perfusate was then drained off, the freshly perfused soil left intact and the reservoir replenished with fresh perfusion fluid plus dichloropropionate. Perfusion was recommenced and continued until the dichloropropionate became minimal. (Sterilized soil crumbs were perfused with and without dichloropropionate addition.)

Auxotrophic micro-organisms requiring dichloropropionate were isolated by streaking a loopful of the soil perfusate on to solid medium. This was a 2 % washed-agar gel of the perfusion fluid with sterile dichloropropionate rubbed into the gel before inoculation. After incubation for 5 days at 28°, colonies were picked off and inoculated into tubes of the perfusion fluid containing dichloropropionate to 0·5 %, w/v. The tubes were examined for growth after similar incubation.

Inoculated soil. Sterilized soil crumbs were brought to maximal moisture-holding capacity with dichloropropionate-perfusion fluid and inoculated with a visibly turbid suspension of each strain isolated as above. Perfusion was continued for 4 days. After this perfusion, all strains were re-isolated in pure culture.

Treated soil. Soil crumbs from the plots of land sprayed at 5 and 10 lb./acre respectively with Dalapon, were perfused and micro-organisms with a dichloropropionate requirement isolated.

RESULTS

Untreated soil. By plotting the concentration of dichloropropionate in the perfusate of the first-perfused soil against time, two phases of disappearance of dichloropropionate were noted: (i) a lag phase lasting 9–10 days; (ii) active dichloropropionate removal lasting a further 6–7 days. These occurred in all perfusion experiments, although the time for each phase varied with the soil (unperfused, reperfused or inoculated soil).

Reperfusion of the initially unperfused soil resulted in more efficient removal of dichloropropionate. The lag phase was decreased to 1–2 days with complete removal

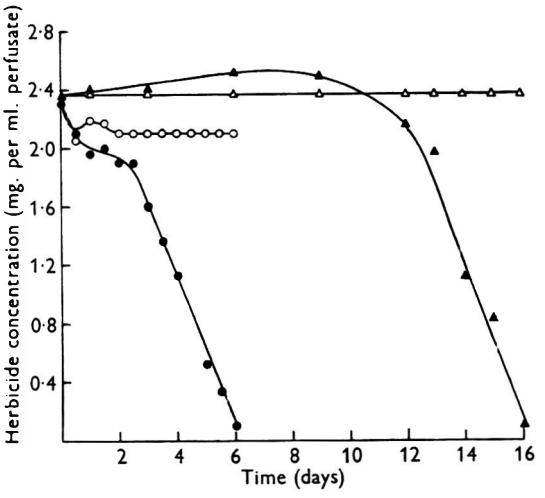

Fig. 1

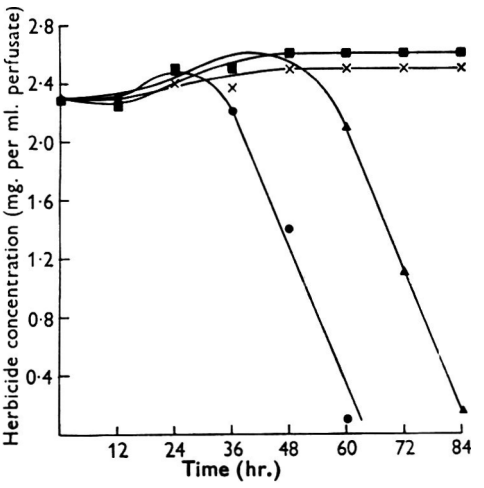

Fig. 2

Fig. 1. Untreated soil. The disappearance of dichloropropionate from various perfused soils. ▲—▲, Unperfused soil + dichloropropionate; ●—●, reperfused soil + dichloropropionate; △—△, ○—○, sterilized soil + dichloropropionate.

Fig. 2. Inoculated soil. The disappearance of dichloropropionate from the perfusates of sterilized soil inoculated with micro-organisms with a requirement for dichloropropionate. ▲—▲, Soil inoculated with isolate A; ●—●, soil inoculated with isolate B; ■—■, soil inoculated with isolate C; × — ×, uninoculated soil.

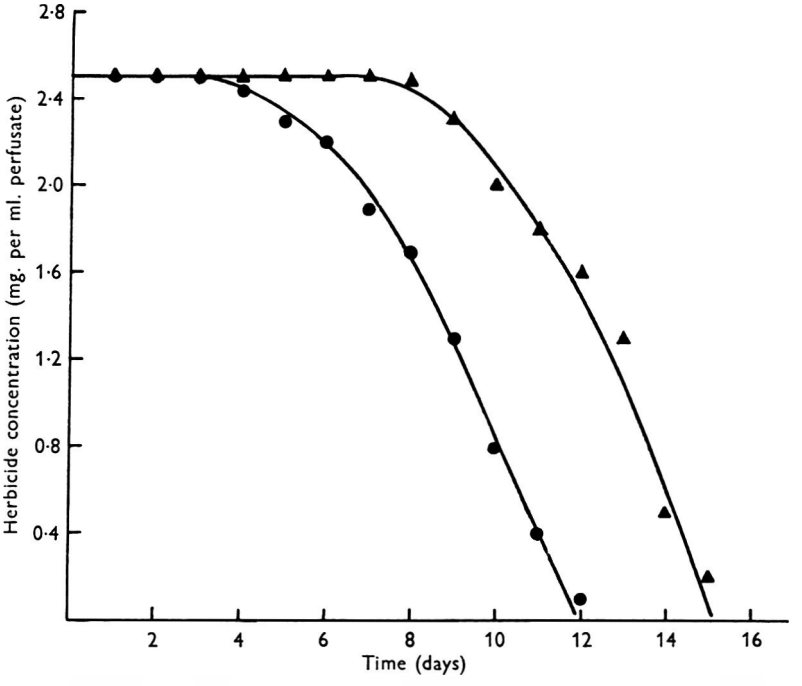

Fig. 3. Treated soil. The disappearance of dichloropropionate from the perfusates of treated soils. ▲—▲, Soil dosed at 5 lb./acre of Dalapon; ●—●, soil dosed at 10 lb./acre of Dalapon.

after a further 4–5 days (Fig. 1). In both the perfusion of unperfused and reperfused soil, sterilizing the soil failed to minimize dichloropropionate.

Inoculated soil. The disappearance of dichloropropionate from the inoculated soils followed the trend of disappearance from the reperfused soil. However, after 4 days, dichloropropionate disappeared in only two soils (Fig. 2).

Three micro-organisms exhibiting a requirement for dichloropropionate were isolated. They were Gram-negative motile rods with single polar flagella and measured $1{\cdot}5–1{\cdot}0 \times 0{\cdot}5\ \mu$. They were unable to grow anaerobically and showed growth at 30–35°. A zone of blue-green fluorescence occurred around the growth on Difco nutrient agar. All three isolates hydrolysed gelatine, produced an alkaline reaction in litmus milk but with no clot formation, and produced nitrite from nitrate. They did not digest cellulose or produce indole. Although growth occurred in glucose-, lactose- and maltose-peptone media, no acid or gas was produced after 4 days incubation. Slight variations in colony morphology occurred on Difco nutrient agar and dichloropropionate agar. These characteristics suggest that the strains may be closely related members of the genus *Pseudomonas* (*Bergey's Manual* 1957).

Treated soil. The pasture soils treated with Dalapon were sampled 20 days after the last spraying. No residual dichloropropionate was detected and it was therefore anticipated that dichloropropionate added to the fluid perfusing these would be removed in less than 20 days. No dichloropropionate was detected in either soil perfusate after 16 days perfusion (Fig. 3).

Seven micro-organisms were isolated from treated soils and shown to have a requirement for dichloropropionate. Five were Gram-negative rods and two branched in young cultures, and formed a pellicle in Difco nutrient broth.

DISCUSSION

The kinetics of the disappearance of dichloropropionate from the untreated soil perfusate are consistent with the hypothesis that a soil enrichment causes some segment of the microbial population to proliferate. It then adapts to the conditions imposed by the enrichment material (Audus, 1951). The graphs illustrating the removal of dichloropropionate from the various perfusates reflect the growth of suitably adapted micro-organisms.

Ten micro-organisms have been isolated during the course of the investigation that require dichloropropionate for growth, perhaps as a sole source of carbon. These micro-organisms present an interesting facet of bacterial metabolism in view of the structural similarity of dichloropropionate to other halogenated fatty acids, e.g. trichloroacetate, iodoacetate and fluoroacetate. The site of dehalogenation of the dichloropropionate molecule has recently been investigated by Hirsch & Alexander (1960).

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Autoradiographic Studies of the Differential Incorporation of Glycine, and Purine and Pyrimidine Ribosides by *Paramecium aurelia*

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SUMMARY

Autoradiographs were prepared from organisms of *Paramecium aurelia* which were grown in an axenic medium to which one of the following compounds was added: [carboxy-^{14}C]glycine, [8-^{14}C]adenosine, [8-^{14}C]-guanosine, [2-^{14}C]cytidine, [^{3}H]thymidine, and [^{3}H]uridine. Trichloro-acetic acid extracts of organisms grown with the isotopically labelled glycine, adenosine and cytidine were prepared and the radioactivity found either in the protein or in the acid-soluble fractions was determined in a scintillation counter. *P. aurelia* was unable to incorporate glycine into the nucleic acids of the macronucleus; however, this compound was utilized in the formation of the cytoplasmic proteins. Thymidine was preferentially taken up by the macronucleus; uridine was found mainly in the cytoplasm. The other purine and pyrimidine ribosides were found both in the macro-nucleus and cytoplasm.

INTRODUCTION

Suggestive evidence that *Paramecium aurelia* might be unable to synthesize its purine and pyrimidine bases came from nutritional studies by Tarantola & Van Wagtendonk (1959) who reported that this organism had an absolute growth requirement for ribosidic purines and pyrimidines. A similar requirement was earlier reported for the closely related species *P. multimicronucleatum* (Johnson, 1952; Miller & Johnson, 1957). Investigations by Soldo & Van Wagtendonk (1961) of the nitrogen metabolism of *P. aurelia* demonstrated the incorporation of some of the ribosidic purines and pyrimidines into nucleic acids and the failure of the incorporation of glycine into these compounds. The significance of the inability to utilize glycine as a precursor for purine synthesis was not emphasized. Autoradiographic techniques have also been utilized to study several facets of purine and pyrimidine metabolism in *P. aurelia* (Berech & Van Wagtendonk, 1959, 1960, 1962; Kimball & Perdue, 1961). However, no comparative study of the fate of the purine precursor, glycine, and the preferential sites of the ultimate incorporation of purines and pyrimidines has been carried out.

METHODS

Stocks and their maintenance. Stock d-40 of syngen 1 and stock 51 of syngen 4 of *Paramecium aurelia* were examined in these experiments. Stock d-40 is a derived stock, homozygous for the recessive gene mt^{I}, limiting it to the production of mating type I (Butzel, 1955). Stock 51 is a sensitive stock, homozygous for the

gene *K*, which has lost the cytoplasmic particle, kappa (Sonneborn, 1946). Both stocks were maintained axenically at 27° in the medium described by Soldo (1961).

The isotopically-labelled compounds, their specific activities and the dosage level in each medium are shown in Table 1. Glycine was obtained from The California Corporation for Biochemical Research, and the other compounds were purchased from Schwarz BioResearch Incorporated.

Table 1. *Isotopically-labelled compounds used*

Isotopically-labelled compound	Specific activity	Concentration in medium (μcuries/ml.)
[Carboxy-^{14}C]glycine	7·8 mc./mmole	10
[8-^{14}C]adenosine	7·2 μc./mg.	3·3
[8-^{14}C]guanosine	15·25 μc./mg.	3·3
[2-^{14}C]cytidine	4·0 μc./mg.	3·3
[^{3}H]thymidine	3·0 c./mmole	25
[^{3}H]uridine	0·31 c./mmole	25

Autoradiographs. The organisms were grown in the presence of each isotopically labelled compound for 3 to 5 days. The organisms were harvested by centrifugation, washed 4 times with a sterile baked lettuce infusion, adjusted to a pH value of 6·9 (Sonneborn, 1950), fixed to previously 'subbed' slides (Comar, 1955) and allowed to dry in air. Staining was by the Dippell-Chao modification of Delamater's basic fuchsin technique (Sonneborn, 1950). Eastman Kodak nuclear emulsion NB-2 was used and processed according to standard techniques (Pelc, 1947). Exposure times ranged from 10 to 14 days.

Assays for radioactivity of the protein and TCA-soluble fractions. Organisms were concentrated from the isotopically-labelled medium by centrifugation and washed 4 times in the sterile baked lettuce infusion. The final 1·0 ml. of centrifugate was homogenized and an equal volume of 10 % (w/v) trichloroacetic acid (TCA) added. The mixture was heated for 15 min. at 90°, cooled, and then centrifuged at 0° and 31,000*g* for 10 min. The supernatant fluid was poured off and saved. The sediment was extracted with an additional 2·0 ml. of TCA solution and recentrifuged. The two supernatant solutions were combined and extracted several times with diethyl ether in order to remove TCA. The sediment from the TCA extractions was dissolved in 1·0 ml. of 2 % (w/v) NaOH solution and heated in a boiling water bath for 15 min. The radioactivity of the TCA soluble- and protein-fractions was determined in a Tricarb Scintillation Counter by adding 0·01 ml. of each preparation to the standard solute (Ott, 1958).

Determination of the presence of nucleic acid in the TCA-soluble fraction. Before counting the TCA-soluble fraction in the Tricarb Counter, the presence of nucleic acid was ascertained by determining the absorption spectrum at 254–260 mμ in a Beckman model DU. spectrophotometer.

RESULTS AND DISCUSSION

The autoradiographs show that organisms grown in the presence of isotopically-labelled glycine did not incorporate the label into the Feulgen-positive material of the macronucleus (Pl. 1, fig. 1); scintillation counts (Table 2) also showed that

no radioactivity was present in the acid-soluble fraction derived from these organisms. In contrast, the labelling of the cytoplasm was very heavy and the protein fraction was highly radioactive. These findings indicate that the organisms were unable to use glycine for the formation of the purine bases of the nucleic acids, but that this amino acid was involved in protein synthesis. This is in accord with the observations by Soldo & Van Wagtendonk (1961) and demonstrates that in *Paramecium aurelia* there is a metabolic block at the formation of glycinamide ribotide. These data therefore explain the long known need for preformed purine ribosides as essential nutrilites for this organism.

Table 2. *Radioactivity of nucleic acid and protein fractions*

Isotopically-labelled compound	Fraction	Average of eight 10 min. counts/0·01 ml. sample ± standard deviation
[Carboxy-^{14}C]glycine	Protein	53,081 ± 322
	TCA-soluble	– *
[8-^{14}C]adenosine	TCA-soluble	12,649 ± 79
[8-^{14}C]guanosine	TCA-soluble	38,664 ± 101
[2-^{14}C]cytidine	TCA-soluble	9,610 ± 116

* Within the limit of the background count (172–211 counts/10 min.).

The inability of *Paramecium aurelia* to utilize glycine makes this organism dependent on preformed purines. Tarantola & Van Wagtendonk (1959) found that the free bases were not metabolized; therefore the purine ribosides were used in these experiments. Adenosine and guanosine were taken up by the organism, as shown by the autoradiographs (Pl. 1, figs. 2, 3). The label was found over the macronucleus and the cytoplasm. The radioactivity found in the TCA-soluble fraction (Table 2) confirms these autoradiographic observations. Adenine and guanine are constituents of ribonucleic acids and deoxyribonucleic acids, and the presence of the label in the macronucleus and the cytoplasm is to be expected. *P. aurelia* thus appears to possess the enzymes responsible for the incorporation of the purine nucleosides into their DNA and RNA. Whether any precursors beyond glycinamide ribotide in the synthetic chain leading to the formation of the purine skeleton can be utilized by this organism remains to be investigated.

Each of the three isotopically-labelled pyrimidine ribosides had different ultimate sites of localization in the organism, in accordance with the differences in base composition found in deoxyribonucleic acid and ribonucleic acid. The label of the pyrimidine riboside, cytidine, which is common to both types of nucleic acid, was concentrated in the macronucleus as well as in the cytoplasm. No definite distinction can, however, be made between deoxyribonucleic acid and ribonucleic acid on the basis of autoradiographic data alone. This may also apply to the observations of Kimball & Perdue (1961), who reported, on the basis of similar data, that cytidine is incorporated into the macronuclear ribonucleic acid alone. The label from the pyrimidine riboside, thymidine, which is incorporated only in DNA was concentrated in the macronucleus (Pl. 1, fig. 5); this is in accord with the findings of Berech & Van Wagtendonk (1962). On the other hand, the label from uridine which was

found only in RNA was heavily concentrated throughout the cytoplasm of the cell (Pl. 1, fig. 6).

It is of interest to note that each member of the Phylum Protozoa so far examined has shown an inability to synthesize purine bases. *Tetrahymena pyriformis* (Heinrich, Dewey & Kidder, 1953), *Paramecium aurelia* (Soldo & Van Wagtendonk, 1961), *P. multimicronucleatum* (Johnson 1952, Miller & Johnson, 1957) among the ciliates and *Trypanosoma mega* (Boné & Steinert, 1956) of the flagellates are dependent on an exogenous source of purine ribosides for nucleic acid synthesis. Of these, *T. pyriformis*, *P. aurelia*, and *Trypanosoma mega* were unable to carry out the synthesis of glycinamide ribotide and it seems probable that the same metabolic block will be shown to be present in *P. multimicronucleatum*. It would seem more than a chance occurrence that of the four forms examined, all require purines as essential nutrilites. Perhaps it will be found that a biochemical distinction of the Phylum Protozoa will be the inability to form their nucleic acids from simple precursors, resulting in a dependence upon exogenous preformed purines for growth.

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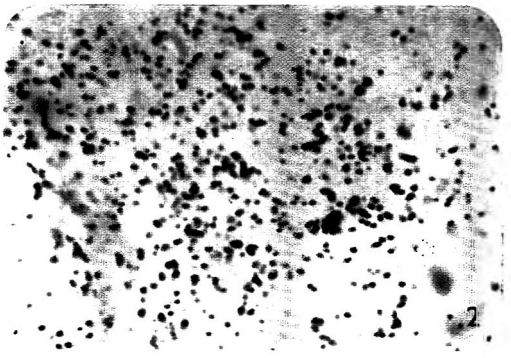

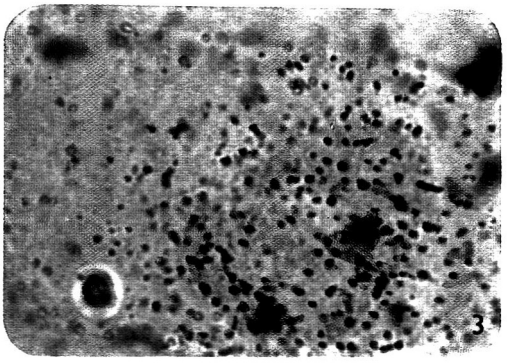

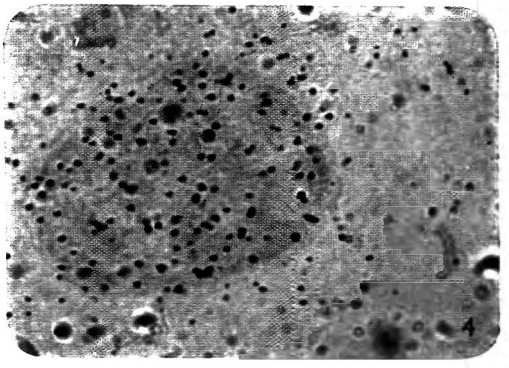

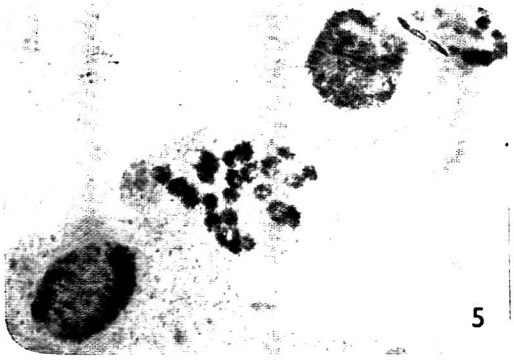

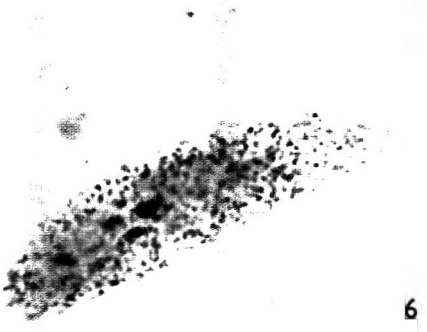

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EXPLANATION OF PLATE

Autoradiographs of *Paramecium aurelia* showing the distribution pattern of the incorporation of labelled glycine and labelled purines and pyrimidines.

Fig. 1. Organisms of stock 51 grown for 5 days in the presence of [carboxy-^{14}C]glycine. One organism is in division. Note absence of grains over the stained macronucleus and the dense concentration over the cytoplasm. Magnification, × 126 on film.

Fig. 2. Organism of stock d-40 after a similar period of growth in the presence of [8-^{14}C]adenosine. Only part of one organism is shown. Note grains over the macronucleus and the cytoplasm. Magnification, × 250.

Fig. 3. An organism of stock d-40 grown in the presence of [8-^{14}C]guanosine. Only part of one organism is shown. Note similar distribution pattern of the label to the previous illustration. Magnification, × 250.

Fig. 4. A stock d-40 organism grown in the presence of [2-^{14}C]cytidine. Part of one organism is shown. The distribution of label is similar to that shown in *b* and *c*. Magnification × 250.

Fig. 5. Stock 51 organisms grown in the presence of [^{3}H]thymidine. The middle organism is undergoing autogamy. Note dense concentration of label over the macronucleus and macronuclear fragments. Magnification × 126.

Fig. 6. A stock 51 organism grown in the presence of [^{3}H]uridine. The organism is in autogamy Note the dense concentration of label throughout the cytoplasm. Magnification, × 250.

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