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Notes on the Phylogenetic Background to Lactobacillus Taxonomy

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SUMMARY

Speculations about the phylogeny and natural relationships of and within the genus *Lactobacillus* are made, based upon current taxonomic knowledge.

Phylogenetic speculation has long held a fascination for biologists who interest themselves in taxonomy and it remains to be seen whether the recent invasion of this field by computers will render this fascination fatal. The present notes are an attempt to extend certain speculations which occur in the literature. For example Davis (1955) suggested that the difficulty found in differentiating species of heterofermentative lactobacilli (see Rogosa & Sharpe, 1959; Cheeseman & Berridge, 1959) might indicate that these organisms are of more recent origin than the homofermenters and might be derived from them. De Ley (1962) stated 'one can thus imagine that the "heterolactic" (i.e. heterofermentative) bacteria could phylogenetically be derived from the "homolactic" (i.e. homofermentative) bacteria by the loss of aldolase and transketolase'.

Lactobacilli have been much studied and the taxonomy is comparatively well developed. The subgeneric classification proposed by Rogosa & Sharpe (1959). with its revival of the species groups recognized by Orla-Jensen (1919), incorporat most of the recent taxonomic ideas about the genus. Newly developed an lytical techniques have been successfully applied to lactobacilli and one of these, c ll-wall analysis, yielded an unexpected result. Cummins & Harris (1956) found t at the homofermentative species, *Lactobacillus plantarum*, differed from all other known Lactobacillus species by the presence of diaminopimelic acid and absence of aspartic acid in cell-wall hydrolysates. It was subsequently found by Baddiley & Davison (1961) that strains of *L. plantarum* also contained ribitol-teichoic acid in their cell walls, whereas other species of lactobacilli contained either glycerol-teichoic acid or none. As Baddiley & Davison pointed out, Sharpe (1955) had found that *L. plantarum* strains formed a distinct serological group with only slight cross-relationship with one other group. It is possible that the latter finding reflects the former. Cummins & Harris (1956) remarked that no other characters indicated that *L. plantarum* is not a typical member of the genus *Lactobacillus*. However, there are numerous reports of lactobacillus strains which in some character are atypical of the genus. Most of these reports are of motility, catalase production and nitrate reduction; by generic definition lactobacilli are negative in these three characters.

When taken in isolation these reports can be viewed merely as further examples of Nature's disregard of man-made classifications. When viewed as a group, however, their significance lies in the fact that strains with these atypical characters have almost invariably been classified as *L. plantarum*, variants of it or as 'atypical streptobacteria' with inferred relationship to it. Table 1 lists many of these reports.

Table 1. *Reports of atypical lactobacilli*

Authors	Source of isolates	Atypical characters	Other characters	Name and/or relationships suggested
Cunningham & Smith (1940)	Silage	Motile	Low growth temp. Weak carbohydrate metabolism	Streptobacterium
Harrison & Hansen (1950*a*, *b*)	Turkey faeces	Motile	High growth temp.	*L. plantarum* var. *mobilis*
Hays & Reister (1952)	Orange juice	Motile	—	*L. plantarum*
Mann & Oxford (1954)	Calf rumen	Motile	High growth temp.	Homofermenter
Deibel & Niven (1958)	Ham brine	Motile	Low growth temp. Weak carbohydrate metabolism	*L. casei–plantarum* intermediate
Keddie (1959)	Silage	Motile	—	Atypical low temperature homofermenters
Vankova (1957)	Fermentation mash	Motile	Catalase	See text
Dacre & Sharpe (1956)	Cheese	Catalase	—	*L. plantarum*
Whittenbury (1960)	Various	Catalase-like	—	*L. plantarum*, *L. brevis*
Costilow & Humphreys (1955)	Cucumbers	Nitrate	—	*L. plantarum*
Rogosa (1961)	—	Nitrate	—	*L. plantarum*, *L. fermenti*

About motility in lactobacilli, Harrison & Hansen (1950*a*) pointed out that 'Orla-Jensen (*Zentr. Bakt. Parasitensk.* II, **22**, 305, 1909) listed *Lactobacillus* as the fourth genus "*Caseobacterium*" in the order "*Petritrichinae*" indicating that, although the genus did not possess flagella, its place in his system would be among the peritrichous rather than the cephalotrichous bacteria'. Pederson (1952) identified a strain isolated by Hays & Reister (1952) as *Lactobacillus plantarum*, without realizing that it was motile. Deibel & Niven (1958) compared their isolates with previous reports and found little common ground. They concluded that their strains were intermediate between *L. casei* and *L. plantarum*, but distinct from both. In common with other workers (e.g. Harrison & Hansen, 1950*b*) Deibel & Niven isolated non-motile strains which were otherwise identical with the motile ones. The growth temperature range of motile strains appears to be variable. *Lactobacillus plantarum* grows at 15° and often 45°, but Hayward (1957) noted marked variability in this character in non-motile strains and related it (see Harrison & Hansen, 1950*b*) to environmental adaptation. (*Note: L. salivarius*, which is usually considered as a member of the low-temperature homofermentative group, i.e. streptobacterium, grows at 45° but not at 15°.) The motile isolates of Harrison & Hansen and Mann &

Oxford (1954) (Table 1) appear to share this character. Both these reports dealt with isolates from warm-blooded animals, whereas the other reports concern isolates from saprophytic sources. The report of Vankova (1957) is unusual in several respects. The strains isolated were motile and catalase-positive and were classified as *L. delbrueckii* (thermobacterium). The detection of cytochromes *a* and *b* (see Deibel & Evans, 1960) in Vankova's strains supports the suggestion of Dr M. E. Sharpe (pers. commun.) that these strains may in fact belong to the genus *Bacillus*. As Sharpe (1962) pointed out, there is often difficulty in distinguishing between certain Bacillus species and members of the high-temperature (thermobacterium) group of lactobacilli. To summarize these reports of motility in lactobacilli it is reasonable to say that such organisms probably occur more commonly than the records indicate in a wide range of habitats. The flagellation pattern requires more study but is probably always peritrichous. The strains do not form a homogeneous group but all resemble *L. plantarum* more closely than any other species.

Concerning catalase activity, Whittenbury (1960) described two 'catalase-like' peroxide-decomposing mechanisms found in various lactic acid bacteria. One is a strong activity, not acid-sensitive but requiring an external haemin source. This suggests that some organisms usually considered as devoid of catalase may retain the ability to synthesize the apoenzyme. Failure in prosthetic group synthesis could also account for the absence of cytochromes in these bacteria. The other activity is weaker, is acid-sensitive and does not need added haemin. The former activity occurred in certain streptobacteria, notably *Lactobacillus plantarum*, and certain heterofermenters, notably *L. brevis*. The latter weaker activity occurred commonly in pediococci and leuconostocs and occasionally in *L. plantarum*. It is likely that this activity was the one detected by Dacre & Sharpe (1956). In confirming Costilow & Humphrey's (1955) work, Rogosa (1961) found nitrate reduction in seven of twelve *L. plantarum* strains and two of five *L. fermenti* strains to be primarily dependent upon the pH value. Nitrate reduction was not detected in any other Lactobacillus species.

The regular occurrence of atypical strains closely related to *Lactobacillus plantarum*, together with the known characters of the species, such as its wide range of biochemical activities, relatively unexacting nutritional requirements and ubiquitous nature, lead to the present thesis, which is that *L. plantarum* and its atypical relatives represent the least specialized members of the genus and constitute a link between it and some other bacterial group, extant or otherwise. The atypical generic characters referred to above can be regarded as vestigial. It is not important whether one considers the atypical character as retrograde or advanced: the genetic potential behind them can be interpreted as vestigial in either case. On this assumption, and also assuming that the bacterial group from which the *L. plantarum* genotype originated is just as likely to be extant as not, it is reasonable to suggest the genus *Bacillus* as a convenient reservoir of biological potential from which lactobacilli, among others, may have originated (see Bisset & Davis, 1960). *Bacillus subtilis* for example exhibits most if not all the characters found in *L. plantarum*, including the atypical characters noted in Table 1 (Knight & Proom, 1950). Possible confusion between *B. coagulans* and certain thermobacteria on more than mere morphological grounds was noted by Sharpe (1962). Dr C. S. Cummins has pointed out that in *B. sphaericus* the vegetative form possesses cell walls which contain

aspartic acid but no diaminopimelic acid, as in most lactobacilli, whereas the walls of the spore do contain diaminopimelic acid, as in most Bacillus species and *L. plantarum* (Powell & Strange, 1957). Organisms described by Thornley & Sharpe (1959) possessed characters apparently relating them to the genus *Bacillus* and to the genus *Lactobacillus*. Similarly, the organism known as *Leptotrichia buccalis* (Hamilton & Zahler, 1957; Davis & Baird-Parker, 1959) and those described by Theilade & Gilmour (1961) are also of possible significance. Bisset (1962) pointed out the possible significance of certain bacterial genera such as *Bacillus* and *Pseudomonas* as the phylogenetic origins of other bacteria and the evidence quoted above conforms with this suggestion.

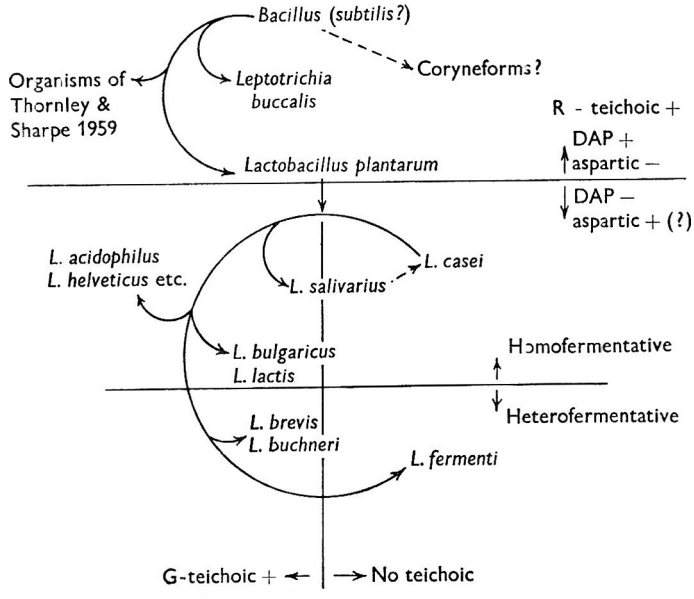

Fig. 1. Phylogenetic scheme for lactobacilli.

So far as relationships within the genus *Lactobacillus* are concerned, Fig. 1 shows one interpretation of the evolution of the various species. The basic assumptions of this scheme are: (*a*) heterofermenters evolved from homofermenters; (*b*) *L. plantarum* is the primitive lactobacillus. The reasoning behind Fig. 1 is that *L. plantarum* links the genera *Lactobacillus* and *Bacillus* as discussed above. The streptobacterium species, *Lactobacillus casei*, represents the further evolution of *L. plantarum* and the shared characters of these two species lead to their classification together as streptobacteria. *Lactobacillus casei* is a successful and ubiquitous species and its derivation from *L. plantarum* is quite a reasonable supposition. *Lactobacillus salivarius* exhibits some characters relating it to the thermobacteria, but from the evidence of Cheeseman (1959) and Keddie (1959) it is probably best considered as a high temperature streptobacterium species. As indicated in Fig. 1 it is possible that *L. salivarius* represents an offshoot of an evolutionary line leading from *L. plantarum* to the true thermobacteria. *Lactobacillus salivarius* certainly exhibits more characters of the streptobacteria than of the true thermobacteria, and its ability to grow at 45° but not at 15° probably reflects the plasticity of these characters seen in *L. plantarum*

(see above) and leads to the next step in Fig. 1, namely the origins of the thermobacteria. This group contains a number of species, each more or less specialized to a certain habitat, and probably includes the most highly specialized versions of the homofermentative lactobacillus on the evidence of ecology and synthetic abilities. In Sharpe's (1955) serological studies the thermobacterium species *L. lactis* and *L. bulgaricus* shared a common group antigen with the betabacterium (heterofermentative) species *L. brevis* and *L. buchneri*. These four species also resemble each other in possessing glycerol teichoic acid as a cell-wall component. The production of ammonia from arginine is an almost universal character of the heterofermenters, but certain strains of *L. brevis* do not carry out this reaction, whereas certain thermobacteria (e.g. *L. leichmannii* and *L. delbrueckii*) are unusual in this respect and resemble betabacteria. The occurrence of a slight but definite serological relationship between *L. plantarum* and the 'lactis–brevis' group (Sharpe, 1955) provides a link between all three of the subgeneric groups and further grounds for proposing that the heterofermenters evolved via the 'plantarum–lactis–brevis' pathway. It is of interest to note that *L. brevis* resembles *L. plantarum* in fermenting pentoses; this is a fairly common character among heterofermenters but is restricted to *L. plantarum* in the homofermenters. *L. brevis* also shares with *L. plantarum* a degree of resistance to penicillin not found in other lactobacilli (Davis, 1959). The fact that *L. brevis* commonly shares habitats with *L. plantarum* is interesting in the present context, but also suggests that their apparent similarities may be explicable in terms of parallel evolution. *Lactobacillus fermenti* is probably the most successful and ubiquitous heterofermenter. It comprises a separate serological group and teichoic acid has not been detected in its cell wall. In speculations of the type outlined above there is always more than one way of interpreting available knowledge. For example, it could be argued that the thermobacteria show signs of closer relationship to the genus *Bacillus* than do the streptobacteria. Similarly it might be feasible to derive *L. fermenti* direct from *L. casei*.

The possible significance of atypical or vestigial characters in bacterial phylogeny is interesting and it is relevant to the present notes to mention the case of *Streptococcus faecalis*. Hirsch (1952) and Orla-Jensen (1943) suggested that *S. faecalis* represents the ancestor of more recently evolved streptococci, e.g. *S. lactis*. Catalase production and motility in *S. faecalis* strains have been reported by Langston, Guttierez & Bouma (1960*a*, *b*) and Steel (1962). Orla-Jensen (1943) represented his ideas about the relationships within the lactic acid bacteria in the form of a diagram with *Lactobacillus plantarum* in a key position and although my Fig. 1 differs considerably from Orla-Jensen's scheme the basic assumption is the same; i.e. '...it is necessary to consider these (streptobacteria) and especially *Streptobacterium plantarum* as the ancestors of the other lactic acid bacteria.'

The inclusion in Fig. 1 of a tentative linkage between the genera *Bacillus* and *Corynebacterium*, or coryneforms in general, should be supplemented by an equally probable linkage between Lactobacillus and coryneforms (compare 'Lactomyces' of von Magnus, 1947, and also 'Lactobacillus bifidus').

Computer taxonomy was mentioned earlier and it should be possible to test, to some extent, speculations about bacterial phylogeny, by applying statistics such as proposed by Sneath (1957). The evidence of similarity values based upon many unweighted characters is likely to be a fairly good indication of phyletic relationships.

Differences in few characters will generally imply closeness of ancestry. Table 2 shows the results obtained after cluster analysis of similarity values calculated manually for the organisms listed. Unfortunately directly comparative information for all these organisms is not available. For example, of the sixty to seventy characters (not character states; see Sneath, 1962) for which results are available for lactobacilli, only twenty to thirty comparable results are available for *Leptotrichia buccalis* or the organisms of Theilade & Gilmour (1961). It would be likewise difficult to compare lactobacilli with Bacillus or Corynebacterium species

Table 2. *Rearrangement of organisms after cluster analysis of S-values*

	2	3	8	15	9	7	1	5	4	6	13	14	10	11	12	
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Thornley & Sharpe Group 2
3	72	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Thornley & Sharpe Group 3
8	67	63	.	.	.	.	.	.	.	.	.	.	.	.	.	*Lactobacillus plantarum* (S)
15	56	59	68	.	.	.	.	.	.	.	.	.	.	.	.	*Leptotrichia buccalis*
9	62	66	58	56	.	.	.	.	.	.	.	.	.	.	.	*Lactobacillus casei* (S)
7	52	48	58	63	55	.	.	.	.	.	.	.	.	.	.	*Lactobacillus salivarius* (S)
1	62	55	60	62	55	58	.	.	.	.	.	.	.	.	.	Thornley & Sharpe Group 1
5	58	58	51	62	57	59	60	.	.	.	.	.	.	.	.	*Lactobacillus acidophilus* (T)
4	37	35	35	50	40	44	36	62	.	.	.	.	.	.	.	*Lactobacillus helveticus* (T)
6	42	41	36	57	41	44	50	60	58	.	.	.	.	.	.	*Lactobacillus delbrueckii* (T)
13	48	44	45	58	41	40	35	38	37	40	.	.	.	.	.	*Lactobacillus viridescens* (B)
14	46	50	58	50	50	56	37	45	42	34	50	.	.	.	.	Theilade & Gilmour
10	48	45	41	47	42	48	36	47	53	50	50	63	.	.	.	*Lactobacillus fermenti* (B)
11	42	34	51	55	41	43	30	33	34	36	54	65	61	.	.	*Lactobacillus buchneri* (B)
12	47	45	46	50	44	44	37	43	45	43	59	54	66	73	.	*Lactobacillus brevis* (B)

Figures across top and down left-hand side of Table 2 are the numbers originally allotted to the organisms listed on the right-hand side. (S) indicates streptobacterium; (T) thermobacterium; (B) betabacterium.

upon our present knowledge. In view of the known occurrence of unusual results when inadequate data are used in such analyses it would be unwise to place much reliance upon Table 2. It was not possible to construct a dendrogram from these results but three groups of elevated S-values can be detected, namely the 'fermenti-buchneri–brevis' group, the 'helveticus–delbrueckii' group, and the group comprising *Lactobacillus acidophilus* and the seven organisms above it. The order of the organisms in Table 2 is, however, in good agreement with the ideas put forward in these notes as a comparison between Table 2 and Fig. 1 will show.

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Gamma Irradiation of *Bacillus subtilis* Spores in the Presence of Sugars

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SUMMARY

Spores of *Bacillus subtilis* were gamma-irradiated (^{60}Co source) and viable counts performed by surface spread and tube dilution methods. Surface spread counts were greater than tube dilution counts by factors varying from 1·57 to 3·90 (aqueous suspensions) and 2·36 to 6·10 (freeze-dried). In 8 cases out of 10 there was no significant difference in the regression coefficients of log % survivors against radiation dose for the two counting methods under identical conditions of irradiation. Freeze-drying from 5 % (w/v) aqueous solutions of glucose, lactose or fructose had a significant protective effect on the radiation resistance of the spores. Freeze-drying from aqueous suspension, or from 5 % maltose had no effect on the resistance.

INTRODUCTION

Hill & Phillips (1959) used a tube dilution method to assess the inactivation of *Bacillus subtilis* spores in penicillin by gamma-irradiation, claiming that in 'conventional nutrient plate colony counts' the viable count apparently increased as dilution increased. This was attributed to the presence of an inhibitor of spore germination, the tube dilution method being used to dilute such an inhibitor. Direct comparison of surface spread and tube dilution methods for assessing inactivation of irradiated organisms has not been previously reported, although plating methods are generally used. Freeze-drying from 5 % glucose solution has been shown to have a significant protective effect on *B. subtilis* spores subsequently subjected to gamma-irradiation (Roberts, 1961). This work has now been extended to include maltose and fructose, and the results with glucose and lactose confirmed, by using tube dilution and plate count methods.

METHODS

Preparation of spore suspension. Bacillus subtilis NCTC 8236 was grown on Lemco agar containing 0·0001 % manganous sulphate, with incubation at 37° for 14 days. The resultant spores were washed five times with sterile water, heated at 78–80° for 20 min. to kill vegetative forms, and stored in aqueous suspension at 0–4°.

Media. 'Oxoid' peptone agar, and 'Oxoid' peptone water (Oxo Ltd., London).

Diluent. The diluent for counting suspensions was sterile distilled water.

Solutions. Solutions of carbohydrates (5%, w/v) in sterile distilled water were used throughout. Glucose was of 'Analar' quality (British Drug Houses Ltd., London), maltose, lactose and fructose were 'Biochemical Reagents' (Thos. Kerfoot and Co.

Ltd., Vale of Bardsley, Lancs.). (These solutions will be referred to as '5 % glucose', etc.)

Freeze-drying. Samples (0·1 ml.) of spore suspensions in 0·5 ml. freeze-drying tubes (B.S.S. 795, 1961) were snap-frozen, and primary-dried over P_2O_5 at 0·01 mm. Hg in an Edwards and Co. Ltd., London, Model L.T. 5 apparatus for 6–8 hr, then maintained over fresh P_2O_5 for a further 18 hr at 0·01 mm. Hg. Ampoules were sealed at atmospheric pressure under air.

Irradiation. Samples were irradiated in a 'hot-spot' ^{60}Co source at the Wantage Research Laboratory, A.E.R.E., at a dose-rate of $2{\cdot}68 \times 10^5$ rads/hr.

Ampoules containing spores were stored at 0° before and after irradiation, which was done at room temperature. Radiation dosimetry was performed by members of the Wantage laboratory staff by the method of oxidation of Fe^{2+} in 0·8 N-sulphuric acid.

Viable count methods

Spread-plate count. A sample (0·5 ml.) of a suspension containing 40–80 viable spores (determined by a preliminary count) was spread on the surface of each of six overdried peptone agar plates (20 ml.) and incubated at 37° for not less than 36 hr.

Tube dilution count. The method used was that described by Cochran (1950) and Finney (1951) for estimating bacterial populations by dilution series. Twofold dilutions were used. Ten samples of 5 drops from calibrated needles were taken from each dilution, and inoculated into peptone water (10 ml.). Tubes were incubated at 37° shaken daily, and read at intervals during 2 weeks. Increase in number of positives was never observed after 7 days, and about 90 % of positives were evident after 48 hr. Five two-fold dilutions were used for each count planned to give an about average of one organism/sample in the third dilution. This gave about equal numbers of positives and negatives, from which maximum information was obtained.

Table 1. *Gamma-irradiation of spores of Bacillus subtilis NCTC 8236, freeze-dried from 5% glucose*

Comparison of the methods of Finney (1951) and Fisher & Yates (1957) for calculating most probable numbers (MPN) from the same dilution series data.

Radiation dose (rads × 10^5)	Fisher & Yates method		Finney's method	
	MPN	Survivors (%)	MPN	Survivors (%)
0	$1{\cdot}6948 \times 10^6$	100	$1{\cdot}9257 \times 10^6$	100
0·66	$1{\cdot}1844 \times 10^6$	69·88	$1{\cdot}0346 \times 10^6$	53·73
1·33	$5{\cdot}9219 \times 10^5$	34·94	$5{\cdot}2578 \times 10^5$	27·30
2·65	$2{\cdot}8296 \times 10^5$	16·70	$2{\cdot}7514 \times 10^5$	14·29
3·97	$6{\cdot}1425 \times 10^4$	3·624	$6{\cdot}4648 \times 10^4$	3·357
5·29	$3{\cdot}1067 \times 10^4$	1·833	$2{\cdot}2266 \times 10^4$	1·156
r (correlation coefficient)		−0·9924		−0·9941
b (regression coefficient rads × 10^{-5})		−0·3476		−0·3571

Comparison of slopes: calculated 'd' = 0·2812, tabulated 't' = 2·447 at $P = 0{\cdot}05$. There is therefore no significant difference in slope.

Calculation of most probable number of viable organisms/ml. Finney (1951) estimated the population density of suspensions of organisms by solving the equation of maximum likelihood by use of a log-log transformation. This method is longer

and more tedious than that of Fisher & Yates (1957) who showed that 87·7 % of the available information is contained in the total number of positive or negative tubes counted without regard to dilution level. A comparison of identical data by using both the Finney and the Fisher & Yates methods of calculation (Table 1) resulted in no significant difference in the slopes of the regressions of log % survivors against radiation dose being established.

The Fisher & Yates method of calculation of the most probable number (MPN) was therefore used.

Statistical analysis. Results from irradiation experiments were expressed as % survivors, using counts from unirradiated ampoules as representative of 100%. In every case a plot of log % survivors against dose was linear. Pairs of calculated regressions were compared by a modified '*t*' rest (Bailey, 1959). Blocks of regression were tested for parallelism by an analysis of variance, and when parallelism was established a further analysis showed whether a common regression line could be plotted (Roberts, 1961).

RESULTS

A summary of results is given in Table 2.

It was found (Table 3) that in 8 cases out of 10 the regressions of log % survivors against dose did not differ significantly whether counted by surface-spread or tube-dilution counts. Protection after freeze-drying, where it occurred, was evident by either counting method (Table 4). Block analyses of variance were performed only on plate-count results.

Table 2. *Survival of Bacillus subtilis* NCTC *8236 spores after gamma irradiation under various conditions*

Slopes (*b*) and *D* values for regressions of log % survivors against radiation dose in aqueous and freeze-dried carbohydrates. aq., suspension of spores in 5 % aqueous solution of...; f.d., spores freeze-dried from a 5 % aqueous solution of...; *D* value, that radiation dose required to decrease viability to 10 % of the initial value.

	Plate counts		Tube counts	
Spore suspension treatment	*b* (rads × 10^{-5})	*D* (rads × 10^{5})	*b* (rads × 10^{-5})	*D* (rads × 10^{5})
Aqueous suspension	−0·6298	1·59	−0·5744	1·74
f.d. from aqueous suspension	−0·6076	1·65	−0·6462	1·55
aq. maltose	−0·6308	1·59	−0·6322	1·58
f.d. maltose	−0·5265	1·90	−0·5243	1·91
aq. glucose	−0·7135	1·40	−0·5320	1·88
f.d. glucose	−0·3962	2·52	−0·3476	2·88
aq. lactose	−0·5607	1·78	−0·5825	1·72
f.d. lactose	−0·3177	3·15	−0·2525	3·96
aq. fructose	−0·6748	1·48	−0·6175	1·62
f.d. fructose	−0·2359	4·24	−0·1871	5·35
correlation coefficient (*r*)	all > −0·9790		all > −0·9800	

For each carbohydrate separate survivor curves were determined thus: (*a*) irradiated spore suspension by plate count; (*b*) irradiated spore suspension by tube count; (*c*) irradiated freeze-dried sample of spores by plate count; (*d*) irradiated freeze-dried sample of spores by tube count.

Spores irradiated in aqueous suspension, and freeze-dried from aqueous suspension. The slopes of four survivor curves (*a–d*) were not significantly different ($F = 3{\cdot}73$, $P = 0{\cdot}10$–$0{\cdot}20$) and could be represented by a common regression line ($F = 1{\cdot}15$, P = greater than 0·20) of slope $-0{\cdot}6588$ (rads $\times 10^{-5}$).

Table 3. *Comparison of counting methods*

Modified '*t*' tests on regressions of log % survivors against radiation dose determined by plate and tube counts in aqueous and freeze-dried states.

	Calculated '*d*'				
	Water	Maltose	Glucose	Lactose	Fructose
*aq. plates *versus* aq. tubes	1·12	0·017	*3·71*	0·59	0·83
f.d. plates *versus* f.d. tubes	0·26	0·033	1·62	*4·11*	0·84

Those '*d*'s in italics indicate a significant difference between slopes.

* aq. plates, suspension of spores: surface spread-count; aq. tubes, suspension of spores: tube dilution count: f.d. plates, spores freeze-dried: surface spread count; f.d. tubes, spores freeze-dried tube-dilution count.

Table 4. *Comparison of regressions of log % survivors against radiation dose in aqueous and freeze-dried states by plate and tube counts*

	Calculated '*d*'				
	Water	Maltose	Glucose	Lactose	Fructose
*aq. plates *versus* f.d. plates	0·31	1·99	*5·83*	*12·98*	*11·88*
aq. tubes *versus* f.d. tubes	0·53	1·18	*4·46*	*9·35*	*5·25*

Those '*d*'s in italics show a significant difference in slopes, indicative of a protective effect upon free-drying.

* aq. plates, suspension of spores: surface spread count; aq. tubes, suspension of spores: tube-dilution count; f.d. plates, spores freeze-dried: surface spread count; f.d. tubes, spores freeze-dried: tube-dilution count.

Spores irradiated in 5 % maltose and freeze-dried from 5 % maltose. The slopes of the four survivor curves were not significantly different ($F = 3{\cdot}80$, $P = 0{\cdot}10$–$0{\cdot}20$) and could be represented by a common regression line ($F = 1{\cdot}16$, $P > 0{\cdot}20$) of slope $-0{\cdot}5921$ (rads $\times 10^{-5}$) (Fig. 1).

Spores irradiated in 5 % glucose and freeze-dried from 5 % glucose. The results are shown in Fig. 2. Freeze-drying produced a significant protective effect, but not as great as previously reported (Roberts, 1961). The reason for the less marked protection here is not known. It might be due to slight variation in the conditions of freeze-drying, or the observed increase in radiation resistance of the spore suspension on prolonged storage at 0–4°.

Spores irradiated in 5 % lactose, and freeze-dried from 5 % lactose. A significant protective effect on freeze-drying was shown when the slopes alone were considered (Fig. 3). Extrapolation of the regression for freeze-dried samples to zero irradiation dose indicates that only 4–8 % of the spores were protected. This is almost exactly the previous result. The 'glass' formed by lactose was less obvious than those formed by glucose and fructose. It is possible that only 4–8 % of the spores are actually in the lactose 'glass' and that only these were protected. However, $0{\cdot}66 \times 10^5$ rads resulted in greater inactivation (96·5 %) in freeze-dried lactose preparations than

in any other system used. This dose gave mean inactivations of 57% in water, 66 % in maltose, 66 % in aqueous glucose, and 55 % in aqueous fructose.

Spores irradiated in 5 % fructose and freeze-dried from 5 % fructose. A significant protective effect of freeze-drying was shown (Fig. 4). The degree of protection was estimated by:

$$\frac{D\text{-value protected (freeze-dried)}}{D\text{-value unprotected (aqueous)}}.$$

Values obtained were: glucose, 1·53–1·78; lactose, 1·77–2·30; fructose, 2·86–3·30. Although glucose, lactose, and fructose all gave protection on freeze-drying, the regressions were not parallel ($F = 9{\cdot}58$, $P = 0{\cdot}001$–$0{\cdot}01$).

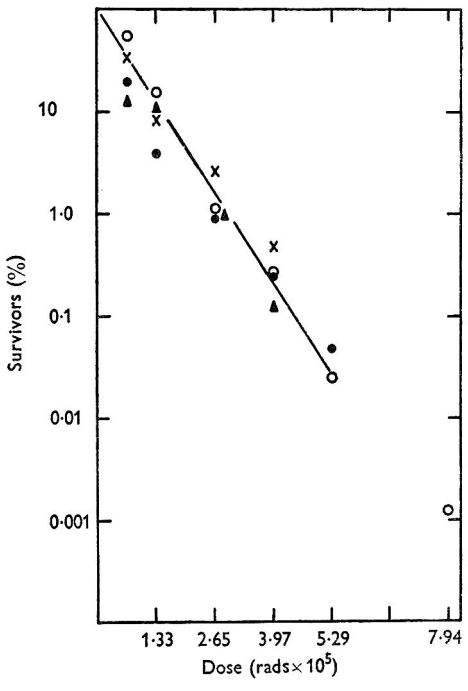

Fig. 1

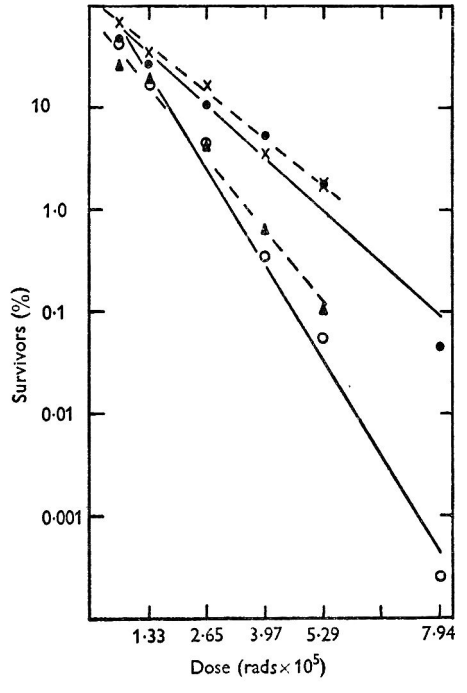

Fig. 2

Fig. 1. —●—, Spores freeze-dried from 5 % maltose: surface spread count; —○—, spores suspended in 5 % maltose: surface spread count; — — × — —, spores freeze-dried from 5 % maltose: tube-dilution count; — —▲— —, spores suspended in 5 % maltose: tube-dilution count.

Fig. 2. —●—, Spores freeze-dried from 5 % glucose: surface spread count; —○—, spores suspended in 5 % glucose: surface spread count; — — × — —, spores freeze-dried from 5 % glucose: tube-dilution count; — —▲— —, spores suspended in 5 % glucose: tube-dilution count.

Regressions for the aqueous suspension, those suspensions in aqueous maltose, lactose, glucose and fructose, and the sample freeze-dried from water were parallel ($F = 1{\cdot}93$, $P = 0{\cdot}10$–$0{\cdot}20$) but could not be represented by a common regression line ($F = 9{\cdot}90$, $P < 0{\cdot}001$). Addition of the regression for freeze-dried maltose to the former analysis diminishes the significance level ($F = 2{\cdot}24$, $P = 0{\cdot}05$–$0{\cdot}10$).

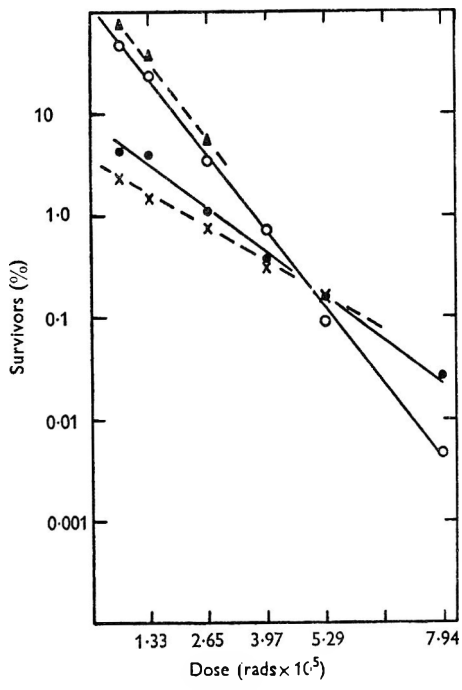

Fig. 3

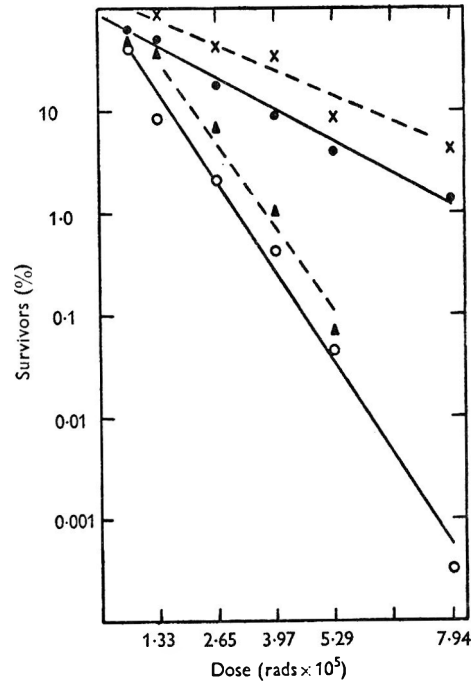

Fig. 4

Fig. 3. —●—, Spores freeze-dried from 5 % lactose: surface spread count; —○—, spores suspended in 5 % lactose: surface spread count; — — × — —, spores freeze-dried from 5 % lactose: tube dilution count; — —▲— —, spores suspended in 5 % lactose: tube-dilution count.

Fig. 4. —●—, Spores freeze-dried from 5 % fructose: surface spread count; —○—, spores suspended in 5 % fructose: surface spread count; — — × — —, spores freeze-dried from 5 % fructose: tube-dilution count; — —▲— —, spores suspended in 5 % fructose: tube-dilution count.

DISCUSSION

It should be borne in mind that, while no significant difference in the slopes of log % survivors against dose counted by surface-spread and tube-dilution methods has been established, the actual number of survivors was, with 2 exceptions in 54 cases, greater on plates than in tubes. This is illustrated in Table 5.

Linear log survivor/dose curves have been obtained by Donnellan & Morowitz (1957) and by Woese (1958) for the radiation inactivation of *Bacillus subtilis* spores. There is no precedent for the type of protection we found above. Protection was evidently associated with the presence of a glass, since maltose produced no obvious glass and no protection, and fructose produced the most marked glass and the greatest protection. Although glass formation is a well-known phenomenon, its characteristics are not readily available. Freeze-drying solutions of glucose from 1 to 30 % (w/v) all produced glasses, and presumably should therefore protect. Whether the protection is concentration-dependent has not yet been examined. It has been reported (Proctor, Goldblith, Oberle & Miller (1955)) that the radiation sensitivity of *B. subtilis* is less *in vacuo* than in air. Since the glass forms during the

freeze-drying process, it appears possible that the spores encased in the glass are present in a localized vacuum and exhibit typical *in vacuo* resistance. In the absence of glass formation (maltose) and in suspensions, spores were irradiated in the presence of air. The protective effect demonstrated in freeze-dried sugars is in each case greater than that shown by Proctor *et al.* (1955), and it seems unlikely that this is the complete explanation.

Table 5. *Plate count/tube count of suspensions of spores of Bacillus subtilis NCTC 8236 in 5 % carbohydrate solutions and samples freeze-dried from 5% carbohydrate solutions at different irradiation doses*

Suspension medium	Unirradiated		Radiation dose (rads × 10^5) 0·67		1·33		2·67		3·97		5·29		Mean	Mean
Sugar	aq.	f.d.	aq.	f.d.	aq.	f.d.	aq.	f.d.	aq.	f.d.	aq.	f.d.	aq.	f.d.
Water	4·05	4·28	2·86	3·31	2·44	2·11	1·34	2·36	2·06	1·58	—	3·56	2·55	2·87
Maltose	3·20	7·05	1·40	3·97	4·56	3·29	3·32	2·46	7·02	3·58	—	—	3·90	4·07
Glucose	1·98	2·42	3·15	1·66	1·98	1·84	1·84	1·46	1·07	5·38	1·05	2·37	1·85	2·52
Lactose	2·60	3·56	1·63	7·38	1·68	10·92	1·63	5·88	—	4·97	—	3·91	1·89	6·10
Fructose	2·71	4·40	2·35	—	0·63	2·43	0·85	1·79	1·10	1·13	1·75	2·05	1·57	2·36
Mean	2·91	4·34	2·28	4·08	2·26	4·12	1·80	2·79	2·79	3·33	1·40	2·97	—	—

Formation of toxic radicals outside the cell may be neglected as a factor in the mechanism of inactivation by ionizing radiations (Davis, 1954; Hutchinson, 1955, 1957). In addition to the 'direct' effect of ionizing radiation, toxic free-radicals produced within spores contribute to their inactivation (Powers, Webb & Ehret, 1960). The latter damage is dependent upon conditions and may be largely avoided by the prevention of toxic free-radicals reactions. The former is independent of conditions, and is now assumed to include ionizations within 'bound' water (Alexander, 1959) since the water forms an integral part of the protein, and radicals formed within it are not freely diffusible.

No evidence is available to indicate whether the sugar glass forms only outside the spore, or inside the spore wall. High concentrations of glucose external to cells of *Saccharomyces cerevisiae* led to intracellular dehydration resulting in a marked decrease in radiation sensitivity (Wood, 1959). The importance of intracellular water has also been stressed in work with bacterial spores (Tallentire & Powers, 1961; Webb & Powers, 1961).

Conflicting results have been reported which suggest that drying may protect against (Moos, 1952) or, when oxygen is present, sensitize to, subsequent irradiation (Tallentire, 1958). Experiments with rigidly controlled water content show sensitization of spores to X- and gamma-radiation in the presence of, and when stored in the presence of, oxygen. Sensitivity is a function of water content, decreasing sensitivity occurring with increasing water content (Tallentire & Davies, 1961; Tallentire & Powers, 1963). Dehydration may therefore explain the sensitization to irradiation of 94 % of the population of spores of *Bacillus subtilis* in freeze-dried lactose, but does not account for the increased radiation resistance of the remaining 6 %, or for the protection afforded to spores in freeze-dried glucose and in freeze-dried fructose. Further, the reason for sensitization occurring only in freeze-dried

lactose is obscure. Black & Gerhardt (1961) showed that *B. cereus* spores were permeated by glucose, principally by passive diffusion. Formation of a sugar glass within the spore therefore seems possible.

On present evidence the most probable site for the primary cellular lesion of radiation is deoxyribonucleic acid (DNA), no other macromolecule within the cell being so radiation sensitive and so indispensable to the cell. Alexander (1959) stated that reactions causing change in shape or size of macromolecules, such as main-chain scission, or cross-linking, are more likely to cause inactivation than reactions involving slight modification of chemical structure. A cross-linked macromolecular gel structure in the spore core has been proposed by Black & Gerhardt (1962). Even if glucose is unable to diffuse into this core, glass formation round the core might maintain its integrity and the viability of the spore.

The irradiation of carbohydrates has been the subject of much research, but aqueous solutions have almost always been used (Phillips & Moody, 1959; Barker, Grant, Stacey & Ward, 1959). Khenokh, Kuzicheva & Evdokimov (1961) detected long-life radicals in the crystal lattice up to 1 year after the irradiation of dry carbohydrates. Collins (1962) irradiated 'Analar' samples of carbohydrates, and determined electron spin resonance (ESR) spectra 3 months after irradiation; the results indicated the presence of long-lived radicals. Room-temperature stabilization of organic free-radicals of barbituric acid derivatives in a boric acid glass was reported by Cloutier (1961); carbohydrate glass with similar properties therefore seems possible. The protection would then be due to the prevention of toxic free-radical reactions.

Subsequent work to that reported in this paper, with *Escherichia coli* CN1539, in the stationary phase, indicates that surface-spread ('Oxoid' peptone agar) and tube-dilution ('Oxoid' peptone water) counts give nearly identical numerical recoveries. The *D*-value in aqueous suspension was $1{\cdot}93 \times 10^4$ rads. Using *E. coli* CN 1539 a similar protective effect was found after freeze-drying from 5 % glucose (*D*-value $8{\cdot}10 \times 10^4$ rads), with no protection either in 5 % glucose (*D*-value $1{\cdot}06 \times 10^4$ rads) or after freeze-drying from water (*D*-value $1{\cdot}3 \times 10^4$ rads).

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Microbiological Fractionation of Sulphur Isotopes

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SUMMARY

A fractionation of sulphur isotopes was found in all metabolic processes investigated except those in which elemental sulphur was the starting substrate for growth of *Thiobacillus concretivorus* and *Chromatium* sp. and for reduction by *Saccharomyces cerevisiae*. Except for polythionates formed during sulphide oxidation by *T. concretivorus* or *Chromatium* sp., the products of metabolism were enriched in ^{32}S relative to the starting substrates. The magnitudes of the enrichment differed for different processes and for the same overall process carried out by different organisms. The $\delta^{34}S$ values (‰) ranged from −46·0 for sulphide from sulphate reduction by *Desulfovibrio desulfuricans* to +19·0 for polythionate formed during growth on sulphide by *T. concretivorus*. Fractionation during sulphate reduction was inversely proportional to rate of reduction when lactate and ethanol were electron donors and directly proportional with molecular hydrogen as the electron donor. Temperature and sulphate concentration, within the normal physiological ranges of these parameters, influenced fractionation only in so far as they influenced rate of reduction. However, anomalous fractionation effects were obtained at low temperatures and when a resting suspension reducing sulphite was subjected to changes in temperature. The data are discussed with reference to the mechanism(s) of fractionation.

INTRODUCTION

The ability of micro-organisms to enrich one isotope of sulphur in preference to others during metabolic function was first demonstrated by Thode, Kleerekoper & McElcheran (1951). They found that the sulphide released during sulphate reduction by growing cells of *Desulfovibrio desulfuricans* was enriched in the lighter isotope of sulphur (^{32}S) by a factor of 10–12‰. Subsequent observations by Jones & Starkey (1957) showed that the degree of fractionation could be increased to 27‰ by growing cultures at reduced temperature (14–20°) and high sulphate concentration. They suggested that temperature regulated the rate of reduction, thereby controlling the enrichment. Similar results were obtained by Kaplan, Rafter & Hulston (1960), who also found that the rate of sulphate reduction was the most important parameter controlling enrichment, with sulphate concentration playing a minor role. The fractionation phenomenon was critically studied by Harrison & Thode (1958) using resting suspensions of *D. desulfuricans*. Their results demonstrated that the degree of fractionation was inversely proportional to the rate of reduction (controlled by temperature and electron donor concentration) and was influenced by sulphate only at limiting concentrations. Apart from some

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experiments by Kaplan *et al.* (1960) with steel wool, lactate served as the electron donor in all the studies cited above. Jones & Starkey (1957), Kaplan & Rafter (1958) and Kaplan *et al.* (1960) could not demonstrate significant isotope fractionation during sulphur oxidation to sulphate by thiobacilli. The oxidation of hydrogen sulphide, however, yielded elemental sulphur and sulphate enriched in ^{32}S (Kaplan & Rafter, 1958).

Although many attempts have been made to interpret processes in nature in terms of microbiological fractionation of sulphur isotopes (Thode, Macnamara & Fleming, 1953; Feely & Kulp, 1957; Jensen, 1958; Kaplan *et al.* 1960, and others), only one attempt has been made to explain the underlying physiological events (Harrison & Thode, 1958). The present study had as its objectives a re-investigation of fractionation during sulphate reduction and an extension of our knowledge of this phenomenon to other important metabolic processes in the sulphur cycle. A brief report of the work has been made (Kaplan & Rittenberg, 1962*b*).

METHODS

Microbiological

Organisms and growth procedures. Desulfovibrio desulfuricans, Hildenborough strain, was used for most sulphate reduction studies. A few experiments, specifically mentioned in the text, were done with a second strain isolated in the authors' laboratory. Resting suspensions were prepared from cultures grown at 30° in 12 l. flasks, using the mineral salts solution of Mechalas & Rittenberg (1960) to which yeast extract (1·0 g./l.) and sodium lactate (3·5 g./l.) were added. The yeast extract was omitted from the medium when fractionation measurements were made on growing cultures so that the added sulphate was the only sulphur source.

Thiobacillus concretivorus (Parker, 1947): isotope fractionation measurements were made with growing cultures oxidizing elemental sulphur or sulphide. In both instances, the base salts solution used contained (g./l. distilled H_2O): KH_2PO_4, 3·0; $MgCl_2$, 0·1; $CaCl_2$, 0·1; NH_4Cl, 0·2; pH 4·6. About 50 ml. of this solution plus 300 mg. elemental sulphur in 250 ml. Erlenmeyer flasks were used for sulphur oxidation studies. The sulphur was sterilized separately by heating on a steam bath on 3 consecutive days for 1 hr each time. For growth on hydrogen sulphide, either several small flasks of the basal salts solution were incubated in a 20 l. container continually flushed with a mixture of H_2S + air (3 + 3000 ml./hr), or the gas mixture was blown directly over thin layers of medium in Fernbach flasks.

Chromatium sp.: photosynthetic sulphide and sulphur oxidation were studied. The base medium contained (g./l. distilled H_2O): KH_2PO_4, 1·0; NH_4Cl, 1·0; $MgCl_2$, 0·5; NaCl, 10; trace elements (with no sulphate anions) in the concentrations suggested by Larsen (1953). To this was added 20 ml. of 10 % (w/v) $NaHCO_3$ separately autoclaved, cooled and aseptically saturated with CO_2; also either 15 ml. 10 % (w/v) $Na_2S.9H_2O$ or 250 mg. sulphur, separately autoclaved. The pH was adjusted to 8·2–8·4. The cultures were grown at 30° in 500 ml. glass-stoppered bottles illuminated by tungsten filament lamps.

Saccharomyces cerevisiae was used to investigate fractionation while incorporating sulphate into organic sulphur during growth and also to study sulphite and elemental sulphur reduction by resting organisms. For the former purpose, yeasts were

harvested for analysis after 2 days growth at 30° on the following medium (g./l. distilled H_2O): glucose, 20·0; KH_2PO_4, 1·0; NH_4Cl, 2·0; yeast extract, 0·5; Na_2SO_4, 8·5; $CaCl_2.6H_2O$, 0·5; $MgCl_2$, 0·5; $FeCl_3$, 0·02. For the latter experiments resting suspensions were prepared from commercially grown, starch-free cakes of Fleishmann's baker's yeast.

Ankistrodesmus sp. was grown on a modification of Knops medium (Curtis & Clark, 1950, p. 384) having a total sulphur content (as Na_2SO_4) of 55 mg./l. The cultures were harvested after incubation at 30° for 8 days and the isotopic composition of the organic sulphur determined.

Escherichia coli was grown on the medium suggested by Roberts *et al.* (1955, p. 5) having a sulphur content (as Na_2SO_4) of 500 mg./l. Bacteria were harvested after 40 hr incubation at 30° and the organic sulphur analysed.

Proteus vulgaris was grown in a 3% (w/v) trypticase soy broth (Baltimore Biological Laboratories) with vigorous aeration. Cultures were harvested after 36 hr at 30° and used for resting suspension studies of cysteine degradation.

Resting suspension experiments. Desulfovibrio desulfuricans was separated from cultures by continuous centrifugation, rapidly washed twice with 0·01 M-potassium phosphate buffer (pH 7·2) suspended in deoxygenated buffer, and adjusted to the desired optical density. Known volumes of suspension were added to three-neck Woulfe bottles each fitted with a gas inlet and exit tube and a dropping funnel. The desired quantities of standard sodium sulphate or sulphite solution and, usually, sodium lactate or ethanol were then added, and the flasks incubated at controlled temperatures with constant flushing by a stream of deoxygenated nitrogen. When molecular hydrogen was used as the electron donor, it replaced nitrogen gas as the flushing agent.

A similar procedure was used for resting suspension studies with *Saccharomyces cerevisiae* and *Proteus vulgaris*. The yeasts were suspended in a potassium phosphate buffered 4% (w/v) glucose solution at pH 5·5; the bacteria were suspended in phosphate buffer (pH 7·2) containing 350 mg. cysteine hydrochloride/100 ml.

Optical density of suspensions. All resting suspension studies with *Desulfovibrio desulfuricans* were made at an optical density of 400 Klett–Summerson units (No. 54 filter) which corresponded to a direct microscopic count of $3{\cdot}0 \times 10^9$ organisms/ml. Harrison & Thode (1958) expressed their results in terms of a population equal to 100 ml. suspension of 0·1 optical density which is equivalent to 3×10^8 organisms/ml. The *Proteus vulgaris* suspensions had an optical density of 475 Klett–Summerson units (No. 54 filter). The population of this suspension was not determined. *Saccharomyces cerevisiae* suspensions contained 2·5 g. yeast cake/100 ml., equivalent to 4×10^8 cells/ml.

Chemical

Collection of metabolites. Hydrogen sulphide. During sulphate, sulphite and elemental sulphur reduction, as well as cysteine hydrolysis, hydrogen sulphide is the end product. In the experiments using lactate, ethanol or glucose as electron donors, and in the experiments with cysteine, the sulphide released was removed by a stream of deoxygenated nitrogen and trapped in two consecutive chambers containing $AgNO_3$. When hydrogen was employed, both as an electron donor and to sweep out H_2S, $AgNO_3$ could not be used because of the deposition of metallic silver, and Cd acetate was substituted.

To determine the rate of sulphate reduction, sequential analyses were made of individual reaction vessels by periodically removing and assaying the precipitated sulphide in the traps. Some sulphide remains in the reaction vessel with this technique, and since there could be an isotope effect in the distribution of sulphide between the liquid and gaseous phases, this procedure was not followed when isotopic analyses were made. Instead, a single determination was made from each reaction vessel.

The fractionation experiments were designed to permit utilization of less than 5% of the added sulphur source. The quantity of precipitant required to bind the desired amount of sulphide was placed in the first trap with an equal quantity in the second. When precipitation was first observed in the second trap the microbial process was stopped by adding sufficient hydrochloric acid to make the culture about pH 2 in experiments concerned with sulphate or sulphur reduction or cysteine decomposition. With sulphite, the pH value was adjusted to about 5, since at low pH values SO_2 is liberated which reacts with sulphide. After acidification, the vessels were placed in a hot water bath and bubbling was continued for about 15 min. to ensure complete liberation of sulphide from solution.

Elemental sulphur, sulphate and polythionates. When formed, these were successively determined, after freeing the cultures of sulphide as above. The sulphur was collected by filtration through sintered glass; the sulphate was precipitated from the boiling filtrate as the barium salt; and the polythionates remaining were then oxidized with bromine and collected as barium sulphate.

Organic sulphur. Sulphur assimilated by organisms grown with sulphate as the sulphur source was converted to sulphate by wet combustion of the harvested cells and determined as the barium salt (Kaplan, 1962).

Preparation of samples for isotope measurements. Detailed descriptions of the methods have appeared elsewhere (Thode, Wanless & Wallouch, 1954; Thode, Monster & Dunford, 1961; Feely & Kulp, 1957; Rafter, 1957; Hulston & Shilton, 1958). The procedures followed in the present work were modifications of those described by Rafter and by Hulston & Shilton (Kaplan, 1962).

Mass spectrometer analyses were made on sulphur dioxide prepared by combustion of silver sulphide or elemental sulphur in the presence of oxygen. All sulphate samples were first converted to sulphide by reduction with spectrographically pure graphite and reacted with silver nitrate to form silver sulphide. Elemental sulphur was burnt at 800°, while silver sulphide combustion was initiated at 600° with rapid increase in temperature to 1200°.

Half the mass spectrometer analyses were made at the Institute of Nuclear Science, Lower Hutt, New Zealand, the rest at the University of California, La Jolla. Both instruments used are Nier 60° sector types with double collection tubes and rapid sample switching devices. The instrumental error amounted to a standard deviation in $\delta^{34}S$ of 0·1–0·2‰.

By convention, enrichment or depletion of ^{34}S is expressed relative to a standard as δ values defined as follows:

$$\delta^{34}S‰ = \frac{(^{34}S/^{32}S)\ \text{sample} - (^{34}S/^{32}S)\text{standard}}{(^{34}S/^{32}S)\ \text{standard}} \times 1000.$$

All $\delta^{34}S$ values are given relative to the starting substrate as the standard.

RESULTS

Sulphate reduction by Desulfovibrio desulfuricans

Since previous investigators had established that fractionation is related to the rate of sulphate reduction, it was necessary to determine whether the metabolism of resting suspensions would be constant under the experimental conditions to be imposed and also to determine the effect of certain parameters on the rate of reduction. In general, sulphide production was linear over the time intervals investigated (Fig. 1). Sulphate concentration had little effect on the rate over the range of 0·02–0·06 M but was inhibitory at 0·15 M. The electron donor markedly influenced rate of reduction. Molecular hydrogen, whose utilization followed a short lag period, gave the fastest rate. The rate with lactate was generally about one-half and that with ethanol about one-tenth the hydrogen rate.

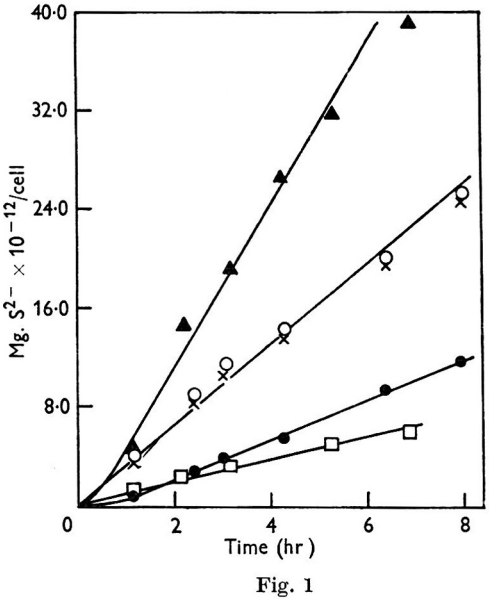

Fig. 1

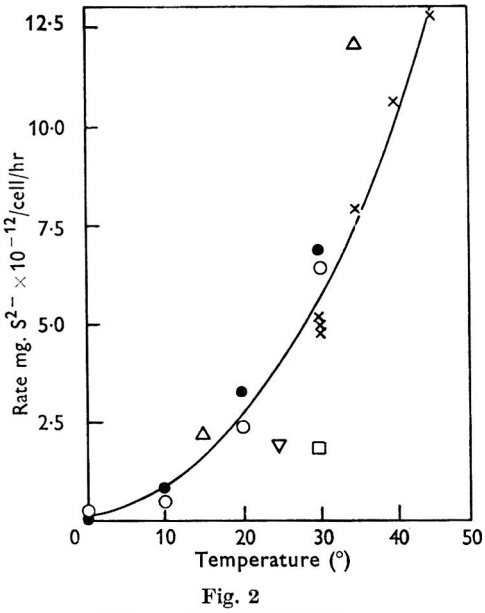

Fig. 2

Fig. 1. Rate of sulphate reduction by resting suspensions of *Desulfovibrio desulfuricans* at 30°. ○, Lactate + 0·02 M-SO_4^{2-}; ×, lactate + 0·06 M-SO_4^{2-}; ●, lactate + 0·15 M-SO_4^{2-}; ▲, hydrogen + 0·06 M-SO_4^{2-}; □, ethanol + 0·06 M-SO_4^{2-}.

Fig. 2. Rate of sulphate reduction by resting suspensions of *Desulfovibrio desulfuricans* as a function of temperature; lactate as electron donor, 0·06 M-sulphate. Each symbol denotes a separate experiment conducted with a single batch of organisms.

The reduction process had a Q_{10} of about 2 over a temperature range of 10–40°. A plot of rate against temperature for all resting suspension experiments using lactate and 0·06 M sulphate (Fig. 2) shows a dR/dT of about 5×10^{-12} mg. sulphide/organism/hr.

Effect of rate of reduction on isotope fractionation. The rate of reduction in individual experiments was altered by choice of temperature and hydrogen donor and was influenced by uncontrolled variations in different batches of resting organisms. Table 1 presents the rate and fractionation data obtained with three hydrogen

donors and 0·06 M-sulphate over a temperature range of 10–45°. Considering only lactate and ethanol, the best fit curve through the data is hyperbolic (Fig. 3) indicating that, with exceptions to be mentioned later, for these two hydrogen donors the isotope effect is inversely proportional to the rate of reduction, with the lighter

Table 1. *Isotopic fractionation during sulphate reduction by resting suspensions of Desulfovibrio desulfuricans*

Each experiment involved a different lot of organisms using 0·05 M-sulphate and lactate, ethanol or hydrogen at the temperatures indicated. $\delta^{34}S$ measured relative to the $^{34}S/^{32}S$ of the starting sulphate. Expts. 1–5 with strain 'Hildenborough'; 6–8 with strain '2'.

Expt. No.	Temp. (°)	Electron donor	Sulphate reduced (%)	Rate of reduction, mg. $S^{2-} \times 10^{-12}$/ organism/ hr	$\delta^{34}S$ of S^{2-} (‰)
1	30	Ethanol	2·61	2·17	−26·0
	30	H_2	2·17	7·18	−10·8
	30	Lactate	3·04	6·48	−25·5
	20	Lactate	2·37	1·85	−30·3
	10	Lactate	2·31	0·50	−35·5
	0	Lactate	1·19	0·13	−18·6
2	30	Ethanol	2·68	5·06	−17·1
	30	H_2	3·19	14·05	−10·1
	30	Lactate	2·24	6·85	−14·8
	20	Lactate	2·24	2·52	−15·1
	10	Lactate	2·55	0·75	−10·0
	0	Lactate	0·85	0·08	−5·2
3	30	Ethanol	2·44	0·41	−42·3
	20	Ethanol	2·10	0·17	−44·0
	30	H_2	2·20	2·64	−8·4
	10	H_2	2·20	0·52	−3·0
4	30	Ethanol	2·00	0·43	−34·9
	20	Ethanol	2·00	0·20	−46·0
	30	H_2	2·55	12·10	−11·2
	20	H_2	3·39	7·65	−6·4
	10	H_2	2·19	1·60	−5·0
5	45	Lactate	2·70	12·80	−16·1
	40	Lactate	2·95	10·80	−17·3
	35	Lactate	3·01	7·95	−19·3
	30	Lactate	2·64	4·85	−21·4
	30	Lactate	2·67	4·90	−20·4
	30	Lactate	2·77	5·10	−20·9
	30	H_2	2·40	3·85	−8·9
	20	H_2	3·03	4·20	−6·3
6	30	Lactate	3·30	1·74	−18·3
	30	H_2	3·43	13·30	−16·5
	20	H_2	2·63	4·70	−14·3
	10	H_2	2·21	1·12	−13·8
7	25	Lactate	3·06	1·87	−16·3
	25	H_2	3·01	7·25	−13·6
	15	H_2	3·14	2·05	−12·9
	5	H_2	0·91	0·14	−14·4
8	35	Lactate	1·43	12·30	−15·5
	15	Lactate	1·40	2·42	−16·6

isotope always being enriched in the sulphide produced. The shape of the curve is similar to that obtained by Harrison & Thode (1958). Of particular interest is that several enrichments of ^{32}S greater than 30‰ were measured, with the maximum reaching 46‰. The maximum reported in previous laboratory experiments (Jones & Starkey, 1957; Harrison & Thode, 1958) was 27‰, yet considerably higher enrichments are found in nature (Kaplan *et al.* 1960; Kaplan, Emery & Rittenberg, 1963).

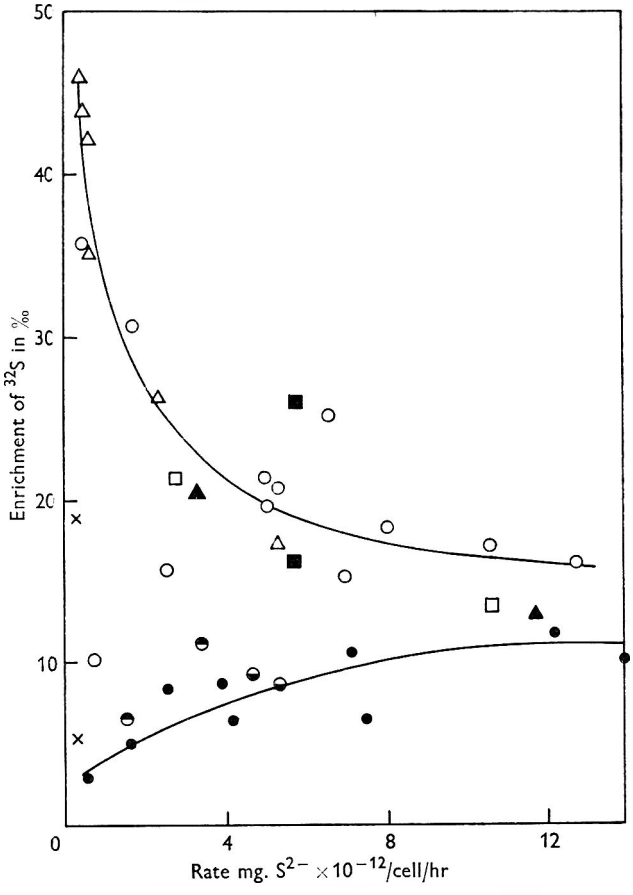

Fig. 3. Enrichment of ^{32}S in hydrogen sulphide as a function of rate of sulphate reduction by *Desulfovibrio desulfuricans*. Temperature 10–45°, 0·06 M-SO_4^{2-}; and lactate, ○; ethanol, △; or hydrogen, ●. Exceptions are: ×, lactate at 0°; ▲, lactate + 0·01 M-SO_4^{2-}; □, lactate + 0·02 M-SO_4^{2-}; ■, lactate + 0·12 M-SO_4^{2-}; ◒, hydrogen + 0·02 M-SO_4^{2-}; ◓, hydrogen + 0·18 M-SO_4^{2-}.

Effect of electron donor. Although at a particular temperature rates of reduction with ethanol were lower than those with lactate (Fig. 1), over the temperature range tested there was an overlapping of rates (Table 1). Since the fractionations obtained with both lactate and ethanol fall on the same curve when plotted against rate (Fig. 3), it is probable that these two electron donors influence fractionation only insofar as they influence rate of reduction.

However, isotope fractionation was always lower with molecular hydrogen as the electron donor than with the other donors at a comparable rate of reduction. In

addition, isotope fractionation with hydrogen is directly related to rate, rather than inversely as with lactate and ethanol (Fig. 3). It is obvious that with molecular hydrogen the mechanism controlling isotope fractionation must be different.

Effect of temperature. Fractionation decreased with increasing temperature when ethanol (Expts. 3 and 4, Table 1) or lactate (Expts. 1 and 5, Table 1) was electron donor and increased with increasing temperature with molecular hydrogen (Expts. 3–5, Table 1). With the same donor and temperature, rates of reduction and fractionation differed with different batches of bacteria (Table 1). Thus over the range of 10–45°, temperature *per se* appears to influence isotope selection only through its influence on rate of sulphate reduction.

Some experiments were run with lactate at 0–10°. Sulphide production was very slow and appeared to diminish in rate over the long incubation period required to collect the necessary amount of sulphide for analysis. In these experiments ($\times$, Fig. 3) the enrichments observed fell far below the normal lactate–ethanol curve. It is apparent that the usual mechanism controlling fractionation no longer functioned at the low temperatures imposed.

Effect of sulphate concentration. Several experiments were performed with lactate and hydrogen in which the sulphate concentration was varied over the range of 0·01–0·18 M. The enrichments show about the same relation to rate of reduction as those obtained with 0·06 M-sulphate (Fig. 3). Thus no independent sulphate concentration effect on fractionation over the range studied is suggested.

Strain variation. A few experiments were done with a second strain of *Desulfovibrio desulfuricans*, and the data are included in Table 1. The enrichment patterns observed were similar, i.e. increasing fractionation with decreasing rate with lactate as the hydrogen donor and the reverse effect with molecular hydrogen. However, the magnitude of enrichment was higher with molecular hydrogen and lower with lactate as compared to the first strain, and the range, 12–19‰, was smaller.

Fractionation by growing cells of Desulfovibrio desulfuricans. Cultures were grown at 30° using 0·02, 0·04, and 0·06 M-lactate, and 0·06 M-SO_4^{2-}. Experiments were terminated when 3·32, 5·05 and 8·96% respectively of the sulphate had been reduced. The corresponding $\delta^{34}S$ values of the sulphide formed were −12·9, −11·2, and −12·8‰ with respect to the starting sulphate. The fractionations were lower than those obtained with resting cells using the same electron donor and temperature (Table 1). Previous data (Jones & Starkey, 1957; Harrison & Thode, 1958; Kaplan *et al.* 1960) also show lower fractionations with growing cultures than those reported here for resting suspensions. Although this might suggest a different control mechanism for fractionation under the two physiological conditions, it must be emphasized that comparisons would only be valid on a rate of reduction per cell basis. Although this absolute rate was not determined for the exponentially growing cultures, experimental conditions were optimum, and a rapid rate of sulphide production per organism and a low fractionation would be expected. Such results were obtained by Harrison & Thode (1958) whose data for resting and growing cells, expressed in terms of unit cell populations, fall on the same fractionation-rate curve.

Sulphite reduction by resting Desulfovibrio desulfuricans

Under comparable conditions of concentration, temperature and electron donor, sulphite was reduced more rapidly than sulphate (cf. Postgate, 1951). The enrichment factor was always smaller than that during sulphate reduction (Table 2). With the exception of an experiment at 5°, in which rate of reduction was very slow and enrichment relatively high, there was no marked change in enrichment with reduction rate. These findings differ from those of Harrison & Thode (1958) who found that both the rate of reduction and the fractionation were the same at equal sulphite and sulphate concentrations.

Table 2. *Isotopic fractionation during sulphate and sulphite reduction by resting suspensions of Desulfovibrio desulfuricans, strain 2*

Lactate as electron donor; 0·06 M-sulphate or sulphite. $\delta^{34}S$ measured relative to $^{34}S/^{32}S$ of starting sulphate or sulphite.

Expt. no.	Temp. (°)	Substrate	Sulphate or sulphite reduced (%)	Rate of reduction, mg. $S^{2-} \times 10^{-12}$/ organism/hr	$\delta^{34}S$ of S^{2-} (‰)
1	25	SO_4^{2-}	3·06	1·87	−16·3
	25	SO_3^{2-}	2·04	3·28	−10·0
	15	SO_3^{2-}	1·56	0·92	−10·0
2	35	SO_4^{2-}	1·43	12·30	−15·5
	15	SO_4^{2-}	1·40	2·42	−16·6
	35	SO_3^{2-}	2·40	14·30	−9·4
	25	SO_3^{2-}	2·72	6·45	−9·4
	15	SO_3^{2-}	3·38	3·58	−7·9
	5*	SO_3^{2-}	1·40	0·50	−14·3
	35*	SO_3^{2-}	1·67	10·50	−1·9
	15*	SO_3^{2-}	5·60	6·76	+0·3

* Temperature raised to 35° after 18·5 hr at 5° and then lowered to 15° after 1 hr at 35°. The sulphide formed in each interval was analysed separately.

The experiment at 5° (Table 2) required 18·5 hr incubation to collect enough sulphide for isotope analysis. The temperature of the reaction mixture was then rapidly raised to and held at 35° for 1 hr and then lowered to and held at 15° for 5 hr. The sulphides produced during the three intervals were collected separately and analysed. With the rise in temperature, fractionation dropped markedly from $\delta^{34}S$−14·3‰ to $\delta^{34}S$−1·9‰, and was completely suppressed by the second lowering (Table 2). As in the low-temperature experiments with sulphate, the normal mechanisms controlling fractionation were disrupted by the environmental changes imposed.

Sulphite reduction by resting Saccharomyces cerevisiae

Attempts to demonstrate sulphate reduction by *S. cerevisiae* suspensions were unsuccessful although cell-free extracts catalyse the process (Wilson & Bandurski, 1958). Sulphite reduction, however, was obtained with glucose as the electron donor. The rate of reduction was difficult to determine accurately since it was inconsistent and diminished with time, possibly because of toxicity of either the sulphite or sulphide. It was, however, slower than sulphite reduction by *Desulfovibrio*

desulfuricans at the same temperature by approximately one order of magnitude. Six separate experiments yielded an average rate of $0{\cdot}93 \times 10^{-12}$ mg. S^{2-}/organism/hr. Temperature did not appear to have a great effect on the rate over the range 22–38° (Table 3), possibly because of opposing effects on rate of reduction and toxicity.

The isotope effect was very large, varying from 34‰ to a maximum enrichment of 41‰ (Table 3). Although the data suggest increasing fractionation with increasing rate of reduction, this cannot be given much significance since the ranges for both parameters were small and the rate measurements were approximations. It is apparent, however, that much greater fractionations are obtained during sulphite reduction by yeast than by *Desulfovibrio desulfuricans* even at comparable rates of reduction.

Table 3. *Isotopic fractionation during sulphite reduction by resting suspensions of Saccharomyces cerevisiae*

Glucose as electron donor. $\delta^{34}S$ measured relative to $^{34}S/^{32}S$ of starting sulphite.

Expt. no.	Temp. (°)	Sulphite (M)	Sulphite reduced (%)	Rate of reduction, mg. $S^{2-} \times 10^{-12}$/ organism/ hr	$\delta^{34}S$ of S^{2-} (‰)
1	30	0·03	0·50	0·63	−37·1
2	30	0·02	0·09	0·81	−36·0
	30	0·06	0·08	0·57	−34·0
3	22	0·02	0·11	1·06	−36·1
	30	0·02	0·11	1·47	−41·0
	38	0·02	0·08	1·03	−39·4

Fractionation during assimilation of sulphate

A heterotrophic bacterium, *Escherichia coli*, a green alga, *Ankistrodesmus* sp., and a yeast, *Saccharomyces cerevisiae*, were grown with sulphate as the sole sulphur source, and the isotopic ratios of the assimilated sulphur determined. Only a very small fractionation was observed, but it was always in the same direction, i.e., an enrichment of ^{32}S in the assimilated sulphur (Table 4).

Table 4. *Isotopic fractionation during incorporation of sulphate sulphur into organic sulphur during growth*

Growth conditions and media given in text. $\delta^{34}S$ measured relative to $^{34}S/^{32}S$ of starting sulphate.

	Total sulphur	
Organism	% dry weight organisms	$\delta^{34}S$ (‰)
Escherichia coli	0·62	−2·2
	0·60	−2·8
Ankistrodesmus sp.	0·46	−0·9
	0·46	−1·8
Saccharomyces cerevisiae	0·30	−2·8

Fractionation during mineralization of organic sulphur

The cleavage of the sulphydryl group of cysteine by *Proteus vulgaris* (Tarr, 1933, 1934) resulted in an enrichment of the lighter isotope in the hydrogen sulphide with a maximum $\delta^{34}S$–5·1‰ (Table 5).

Table 5. *Isotopic fractionation during the liberation of hydrogen sulphide from cysteine by resting suspensions of Proteus vulgaris*

A solution of 350 mg. cysteine hydrochloride per 100 ml. of phosphate buffer, pH 7·2, and a cell density of 475 Klett–Summerson units (No. 54 filter) used. $\delta^{34}S$ measured relative to $^{34}S/^{32}S$ of cysteine sulphur.

Temp. (°)	Sulphide formed (%)	Rate of S^{2-} release, mg. × 10^{-3}/ml./hr	$\delta^{34}S$ of S^{2-} (‰)
40	1·9	3·2	−5·1
25	4·7	1·8	−4·3

Chemosynthetic oxidation of sulphide by Thiobacillus concretivorus

The studies on hydrogen sulphide oxidation yielded results (Table 6) similar to those of Kaplan & Rafter (1958). Both the sulphur and sulphate formed as products during the oxidation were enriched in ^{32}S relative to the starting sulphide. The sulphur was only slightly enriched, the maximum measured being about 2·5‰. The sulphate, however, showed a greater enrichment. Neither sulphur nor sulphate was formed in the uninoculated controls, showing that the oxidation was biological.

Table 6. *Isotopic fractionation during the oxidation of sulphide by growing cultures of Thiobacillus concretivorus and Chromatium sp.*

Culture conditions given in text. $\delta^{34}S$ measured relative to the $^{34}S/^{32}S$ of the starting sulphide.

Expt. and organism	Sulphide oxidized (%)	$\frac{SO_4^{2-}-S}{S_xO_6^{2-}-S}$	$\frac{S^\circ}{S_xO_6^{2-}-S}$	$\delta^{34}S$ (‰) SO_4^{2-}	$\delta^{34}S$ (‰) S°	$\delta^{34}S$ (‰) $S_xO_6^{2-}$
T. concretivorus						
1*a*	—	—	—	−11·2	0	—
b	—	—	—	−10·5	−1·0	—
c	—	—	—	−11·1	−0·7	—
1*	—	4·8	20·3	—	—	+3·2
2*a*	—	—	—	−18·0	−2·5	—
b	—	—	—	−15·6	−1·2	—
c	—	—	—	−16·3	−1·4	—
d	—	—	—	−16·7	−2·3	—
2*	—	2·9	35·6	—	—	+0·6
3*a*	—	1·1	44·6	−10·6	+1·2	+19·0
b	—	1·5	75·0	−13·4	−1·2	+10·6
c	—	1·2	50·0	−13·1	−1·3	+7·5
Chromatium sp.						
4*a*	27·0	1·94	1·35	+0·5	−5·1	+10·5
b	46·0	2·14	2·47	+0·9	−3·6	+8·4
c	22·0	1·00	0·55	+0·7	−10·0	+11·2
d	17·0	1·50	0·94	−2·9	−8·5	+4·9

* Cultures 1*a–c* and 2*a–d* pooled for analyses.

Previous experiments by Kaplan & Rafter (1958) indicate that the abiological oxidation of sulphide to sulphur leads to a slight enrichment of ^{34}S in the product of about 3‰. The enrichment of the lighter isotope in the present experiment must therefore be considered significant.

In most experiments, a large enrichment of ^{34}S was found in a sulphur fraction designated $S_xO_6^{2-}$ (Table 6). The exact composition of this moiety is not known but it is presumed to be a polythionate from the method employed for its determination. Under the conditions of the experiments, $S_xO_6^{2-}$ accumulated in the medium in amounts one-fifth to one times that of sulphate and was absent from uninoculated controls.

Photosynthetic oxidation of sulphide by Chromatium sp.

Three products, elemental sulphur, polythionate and sulphate, were obtained from the photosynthetic oxidation of sulphide by a *Chromatium* sp. when growth of cultures was halted at an intermediate stage in substrate oxidation. Elemental sulphur accumulated as intracellular globules while the other two products were in the medium. As with chemosynthetic sulphide oxidation, the polythionate fraction was markedly enriched in ^{34}S (Table 6). In contrast to the thiobacillus oxidation, however, no significant fractionation was found in sulphate and a marked enrichment of ^{32}S occurred in elemental sulphur.

Metabolism of elemental sulphur

Sulphur oxidation by *Thiobacillus concretivorus* and by *Chromatium* sp., and reduction by *Saccharomyces cerevisiae*, did not result in significant fractionation in the products, sulphate and sulphide respectively. The observed $\delta^{34}S$ values ranged from $-0{\cdot}1$ to $+1{\cdot}4$ for *T. concretivorus*, $+0{\cdot}3$ to $+0{\cdot}4$ for *Chromatium* sp., and $-0{\cdot}6$ to $+0{\cdot}3$ for *S. cerevisiae*. The significance of this lack of isotope discrimination in the oxidation of elemental sulphur to the question of sulphur transport has been discussed elsewhere (Kaplan & Rittenberg, 1962*a*).

DISCUSSION

The data presented here show that discrimination between sulphur isotopes commonly occurs in metabolic function. Significant fractionation was found in all processes examined except those in which elemental sulphur served as a starting substrate. The magnitude of the effect differed in different processes, being greatest during sulphate reduction by *Desulfovibrio desulfuricans* and sulphite reduction by *Saccharomyces cerevisiae*. Metabolic reactions represented by identical overall equations did not necessarily yield the same isotope effect, e.g. sulphite reduction by *D. desulfuricans* and by *S. cerevisiae*. This finding, in itself, shows that an understanding of the fractionation mechanisms in organisms requires more than a consideration of a single rate-limiting step in the non-enzymic reaction sequence leading from the same initial to final states.

Fractionation during sulphate and sulphite reduction was investigated most completely in this study and it is only for these processes that a significant body of information exists in the literature. Our data show that with lactate and ethanol as electron donors, fractionation decreases with increasing rate of sulphate reduction. Temperature and sulphate or lactate concentration, within the 'physiological'

range, appear to influence fractionation only in so far as they influence rate of reduction. These findings are in complete agreement with the results of Harrison & Thode (1958). In contrast, when hydrogen is the electron donor, fractionation increases with increasing rate of reduction. With hydrogen, the rate of sulphate reduction is more rapid than with ethanol or lactate at the same temperature.

An attempt was made by Harrison & Thode (1958) to explain the mechanism controlling fractionation during sulphate and sulphite reduction by postulating only two rate-controlling steps; the entrance of sulphate into the cell and the breaking of the sulphur-oxygen bond during reduction of sulphate to sulphite. The former was assumed to have a very small isotope effect and to regulate fractionation at very high rates of sulphate reduction or at very low sulphate concentrations. The latter was believed to have a kinetic isotope effect similar to that for the chemical reduction of sulphate, 22‰ (Harrison & Thode, 1957), and to be important at low rates of sulphate reduction.

This explanation is not adequate to explain the data now available. Fractionations much greater than 22‰ occur, and there is no reason to believe that the highest observed laboratory value, 46‰, is a maximum since enrichments of 62‰ have been observed in nature under conditions where recycling of sulphur is unlikely (Kaplan *et al.* 1963). Thus the breaking of the S—O bond in sulphite formation from sulphate cannot be the dominant process controlling fractionation at low rates of sulphate reduction.

That sulphate entrance into the cell is the rate- (and fractionation-) controlling step at high rates of sulphate reduction is equally unlikely. Since, with all other conditions the same, the rate of reduction is always much greater with hydrogen than with lactate or ethanol, then the availability of electrons at the reduction site and not penetration of sulphate must be the limiting factor for the organic donors mentioned. What is rate-limiting with hydrogen as the donor is unknown. However, with this electron source fractionation increases with increased rate of reduction.

Mass difference allows discrimination between isotopes in both chemical and physical processes. In chemical reactions, the difference in mass of the nuclides results in chemical bonds of different stability and consequently the reaction rates are different for each mass. The fractionation factor depends on the nature of the reaction(s) and will differ for steady state or equilibrium processes as compared to unidirectional ones. Thus, for the unidirectional reduction of sulphate to sulphide, with the step to sulphite rate-limiting, a kinetic isotope effect of 1·010 or 1·035 was calculated, depending on the assumed nature of the activated complex, and an experimental value of 1·021 was observed (Harrison & Thode, 1957). However, for the equilibrium reaction

$$^{32}SO_4^{2-} + H_2{}^{34}S \rightleftharpoons {}^{34}SO_4^{2-} + H_2{}^{32}S$$

a much greater fractionation was calculated (K = 1·085 at 0° and 1·074 at 25°; Tudge & Thode, 1950).

In attempting to interpret the fractionation data in biological sulphate reduction one must decide whether a unidirectional or equilibrium situation exists and this requires a consideration of the mechanism of penetration of sulphate into the cell.

Littlewood & Postgate (1957) studying gross permeability of resting organisms by the 'thick suspension' technique of Mitchell & Moyle (1956) and Furusaka (1961)

measuring uptake of radioactive sulphate concluded that *Desulfovibrio desulfuricans* is not freely permeable to sulphate. Furusaka showed, however, that sulphate could reach an internal concentration appreciably greater than that of the suspending medium and found that the kinetics of accumulation was influenced by oxygen and selenate. He concluded that a significant accumulation of SO_4^{2-} occurs in the organism and that the transport of sulphate is associated with its reduction. Whether transport is in ionic form remains unanswered. If so, a fractionation could occur during the process of penetration which, following the laws for a unidirectional reaction, would yield a maximum enrichment factor of $(^{34}SO_4^{16}/^{32}SO_4^{16})^{\frac{1}{2}}$ 1·010 or $\delta^{34}S$ $-10‰$. This factor would probably be negligible especially if activation occurred previous to entry.

If there is no equilibrium between external and internal sulphate and all the sulphate that enters is reduced to sulphide then the maximum enrichment would be 10‰ in favour of ^{32}S. Since considerably greater fractionations have been observed, it is apparent that for the non-equilibrium assumption to be valid a sink for heavy sulphur would have to exist, perhaps a side reaction draining off ^{34}S or a residual pool of sulphate within the cells greatly enriched in ^{34}S. As no side reactions have ever been demonstrated during sulphate reduction by *Desulfovibrio desulfuricans* it is pointless to speculate on this possibility further. The pool alternative is very unlikely, despite the demonstration by Furusaka (1961) that one exists during sulphate reduction, since calculations show that an approximately 2 M sulphate concentration of $\delta^{34}S + 36‰$ would be required within the organisms to account for some of the data, and much lower extracellular sulphate levels are inhibitory. This is not to imply that an intracellular sulphate pool does not exist but only that it could not be large enough to account for observed fractionations.

If it is assumed that the intracellular sulphate is in equilibrium with the external sulphate reservoir, as is suggested by the data of Furusaka (1961), then the kinetic fractionation effect across the boundary will be lessened, since the lighter isotope will emerge as well as enter more readily than the heavier one. However, under these conditions one is no longer dealing with strictly a unidirectional process and, to the extent that the enzymic reactions in the multiple steps of sulphate reduction are reversible, the situation becomes a steady-state condition up to the point of the rate-limiting reaction. The resulting fractionation would be the sum of the equilibrium effects plus the kinetic effect of the rate-limiting step.

The detailed intermediary metabolism of sulphate reduction is not yet known. For *Desulfovibrio desulfuricans* the first two reactions are (Peck, 1962):

$$ATP + SO_4^{2-} \overset{\text{ATP-sulfurylase}}{\rightleftharpoons} APS + PP; \quad (1)$$

$$APS + 2e \overset{\text{APS-reductase}}{\rightleftharpoons} AMP + SO_3^{2-}. \quad (2)$$

The equilibrium for (1) is far to the left ($K \sim 10^{-8}$, Robbins & Lipmann, 1958) and, in cell-free systems, at least, pyrophosphatase is needed to pull the reaction. The equilibrium constant for reaction (2) has not yet been determined, although the reaction is reversible (Peck, 1961). The reduction of sulphite has not been thoroughly investigated but it is known that cytochrome c_3 functions here (Postgate, 1956) as well as in sulphate reduction (Peck, 1959). The extent to which the reactions in sulphite reduction are reversible is completely unknown, although a tenuous

argument for reversibility is that organisms oxidizing reduced sulphur compounds as energy sources do so *via* APS (Peck, 1960).

Assuming the reversibility of the entire sequence then an active exchange between SO_4^{2-} and H_2S could occur, and a fractionation factor of 1·074 might be approached. In actuality, however, sulphate reduction must be considered unidirectional beyond the rate-limiting step. The further along in the reaction sequence this step occurs and the slower it is relative to the preceding steps the greater the expected fractionation. Since this step and/or its relative rate could differ under different physiological or environmental conditions, the physiological functioning of the organism must be of great significance in the fractionation process. The existence of strain differences, as was found, would thus be expected. Further, any imposed condition which disrupts the normal functioning of the organism should have a marked influence on fractionation. The anomalous results at 0° and the suppression of isotope discrimination in the temperature variation experiments during sulphite reduction are cases in point. In the former instance, the temperature was well below the normal growth range; in the latter, the abrupt changes of temperature might well have caused cell damage.

As has already been pointed out, the transfer of electrons from lactate and ethanol must have been rate-limiting in most experiments with these donors and thus reaction (2) or a similar reductive step further in the sequence would control the flow of sulphur through the cell. The faster the rate-limiting step, the smaller the steady state pool of preceding intermediates and the smaller the equilibrium effect on fractionation. This would account for the inverse relation between rate of reduction and fractionation observed with these donors. Since the isotope effect was smaller with hydrogen as compared to the organic donors and increases with rate of reduction, it might be assumed that reaction (1) is rate-limiting.

Furusaka (1961) has shown that at 37° the rate of sulphate entry into the cell is somewhat greater than the rate of reduction of sulphate by hydrogen whereas at 0° reduction is much more rapid than transport. Thus with decreasing temperature, control of fractionation would shift from some intracellular process to the permeation step. The small fractionations in the low-temperature experiments of the present study and those at very low sulphate concentrations observed by Harrison & Thode (1958) could be explained in this manner.

Because of the limited data available, little can be said about the factors controlling fractionation in the other processes investigated. Except for a single experiment in which a relatively high fractionation was observed, rate of reduction did not appear to be a factor in sulphite reduction by either *Desulfovibrio desulfuricans* or *Saccharomyces cerevisiae*. The rate of reduction, however, was much lower with the latter organism and the isotopic effect strikingly higher. It is known that sulphite reduction is linked to $NADPH_2$ in *S. cerevisiae* (Wainwright, 1961) and to cytochrome c_3 in *D. desulfuricans* (Postgate, 1956) and this difference in mechanism may be the basis of the different fractionation factors. The difference in fractionation during sulphate assimilation and sulphite reduction by *S. cerevisiae* is also marked, and implies a major dissimilarity in either transport or metabolism of the two ions.

Escherichia coli, *Saccharomyces cerevisiae* and *Ankistrodesmus* sp. yielded a similar, and very small, enrichment of ^{32}S in cellular organic sulphur when grown on sulphate as the sole sulphur source. Ishii (1953) in a similar study found no fractionation

during sulphur assimilation by higher plants. The data suggest that there is no intracellular sulphate pool and that all the sulphate that enters during growth is rapidly assimilated. Fractionation in this event would occur only at the cell boundary and only a small effect would be expected.

A consideration of the isotope distribution data in chemo-synthetic and photosynthetic sulphide oxidation leads to some interesting, and somewhat unorthodox, suggestions with respect to the intermediary metabolism of these processes. In both instances a compound(s) markedly enriched in ^{34}S and reacting like a polythionate is found in appreciable amounts in the media. In chemosynthetic oxidation, the sulphate formed is correspondingly enriched, but in ^{32}S. Since the heavier isotope accumulates in the polythionate and the lighter in the sulphate it can be argued that the former represents a residual component from which sulphate is directly formed. The elemental sulphur, deposited extracellularly, is only slightly enriched in ^{32}S. Since the oxidation of extracellularly supplied sulphur does not result in any isotope discrimination, the metabolically deposited sulphur cannot be an intermediate in the formation of the light sulphate. The isotope data thus put polythionate in the direct pathway of sulphide oxidation to sulphate and relegate elemental sulphur to a product of a side reaction. The first conclusion at least has been reached by many investigators on the basis of more conventional data (see review by Vishniac & Santer, 1957).

If one follows the same argument with the data from photosynthetic oxidation, the conclusion with respect to elemental sulphur is the same. Here sulphur and not sulphate is the light component and one would assume it arises from the polythionate. Although this has not previously been proposed for photosynthetic bacteria, it has frequently been suggested for sulphur formation by the thiobacilli (Vishniac & Santer, 1957). If the path of oxidation of sulphide to sulphate involved the initial formation of elemental sulphur, it would be expected that the fractionation process would lead to an enrichment of ^{32}S in the sulphate, which would have the same fractionation factor as the sulphur relative to sulphide (if no further enrichment occurred) or a greater one. Thus, the data also seem to exclude elemental sulphur as an intermediate in sulphate formation from sulphide.

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Carbon Isotope Fractionation during Metabolism of Lactate by *Desulfovibrio desulfuricans*

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SUMMARY

Lactate oxidation during sulphate and sulphite reduction by growing or resting *Desulfovibrio desulfuricans* resulted in an enrichment of ^{12}C in the CO_2 released and also in the cell carbon during growth. The enrichments observed varied between $\delta^{13}C$ $-5{\cdot}5$ to $-12{\cdot}8$‰. With sulphite as the oxidant, the fractionation appeared proportional to rate of metabolism of lactate; no definite rate effect on fractionation was suggested by the limited data obtained with sulphate as oxidant.

INTRODUCTION

More studies have probably been made of the distribution of stable carbon isotopes in nature than of any other element (Craig, 1953; Silverman & Epstein, 1958). The $^{13}C/^{12}C$ content of animals, plants, algae and fungi have been measured precisely, yet few experiments have been made to determine fractionation patterns during metabolism. Nier & Gulbransen (1939) recognized that formation of plant material tended to concentrate the light carbon isotope and Urey (1948) showed that laboratory-cultured algae contained 2·97% less ^{13}C than the CO_2 utilized for growth. Recently Park & Epstein (1960, 1961) and Abelson & Hoering (1961) have shown with the tomato plant and with *Chlorella* sp., respectively, that the lighter isotope is preferentially incorporated into lipids, carbohydrates and proteins during photosynthesis and released during respiration. Using growing cultures of a methane producing bacterium, Rosenfeld & Silverman (1959) showed that an enrichment of 94‰ occurred in the methane liberated during methanol fermentation at 23°. At 30° there was an increased rate of methane production and a drop in enrichment to 81‰. In contrast, metabolic CO_2 was only slightly enriched in ^{12}C relative to the methanol (< 4‰) and the enrichment factor was not related to its rate of production.

In the course of investigating the fractionation of stable sulphur isotopes during various processes in the sulphur cycle (Kaplan & Rittenberg, 1963) some measurements were also made on carbon isotopes, the results of which are reported here. The reactions studied were the oxidation of lactate during sulphate and sulphite reduction by growing and resting *Desulfovibrio desulfuricans*. The equations representing the overall reactions are:

$$2CH_3.CHOH.COOH + SO_4^{2-} \rightarrow 2CH_3.COOH + 2CO_2 + S^{2-} + 2H_2O, \quad (1)$$

and

$$3CH_3.CHOH.COOH + 2SO_3^{2-} \rightarrow 3CH_3.COOH + 3CO_2 + 2S^{2-} + 3H_2O. \quad (2)$$

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METHODS

Procedures for growth and resting suspension experiments have been described previously (Kaplan & Rittenberg, 1963).

Metabolic CO_2 was swept continuously from experimental flasks by a stream of nitrogen which then passed through two $AgNO_3$ traps to remove sulphide and two traps containing saturated solutions of $Ba(OH)_2$. Experiments were terminated by addition of acid to reaction mixtures which also insured complete release of CO_2. The precipitated carbonate was collected on a sintered glass filter, washed with boiling water and dried at 105°. The $BaCO_3$ was treated with sulphuric acid in vacuo to release CO_2 for isotopic assay.

The carbon in centrifuged, washed and dried bacteria was converted to CO_2 by combustion with oxygen in an apparatus described by Craig (1953).

Isotopic measurements were made on CO_2 using a Nier-type dual collection instrument. Isotopic ratios were measured relative to a standard and are reported as δ values (Kaplan & Rittenberg, 1963).

Since the studies of Stuven (1960) show that all of the CO_2 released during lactate metabolism by *Desulfovibrio desulfuricans* arises from the carboxyl group, the standard used in the present study was the $^{13}C/^{12}C$ of this group. This ratio was determined by measurements on metabolically released CO_2 in experiments in which lactate concentration was limiting and its complete degradation occurred.

RESULTS AND DISCUSSION

Two experiments were run, one with growing cultures and the other with resting suspensions (Table 1). In the growth experiment, three concentrations of lactate were used while sulphate (0·06 M) and temperature (30°) were constant. Both the CO_2 and the cell carbon were enriched in the light carbon isotope to approximately the same extent. The range of fractionations observed was small, between $\delta^{13}C - 5{\cdot}5$ and $-7{\cdot}8‰$, and the variations did not correlate with either the initial lactate concentration or with the rate of CO_2 release. The latter, however, varied by less than a factor of two in the three cultures which may have been insufficient to detect a rate effect on fractionation.

The resting suspension experiments were run at constant lactate (0·12 M) and sulphate (0·06 M) or sulphite (0·06 M) concentration but at different temperatures to give a range of rates of lactate oxidation. As with growing cultures, the metabolic CO_2 was enriched in the light carbon isotope in all instances. With sulphate as the oxidant, there was an almost fivefold difference in rate of CO_2 release at the two temperatures tested but very little difference in the fractionation. With sulphite, however, the degree of fractionation was proportional to the rate of lactate oxidation (Fig. 1).

Lactate oxidation was very slow at 5°, and 18·5 hr were required to collect the desired amount of CO_2 for analysis. To determine whether this prolonged contact with sulphite had affected the enzymic activity of the resting bacteria, the temperature of the reaction mixture was raised to and held at 35° for 1 hr, and then lowered to 15° for the remaining 5 hr of the experiment. The rate of lactate oxidation responded, qualitatively, in the expected manner to these temperature

manipulations by first increasing and then decreasing. Concurrent with the changes in rate, the fractionation factor for CO_2 first increased from $\delta^{13}C - 6{\cdot}9$ to $-9{\cdot}8$, and then fell to $-9{\cdot}1$‰. It is interesting that in the same experiment the temperature manipulations had quite a different effect on the fractionation of sulphur isotopes, suppressing this phenomenon almost completely (Kaplan & Rittenberg, 1963).

Table 1. *Isotopic fractionation of carbon during lactate metabolism by Desulfovibrio desulfuricans*

$\delta^{13}C$ measured relative to the $^{13}C/^{12}C$ of the carboxyl-carbon of starting lactate.

Oxidant	Lactate (M)	Temp.°	Lactate used (%)	Rate*	$\delta^{13}C$-CO_2 (‰)	$\delta^{13}C$-cell carbon (‰)
			Growth experiments			
SO_4^{2-}	0·02	30	7·9	$1{\cdot}4 \times 10^{-8}$	−5·5	−5·9
SO_4^{2-}	0·04	30	6·4	$2{\cdot}3 \times 10^{-8}$	−5·8	−6·3
SO_4^{2-}	0·08	30	4·2	$1{\cdot}9 \times 10^{-8}$	−7·8	−5·8
			Resting suspension experiments			
						CO_2/S^{2-}§
SO_4^{2-}	0·12	35	0·6	$9{\cdot}1 \times 10^{-12}$	−7·4	2·0
SO_4^{2-}	0·12	15	0·6	$1{\cdot}9 \times 10^{-12}$	−8·8	2·1
SO_3^{2-}	0·12	35	0·6	$6{\cdot}6 \times 10^{-12}$	−12·8	1·2
SO_3^{2-}	0·12	25	0·8	$3{\cdot}3 \times 10^{-12}$	−10·8	1·3
SO_3^{2-}	0·12	15	1·0	$2{\cdot}0 \times 10^{-12}$	−9·2	1·4
SO_3^{2-}†	0·12	5	0·5	$0{\cdot}3 \times 10^{-12}$	−6·9	—
SO_3^{2-}†	0·12	35	0·4	$5{\cdot}3 \times 10^{-12}$	−9·8	—
SO_3^{2-}	0·12	15	2·1	$4{\cdot}6 \times 10^{-12}$	−9·1	—
SO_3^{2-}‡	0·12	—	3·0	$1{\cdot}4 \times 10^{-12}$	−8·9	1·6

* Rate: mg. carbon released as CO_2/ml. culture/hr for growth experiments; mg. carbon released as CO_2/organism/hr for resting suspension.

† Temperature held at 5° for 18·5 hr, raised to 35° for 1 hr and lowered to 15° for 5 hr.

‡ Weighted averages of the three preceding lines.

§ Molar ratio of products.

The changes in $\delta^{13}C$ with the temperature fluctuations are in the proper direction if fractionation is proportional to rate of lactate oxidation but the magnitudes of change are less than what would be predicted from the curve established by the other experiments in the series (see Δ's, Fig. 1). In the temperature fluctuation experiment it was impossible to add acid to the flask at the end of each CO_2 collection period to insure complete release of the gas from the medium. Consequently, part of the CO_2 formed at 5° could have been retained in solution and then released at the higher temperatures. Since such CO_2 had a relatively low enrichment of ^{12}C, it would have depressed the observed enrichments of the two samples collected at the higher temperatures. Acid was added at the end of the experiment, so the sum of the three portions of CO_2 collected represents the total formed. The average rate of CO_2 formation over the entire experiment and its $\delta^{13}C$ were calculated (last line, Table 1). The point determined by these two values falls very close to the curve established by the other data (○, Fig. 1). This suggests that, as with fractionation of the sulphur isotopes (Kaplan & Rittenberg, 1963), it is the rate of the process and not the temperature *per se* that influences the fractionation of the carbon isotopes.

It is of interest that the ratio of CO_2/S^{2-} produced, which from equation (2) should be 1·5, was low at high rates of sulphite reduction and increased as rate of reduction fell (Table 1). No attempt was made to determine whether this phenomenon is related to the pattern of fractionation observed.

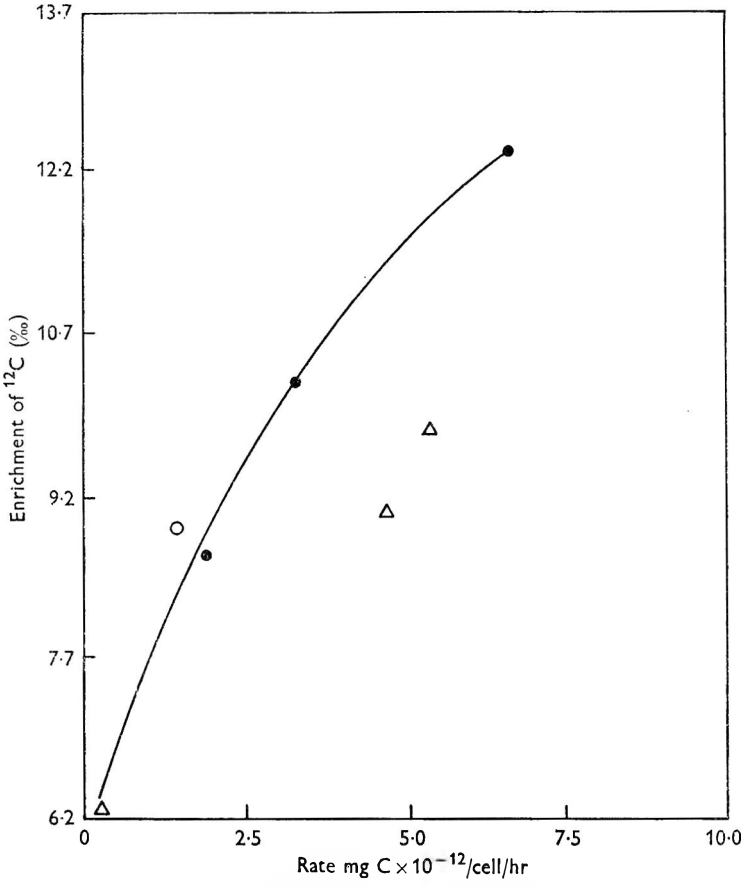

Fig. 1. Enrichment of ^{12}C in metabolic CO_2 as a function of its rate of formation during lactate oxidation by *Desulfovibrio desulfuricans* with sulphite as the oxidant. ●, Individual experiments at different temperatures; △, temperature fluctuation experiment; ○, weighted average of temperature fluctuation experiment.

It is clear that isotope discrimination occurs during decarboxylation of lactate by *Desulfovibrio desulfuricans* with the lighter carbon isotope being concentrated in the CO_2 released and in carbon assimilated during growth. Fractionations during other decarboxylation reactions have been reported (Lindsay, Bourns & Thode, 1952; Rabinowitz, Lafair, Strauss & Allen, 1958) and in all cases the $-C-^{12}C$ bond cleaved more rapidly than the $-C-^{13}C$ or $-C-^{14}C$ bonds. The magnitudes of the enrichments observed during the biological oxidation of lactate ($\delta^{13}C$ −5·5 to −12·8‰) are much lower than those reported for methane produced biologically from methanol (Rosenfeld & Silverman, 1959) but are similar to the 15‰ enrichment of ^{12}C in CO_2 during non-enzymatic decarboxylation of pyruvic acid (Taylor, 1955).

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On the Unity of Bacterial Ribosomes

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SUMMARY

Reported sedimentation coefficients of bacterial ribosomes display noticeable variation. Since this might be due to differences in experimental conditions or to actual differences amongst the bacteria this problem needed re-investigation. The sedimentation coefficients of ribosomes from seven widely divergent bacteria (Streptomyces, Acetobacter, Pseudomonas, Azotobacter, Escherichia, Streptococcus and Bacillus) were determined by analytical ultracentrifugation. The bacteria were selected to cover nearly the entire range of molar guanine+cytosine content of DNA. Correction for pressure and dilution was always negligible; correction for temperature was eliminated by working directly at 20·0°. The correction for concentration was the most important one. Further reduction to standard conditions (from dilute buffer to water) resulted in a change of about 3%. The corrected sedimentation coefficients, expressed as $(s_{20,\,w})_0$, were nearly the same for all these bacteria, being in the ranges $29{\cdot}5 \pm 2$, $37{\cdot}5 \pm 2{\cdot}8$, $56{\cdot}3 \pm 1{\cdot}9$, $76{\cdot}7 \pm 2{\cdot}5$, $110{\cdot}5 \pm 1{\cdot}6 \times 10^{-13}$ sec. It thus seems unlikely that these coefficients will be useful as an aid in bacterial taxonomy. Disruption of samples of the same bacterial suspension by ultrasonic treatment or with the French pressure cell frequently resulted in quantitative, and sometimes qualitative, differences in the yield of ribosomes.

INTRODUCTION

Living cells appear to contain about five or six types of ribosomal particles, which can be differentiated by their sedimentation coefficient. The ribosomes of *Saccharomyces cerevisiae*, higher plants and animals appear to be slightly larger than those of *Escherichia coli*, since they sediment somewhat faster; the former sediment at about 27, 40, 60, 80 and 120s (Svedberg units), whereas the latter do so at about 20, 30, 50, 70 and 100s. The basis for the widespread opinion that all bacteria would have identical ribosomes resides on the pioneering paper of Schachman, Pardee & Stanier (1952), who discovered that *E. coli*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Rhodospirillum rubrum*, *Staphylococcus aureus* and *Clostridium kluyverii* contain RNA particles sedimenting at 20–29s, 32–48s and 55–58s (uncorrected). Tissières, Watson, Schlessinger & Hollingworth (1959) established the corrected s values of 'coli' ribosomes as being 20, $30{\cdot}6 \pm 1$, $50{\cdot}0 \pm 1$, $69{\cdot}1 \pm 1$ and $100{\cdot}0 \pm 2$. Subsequent measurements have sometimes been quite close to these values, others agree less well, possibly because not all the corrections were applied. From the results of Bolton *et al.* (1959) one can calculate $34{\cdot}3 \pm 0{\cdot}9$, $53{\cdot}3 \pm 1{\cdot}9$, $69{\cdot}4 \pm 5{\cdot}4$ and 84–100s (partially corrected) depending upon Mg^{2+} concentration. Dagley & Sykes (1958) found 20, 29 and 40s, Elson (1959) found 35 and 52s, whereas Bowen, Dagley & Sykes (1959) and Bowen, Dagley, Sykes & Wild (1961)

mentioned a whole series of values between 17 and 90s, depending on external conditions. Relatively little information is available about the exact sedimentation coefficients of ribosomes from other bacteria. Corrected s values of 10·4, 33·2, 49·7, 54·5, 69·7, 84·8 and 106 were reported by Brown & Rosenberg (1962) for *Sarcina lutea* and 10, 39, 58, 77 and 86 by Gillchriest & Bock (1958) for *Azotobacter vinelandii*. Other values, chiefly uncorrected or partially corrected, have been reported for *Bacillus cereus* (30 and 50s; Bowen *et al.* 1961), with vegetative forms of *B. subtilis* (33 ± 2, 50 ± 2, 68 ± 1, 99 ± 3s) and spores of the same strain (27, 33, 50, 70s; Woese, Langridge & Morowitz, 1960), for *Salmonella typhimurium* (23, 26, 35, 55, 70, 75 and 85s; Ecker & Schaechter, 1963) and for *Mycoplasma gallisepticum* (17, 38, 58, 76, 84, 117s; Morowitz *et al.* 1962). Thus some of the above values indeed centre around 20, 30, 50, 70 and 100 s, but others seem considerably divergent. This casts some doubt on the validity of the general belief that bacterial ribosomes are identical. Alternatively, on the basis of the above numbers, it is not excluded that differences may exist between ribosomes of different bacterial taxa which might have taxonomic implications. It therefore seemed interesting to re-investigate the sedimentation coefficients of bacterial ribosomes by using several widely divergent bacteria, but with the same experimental conditions and the same analytical ultracentrifuge. Two different methods of cell disruption were used (ultrasonic treatment and French pressure cell) in order to increase the chance of detecting all ribosomal types.

METHODS

Organisms and culture conditions

Most strains were from the collection in our department; bacteriological controls and precautions were taken to ensure that only pure cultures were used.

Streptomyces lavendulae strain 265 was grown in a liquid medium containing (% w/v): 0·5, peptone; 0·5, glucose; 0·25, yeast extract (Ned. Gist- en Spiritusfabriek, Brugge). 200 ml. volumes of medium were shaken in 1 l. Erlenmeyer flasks for 24 hr at 30°. Yield: 7·5 g. wet wt. organisms/l. culture medium.

Acetobacter aceti (*xylinum*) strain NCIB 8747 was grown in Roux flasks on 150 ml. solid medium containing (%, w/v): 10, glucose; 1, yeast extract; 3, $CaCO_3$; 2·5, agar. Yield after 2 days at 30°: 1 g. wet wt. organisms/flask.

Pseudomonas fluorescens strain 488 (obtained through the courtesy of Dr O. Lysenko Prague) was grown for 24 hr at 30° in Roux flasks with 150 ml. solid medium containing (%, w/v): 1, yeast extract; 1, glucose; 2, $CaCO_3$; 2·5, agar. Yield: 1 g. wet wt. organisms/flask.

Escherichia coli strain 101 was grown for 21 hr at 30° in Roux flasks on solid medium containing (%, w/v): 0·5, peptone; 0·25, yeast extract; 0·5 glucose; 2·5, agar. Yield: 700 mg. wet wt. organisms/flask.

Azotobacter vinelandii strain 7492 (obtained through the courtesy of Professor J. Voets, Department of Soil Microbiology, Ghent) was grown with continuous shaking for 24 hr at 30° in a liquid medium containing (%, w/v): 1, glucose, 0·1, K_2HPO_4; 0·25, KH_2PO_4; 0·25, NaCl; 0·25, $MgSO_4.7H_2O$; 0·125, $CaSO_4$; 0·012, $Fe_2(SO_4)_3$; in tap water. Yield: 8·2 g. wet wt. organisms/l. The organisms were also grown on the same medium, but solidified with agar, in Roux flasks for 24 hr.

Bacillus cereus strain BCV was grown for 2 days at 30° in Roux flasks on a solid medium containing (%, w/v): 0·5, peptone; 0·25, yeast extract; 0·5, glucose; 0·01, $MgSO_4.7H_2O$; 0·036, KH_2PO_4; 0·052, Na_2HPO_4; 2·5, agar. Yield: 1·7 g. wet wt. organisms/flask.

Streptococcus faecalis (obtained through the courtesy of Professor J. Voets, Department of Soil Microbiology, Ghent) was grown for 53 hr at 30° in flasks filled to the neck with a liquid medium containing (%, w/v): 1, yeast extract; 0·01, $MgSO_4.7H_2O$; 0·054, KH_2PO_4; 0·085, Na_2HPO_4; in tap water. The medium was sterilized at 120° for 20 min., filtered and sterilized again. To it were added sterilized solutions of glucose (to final 1 %) and Liebig meat extract (to final 0·5 %). Yield: 1·36 g. wet wt. organisms/l.

Preparation of cell-free extracts

Organisms were harvested by centrifugation and washed two or three times by suspending in TMS buffer pH 7·1 (0·01 M-tris, 0·004 M-succinic acid; 0·005 M-$MgSO_4$). The same buffer was used in all the work. The organisms were finally suspended at a concentration of about 100–200 mg. wet wt. organisms/ml. buffer. Half of each suspension was disrupted in a French pressure cell with Wabash hydraulic press (American Instruments Co., Silver Spring, Md., U.S.A.) at 7600 lb./in.^{2}; the other half was treated in the 10 kcyc., 250 W. Raytheon Sonic Oscillator at 4° in H_2 atmosphere. In the former procedure the suspension was usually passed twice through the pressure cell, which sufficed for nearly complete breakage. With the Raytheon instrument the treatment was continued until nearly all the organisms were broken. In both cases the disruption was followed by phase-contrast microscopy. After disruption all suspensions were centrifuged at 13,000 *g* for 10–15 min. at 4° to eliminate unbroken organisms and large pieces of débris. In many cases a minute crystal of crystalline deoxyribonuclease (Worthington, Freehold, N.J., U.S.A.) was added to the supernatant fluid, which was then incubated for about 1 hr at 22° and then at once subjected to ultracentrifugal analysis.

Ultracentrifugation

Analytical ultracentrifugation was done in a Spinco Ultracentrifuge, model E, with Rotor Temperature Indicator Control (RTIC) at 20·0°, schlieren optics, rotor An-E at 37,020 rev./min. and 18 mm. optical cells (one standard and one wedge). Each optical cell contained 1·7 ml. of crude cell-free extract. The rotor and the optical cells were equilibrated thermally with the RTIC system in the centrifuge before each run. About 7–10 min. after the desired speed had been reached, photographs were taken, at 65° bar angle, every 2 min. with 4–7 sec. exposure time, on Kodak Metallographic plates. Measurements of the position of the schlieren peaks on the photographic plates were at first determined with a Leitz TP comparator (to 0·001 mm.), but this was found too time-consuming for routine use. Later we used our own method with a photographic enlarger, which projected the picture, 10 times enlarged, on a sheet of millimetre paper (28 × 32 cm.). Both the millimetre paper and the position of the enlarger were calibrated so that the position of the schlieren peaks in the rotor cell could be determined directly, with an accuracy of 0·5 mm. on the paper, which was equivalent to 0·025 mm. in the rotor. This method was found to be reproducible, and faster and more convenient than the one with the comparator. A similar method was described by Schachman (1957).

RESULTS

Corrections to be applied to experimental s values

Correction for the effect of pressure and dilution due to the pressure gradient in the rotor cell and its sector shape. Elias (1961) pointed out that this correction can be found by plotting $(s_c)_{app.}$ against $1-r_m^2/\bar{r}^2$, in which $(s_c)_{app.}$ is the experimentally determined sedimentation coefficient, r_m the distance of the meniscus to the rotor axis (in cm.) and $\bar{r}$ the average distance of the schlieren peaks to the rotor axis (in cm.). The value obtained by correction for these effects is called s_c. Figure 1 illustrates one case and shows that this correction did not apply in our case. This was found to be so in every experiment.

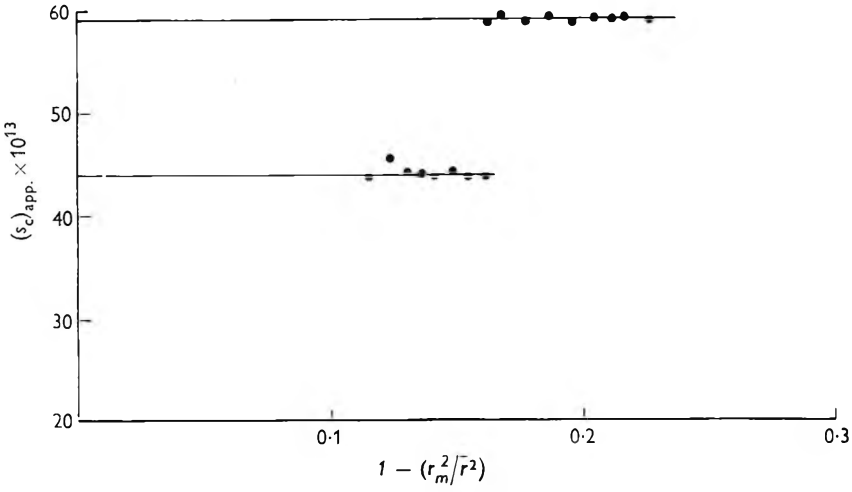

Fig. 1. The sedimentation coefficients of bacterial ribosomes are independent of the effect of pressure and dilution. Example of an extrapolation with ribosomes of *Acetobacter aceti* (*xylinum*). $(s_c)_{ap}$ was determined as described in the text and calculated according to Svedberg & Pedersen (1940). r_m and $\bar{r}$ were determined with a photographic enlarger.

Correction for the effect of temperature. Sedimentation coefficients are often determined at 4° or at least below room temperature. Since ribosomes are quite stable for several hours at room temperature, we eliminated the temperature correction by measuring directly in the standard conditions of 20·0°. The speed of 37,020 rev./min. was selected to make the duration of the experiment as short as possible and still to allow measurable speeds of sedimentation.

Correction for the effect of concentration. This was found to be most important. From each crude extract dilutions of 3/4, 1/2, 1/4 and usually also 1/8 in TMS buffer were made and the sedimentation coefficients determined. These values were extrapolated to zero concentration. The extrapolated value will be called $(s_{20,TMS})_0$. Figure 2 illustrates one experiment. In this particular case the difference between the sedimentation coefficient s_c in the crude extract and $(s_{20,TMS})_0$ at infinite dilution amounted to 31 %. With the other bacteria, differences of 10–38 % were observed. This means that quite considerable errors can be introduced when this correction is not taken into account. For example, direct estimation of a ribosome in bacterial

extract always gives too low s values, with the correspondingly incorrect s nomenclature. Also, when the concentration of the bacterial extract is rather high, the s_c of one type of ribosome may be lower than the $(s_{20,\text{buffer}})_0$ value of the following one, resulting in its classification in the wrong group. The larger the ribosome, the more pronounced is this effect.

Not infrequently one or two of the ribosomal types were only visible at the highest concentration and practically disappeared on dilution. The $(s_{20,\text{TMS}})_0$ value could thus not be determined by extrapolation. A fair approximation, however, was given by the following procedure. It had been observed that, at each concentration c, a linear relationship existed between s_c and $(s_{20,\text{TMS}})_0$ for all the ribosomes

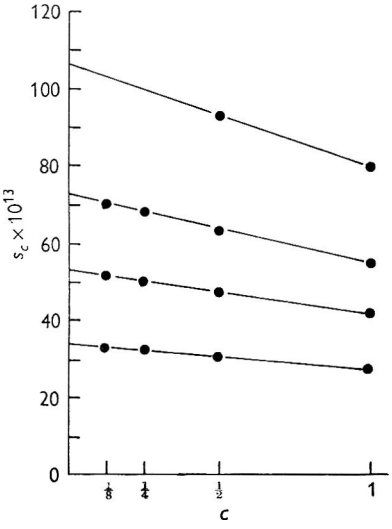

Fig. 2

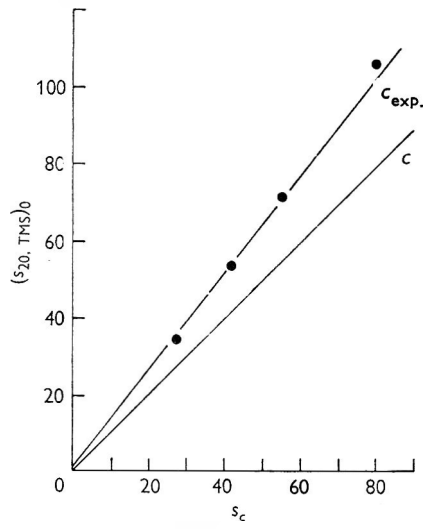

Fig. 3

Fig. 2. The dependence of the sedimentation coefficients of bacterial ribosomes on the concentration. The crude extract of *Streptococcus faecalis* was prepared from a suspension with 112 mg. of living cells/ml. TMS buffer (equals concentration $c = 1$ in the graph). The extrapolation of s_c to $c = 0$ yields $(s_{20,\text{TMS}})_0$.

Fig. 3. Linear relationship between $(s_{20,\text{TMS}})_0$ and s_c of several ribosomes from the same preparation. The results are taken from Fig. 2. The curve $c_{exp.}$ represents the actual relationship found with s_c at concentration c (crude extract of a suspension of 112 mg. of living cells/ml. TMS buffer). Smaller concentrations yield curves which approach the theoretical one at c_0, where s_c equals $(s_{20,\text{TMS}})_0$.

of each extract (Fig. 3). In case only a very small amount of any one ribosome was present in an extract, a plot of s_c against the $(s_{20,\text{TMS}})_0$ values of the other ribosomes was made, upon which the $(s_{20,\text{TMS}})_0$ value of the ribosome present in trace amount could be deduced from its s_c value in the crude extract. The results obtained by this procedure corresponded closely to the $(s_{20,\text{TMS}})_0$ value of the same ribosome from other bacteria, obtained by the regular extrapolation (for examples see Table 1).

Reduction to standard conditions. This is usually carried out by means of the formula of Svedberg & Pedersen (1940):

$$(s_{20,w})_c = (s_{t,\text{solv.}})_c \frac{\eta_{t,w}}{\eta_{20,w}} \frac{\eta_{t,\text{solv.}}}{\eta_{t,w}} \frac{1 - V_{20,\text{solv.}} \cdot \rho_{20,w}}{1 - V_{t,\text{solv.}} \cdot \rho_{t,\text{solv.}}},$$

This equation has been verified experimentally for solutions of proteins in buffer and for synthetic high polymers. According to Elias (1961) it is applicable only in narrow ranges of temperature and with bad approximation. In our case we have only to reduce $(s_{20,\,TMS})_0$ from buffer to water and the above equation becomes:

$$(s_{20,\,w})_0 = (s_{20,\,TMS})_0 \frac{\eta_{20,\,TMS}\,(1 - V_{20,\,TMS} \cdot \rho_{20,\,w})}{\eta_{20,\,w}\,(1 - V_{20,\,TMS} \cdot \rho_{20,\,TMS})},$$

in which $(s_{20,\,w})_0$ is the value reduced to water at 20° and at infinite dilution; $\eta_{20,\,TMS}$ is the viscosity of the TMS buffer at 20°; $\eta_{20,\,w}$ is the viscosity of water at 20°; $V_{20,\,TMS}$ is the partial specific volume of the ribosomes at 20° in TMS buffer; $\rho_{20,\,TMS}$ is the specific gravity of the TMS solution at 20°; $\rho_{20,\,w}$ is the specific gravity of water at 20°. It can be expected theoretically that $\eta_{20,\,TMS}/\eta_{20,\,w}$ and $\rho_{20,\,w}/\rho_{20,\,TMS}$ will be close to unity. In fact, the former ratio was experimentally found to be 1·027 by using an Oswald viscosimeter and $\rho_{20,\,TMS}$ was determined with a pycnometer to be 1·0023. On the assumption that the partial specific volume of the ribosomes was 0·66, identical in TMS buffer and water and identical for all types of ribosomes, it was calculated that $(s_{20,\,w})_0 = 1{\cdot}035(s_{20,\,TMS})_0$.

Table 1. *Some examples of corrected sedimentation coefficients, expressed as* $(s_{20,\,TMS})_0 \times 10^{13}$ *sec of ribosomal particles of several bacteria*

(Values in parentheses are from particles present in very small amount only in the highest concentration of crude extract; they could not be determined by extrapolation but the s_c–$(s_{20,\,TMS})_0$ plots were used, as exemplified by Fig. 3. The percentage guanine+cytosine of Pseudomonas and Acetobacter was determined in the laboratory; the other values are taken from the literature.)

Strain	Molar percentage of guanine + cytosine in DNA	Method of disruption	$(s_{20,\,TMS})_0 \times 10^{13}$ sec.							
Streptomyces lavendulae	74	French pressure cell	(28·5)	36·6	(56)	74·4	—	—	—	—
Acetobacter aceti (*xylinum*)	61	French pressure cell	(28·3)	—	53·8	72·5	(111)	—	—	—
		Raytheon	—	—	54·0	74·0	112·2	139	168	(188)
Pseudomonas fluorescens	60	Raytheon	28·6	38·0	(58·5)	74·1	(107)	—	—	—
Azotobacter vinelandii	57	Raytheon	—	(37·5)	—	74·5	—	—	—	—
		Raytheon	27·3	35·5	54·0	72·7	108·5	—	—	—
Escherichia coli	50	French pressure cell	28·5	35·3	55·0	74·3	106·7	—	—	—
		Raytheon	(26·2)	37·7	54·7	75·8	107·0	—	—	—
Streptococcus faecalis	34	French pressure cell	—	34·0	53·0	72·7	106·9	—	—	—
			—	37·6	55·3	73·8	105·3	—	—	—
		Raytheon	—	—	54·1	74·5	105·7	—	—	—
Bacillus cereus	33	French pressure cell	28·7	33·6	52·6	76·4	—	—	—	—
		Raytheon	28·7	36·9	54·2	75·1	108·5	—	—	—

The corrected sedimentation coefficients of bacterial ribosomes

Some representative results are collected in Table 1. The average $(s_{20,\mathrm{TMS}})_0$ values (expressed as Svedberg units) were calculated to be: 28·5 ± 2, 36·3 ± 2·7, 54·4 ± 1·8, 74·2 ± 2·4, 106·9 ± 1·6, or, reduced to standard conditions as $(s_{20,w})_0$: 29·5 ± 2, 37·5 ± 2·8, 56·3 ± 1·9, 76·7 ± 2·5, 110·5 ± 1·6. The complete set of ribosomes was not always detectable with every strain. For example there appeared to be no 110 s ribosomes in Streptomyces, no 37 s ribosomes in Acetobacter and sometimes no 29 and 37 s ribosomes in Streptococcus. It is very likely that each strain had indeed the complete set, but that some of the ribosomal types were occasionally present in very small concentrations. Furthermore, it is well known that the

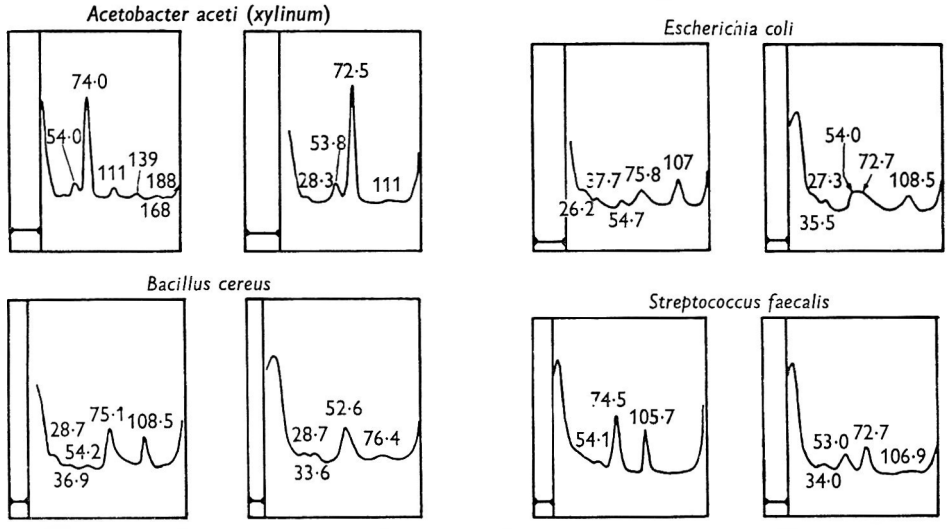

Fig. 4. Comparison of schlieren patterns of ribosomes from cells broken with the French pressure cell and by ultrason. Methods, see text. For each strain two pictures are given: the left one was made with the crude extract from cells disrupted by ultrason, the right one with a sample of the same cell suspension disrupted with the French pressure cell. Tracings do not represent pictures taken at the same time but are selected to show all the peaks. The suspensions of intact cells used for disruption contained, for *Acetobacter aceti* (*xylinum*): 124 mg. of wet wt. cells/ml.; *Bacillus cereus*: 210 mg. of wet weight cells/ml.; *Escherichia coli*: 104 mg. of wet wt. cells/ml.; *Streptococcus faecalis*: 112 mg. of wet wt. cells/ml. The numbers accompanying the peaks represent the extrapolated sedimentation coefficients at infinite dilution, expressed as $(s_{20,\mathrm{TMS}})_0$.

relative amounts of the different ribosomes change with the physiological state of the organisms (young cells usually having more of the smaller ribosomes and resting cells mainly the larger ones) and depend on the composition of the growth medium (particularly the Mg^{2+} concentration).

It will be seen from Table 1 and Fig. 4 that *Acetobacter aceti* (*xylinum*) released large particles (139, 168, 188 s) which were not encountered with other strains. It is possible that these were polysomes, but we held it to be far more likely that they represented fragments of the cell hull, which is indeed rather unusual in these bacteria, since it contains much cellulose and other polysaccharides. We showed that purified cell hulls of this strain which had been prepared with the French pressure cell released identical large particles when treated with the Raytheon intruments.

Effect of growth conditions and physiological state of the organisms. The effect of both factors on the relative amounts of ribosomes is well known. However, they appear to have little effect on the sedimentation coefficients. In spite of the fact that the growth conditions varied greatly (some organisms were grown on solid media, others in liquid ones; the amount of Mg^{2+} added varied between 0 and 0·25 %, w/v) and that the physiological state of the organisms was different (age of the cells between 21 and 53 hr; variation from early exponential to stationary phase), the sedimentation coefficients are nearly identical. An experiment with *Azotobacter vinelandii* of the same age, grown either on solid or in liquid medium, showed that the relative amounts of the ribosomes were different, as expected, but that the sedimentation coefficients were nearly indistinguishable.

As the Mg^{2+} concentration of the suspending medium is of primary importance, we selected 0·005 M-$MgSO_4$, because both the large and the small ribosomes are detectable under these conditions (see Bolton *et al.* 1959).

Effect of the method of cell disruption on the amount and the nature of the ribosomes released. With some organisms (Streptomyces, Pseudomonas, Azotobacter) there was hardly any difference between the schlieren pictures of cells broken by either method used. With other organisms (see Fig. 4) quite often there were more of the larger ribosomes when the cells had been disrupted with the Raytheon instrument and more smaller ones when they had been treated with the French pressure cell. An unusual phenomenon was observed with *Escherichia coli* disrupted with the French pressure cell, namely, a broad peak which extended from 54·0 to 72·7 s, as if a family of ribosomes were present.

DISCUSSION

The results show that the ribosomes appear to be identical, or nearly identical, in the examples of seven widely divergent genera of bacteria investigated. It thus seems likely that the s values will be the same for most, if not all, bacteria, including the actinomycetes. If there are small differences, they fall within the experimental error. There seems to be little hope that ribosomal sedimentation coefficients will be of use in bacterial taxonomy for distinguishing between several groups. In our conditions the s values were: 29, 37, 56, 77, 110. The three middle values are near to the values reported by Gillchriest & Bock (1958) for Azotobacter, by Morowitz *et al.* (1962) for Mycoplasma and by Ecker & Schaechter (1963) for Salmonella; they agree less well with the results of other authors. These 37, 56 and 77 s peaks correspond to the 30, 50 and 70 s ribosomes of Tissières *et al.* (1959). Our 110 s peak corresponds to the 100 s ribosomes of Tissières *et al.* (1959); it is the dimer of the 77 s particle (Huxley & Zubay, 1960) and is regarded as inert ribonucleoprotein (McCarthy, 1960). Our 29 s peak is probably similar to the 20 s particle of Tissières *et al.* (1959) and to the 25 s particle of Ecker & Schaechter (1963) and Takai, Oota & Osawa (1962), which was shown by the latter authors to be a protein-rich RNA-poor ribosome-like particle. In our experiments we did not detect the 85 s peak, in agreement with other authors (Tissières *et al.* 1959; Dagley & Sykes, 1958; Elson, 1959; Woese *et al.* 1960). In some cases it was detected as a very small peak (Bowen *et al.* 1961; Brown & Rosenberg, 1962). A distinct 85 s peak was observed with *Escherichia coli* (Bolton *et al.* 1959), Salmonella (Ecker & Schaechter, 1963) and Mycoplasma

(Morowitz *et al.* 1962). It should be remembered, though, that the 85s value may be too small, since it was usually reported uncorrected. According to Huxley & Zubay (1960) the 85s particle is the dimer of the 56s particle and, since we found the latter to be identical in all strains investigated, there seems no reason to believe that its dimer would vary in the bacterial world.

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On the Synthesis of Ornithine Carbamoyltransferase in Biotin-deficient *Saccharomyces cerevisiae*

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SUMMARY

Synthesis of ornithine carbamoyltransferase by yeast grown in a medium containing a suboptimal concentration of biotin was less than in biotin-optimal yeast and was diminished still further when L-aspartate was included in the biotin-deficient medium, although the presence of this amino acid is known to cause restoration of nucleic acid and total protein synthesis. Addition of L-ornithine to biotin-deficient medium increased synthesis of the enzyme to a value approximately half of the maximum recorded in biotin-optimal yeast, but DL-citrulline, L-glutamate, L-proline, oleate, or cytosine and uracil and their nucleosides and nucleotides had little or no stimulatory effect on synthesis of the enzyme. L-Arginine had little effect on enzyme synthesis by biotin-optimal yeast but caused a marked decrease in synthesis of the enzyme by yeast grown in biotin-deficient medium containing L-aspartate. With yeast grown in unsupplemented biotin-deficient medium, but not in this medium supplemented with aspartate + ornithine, norbiotin (10^{-8} M) restored synthesis of the enzyme to a level greater than the maximum recorded in biotin-optimal yeast. Homobiotin (10^{-8} M) had a similar though less marked effect.

INTRODUCTION

The major established metabolic role of the B group vitamin biotin is in the fixation and transfer of CO_2 (Lynen *et al.* 1959; Ochoa & Kaziro, 1961; Wakil, 1961), and many of the reported effects of biotin deficiency in micro-organisms can be interpreted in terms of an impairment of CO_2 metabolism. One of the most striking results of biotin deficiency in the yeast *Saccharomyces cerevisiae* is a diminished synthesis of ribonucleic acid and total protein (Ahmad, Rose & Garg, 1961). These would seem to be secondary effects of an impaired ability of the yeast to fix and transfer CO_2 since, by growing the yeast in a biotin-deficient medium containing aspartate, synthesis of these major cell constituents can be partially restored (Ahmad & Rose, 1962*a*). Similarly, an altered lipid metabolism in biotin-deficient micro-organisms appears to be due to some extent to the inability of the organisms to carry out the biotin-dependent carboxylation of acetyl-CoA (Wakil, 1961); this would explain the biotin-sparing action of certain fatty acids (Williams & Fieger, 1946; Hofmann, O'Leary, Yoho & Liu, 1959).

Biotin deficiency is also known to affect adversely the synthesis of some enzymes, including the malic enzyme (Blanchard, Korkes, Del Campillo & Ochoa, 1950) and ornithine carbamoyltransferase (OCT; E.C. 2.1.3.3; Ravel, Grona, Humphreys & Shive, 1959), although synthesis of other enzymes is not restricted (Ahmad & Rose,

1962*b*). The reason for this specific requirement for biotin in the synthesis of certain enzymes has not been fully explained. Studies on the role of biotin in synthesis of the malic enzyme in various micro-organisms suggested that the vitamin probably acts indirectly through its role in the synthesis of adenine (Duerre & Lichstein, 1961), and aspartate and related compounds (Plaut, 1961; Ables, Ravel & Shive, 1961). These findings do not explain, however, why provision of these compounds has such a profound effect on the synthesis of this particular enzyme. This applies also to the claim by Ravel, Mollenhauer & Shive (1961) that, during synthesis of OCT in *Streptococcus lactis*, biotin functions in the synthesis of a 4-carbon unit, which can either be synthesized by the bacterium through a reaction involving biotin or supplied exogenously by derivatives of aspartate or by aspartate itself. This conclusion was at variance with the results reported by Ahmad & Rose (1962*b*), who found that addition of L-aspartate to biotin-deficient medium caused a diminution in the synthesis of OCT by a strain of *Saccharomyces cerevisiae*. The present paper reports further results on the synthesis of OCT in biotin-deficient yeast. Certain of the data included in this paper have already been reported elsewhere in abstract form (Dixon & Rose, 1963).

METHODS

Organism. The strain of *Saccharomyces cerevisiae* (Fleischmann) used was obtained from the Division of Applied Biology, National Research Council of Canada, Ottawa, and was maintained on slopes of malt wort agar: 10% (w/v) spray-dried malt extract ('Muntona', Munton and Fison, Ltd., Stowmarket, Suffolk) + 2% (w/v) agar. Cultures were stored at 3°.

Experimental cultures. All experiments were conducted using the glucose–salts–vitamins medium (pH 4·5) of Rose & Nickerson (1956). This medium, which in most experiments contained either an optimal ($8{\cdot}0 \times 10^{-10}$ M) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) concentration of D-biotin, was supplemented with various other compounds. Biotin-sparing substances and amino acids were incorporated into media as solutions (pH 4·5). Oleic acid was added as a solution in 95% (v/v) ethanol in water; the concentration of ethanol in media never exceeded 0·1% (v/v). Portions (95 ml.) of medium were dispensed into 350 ml. conical flasks which were then sterilized by autoclaving momentarily at 115°. Flasks were inoculated with 5 ml. of a suspension containing 1·0 mg. dry weight equivalent of washed biotin-deficient yeast per ml. M/15 KH_2PO_4; these organisms were harvested from 120 hr cultures of the yeast grown in biotin-deficient medium. Experimental cultures were incubated statically at 25°. Yeast was harvested from these cultures by centrifuging in a refrigerated (0°) centrifuge and was washed three times at 0° with M/15 KH_2PO_4 before being analysed. Growth was measured turbidimetrically as described by Rose (1960), extinction readings being related to mg. dry weight per ml. by a calibration curve.

Assays of OCT activity. Cell-free extracts of yeast were prepared as described by Ahmad & Rose (1962*b*) using a Mickle tissue disintegrator (Mickle, 1948). The protein contents of the extracts were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using crystalline bovine plasma albumin as a standard. OCT activity in cell-free extracts was determined by the method of Estes, Ravel & Shive (1956) and the citrulline formed was estimated by the method of

Archibald (1944). There was no detectable effect on the OCT activity of cell-free extracts when biotin ($16{\cdot}0 \times 10^{-10}$M), norbiotin ($2{\cdot}0 \times 10^{-8}$M) or homobiotin ($2{\cdot}0 \times 10^{-8}$M) was included in the reaction mixture. One unit of OCT activity was taken as the number of μmoles ornithine converted/mg. extract protein/hr at 30°.

Analytical methods. Water-soluble ninhydrin-positive substances were extracted from the yeast by suspending the equivalent of 10 mg. dry weight of washed organisms in 10 ml. water and placing the suspension in a boiling water bath for 15 min. On cooling, the supernatant fluid was removed from the cell debris by centrifugation and, after being supplemented by washings (2·0 ml.) from the debris, the volume of extract was made up to 10 ml. with water. Total ninhydrin-positive substances in these extracts were estimated by a modification of the method of Smith & Agiza (1951) described by Hagen & Rose (1962), using glycine as a standard. Results are expressed as μg. NH_2/10 mg. dry weight yeast. The ornithine content of water extracts of the yeast was determined by the method of Chinard (1952) and the arginine content by the method of Rosenberg, Ennor & Morrison (1956).

The contents of acid-soluble ultraviolet (u.v.)-absorbing substances and RNA in the yeast were determined spectrophotometrically using the procedure described by Ahmad *et al.* (1961). Protein in the residue remaining after nucleic acids and related substances had been extracted from the yeast was determined by the micro-Kjeldahl method (Markham, 1942) using a mercuric oxide catalyst (Miller & Houghton, 1945).

Chemicals. All compounds used in this study were screened for possible contamination with biotin by examining the ability of the compound to stimulate growth of the biotin-requiring yeast in biotin-free basal medium (Ahmad & Rose, 1962*a*); contaminated compounds were washed or recrystallized until shown to be free of biotin. Amino acids were examined chromatographically for purity and, with the exception of citrulline which contained a trace of ornithine, all were found to be free of contaminating ninhydrin-positive compounds. The sample of oleic acid used was shown by gas chromatography to contain 76% (w/w) oleic acid, the principal contaminant being elaidic acid (22% w/w), the *trans* isomer of oleic acid. Asparagine, citrulline and oleic acid were obtained from British Drug Houses Ltd, Poole, Dorset. All other amino acids, together with vitamins, carbamoyl phosphate, cytosine and uracil were purchased from L. Light and Co. Ltd, Colnbrook, Buckinghamshire, and nucleosides and nucleotides from C. P. Boehringer und Soehne, G.m.b.H., Mannheim, West Germany.

RESULTS

Growth and synthesis of ornithine carbamoyltransferase under conditions of biotin deficiency

Growth of the yeast in biotin-optimal medium and in supplemented and unsupplemented biotin-deficient media is shown in Fig. 1; OCT activities of extracts of yeast grown in each of these media are given in Fig. 2.

In yeast grown in biotin-optimal medium, enzyme synthesis increased rapidly during the early part of the exponential phase of growth, but thereafter declined. Growth of, and OCT synthesis by, the yeast were severely restricted in unsupplemented biotin-deficient medium. Supplementing the biotin-deficient medium with

L-aspartate caused an initial stimulation of growth but led to a further diminution of OCT synthesis. In oleate-supplemented biotin-deficient medium, there was a much slower stimulation of growth and OCT synthesis was slightly higher than in yeast grown in unsupplemented biotin-deficient medium. When the yeast was grown in biotin-deficient medium supplemented with aspartate+oleate, there was a stimulation of growth which, especially in the early stage of growth, was slightly greater than the sum of the stimulations caused by aspartate and oleate singly. OCT synthesis in yeast grown in this medium was greater than that in yeast grown in biotin-deficient medium supplemented with only aspartate, but was still below the maximum reached in yeast grown in unsupplemented biotin-deficient medium.

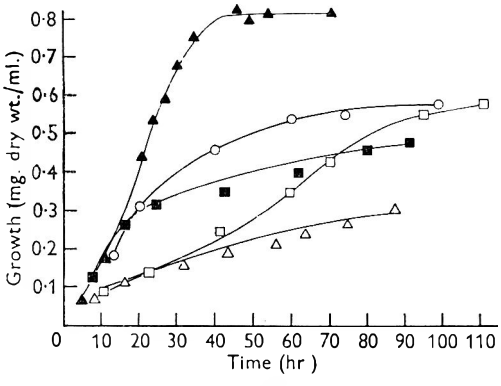

Fig. 1

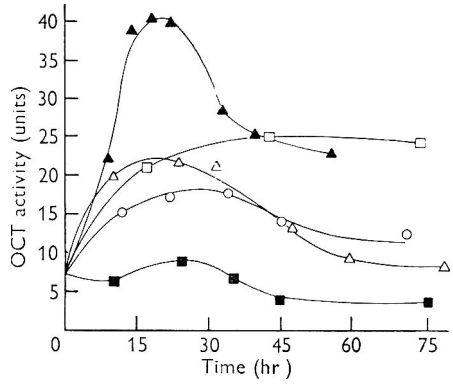

Fig. 2

Figs. 1 and 2. Growth of (Fig. 1) and ornithine carbamoyltransferase activity in extracts of (Fig. 2) yeast grown in media containing an optimal concentration ($8{\cdot}0 \times 10^{-10}$M; ▲) of biotin or a suboptimal concentration ($0{\cdot}4 \times 10^{-10}$M) either unsupplemented (△) or supplemented with L-aspartate ($2{\cdot}0 \times 10^{-3}$M; ■), oleate (100 μg./ml.; □) or aspartate+oleate (○).

The effects of these conditions of biotin deficiency on growth of, and OCT synthesis by, the yeast were therefore similar to those reported in brief by Ahmad & Rose (1962*b*) although these workers used a much smaller inoculum. Yeast grown under the conditions employed in the present study also showed changes in the contents of acid-soluble u.v.-absorbing substances, RNA and intracellular amino acids similar to those reported by Ahmad & Rose (1962*a*, *b*).

Effect of potential inducer and repressor compounds on ornithine carbamoyltransferase synthesis

In order to examine the possible effects of biotin deficiency on feed-back mechanisms controlling the synthesis of OCT, a study was made of the effect of incorporating potential inducer and repressor compounds in the medium. Compounds tested included L-proline, L-arginine and intermediates on the biosynthetic pathway to arginine in yeast, namely L-glutamate, L-ornithine and DL-citrulline (Abelson & Vogel, 1955; Scher & Vogel, 1957). Incorporation of citrulline, glutamate or proline in concentrations up to 500 μg./ml. in biotin-optimal medium or in unsupplemented or aspartate-supplemented biotin-deficient medium had no detectable

effect on growth or OCT synthesis as compared with yeast grown in media lacking these amino acids. Ornithine, however, caused a modest stimulation of enzyme synthesis (Table 1). In yeast grown in biotin-optimal or in biotin-deficient medium containing 100 μg. ornithine/ml., there was an increase of up to 60 % in the synthesis of OCT as compared with yeast grown in the corresponding medium lacking ornithine. OCT synthesis was considerably diminished in yeast grown in aspartate-supplemented biotin-deficient medium as compared with yeast grown in unsupplemented biotin-deficient medium (Fig. 2), but addition of ornithine to the aspartate-supplemented biotin-deficient medium caused an approximately fourfold increase in specific activity. A similar though less marked effect was found using media containing L-asparagine instead of L-aspartate. Addition of ornithine, at the same concentration, to oleate-supplemented biotin-deficient medium caused little or no change in OCT synthesis in the yeast but, when added to biotin-deficient medium containing oleate + aspartate, an increase in specific activity of up to 60 % was recorded.

Table 1. *Effect of L-ornithine on ornithine carbamoyltransferase synthesis in biotin-optimal and biotin-deficient yeast*

Yeast was grown in a biotin-deficient medium with and without the supplements indicated below. After harvesting, the yeast was washed, disintegrated, and the OCT activities of the cell-free extracts determined as described in Methods. Ornithine was included in media at 100 μg./ml.

Supplements in biotin-deficient medium	Age of culture (hr)	OCT activity (units) Medium lacking ornithine	OCT activity (units) Medium containing ornithine
None	17	20·5	29·2
	27	19·3	25·9
	41	18·2	24·6
L-Aspartate (2·0 mM)	10	6·3	25·0
	22	8·4	22·0
	35	6·6	19·0
L-Asparagine (2·0 mM)	12	7·9	20·0
	19	10·5	22·3
	36	8·7	15·5
Oleate (100 μg./ml.)	17	21·0	22·8
	43	25·0	27·2
	75	24·5	26·0
	89	20·5	21·2
L-Aspartate (2·0 mM) + oleate (100 μg./ml.)	12	15·3	24·3
	35	17·5	23·5
	46	14·3	19·6
Biotin (0·76 mμM; optimal)	12	30·5	35·2
	22	42·5	65·5
	35	27·5	39·5

Increasing the concentration of ornithine in the medium to 500 μg./ml. (Fig. 3) had no effect on growth, while synthesis of OCT was either unaffected or diminished. At concentrations lower than 100 μg./ml., OCT synthesis by yeast grown in biotin-optimal medium or in unsupplemented biotin-deficient medium was diminished, but synthesis in yeast grown in aspartate-supplemented biotin-deficient medium

increased and was at a maximum in yeast grown in media containing 50 μg. ornithine/ml.

When arginine (100 μg./ml.) was included in biotin-optimal medium, OCT synthesis by the yeast was slightly but consistently higher than in yeast grown in arginine-free biotin-optimal medium. However, arginine at this concentration had no effect on OCT synthesis in unsupplemented biotin-deficient medium or in this medium supplemented with oleate or oleate + aspartate. In biotin-deficient

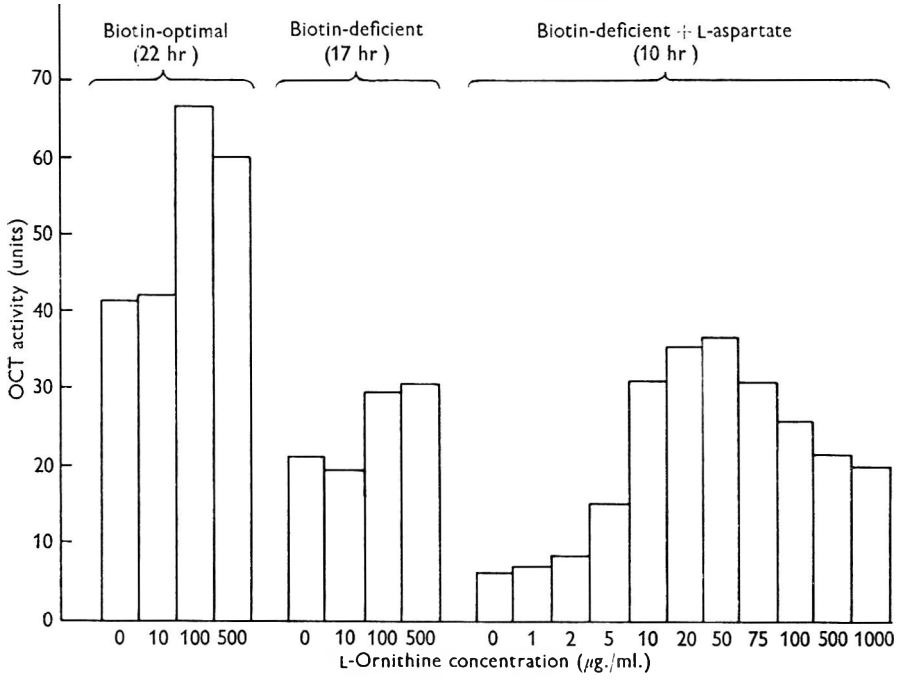

Fig. 3. Effect of L-ornithine concentration on induction of ornithine carbamoyltransferase synthesis in yeast grown in medium containing an optimal concentration ($8{\cdot}0 \times 10^{-10}$ M) of biotin, or a suboptimal concentration ($0{\cdot}4 \times 10^{-10}$ M) with and without L-aspartate ($2{\cdot}0 \times 10^{-3}$ M) together with L-ornithine at the concentration indicated.

Table 2. *Effect of L-arginine on induction of ornithine carbamoyltransferase by L-ornithine in biotin-optimal and biotin-deficient yeast*

Experimental details are as described in Table 1. Arginine and ornithine were each included in media at 100 μg./ml., and aspartate at 2·0 mM.

Medium	Age of culture (hr)	OCT activity (units)	
		Medium lacking arginine	Medium containing arginine
Biotin-optimal	22	42·5	43·5
Biotin-optimal + ornithine	22	62·5	66·0
Biotin-deficient	17	20·5	20·0
Biotin-deficient + ornithine	17	29·2	28·6
Biotin-deficient + aspartate	10	6·3	3·8
Biotin-deficient + aspartate + ornithine	10	25·0	23·2

medium supplemented with aspartate + arginine, there was a diminution in enzyme synthesis as compared with that in yeast grown in arginine-free aspartate-supplemented biotin-deficient medium. As shown in Table 2, the presence in the medium of 100 μg. arginine/ml. did not affect the ability of ornithine (100 μg./ml.) to induce synthesis of OCT.

In all experiments in which ornithine or arginine was included in media, it was shown, by determinations of the concentrations of total ninhydrin-positive substances, ornithine and arginine in the intracellular pools, that these amino acids had penetrated the cell (Table 3). It was also clearly established that arginine was able to enter the yeast in the presence of exogenous ornithine.

Table 3. *Arginine and ornithine contents of intracellular amino acid pools of yeast grown in biotin-optimal medium and in basal and supplemented biotin-deficient media containing different concentrations of* L-*arginine or* L-*ornithine*

Yeast was grown in biotin-optimal or biotin-deficient medium supplemented as indicated in the table. After harvesting the cell crops, a suspension of the washed yeast was placed in a boiling water bath for 10 min. to liberate intracellular amino acids. The concentrations of arginine and/or ornithine in these water extracts were then determined as described in Methods.

Supplement in biotin-deficient medium	Age of culture (hr)	Concn. in medium (μmole/ml.)		Concn. in intracellular pool (μmole/mg. dry wt. cells)	
		Ornithine	Arginine	Ornithine	Arginine
Biotin (0·76 mμM; optimal)	22	0	—	0·05	—
		10	—	0·12	—
		100	—	0·28	0·16
		500	—	—	—
		—	0	—	0·18
		—	100	—	0·37
		—	500	—	0·54
		100	100	—	0·41
None	17	0	—	0·03	—
		10	—	0·03	—
		100	—	0·05	0·31
		500	—	0·14	—
		—	0	—	0·25
		—	100	—	0·41
		—	500	—	0·74
		100	100	—	0·48
L-Aspartate (2·0 mM)	10	0	—	0·02	—
		10	—	0·08	—
		100	—	0·17	0·13
		500	—	0·65	—
		—	0	—	0·10
		—	100	—	0·23
		—	500	—	0·49
		100	100	—	0·20
Oleate (100 μg./ml.)	40	0	—	0·03	—
		10	—	0·05	—
		100	—	0·07	—
		500	—	0·23	—
		—	0	—	0·21
		—	10	—	0·22
		—	100	—	0·34
		—	500	—	0·88

Effect of cytosine and uracil and their ribonucleosides and ribonucleotides on ornithine carbamoyltransferase synthesis

Rogers & Novelli (1960) reported that synthesis of OCT by lysates from protoplasts of *Escherichia coli* strain W was exceptional in requiring cytidine triphosphate (CTP) and uridine triphosphate (UTP) in addition to the normal requirement for guanosine triphosphate (GTP) in protein synthesis. It seemed of interest, therefore, to examine the effect of uracil and cytosine and their ribonucleosides and ribonucleotides on OCT synthesis, since the diminished synthesis of this enzyme in biotin-deficient yeast might be due to a shortage of CTP or UTP. The results of this investigation, which are given in Table 4, showed that, while addition of cytosine + uracil to biotin-deficient medium caused a slight increase in OCT specific activity in cell-free extracts, other combinations tested in both supplemented and unsupplemented biotin-deficient medium failed to stimulate synthesis of the enzyme.

Table 4. *Ornithine carbamoyltransferase synthesis by yeast grown in media supplemented with cytosine, and uracil and their ribonucleosides and ribonucleotides*

Experimental details are as described under Table 1. With the exception of cytidylic acid, each supplement was present at $2{\cdot}0 \times 10^{-5}$ M; cytidylic acid, which is sparingly soluble in water, was present as a saturated solution.

Supplements in biotin-deficient medium	Age of culture (hr)	OCT activity (units) of extracts of yeast grown in medium containing: Cytosine + uracil	Cytidine + uridine	Cytidylic acid + uridylic acid	No additions
None	42	19·7	18·4	17·5	17·0
L-Aspartate (2·0 mM) + oleate (100 μg./ml.)	10	25·8	27·6	26·2	25·8

Table 5. *Ornithine carbamoyltransferase synthesis by yeast grown in media containing biotin homologues*

Supplements in biotin-deficient medium	Age of culture (hr)	OCT activity (units) of extracts of yeast grown in medium containing: Norbiotin (10^{-8} M)	Homobiotin (10^{-8} M)	No additions
None	16·5	69·6	56·2	22·2
	65	102·0	35·5	13·2
Biotin (0·76 mμM; optimal)	18	37·5	38·5	38·6
	42	24·3	25·9	25·5
L-Aspartate (2·0 mM) + L-ornithine (100 μg./ml.)	10	24·5	25·4	26·0
	22	21·5	22·6	22·0

Effect of biotin analogues on synthesis of ornithine carbamoyltransferase

Several analogues of biotin have been reported to replace the biotin requirement of micro-organisms or to act as metabolic antagonists of the vitamin (Goldberg *et al.* 1947). Since it appeared that biotin was essential for maximum synthesis of OCT by the yeast even in media containing aspartate + ornithine, an examination was

made of the ability of certain biotin analogues to replace the biotin requirement for synthesis of the enzyme. Analogues tested included biotinol, the alcohol analogue of biotin, oxybiotin in which the sulphur atom is replaced by oxygen, and norbiotin and homobiotin which contain respectively one fewer and one more CH_2 group in the side chain. At concentrations of $0{\cdot}5 \times 10^{-10}$M and above, both biotinol and oxybiotin stimulated growth of the yeast, but with norbiotin and homobiotin stimulation of growth was not observed until the concentration in the medium was raised to 10^{-7}M. An examination was therefore made of the effect of norbiotin and homobiotin on OCT synthesis by the yeast at a concentration (10^{-8}M) below that which stimulated growth. The results are given in Table 5. Norbiotin, and to a lesser extent homobiotin, caused a very marked stimulation of OCT synthesis in biotin-deficient medium. However, in biotin-optimal medium and in biotin-deficient medium supplemented with aspartate + ornithine, these homologues were without effect on OCT synthesis.

DISCUSSION

Synthesis of ornithine carbamoyltransferase in the strain of *Saccharomyces cerevisiae* used in this study is inducible by ornithine and, under certain conditions, repressible by arginine. It differs, therefore, from the strain of *S. cerevisiae* examined by Bechet, Wiame & De Deken-Grenson (1962) in which end-product (arginine) repression of OCT synthesis was demonstrated but not induction by ornithine. Induction of OCT synthesis has, however, been previously reported by Gorini (1960) in a strain of *Escherichia coli*, in which ornithine was found to induce enzyme synthesis under conditions of partial arginine repression.

The diminution of OCT synthesis caused by growing the yeast in a medium containing a suboptimal concentration of biotin would seem to be due in part to an impairment in the mechanism for the induced synthesis of this enzyme. The data in this paper suggest that this is possibly caused by the presence of a lower concentration of intracellular ornithine in yeast grown in biotin-deficient medium as compared with biotin-optimal yeast, and also a decreased sensitivity of the induction mechanism in biotin-deficient yeast to the presence of exogenous ornithine. Thus induced synthesis of the enzyme in biotin-deficient yeast becomes considerably more sensitive to the presence of exogenous ornithine when the yeast is grown in biotin-deficient medium supplemented with aspartate or asparagine. This may be due to an enhanced ability on the part of the aspartate-grown biotin-deficient yeast to accumulate ornithine, but it is also conceivable that the restoration of nucleic acid and total protein synthesis caused by aspartate has led to an increased synthesis of repressor substances (? protein) (Jacob & Monod, 1961; Chantrenne, 1963), so making the mechanism controlling enzyme synthesis more sensitive to derepression by inducer. Another possible explanation is that ornithine antagonizes a repression of OCT synthesis caused by aspartate. But repression by aspartate would not seem to be an example of end-product repression, such as that caused by arginine, since OCT is not known to catalyse reactions leading to aspartate synthesis. Moreover, aspartate does not repress OCT synthesis in biotin-optimal yeast. It is more likely that the observed effects of aspartate on OCT synthesis in biotin-deficient yeast are the results of changes in the composition and utilization of amino acids in the intracellular pool (Table 3). The inability of oleate, when added to unsupplemented or

aspartate-supplemented biotin-deficient medium, to affect the response of the yeast to exogenous ornithine suggests that any restoration of normal membrane structure brought about by growth in the presence of oleate (Rose, 1963) is not in itself necessary for the response of the OCT-synthesizing system to exogenous ornithine.

However, none of the combinations of biotin-sparing substances tested, in the presence or absence of ornithine, stimulated synthesis of OCT in biotin-deficient yeast to a level approaching that in biotin-optimal yeast, the greatest activity recorded being only one-half of the maximum obtained in biotin-optimal yeast. This suggests that the role of biotin in synthesis of OCT in this yeast may differ from that in *Streptococcus lactis* in which, according to Ravel *et al.* (1961), it acts by mediating synthesis of a 4-carbon compound which can be supplied exogenously by derivatives of aspartate (e.g. asparagine peptides) or less effectively by aspartate. These workers found, however, that high concentrations of aspartate or lower concentrations of asparagine raised OCT synthesis to only about half of that in the presence of optimal biotin. Furthermore, the inability of uracil and cytosine, and their ribonucleosides and ribonucleotides, to spare the biotin requirement for OCT synthesis by the yeast would seem to indicate that the restriction in synthesis of this enzyme is not caused by a deficiency of CTP or UTP which Rogers & Novelli (1960) reported were required for OCT synthesis in extracts of *Escherichia coli*.

The possibility of a direct role for biotin must be considered in view of the ability of norbiotin and homobiotin to replace the biotin requirement for OCT synthesis without affecting growth. These homologues may act by replacing biotin as a coenzyme in one or more biotin-containing enzymes concerned in OCT synthesis, and the fact that growth of the yeast is not affected suggests that these homologues cannot replace biotin in certain other biotin-containing enzymes. Little, if anything, is known of the ability of these biotin homologues to replace biotin as the coenzyme in biotin-containing enzymes, so it is not possible to speculate regarding the nature of any such enzymes which may be acting in the synthesis of OCT. The finding that synthesis of the enzyme in yeast grown in biotin-deficient medium containing one of these biotin homologues was greater than in biotin-optimal yeast can be explained by assuming that the normal repression mechanisms are not operating in the biotin-deficient yeast. The reason for the inability of the homologues to stimulate OCT synthesis in yeast grown in biotin-deficient medium containing aspartate might, on the other hand, be due to an increased synthesis of repressor substances in this yeast, or possibly to the inability of these homologues to catalyse synthesis of a compound capable of antagonizing the repression by aspartate.

One might speculate, therefore, that in this strain of *Saccharomyces cerevisiae*, biotin functions in the synthesis of OCT partly through its role in the synthesis of inducer, ornithine, partly through its role in the synthesis of other components of the induction mechanism, and partly through some other mechanism which is dependent directly or indirectly upon biotin but which is apparently independent of the synthesis of RNA and total protein. Evidence for a direct role for biotin in OCT synthesis is at present only circumstantial. Nevertheless, it is worth noting that Seaman (1958) has reported that biotin is specifically required for the incorporation of glutamate into isolated kinetosomes from *Tetrahymena pyriformis*. It is possible that biotin may have a similar role in OCT synthesis in yeast.

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Control of Valine and Isoleucine Metabolism in *Pseudomonas aeruginosa* and *Escherichia coli*

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SUMMARY

Isoleucine increased the quantity of α-acetolactic acid-forming enzyme which takes part in the synthesis of valine and isoleucine in *Pseudomonas aeruginosa*. Threonine and α-ketobutyric acid, the precursors of isoleucine, had a similar effect. In *P. aeruginosa* during the initial stage of growth after inoculation, a temporary substantial increase of α-acetolactic acid-forming enzyme can be observed. This increase was not so great when valine was present, and was enhanced by isoleucine, which was effective during the whole period of growth. The repressive effect of valine can be counteracted with isoleucine and the end product inductive effect of isoleucine can be inhibited by valine. In extracts of disrupted organisms the activity of the α,β-dihydroxy acid dehydrase changes parallel with the concentration of the α-acetolactic acid-forming enzyme.

In the case of *Escherichia coli* the repressive effect of valine on the α-acetolactic acid-forming enzyme can be counteracted with isoleucine.

INTRODUCTION

It was shown by Umbarger & Brown (1958*a*) for *Escherichia coli*, and then by Halpern & Umbarger (1959) for *Aerobacter aerogenes*, that the quantity of α-acetolactic acid-forming enzyme which took part in the biosynthesis of valine and isoleucine substantially increased during the initial period of growth, and that this increase was repressed by valine. Radhakrishnan & Snell (1960) found no similar effect in the case of *Neurospora crassa*. Horváth, Gadó & Szentirmai (1961, 1962) found that in *Streptomyces rimosus* α-ketobutyric acid inductively increased the quantity of this enzyme. Freundlich, Burns & Umbarger (1962), investigated in auxotroph mutants of *Salmonella typhimurium* the effect of the products upon the quantity of enzymes taking part in the synthesis of valine and isoleucine, and showed that there existed some correlation in the regulation of these processes. In the present experiments the regulation of metabolic processes was investigated in *Pseudomonas aeruginosa* and *E. coli*.

METHODS

Organisms. The experiments were carried out with *Pseudomonas aeruginosa*, laboratory strain No. 132, and *Escherichia coli* strain K 12.

Cultivation. In the experiments Erlenmeyer flasks (500 ml.) each containing 200 ml. of medium were incubated in the case of *Pseudomonas aeruginosa* at 28° and in the case of *Escherichia coli* at 37° on reciprocal shakers (diameter 2 cm., rev./min. 300).

Media and inoculation. *Pseudomonas aeruginosa* was cultivated on media containing (%, w/v): L-glutamic acid, 0·5 (neutralized with sodium hydroxide); $MgSO_4.7H_2O$, 0·05; K_2HPO_4, 0·7; KH_2PO_4, 0·3; glucose, 0·4. The phosphates and glucose were sterilized separately. The other components of the nutrient medium, and their concentrations, are listed in the tables and figures. Inoculation was as follows: 200 ml. of medium was inoculated with a 24 hr broth culture. After 24 hr a sample, 1 ml. or 5 ml. depending on the experiment, was taken for inoculation of the experimental media.

Escherichia coli was cultivated on a minimal medium described by Davis & Mingioli (1950), from which citrate was omitted (Umbarger & Brown, 1958*a*). The additions to the medium are shown in the tables. The bacteria were grown at least three times in minimal medium before the actual experiments. The inoculation was carried out with 10 ml. portions of a 16 hr culture. Samples were taken at 0 hr and the 3rd hr, when the increase of enzyme activity following inoculation was at its peak.

Enzyme methods. The determination of α-acetolactic acid-forming enzyme in toluene-treated organisms was done by the method, slightly modified, of Halpern & Umbarger (1959). Two ml. of the reaction mixture contained 0·4–0·5 mg. dry wt. washed organisms, 50 μmole magnesium chloride, 100 μmole potassium phosphate (pH 8·0), 100 μg. thiamine pyrophosphate (TPP), and 0·02 ml. toluene. After incubation for 30 min. at 28°, 25 μmole sodium pyruvate in 0·5 ml. were added.

N-Sodium hydroxide (0·25 ml.), N-zinc sulphate (0·25 ml.) were added to stop the reaction. Immediately after this α-acetolactic acid was determined in a sample, according to Westerfeld (1945), as acetoin after decarboxylation with N-sulphuric acid.

The determination of α-acetolactic acid forming enzyme in crude extracts was done by the method, slightly modified, of Umbarger & Brown (1958*b*). The composition of the reaction mixture was as follows: 0·5 mmole potassium phosphate pH 8·0, 50 μmole sodium pyruvate, 100 μg. TPP, 10 μmole magnesium sulphate and 4–5 mg. protein; total volume 1 ml. The reaction mixture was incubated at 37° for 10 min., the reaction was stopped by adding 1·8N-sulphuric acid and the α-acetolactic acid determined as acetoin after decarboxylation.

The method of Wixom, Shatton & Strassman (1960) was used for determining the α,β-dihydroxyacid dehydrase activity with DL-α,β-dihydroxyisovaleric acid or DL-α,β-dihydroxymethylvaleric acid as substrates. The resulting keto acids were determined by the method of Cavallini & Frontali (1954), as modified by Strassmann, Shatton & Weinhouse (1960).

Disruption of organisms. After harvesting and washing with buffer the cell paste was suspended in 0·1 M-potassium phosphate (pH 8·0). The suspensions (50 mg. dry wt./ml.) were disrupted by a 12 min. treatment in a MSE Mullard ultrasonic apparatus, at 0–2°, and then centrifuged at 20,000*g* for 15 min. in a refrigerated centrifuge. The clear supernatant fluid was used immediately for enzyme determination. The protein content of the crude extract varied between 20 and 25 mg./ml. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Chemicals. DL-α,β-Dihydroxy-isovaleric and DL-α,β-dihydroxy-β-methylvaleric acid were synthesized according to Sjolander, Folkers, Adelberg & Tatum (1954); α-keto-isovaleric and α-keto-β-methylvaleric acids were prepared from valine and

isoleucine, respectively, with amino acid oxidase, by the method of Meister & Tice (1950).

The other substances were of analytical reagent or *purissimum* grade.

RESULTS

Induction of the α-acetolactic acid-forming enzyme with isoleucine. In the first series of experiments the amount of the α-acetolactic acid-forming enzyme was determined in 14 hr cultures from 1% (v/v) inocula. Results are summarized in Table 1. When different quantities of isoleucine were added to the medium, a gradual increase in the enzyme level up to fourfold at the highest concentration could be observed. Valine did not affect the production of enzyme, but it decreased the inductive effect of isoleucine. No stoichiometric relationship between valine and isoleucine was found in connexion with their antagonistic effect. Isoleucine precursors, e.g. threonine, α-ketobutyric acid, also had an inductive effect and this effect was decreased by valine; in similar concentrations, other natural amino acids had no effect. The different substances added to the medium did not affect the growth of the organisms. The inductive effect of isoleucine was observed with several strains of *Pseudomonas aeruginosa*.

Table 1. *The effect of the composition of the medium upon the α-acetolactic acid-forming enzyme level in cells of Pseudomonas aeruginosa*

Substances added to the minimal medium	μmole α-acetolactic acid/mg. bacteria dry wt./hr
None	0·38
DL-Isoleucine, 100 μg./ml.	0·96
DL-Isoleucine, 300 μg./ml.	1·56
DL-Isoleucine, 900 μg./ml.	1·60
DL-Valine, 600 μg./ml.	0·42
DL-Isoleucine, 300 μg./ml. + DL-valine 300 μg./ml.	1·61
DL-Isoleucine, 300 μg./ml. + DL-valine 600 μg./ml.	0·43
DL-Isoleucine, 900 μg./ml. + DL-valine 300 μg./ml.	1·74
DL-Isoleucine, 900 μg./ml. + DL-valine 600 μg./ml.	0·40
DL-Threonine, 4 mg./ml.	1·22
DL-Threonine 4 mg./ml. + DL-valine 600 μg./ml.	0·56
α-Ketobutyric acid 1 mg./ml.	1·55
α-Ketobutyric acid 1 mg./ml. + DL-valine 600 μg./ml.	0·55

Variation in amount of the α-acetolactic acid-forming enzyme during incubation. When 5 ml. portions of the inoculum were used the α-acetolactic acid-forming enzyme value paralleled the growth rate of *Pseudomonas aeruginosa* and a more intensive synthesis of enzyme was noted during the initial period of growth (Fig. 1). When isoleucine was added to the medium the initial increase of the enzyme value became substantially higher and, during the whole period of growth, the enzyme value of the organisms was much higher than in organisms grown on minimal medium. Valine repressed this enhanced synthesis of enzyme. When valine + isoleucine were added to the medium in similar concentrations the change in enzyme value was identical with that observed in organisms grown on minimal medium; i.e. valine and isoleucine neutralized the effect of each other. Investigations were

carried out to determine the greatest time after inoculation at which the addition of end products still produced an effect. It was observed that additions 1 hr after inoculation from the beginning of the log phase were still effective, but beyond that time neither a repressive effect of valine, nor an inductive effect of isoleucine, was observed.

The parallel change of the amount of α-acetolactic acid-forming enzyme with that of α,β-dihydroxyacid dehydrase. For the further support of the experimental findings mentioned, the activity in cell-free extracts of the α-acetolactic acid-forming enzyme and of the α,β-dihydroxyacid dehydrase taking part in the biosynthesis of

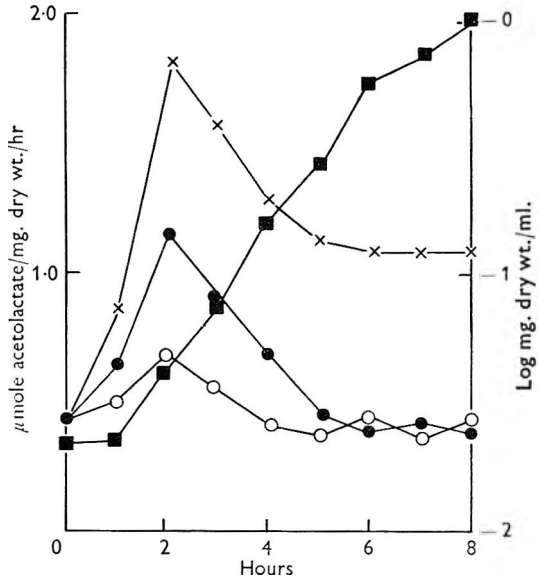

Fig. 1. The effect of the composition of the minimal medium upon the change of the α-acetolactic acid-forming enzyme activity of the cells of *Pseudomonas aeruginosa*. ●—●, Minimal medium; ○—○, minimal medium + 600 μg./ml. DL-valine; ×—×, minimal medium + 900 μg./ml. DL-isoleucine; ■—■, growth.

valine and isoleucine was determined. After the disruption of organisms of different ages and grown on different media, it was found (Table 2) that the activity of the α-acetolactic acid-forming enzyme and that of the α,β-dihydroxyacid dehydrase changed correspondingly.

The inhibition of α-acetolactic acid-forming enzyme with valine. Umbarger & Brown (1958*b*) and Halpern & Umbarger (1959) showed that the α-acetolactic acid-forming enzyme taking part in the biosynthesis of valine and isoleucine is competitively inhibited by valine in *Escherichia coli* and *Aerobacter aerogenes*. The enzyme in *Pseudomonas aeruginosa* can be similarly inhibited by valine (Table 3).

The effect of isoleucine on valine repression of the α-acetolactic acid-forming enzyme in Escherichia coli. It is known that valine has a repressive effect on the synthesis of α-acetolactic acid-forming enzyme, but the antagonistic effects of valine and isoleucine observed in *Pseudomonas aeruginosa* have not been unequivocally demonstrated in the case of *E. coli*. We found, in accordance with the

Table 2. *The activity of α-acetolactic acid-forming enzyme, and α,β-dihydroxyacid dehydrase in cell-free extracts of Pseudomonas aeruginosa*

	μmole acetolactic acid/mg. protein/hr		μmole α-ketoisolvaleric acid/mg. protein/hr		μmole α-keto-β-methylvaleric acid/mg. protein/hr	
Age of culture ...	2 hr	8 hr	2 hr	8 hr	2 hr	8 hr
Medium						
Minimal	2·6	1·0	3·3	1·5	2·8	1·1
Minimal + 900 μg./ml. isoleucine	5·0	2·5	6·4	2·8	6·4	3·2
Minimal + 600 μg./ml. valine	1·8	1·0	2·0	1·6	1·8	1·4

Table 3. *The effect of valine on the α-acetolactic acid-forming enzyme in cell-free extracts of Pseudomonas aeruginosa*

DL-Valine concentration (μmole/ml.)	Acetolactic acid formed (μmole/mg. protein/hr)	Inhibition (%)
0·00	6·20	0
0·05	3·86	38
0·10	2·46	60
0·50	1·19	81

Table 4. *α-Acetolactic acid-forming enzyme in Escherichia coli strain K12*

Medium	Time (hr)	Opt. density at 650 mμ.	α-Acetolactic acid formed (μmole/mg. dry wt./hr)
Minimal	0	42	0·12
Minimal	3	150	0·54
Minimal + 25 μg./ml. DL-valine	3	70	0·29
Minimal + 50 μg./ml. DL-valine	3	65	0·23
Minimal + 50 μg./ml. DL-isoleucine	3	148	0·50
Minimal + 25 μg./ml. DL-valine + 25 μg./ml. DL-isoleucine	3	150	0·75
Minimal + 25 μg./ml. DL-valine + 50 μg./ml. DL-isoleucine	3	150	0·79

Table 5. *Inhibition of α-acetolactic acid-forming enzyme with DL-valine in case of Escherichia coli strain K12*

	α-Acetolactic acid formed (μmole/mg. dry wt./hr)		
Medium		In the presence of 0·1 μmole of DL-valine	Inhibition (%)
Minimal	0·54	0·18	67
Minimal + 50 μg./ml. DL-valine + 50 μg./ml. DL-isoleucine	0·72	0·20	72

published data of Umbarger & Brown (1958*b*) that the growth-inhibiting concentration of valine repressed the enzyme formation. The growth-inhibiting and repressive effect was counteracted by the addition of isoleucine and in the presence of both amino acids the enzyme level was about 40 % greater than in the control culture (Table 4). Isoleucine by itself was without effect on enzyme level. The α-acetolactic acid-forming enzyme grown under different conditions was sensitive to valine (Table 5).

DISCUSSION

The first experimental results suggested that in the regulation of the enzymes taking part in the synthesis of valine and isoleucine valine plays a role. Valine, which inhibits the growth of K12 strain of *Escherichia coli* (Tatum, 1946; Umbarger & Brown, 1955), competitively inhibits the α-acetolactic acid-forming enzyme (Umbarger & Brown, 1958*b*). The enzyme to be found in variants resistant to valine (Leavitt & Umbarger, 1962) cannot be inhibited by valine. It was shown that valine represses the enzyme level which normally increases during the initial period of growth (Umbarger & Brown, 1958*b*; Halpern & Umbarger, 1959). Later, experiments carried out by Freundlich *et al.* (1962), with an auxotroph of *Salmonella typhimurium*, showed that the formation of the enzymes taking part in the synthesis of valine, isoleucine and leucine can be repressed by the end products.

In this paper the regulation of valine and isoleucine metabolism has been investigated in *Pseudomonas aeruginosa* and *Escherichia coli*. It has been observed in the course of experiments that the α-acetolactic acid-forming enzyme substantially increases after inoculation, the phenomenon which has already been demonstrated in *E. coli* (Umbarger & Brown, 1958*b*), and in *Aerobacter aerogenes* (Halpern & Umbarger, 1959). This initial increase can be modified by the addition of the end products to the growth medium. In *P. aeruginosa*, as in *E. coli* and *A. aerogenes*, valine represses this increase in enzyme content of the organisms during the early stages of growth. But, unlike the other two organisms, isoleucine exerts an end-product induction effect which is in evidence during the entire growth period. The end-product induction was first noted by Gorini (1960), and Gorini & Gundersen (1961), in connexion with the biosynthesis of arginine. The induction effect can be demonstrated also with isoleucine, produced endogenously, from threonine and α-ketobutyric acid, or with exogenous isoleucine, and this is the probable cause of the suitability of *P. aeruginosa* strains for the production of isoleucine (Chibata, Kisumi & Ashikaga, 1960). The induction effect of isoleucine was decreased by valine. It could be observed that the repressive effect of valine can be counteracted by isoleucine, an effect which has also been noted with *E. coli*.

Experimental findings in this work are supported by the fact that parallelism is found between the change in the concentration of the α-acetolactic acid-forming enzyme and that of the α,β-dihydroxyacid dehydrase.

It was found in experiments carried out here that in the case of both organisms valine and isoleucine have opposing effects in the regulation, though no stoichiometric relationship between the quantity and the effect of amino acids added to the medium could be detected. Cohen & Rickenberg (1956) observed that the uptake of valine and isoleucine is an active process, and moreover that the uptake of valine can be inhibited by isoleucine. It seems probable that the reason we could not find any

stoichiometric relationship is due to the fact that permease has an effect on intracellular concentrations of valine and isoleucine. The results obtained by Cohen & Rickenberg (1956) do not explain our results, because they studied only the active uptake of valine.

It should be emphasized that the effects found are in evidence only in the initial stage of growth when there is a spontaneous increase of the enzyme levels. The changes can be explained on the basis of theories accepted at present (Szilárd, 1960): the concentration of valine and isoleucine inside the cells regulates the enzyme levels which take part in the synthesis of the two branched chain amino acids.

The initial rise of the enzyme levels can be observed also in other strains, e.g. in *Mycobacterium pellegrino*, *M. smegmatis*, *Streptomyces rimosus* and *S. fradiae* (Horváth *et al.* unpublished results). It seems probable that this rise is important biologically. Attempts have been made to elucidate this problem in our laboratory.

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The Anatomical Distribution of Murine Toxin in Spheroplasts of *Pasteurella pestis*

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SUMMARY

In order to study the distribution of the murine toxin in *Pasteurella pestis*, whole organisms were converted to spheroplasts by treatment with penicillin or glycine in a sucrose medium. The spheroplasts were broken by osmotic lysis and homogenization. After centrifugation and washing, the membrane residues contained about 10% of the total activity of the spheroplast; the remainder of the toxic activity resided within the cytoplasm. Ribosomes were of low toxic activity. Magnesium ions selectively inhibited the destruction of these membranes by sonic oscillation; their treatment with trypsin resulted in the release of large amounts of non-toxic protein and peptides. Varying the temperature during spheroplast formation altered both the distribution of toxin between the anatomical components as well as the amount of toxin synthesized. Preliminary tests indicated that toxin obtained from the membrane and that found within the cytoplasm were identical.

INTRODUCTION

Ajl, Reedal, Durrum & Warren (1955) isolated, purified and described the murine toxin of *Pasteurella pestis* as a non-conjugated protein of about 74,000 molecular weight. It has been observed by a number of workers that this toxin is intracellular and that it can be released following lysis or autolysis of the organisms (Englesberg & Levy, 1954; Goodner, Pannel, Bartell & Rothstein, 1955; Warren, Walz, Reedal & Ajl, 1955). Cocking, Keppie, Witt & Smith (1960) submitted *Pasteurella pestis* grown *in vivo* to sonic oscillation. They found that toxicity for mice resided predominantly in the resulting extract while a small amount of toxic activity remained in the residue. We have observed the bulk of the toxin to be present in the extracts as well as some in the cell wall from sonically treated organisms grown *in vitro* (unpublished data). The present report presents a somewhat different approach to the problem of toxin localization within the organisms. The possibility that certain breaking procedures might lead to erroneous conclusions about toxin distribution in growing organisms prompted our use of spheroplasts, in lieu of parent organisms, in order to eliminate this possibility as well as to demonstrate whether or not toxin resided within the cell membrane, cytoplasm or ribosomes. The results described here represent an attempt to further elucidate the anatomical site of toxin biosynthesis within the bacterial cell. In these investigations we have assumed a spheroplast to be devoid of the characteristic rigid bacterial cell wall of the parent organism from which it was derived.

METHODS

Culture of cells and production of spheroplasts. Organisms of *Pasteurella pestis* strain 'Tjiwidej' used in these studies were obtained from a modified defined medium of Englesberg (1952) and Englesberg & Levy (1954) in which casein hydrolysate was replaced by sodium thiosulphate (500 mg./l.) and five L-amino acids: phenylalanine (1·0 mM), methionine (0·5 mM), isoleucine (1·0 mM), threonine (1·0 mM) and valine (1·0 mM). Vitamins were added in the following amounts (μg./l.): thiamine hydrochloride (200), biotin (10), pyridoxine hydrochloride (100), folic acid (100), and cobalamin (50) containing 3000 μg./g. vitamin B-12. The amino acids and vitamins, sterilized by filtration, and glucose (0·2%, w/v) solution, separately sterilized by heat were added to the autoclaved salt solution.

Organisms were stored on agar slopes composed of the defined medium containing 1·6% agar. Transfers were made from slopes to liquid media and the suspension incubated at 26° for 24 hr. The resulting suspension, stored at 7°, was used for daily inoculations. An inoculum (1·5 ml.) was transferred to 150 ml. of medium to produce actively growing cultures after incubation at 26° for 16–19 hr on a rotary shaker. The organisms were centrifuged down, washed in 0·054 M-potassium phosphate buffer (pH 7·0), and standardized by resuspending in defined medium to a Klett reading of 25 with a no. 42 filter (400–465 mμ); this corresponded to a concentration of 40 μg. Lowry protein/ml. Sucrose to 10% (w/v) and penicillin G (Parke, Davis and Co.), 200 units/ml., were then added. In certain experiments, glycine (1%, w/v) replaced the penicillin. When glycine was used, a concentration of organisms giving a Klett reading of 100 (160 μg. protein/ml.) was achieved and was routinely used to obtain larger quantities of spheroplasts. One-litre flasks containing 250–300 ml. penicillin- or glycine-treated suspensions of organisms were incubated on a rotary shaker. After a period of 7–11 hr at 26° over 95–98% of the organisms were converted to spheroplasts as determined by haemocytometer counts.

Spheroplast fractionation. Spheroplasts were removed by centrifugation (10,000 *g*) from 1 l. of suspension at 4° for 10 min. All subsequent centrifugations were also performed at 4°. The spheroplasts were suspended in 30 ml. of 4×10^{-3} M- or 10^{-2} M-potassium phosphate buffer (pH 7·0) containing 2×10^{-3} M-magnesium chloride and deoxyribonuclease (beef pancreas, 1× crystallized, Mann Research Laboratories) 10 μg./ml. Although considerable breakage was evident, complete spheroplast destruction was obtained by further homogenization with a motor-driven Teflon pestle. The resulting homogenate was spun at 32,000 *g* for 10 min. The pellet obtained was resuspended in the phosphate + magnesium chloride buffer (pH 7·0) without deoxyribonuclease and homogenized until no intact spheroplasts were detected by microscopy. The membrane pellet was washed twice in 20 ml. of the phosphate buffer + magnesium chloride solution and stored at 4° until used. This membrane fraction contained an insignificant number of viable organisms as determined by agar plate counts. The combined supernatant fluids obtained after homogenization were termed the cytoplasmic fraction. Ribosomes were obtained by adjusting the supernatant fluid to 0·02 M-$MgCl_2$ and spinning for 90 min. at 38,000 rev./min. in a no. 40 rotor of a Model L Spinco ultracentrifuge. The ribosomal pellet was washed with 0·02 M-$MgCl_2$ and recentrifuged.

Total ribonucleic acid was determined by extracting the various fractions with

0·5 N-perchloric acid for 30 min. at 70° as modified after Basler & Commoner (1956). The orcinol method of Ceriotti (1955) and absorption at 260 mμ were two methods used to measure ribonucleic acid in the acid extract. Yeast ribonucleic acid (Worthington Biochemical Corporation) served as the standard.

Total protein and toxin determination. Protein was determined by a modified Lowry procedure (Oyama & Eagle, 1956) with bovine albumin (crystallized, Pentex Incorporated, Kanakee, Ill., U.S.A.) as standard. Toxin was assayed by diluting the various fractions with distilled water and injecting 0·5 ml. intraperitoneally into 16–18 g. Swiss Albino mice; deaths which occurred within 24 hr were recorded. Four mice were used for each dilution. The LD 50 dose was averaged from maximal and minimal lethal doses. Toxin concentration in a particular fraction was calculated by dividing the LD 50 dose, in μg., into the total μg. protein of each fraction and expressed as the total LD 50 (total toxic units).

Membrane disruption. To evaluate methods that might be used to isolate membrane-bound toxin and to gain insight into the structural relationship between the toxin and the membranes these were disrupted by various methods. In each case the treatments were concluded by centrifugation of the membrane suspension at 32,000 *g* for 10 min. and assaying the resulting soluble and residue fractions for total toxin. To disrupt by sonic treatment, isolated membranes were suspended in 20 ml. distilled water or dilute phosphate buffer (pH 7·0) and treated in a Raytheon 10 kc. Sonic Disintegrator for 2–6 min. at 5°. Trypsin (2 × crystallized; Worthington Biochemical Corporation) was used for digestion of isolated membranes; digestion was facilitated by slow agitation on a rotary shaker for from 2 to 5 hr. Membranes were also treated by suspending in sodium deoxycholate (0·1 %, w/v) or weak alkaline solutions (sodium hydroxide pH 9–10) and agitating the suspension for 2–5 hr. Membranes were also disrupted by rapidly freezing aqueous membrane suspensions in thin layers, grinding with a pestle for a few minutes, then slowly thawing with further grinding.

RESULTS

Toxin content of spheroplast and spheroplast fractions

The spheroplast homogenate or the recombined isolated cytoplasm + membrane fractions displayed the highest specific toxic activity (lowest LD 50 value). The LD 50 of the recombined fractions decreased 60–70 % compared with the averaged LD 50 of these two components individually. Thus, cytoplasmic and membrane toxin complemented each other in expressing a more lethal effect. Cocking *et al.* (1960) found that the combined soluble extract and residue guinea-pig toxins of *Pasteurella pestis* exhibited a similar synergistic effect.

During a typical spheroplast conversion, total protein in these fragile forms increased three- to fourfold over that of the original culture. It was noted in many experiments that spheroplasts formed with glycine tended to contain more total toxin and protein in all fractions than those obtained by the use of penicillin. Most of the toxic activity appeared to reside within the cytoplasm, but at least 10 % of the total toxin remained in the twice-washed membrane fraction (Table 1). This fraction contained 50–60 % protein on a dry-weight basis. In other experiments five subsequent washings continued to remove small amounts of protein, suggesting that the membrane was being disintegrated. The final washing contained

minute amounts of RNA. It was found that 200 μg. RNA/mg. protein remained in the membrane fraction upon completion of the washing procedure.

A gelatinous opaque pellet consisting almost entirely of ribosomes was obtained when the cytoplasmic fraction was adjusted to 0·02 M with $MgCl_2$ and centrifuged at 38,000 rev./min. The washed pellet contained less than 1% toxin and protein of that of the intact spheroplast.

Table 1. *Distribution of toxin and protein in Pasteurella pestis spheroplast fractions obtained with penicillin and glycine*

The final volume of spheroplast suspension was 600 ml. for each treatment. Fractionation was carried out in 10^{-2} M-potassium phosphate buffer (pH 7·0).

Treatment	Fraction	LD 50 dose (μg. protein)	Total protein (mg.)	Total LD 50 doses
Penicillin	Cytoplasm	53	57·78	1,088
	Membrane	212	23·40	110
Glycine	Cytoplasm	50	63·00	1,260
	Membrane	162	24·60	152

Table 2. *Comparison of the relative effectiveness of various methods in removing toxic protein from spheroplast membranes of Pasteurella pestis*

The data from a series of experiments were used to estimate the relative effectiveness of a particular treatment. The methods listed below are in order of decreasing effectiveness (+ + + + = most effective).

Method	Toxic protein solubilized
Sonic treatment	+ + + +
Sodium deoxycholate (0·1 %)*	+ + +
Aqueous sodium hydroxide (pH 9–10)	+ +
Trypsin	+
Freeze, grinding and thawing	–
Control	–

* Although a great deal of protein was released, the specific toxic activity (LD 50) present in the solubilized fraction was difficult to determine since deoxycholate enhanced toxin activity (also see Goodner *et al.* 1955).

Slight alkalinity favoured the solubility of toxin from lysed whole organisms. Spheroplasts were fractionated at pH 8·8 as described in the Methods by substituting sodium borate buffer for the phosphate buffer in order to avoid possible selective precipitation of cytoplasmic toxin. Membranes so obtained contained over 70% of the total toxic activity of that found in membranes fractionated at pH 7·0. Relatively larger amounts of non-toxic protein were removed from membranes at pH 8·8 as compared to pH 7·0, suggesting that non-toxic particulate protein was also being solubilized. It was concluded that toxin could not be readily removed by washing from the membrane fraction, indicating that toxin was bound to the fraction which sedimented at 32,000 *g*.

Release of toxin from the membranes by various agents

Attempts were made to disrupt the membrane structure to test for the presence of a membrane-bound toxin by partial isolation and to elucidate the relationship of the toxin to the membrane. Sonic treatments of membrane suspensions for 2 min. significantly increased the specific toxic activity of the protein as compared with the untreated original suspension. A comparison of the methods used to remove and solubilize the toxin is shown in Table 2. Sonic treatment for 2–6 min. was the most effective method for the release of toxin. Quick-freezing, grinding and thawing, although releasing toxin from whole organisms, did not release appreciable amounts of toxin and total protein from isolated membranes.

Table 3. *Comparison of the amounts of toxin and total protein released from 'glycine membranes' from Pasteurella pestis spheroplasts during sonic treatment, with and without* 0·02 M-$MgCl_2$

Membrane suspensions prepared as usual were subjected to three 2 min. sonic treatments. Control membranes were resuspended during each 2 min. treatment.

	Membranes in water			Membranes in $MgCl_2$		
Fractions	LD 50 dose (μg. protein)	Total LD 50 doses (toxic units)	Protein (% of total)*	LD 50 dose (μg. protein)	Total LD 50 doses (toxic units)	Protein (% of total)*
Soluble						
Sonically treated (1)	79	80	45	83	20	15
Control	83	22	12	83	19	9
Sonically treated (2)	100	24	17	—	—	3
Control	—	—	2	—	—	2
Sonically treated (3)	—	—	8	—	—	1
Control	—	—	1	—	—	2
Final residue						
Sonically treated	> 205	—	30	250	34	81
Control	242	57	85	224	66	87

* '% of total' refers to the amount of protein present in each fraction relative to the total soluble protein + residue protein.

Release of toxin by sonic treatment

During sonic treatments it was found that an increase in $MgCl_2$ concentration inhibited solubilization of toxin and membrane protein. Table 3 gives the results of a typical experiment with membranes from spheroplasts produced with glycine and submitted to sequential sonic treatments in order to remove the bulk of the toxin. Membrane material was suspended in distilled water or 0·02 M-$MgCl_2$, sonically treated for 2 min. and centrifuged. After resuspension this procedure was repeated twice for each sample before calculation of the data presented. Over 70% of the total protein was released from membranes suspended in water after three sonic treatments; this protein contained 104 LD 50 (total toxin units). By comparison, 20 LD 50 and 20% protein was released in the $MgCl_2$ preparations. It should be emphasized that the LD 50 dose of released protein decreased about threefold below the initial membrane-bound protein. This observation and the

previously mentioned results which showed an increase in the toxic activity of the sonically treated homogenates suggest that the potential toxic activity of bound toxin cannot be adequately expressed until the toxin is solubilized.

When 0·02 M-$MgSO_4$ or 0·02 M-NaCl was substituted for $MgCl_2$ in membrane sonic-treatment experiments, only $MgSO_4$ afforded significant protection. It was concluded from these sonic treatments with membranes from glycine- or penicillin-derived spheroplasts that magnesium ions protect the isolated membranes of *Pasteurella pestis* against disruption by sonic treatment. This effect of Mg^{2+} is interesting in that Grossowicz (1962) reported that Mg^{2+} was needed to stabilize whole *Pasteurella pestis* organisms against lysis by lysozyme. In confirmation we have observed that whole organisms were protected against sonic treatment with higher concentrations of Mg^{2+}. Sonic treatment of the cytoplasmic contents obtained from the spheroplasts and retreatment of toxic protein released from the membrane by sonic treatment did not alter the LD 50 dose of the initial preparations. It does not appear that sonic treatment affected soluble (i.e. unbound) toxin.

Table 4. *The distribution of toxic protein and/or peptides between the soluble and residue fractions of glycine spheroplast membranes of Pasteurella pestis after treatment with trypsin*

Trypsin (300 μg.) was added to a 20·75 mg. membrane protein suspension and incubated at 37° in 0·01 M-potassium phosphate buffer (pH 7·0) for 135 min. The control contained 20·25 mg. membrane protein similarly incubated but without enzyme. The 'total protein' and 'total LD 50' doses (total toxic units) are expressed as % of total soluble protein + residue protein or toxicity.

	Solubilized			Residue		
Treatment	LD 50 dose (μg. protein)	Total protein (%)	Total LD 50 doses (%) (toxic units)	LD 50 dose (μg. protein)	Total protein (%)	Total LD 50 doses (%) (toxic units)
Control	39	12	49	240	88	51
Trypsin	109	40	60	245	60	40

The effect of trypsin on membrane disruption

Ajl, Rust, Hunter, Woebke & Bent (1958) reported that incubation of partially purified murine *Pasteurella pestis* toxin with trypsin did not significantly alter the toxicity of the preparation, although ninhydrin-positive material increased. Trypsin was therefore incubated with membrane suspensions in the hope of selectively releasing membrane toxin or of removing impurities while leaving toxin bound.

The LD 50 dose of solubilized membrane protein increased 2–4 times over that of the control (membrane + buffer; Table 4). This indicated that most of the protein released by trypsin was non-toxic. These results were in contrast to those from sonic treatment experiments, where the protein released was of high toxicity. In this particular experiment (Table 4) an unusually large degree of autolysis of the control membranes occurred which resulted in the release of highly toxic protein.

It would be expected that the loss of large amounts of non-toxic protein would

be detectable as an increase in the remaining residue activity. Such an increase in residue activity was only detectable in experiments where the release of non-toxic protein was most pronounced. Incubation of toxin released by control membranes with trypsin showed no decrease in toxicity, substantiating the fact that trypsin does not destroy toxic activity. These results suggested that toxin may be bound in some manner which makes it inaccessible to trypsin action.

Toxin synthesis at 26° *and* 37°

It has been reported that *Pasteurella pestis* alters its metabolic characteristics and requirements as a result of temperature changes (Surgalla, 1960). Burrows (1960) stated that preliminary results showed that spheroplasts incubated at 37° contained 75% less non-diffusible antigen (apparently complexed material) than did organisms converted to spheroplasts at 28°. Experiments were made to test

Table 5. *The distribution of toxin and protein in Pasteurella pestis spheroplasts produced by penicillin or glycine at* 26° *or* 37°

Spheroplasts were produced at 26° and 37° with penicillin or glycine, and cytoplasm and membranes isolated. The 'total protein (%)' and 'total LD 50 (%)' refer to % of total cytoplasmic protein + membrane protein or toxicity.

	Fractions					
	Cytoplasm			Membrane		
Treatment	LD 50 dose (μg. protein)	Total protein (%)	Total LD 50 (%) (toxic units)	LD 50 dose (μg. protein)	Total protein (%)	Total LD 50 (%) (toxic units)
Penicillin						
26	53	71	90	212	30	10
37	> 140	40*	< 57	272	60	> 43
Glycine						
26	50	71	89	162	29	11
37	119	61	78	276	39	22

* Spheroplast lysis with penicillin is more pronounced at 37° than at 26°. This observation might account in part for the low % yield of cytoplasmic protein, which would be lost when harvesting the spheroplasts.

whether spheroplasts formed at 26° or at 37° differed in toxin content and distribution in the fractions studied earlier. After incubation at 37° total synthesis of toxin and protein was inhibited as compared to 26°. Total protein decreased as much as 70% at the high temperature. Membrane fractions contained a greater percentage of toxin relative to cytoplasm when incubated at 37° than at 26° (Table 5). With penicillin, membrane toxin increased to over 43% of the total toxin in both fractions. This extreme shift in the relative location of toxin activity was seen in many experiments performed with penicillin; the effect was less pronounced when glycine was used in place of penicillin (Table 5). The specific activity of the penicillin membranes decreased 30–40% at the higher temperature (Table 5), while the decrease in specific activity of the cytoplasmic fraction was more drastic, being

almost a threefold loss at 37°. From this decrease in activity of both fractions it was concluded that at 37° there was less protein synthesis and toxin production than at 26°. The increase in LD 50 dose illustrates that less toxin existed relative to total protein at 37° and suggests a selective inhibition of toxin production at this temperature. This selective effect was more pronounced in the cytoplasm than in the membrane fraction. Whether toxin was selectively degraded or its synthesis selectively inhibited remains to be determined. Synthesis inhibition seemed more likely when spheroplasts were examined microscopically (Pl. 1, figs. 1 and 2), because spheroplasts formed at 37° (fig. 2) were smaller and contained a lesser amount of dense cytoplasmic material as compared with those at 26° (fig. 1). By comparison, protein synthesis of whole organisms was inhibited over 50% and the specific toxin activity of the sonically treated organisms decreased 40% at 37° as compared with 26° when these suspensions were incubated under the same conditions but without penicillin or glycine. This suggests that, under the conditions used at 37°, inhibition was not an artifact of spheroplast induction but was a metabolic inhibition characteristic of the whole organisms.

DISCUSSION

The cytoplasmic toxin of *Pasteurella pestis* appears to exist as a non-conjugated non-particulate protein as deduced from the fact that a negligible amount of toxin was found in the ribosomes. In the case of the particulate toxin it may be asked whether the insoluble residue consists mainly of cell membrane. Bacterial cell membranes are composed primarily of protein and lipid (Hughes, 1962). It is, therefore, reasonable to assume that the membrane does contribute a large amount of the total protein of the residue and perhaps the bulk of the total toxin.

Clarke & Lilly (1962) recently reported that Gram-negative bacteria contain a complex consisting of cell wall bounded on both sides by the membrane structure; one of these membranes appears to be attached to the cell wall. Hughes (1962) also suggested a closely associated wall–membrane complex in Gram-negative bacteria. Such a complex in *Pasteurella pestis* would make it difficult to evaluate the results presented here, in terms of two distinct separate envelope structures found in Gram-positive bacteria. It is interesting to note that, after vigorous stirring or homogenization, the *P. pestis* membrane disintegrates into particles some of which float free. A similar fragmentation was reported by Mitchell & Moyle (1957) with *Staphylococcus aureus* membranes. The gradual loss of protein and toxin from the *P. pestis* membrane fraction during the washing procedures demonstrates the fragility of the cell wall+membrane complex in *P. pestis*. This apparent instability makes it difficult to arrive at definite conclusions concerning the precise amount of toxin and protein contained in the membrane and cytoplasm of the intact organisms.

The cytoplasmic membrane of *Pasteurella pestis* and the partially purified toxin released from autolysed whole bacteria apparently are identical. Both autolysed whole-organism toxin and cytoplasmic toxin from spheroplasts are purified by precipitation between 35 and 70% ammonium sulphate saturation (Ajl *et al.* 1955). The toxins (cytoplasmic, membrane, and autolysed whole organism) are increased in specific toxic activity by adding sodium deoxycholate, are not decreased in

specific activity by trypsin digestion and show a similar protein band when chromatographed by acrylamide gel electrophoresis or by agar diffusion analysis (Ouchterlony, 1949). Final confirmation of the identity of the membrane and cytoplasmic toxins is being sought by further purification of each toxin so that their properties can be compared more precisely. The temperature studies suggest that the synthesis of membrane and cytoplasmic toxin, relative to total protein, decreases with an increase in temperature. Most interesting is the fact that the decrease is not proportional in both fractions. This decrease in total protein synthesis appears to be a reflexion of the increase in nutritional requirements of *P. pestis* as a function of the temperature of incubation (Higuchi & Carlin, 1958). In more recent experiments we had indications that, with phenylalanine-depleted spheroplasts, the amount of membrane toxin was decreased. Upon subsequent addition of phenylalanine for a short period of time, the membrane toxin increased 10% to 15% relative to total membrane protein, while an increase in cytoplasmic toxin was undetectable. These findings and the temperature studies indicate that the murine toxin of *P. pestis* may be synthesized selectively as the growth conditions are changed. If we assume that the membrane and cytoplasmic toxins are identical, a plausible hypothesis is that the toxin is synthesized within or on the membrane and is then transferred to the cytoplasm, where it accumulates as a soluble protein. That the membrane is a possible site of protein synthesis has been suggested by several authors (Butler, Crathorn & Hunter, 1958; Hunter & Godson, 1962; Mitsui, 1961; Spiegelman, 1959). Experiments are now in progress with labelled amino acids *in vitro* and *in vivo* with spheroplast fractions in an attempt to establish the exact location of toxin synthesis.

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EXPLANATION OF PLATE

Spheroplasts of *Pasteurella pestis* formed in 1 % glycine medium at 37° and 26°. Phase microscopy, × 2000.

Fig. 1. Larger spheroplasts at 26°.

Fig. 2. Spheroplasts decreased in size at 37°.

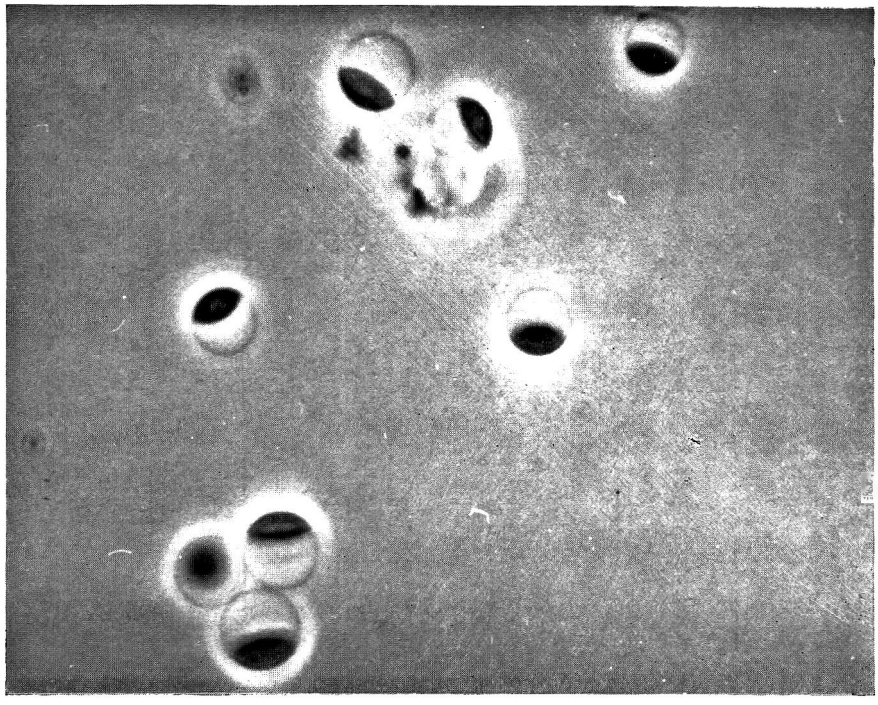

Fig 1

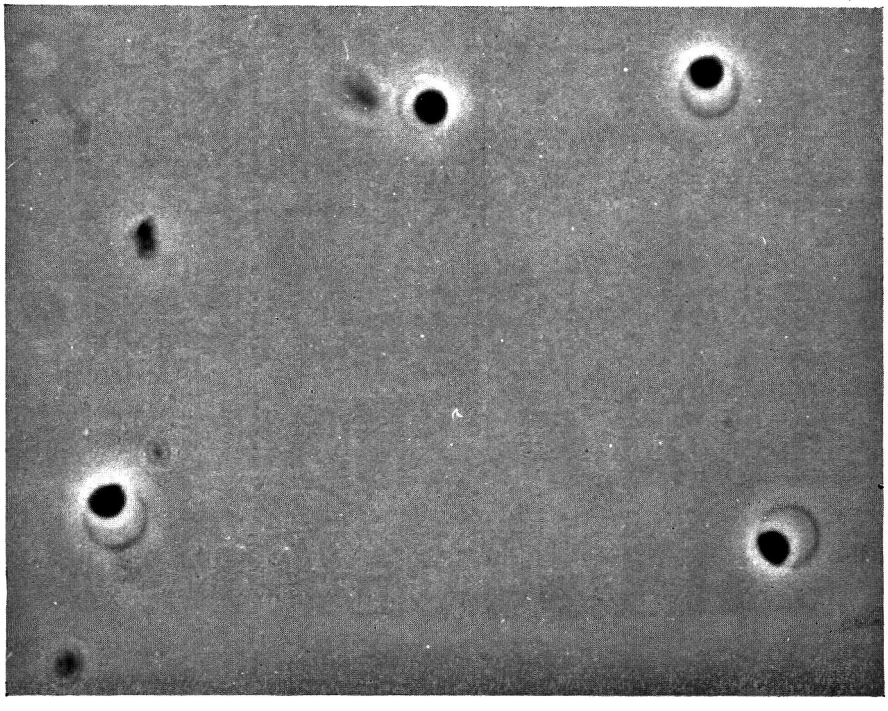

Fig. 2

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The Carotenoids of Algae: Pigments from some Cryptomonads, a Heterokont and some Rhodophyceae

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SUMMARY

The carotenoids of two Cryptophytes, one heterokont and five Rhodophytes have been examined. The main pigments present are, in *Cryptomonas* sp., α-carotene and diatoxanthin; in *Hemiselmis virescens*, α-carotene, and diatoxanthin; in *Vischeria* sp., β-carotene and three unidentified xanthophylls; in *Antithamnion plumula* and *Nemalion multifidum*, β-carotene, lutein and neoxanthin; in *Erythrotrichia carnea*, *Rhodosorus marinus* and *Polysiphonia fastigiata*, β-carotene, zeaxanthin and lutein.

INTRODUCTION

The carotenoids of algae have always attracted biochemists because of their diversity as compared with those present in the leaves of higher plants (see Goodwin, 1952, 1961; Strain, 1958). During the past few years interest has been greatly stimulated because of the isolation for the first time of a number of unicellular algae in pure culture and because of the development of suitable media in which they could be cultivated in the laboratory on a comparatively large scale. Reports have appeared on the carotenoids of the Cryptomonad *Cryptomonas ovata* (Haxo & Fork, 1959) and of a number of Chrysophyceae, *Ochromonas danica* and *Prymnesium parvum* (Allen, Goodwin & Phagpolngarm, 1960), *Isochrysis galbana* (Dales, 1960; Jeffrey, 1961), *Pseudopedinella* sp., *Phaeaster* type *Pavolva gyrans*, *Phaeocystis pouchetti*, *Chrysochromulina ericina*, *Dicracteria inomata* and *Hymenomonas* sp. (Dales, 1960) and *Sphaleromantis* sp. (Jeffrey, 1961). Strain (1958) has described the pigments in a large number of Rhodophytes. The present paper reports our findings on two further Cryptomonads, *Hemiselmis virescens* and *Cryptomonas* sp., the heterokont *Vischeria* sp. and on several previously unexamined Rhodophytes: *Nemalion multifidum*, *Antithamnion plumula*, *Erythrotrichia carnea*, *Rhodosorus marinus*, and *Polysiphonia fastigiata*.

METHODS

Cultivation. Cultures of *Vischeria* sp. were grown in the medium described in Table 1, at 18–20° and illuminated by 'cool white' fluorescent tubes (175 f.c.). The Cryptomonads were grown under conditions described by Allen, Dougherty & McLaughlin (1959). Three species of red algae (*Nemalion multifidum*, *Antithamnion plumula* and *Erythrotrichia carnea*) were collected on the west coast of Sweden and have since been cultivated in 100 ml. Pyrex flasks of an artificial sea water, ASP 6 (Provasoli, McLaughlin & Droop, 1957). Diatoms and other small algae were removed by washing and careful manipulations under microscope, and by transfers

Table 1. *Medium for Vischeria sp.*

Peptone (Difco) (0·1 %) containing the macroelements and microelements indicated (2 ml. of micronutrient mix/l. medium).

Macronutrients	M	Micronutrients	mg./l.
$NaNO_3$	0·003	$B(H_3BO_3)$	0·5
$CaCl_2$	0·00022	$Mn(MnSO_4 \cdot H_2O)$	0·5
$MgSO_4$	0·00062	$Zn(ZnSO_4 \cdot 7H_2O)$	0·005
NaCl	0·00043	$Cu(CuSO_4 \cdot 5H_2O)$	0·02
K_2HPO_4	0·00044	Mo(MoO) 85 %	0·01
KH_2PO_4	0·0013	$V(NH_4VO_3)$	0·01
		Fe(NaFeEDTA)*	4·0

* Diethylenetriaminopentaacetate.

to new flasks. After repeated treatments with antibiotics and fungicides *N. multifidum* was obtained free from bacteria and yeasts (Fries, 1961). The cultures of *A. plumula* and *E. carnea*, however, were not completely freed from bacterial or yeast contaminants. *Nemalion multifidum* was cultivated under a bank of five fluorescent tubes (Philips) consisting of two reflector-backed TL 55, one TL 5, and one TL 17. The light intensity was about 800 lux. The temperature was about 20°. *Antithamnion plumula* was cultivated at 10° under the combined irradiation of an incandescent filament lamp, Luma 500 W., and a mercury-vapour lamp, 265 W., in a light intensity of 700 lux. *Erythrotrichia carnea* was cultivated under the conditions described by Fries (1960). After an incubation time of about 6 weeks the algae were removed from the flasks and freeze-dried immediately without washing. *Rhodosorus marinus* was obtained in axenic culture from Dr G. Giraud; it was collected at Banyuls (Giraud, 1958) and cultivated in artificial sea water ASP 6 at 20° F. (Fries, 1960) and under the light conditions described above for *N. multifidum*.

Polysiphonia fastigiata was collected at Aberystwyth.

Extraction, separation and identification of pigments. Full details of our general procedures have already been reported (Allen *et al.* 1960). In addition thin-layer chromatography with the apparatus designed by Stahl (1962) was used in part of this work.

RESULTS

Carotenoid in Cryptomonads

Cryptomonas sp. strain 35. The carotenoids were chromatographed on a mixture of 3 + 1 (w/w) $ZnCO_3$ + celite and the column developed with light petroleum containing increasing concentrations of ether. Three main carotenoids were obtained (Table 2). Fraction 1 percolated through the column slowly with light petroleum as eluant, but ran through quickly with light petroleum containing 2 % (v/v) diethyl ether. It was purified on activated alumina (Grade H) and exhibited the chromatographic and spectral properties of α-carotene. This was confirmed by co-chromatography on $ZnCO_3$ + celite (3 + 1 by wt.) with an authentic sample of α-carotene prepared from carrots. No separation was obtained. No β-carotene was detected, although a very faint band, with an indeterminate absorption spectrum, closely followed α-carotene on the column.

Table 2. *The carotenoids present in Cryptomonas sp. strain 35*

Pigment extract chromatographed on $ZnCO_3$ celite (3 + 1, by wt.); fractions listed in order of increasing absorptive power.

Fraction	Description	Absorption maxima (mμ) in light petroleum (b.p. 60–80°)	Identification	Concn. (mg./g. dry wt.)	% of total pigment
1	Yellow	447·5, 477·5	α-Carotene	0·28	13·5
2	Orange	430*, 451, 482	Diatoxanthin	1·08	73·5
3	Pale yellow	449, 478	Unknown	0·25	13·0
			Total	1·61	—

* Inflexion.

Table 3. *The carotenoids present in Hemiselmis virescens*

Pigment extract chromatographed on $ZnCO_3$ + celite (3 + 1, by wt.); fractions listed in order of increasing absorptive power.

Fraction	Description	Absorption maxima (mμ) in light petroleum (b.p. 60–80°)	Identification	Concn. (mg./g. dry wt.)	% of total pigment
1	Yellow	447·5*, 477·5,	α-Carotene	0·076	15·9
2	Yellow	425, 447·5, 477·5	(?)	0·108	22·5
3	Yellow-orange	430*, 452, 483	Diatoxanthin	0·160	33·0
4	Bright red†	430*, 450, 480	Unknown	0·140	28·6
			Total	0·484	—

* Inflexion.
† Maxima in ethanol.

Fraction 2 closely resembled diatoxanthin first described in diatoms by Strain, Manning & Hardin (1944) and reported recently in *Prymnesium parvum* (Allen *et al.* 1960). The pigment most closely resembling diatoxanthin is zeaxanthin. Fraction 2 was, however, easily separated from an authentic specimen of zeaxanthin,

prepared from yellow maize, on a $ZnCO_3$ + celite column. Furthermore, the absorption maxima of samples obtained at different times were consistently slightly higher than those of zeaxanthin (448–449, 478 mμ, in light petroleum). Fraction 3 was very tightly absorbed on the $ZnCO_3$ + celite column, and was eluted only with diethyl ether containing a trace of ethanol. It was not identified.

Hemiselmis virescens. The four pigments obtained from this organism (Droop's strain) are described in Table 3. Fraction 1 passed slowly through the column with light petroleum and was shown to be α-carotene by co-chromatography with an authentic sample from carrots on $ZnCO_3$ + celite (1 + 1) and on activated alumina.

Fraction 2 had the spectral properties of lutein. That this was not lucein was demonstrated by co-chromatography on a $ZnCO_3$ + celite column with an authentic sample of lutein isolated from grass; complete separation was obtained.

Fraction 3, the main fraction, resembled diatoxanthin in its properties; on co-chromatography with the corresponding pigment from the Cryptomonas species examined in the previous experiment, no separation was obtained. Fraction 4, which formed a very tightly bound red zone at the top of the column, could only be eluted with ethanol. It was unidentified.

Table 4. *The carotenoids present in Vischeria sp.*

Pigment extracts chromatographed on $ZnCO_3$ + celite (3 + 1, by wt.); fractions listed in order of increasing absorptive power.

Fraction	Description	Absorption maxima (mμ) in light petroleum (b.p. 60–80°)	Identification	Concn. (mg./g. dry wt.)	% of total pigment
1	Yellow	430*, 451, 480	β-Carotene	3·27	64·1
2	Pale yellow	Trace only	Unknown	Trace	—
3	Orange-yellow	422·5, 447, 475	Unknown	0·86	17·0
4	Bright yellow	443, 472	Unknown	0·56	11·0
5	Yellow	425*, 445, 473	Unknown	0·32	7·9
			Total	5·01	—

* Inflexion.

Carotenoids in a heterokont

Vischeria sp. Chromatographic separation of the carotenoids of *Vischeria* sp. yielded five components (Table 4). The first band was shown in the usual way to be β-carotene; no trace of α-carotene could be observed. Fraction 2 is a small yellow band which appears to correspond to that observed in other heterokonts by Strain (1958), who states 'the two principal xanthophylls were often preceded by traces of another xanthophyll which formed a light yellow zone'. Fraction 3 was the main xanthophyll fraction; it was easily separated from lutein, which was less strongly absorbed, and was somewhat less strongly absorbed than diatoxanthin; it resembles the 'moderately sorbed xanthophyll' reported by Strain (1958). Fractions 4 and 5 also correspond to unknown xanthophylls reported by Strain. The very high relative concentration of β-carotene should be emphasized.

Carotenoids in Rhodophytes, subclass Florideae

Nemalion multifidum. This alga, which belongs in the order Nemalionales, family Helminthocladiaceae, contained the pigments indicated in Table 5. Rechromatography of fraction 1 on alumina resulted in its separation into two zones; the major pigment was identified as β-carotene and the minor one as α-carotene by co-chromatography with authentic samples. Fraction 3 was identified as lutein by comparison with an authentic sample from grass. The need to identify lutein unequivocally was important, because it has recently been shown that antheraxanthin (5,6-epoxyzeaxanthin), a pigment with an absorption spectrum almost identical with that of lutein, is the main xanthophyll of Euglena (Krinsky & Goldsmith, 1960). Fraction 3, which could not be separated from authentic lutein on the

Table 5. *The carotenoids present in Nemalion multifidum*

Pigment extract chromatographed on $ZnCO_3$ + celite (3 + 1, by wt.); fractions listed in order of increasing absorptive power.

Fraction	Description	Absorption maxima (mμ) in light petroleum (b.p. 60–80°)	Identification
1	Yellow	425*, 446, 478	β-Carotene
2	Pale yellow	442·5, 443, 472	Unknown
3	Yellow-orange	442·5, 446, 475	Lutein
4	Pale yellow	420, 438, 468	Neoxanthin (probably)

α-Carotene also present in small amounts.
* Inflexion.

Table 6. *Quantitative distribution of carotenoids in Antithamnion plumula and Nemalion multifidum*

Pigment	*A. plumula*		*N. multifidum*	
	mg./g. (dry wt.)	% of total pigment	mg./g. (dry wt.)	% of total pigment
α-Carotene	Traces	—	0·014	1·7
β-Carotene	0·108	14·9	0·103	12·6
Unknown	0·042	5·8	0·050	6·1
Lutein	0·0480	66·5	0·400	49·0
Neoxanthin?	0·092	12·8	0·050	30·6
Total	0·722	—	0·617	—

$ZnCO_3$ + celite column, was therefore mixed with authentic antheraxanthin prepared from *Euglena gracilis*; the two pigments were easily separated on a $ZnCO_3$ + celite column. Confirmation was obtained by running a thin-layer chromatogram of the various pigments on a silicic acid + rice starch plate with a mixture of ethyl acetate + light petroleum (35 + 65, by vol.) as developer. Fraction 3 was not separated from lutein, but was separated from antheraxanthin. Fraction 4 closely resembled neoxanthin and was not separated completely from an authentic specimen of neoxanthin, although very slight separation appeared to take place in occasional

samples. We concluded that fraction 4 was probably neoxanthin, but cannot state this categorically. Fraction 2 occurred only in traces and was not identified.

Antithamnion plumula. The pigments in this alga, which belongs in the order Ceramiales, family Ceramiaceae, are the same as those found in *Nemalion multifidum* and will not be described in detail. They do, however, differ quantitatively as indicated in Table 6.

Polysiphonia fastigiata. The pigments in this alga, a member of the order Ceramiales, family Rhodomelaceae, were a comparatively simple mixture of β-carotene, zeaxanthin and lutein (Table 7); these were identified by comparison with authentic pigments. It is significant that zeaxanthin predominates over lutein.

Table 7. *The carotenoids present in Polysiphonia fastigiata*

Pigment extract chromatographed on $ZnCO_3$ + celite; fractions listed in order of increasing absorptive power.

Fraction	Description	Absorption maxima (mμ) in light petroleum (b.p. 60–80°)	Identification	Concn. (mg./g. dry wt.)	% of total pigment
1	Yellow	430*, 452, 481	β-Carotene	0·024	27·3
2	Orange-yellow	430*, 451, 480	Zeaxanthin	0·040	55·4
3	Yellow	423, 445, 472·5	Lutein	0·024	27·3
			Total	0·088	—

* Inflexion.

Table 8. *The carotenoids present in Erythrotrichium carnea*

Pigment extract chromatographed on $ZnCO_3$ + celite (3 + 1, by wt.); fractions listed in order of increasing absorptive power.

Fractions	Description	Absorption maxima (mμ) in light petroleum (b.p. 60–80°)	Identification	Concn. (mg./g. dry wt.)	% of total pigment
1	Yellow	430*, 452, 481	β-Carotene	0·058	29·0
2	Orange-yellow	430*, 451, 480	Zeaxanthin	0·081	40·5
3	Yellow	423, 445, 472·5	(?) Lutein	0·061	30·5
			Total	0·200	—

* Inflexion.

Carotenoids in Rhodophytes, subclass Bangioideae

Erythrotrichia carnea. Only one very small sample (49 mg./dry wt.) of this alga, which belongs to the order Bangiales, was available. Considerable difficulty was encountered in extracting the carotenoids; they were released slowly with absolute methanol; no extraction occurred with acetone or absolute ethanol. A comparatively low concentration of pigment was present (0·2 mg./g. dry wt. compared with, for example, 0·8 mg./g. in *Nemalion multifidum*). All the separations reported here were done on 40 μg. of pigment. A small column was used (15 × 0·8 cm.) and the absorption spectra were measured in microcells containing 0·3 ml. instead of 3–4 ml. of solution; the separation obtained is recorded in Table 8. Fractions 1,

and 2 were clearly identified by co-chromatography with authentic samples of β-carotene and zeaxanthin, respectively. As far as could be judged with the small amounts available, no α-carotene was present. Fraction 3 was similar in adsorptive power to lutein, but its absorption maxima were at slightly lower wavelengths. Unfortunately, it was not possible to carry out a mixed chromatogram with authentic lutein, but we feel that C is probably lutein which had been isomerized during experimental manipulation.

Rhodosorus marinus. The pigment separation obtained with this alga, a member of the order Porphyridiales, is given in Table 9. By the usual criteria Fractions 1, 2 and 3 were identified as β-carotene, zeaxanthin and lutein, respectively. There was no indication of the presence of α-carotene and there is more than ten times more zeaxanthin present than lutein.

Table 9. *The carotenoids present in Rhodosorus marinus*

Pigment extract chromatographed on $ZnCO_3$+celite (3+1, by wt.); fractions listed in order of increasing absorptive power.

Fractions	Description	Absorption maxima (mμ) in light petroleum (b.p. 60–80°)	Identification	Concn. (mg./g. dry wt.)	% of total pigment
1	Yellow	430*, 452, 480	β-Carotene	0·086	21·0
2	Orange-yellow	430*, 452, 481	Zeaxanthin	0·291	70·9
3	Yellow-orange	427·5, 447·5, 477·5	Lutein	0·024	8·1
			Total	0·401	—

* Inflexion.

DISCUSSION

Cryptomonads. The *Cryptomonas* sp. which was examined in this work is very similar to *Cryptomonas ovata* examined by Haxo & Fork (1959). In both cases, the major carotene is α-carotene, with no β-carotene. The major xanthophyll is diatoxanthin in *Cryptomonas* sp. and in *C. ovata* 'the principal xanthophyll corresponded closely to zeaxanthin; however, identity with diatoxanthin was not precluded' (Haxo & Fork, 1959). Furthermore, the xanthophyll pigments preponderate in both species, the ratio xanthophylls to carotenes being 6·4:1 and 2·4:1 for *Cryptomonas* sp. and *C. ovata*, respectively. In neither species was lutein, fucoxanthin or peridinin observed. The situation in *Hemiselmis virescens* is very similar; diatoxanthin is the main xanthophyll, and α-carotene is the main carotene; β-carotene could not be detected.

Heterokonts (*Xanthophyceae*). Strain (1958) studied a number of heterokonts including *Vischeria stellata*. The present results on our *Vischeria* sp. are in general agreement with his in that β-carotene is the main carotene present and three or four unidentified pigments are present in the xanthophyll fraction. Lutein was not identified in any specimen. This compares with the observation of Jamikorn (1954) that lutein was present in *Tribonema aequale*, *Heterococcus fuoreresis*, and *Ophiocytium majus*. A unique feature of our strain of *Vischeria* sp. is that β-carotene predominates over the xanthophylls.

The Rhodophytes. To facilitate more detailed discussion of the red algae the general classification of the Bangioideae and Florideae is given in Table 10. *Rhodosorus marinus* (order Porphyridiales) is like another member of the same order, *Porphyridium cruentum* studied by Strain (1958) in synthesizing zeaxanthin and lutein but not α-carotene. *Erythrotrichia carnea*, a member of another order, Bangiales, in the subclass Bangioideae, qualitatively resembles *R. marinus*; the two algae differ quantitatively, however, in that the amount of zeaxanthin relative to that of lutein is very much greater in *R. marinus* (9:1) than in *E. carnea* (1·3:1). *Erythrotrichia carnea* is generally similar to the two Bangiales, *Porphyra naiadum* and *P. perforata*, studied by Strain (1958) in synthesizing zeaxanthin and lutein; however, *P. perforata* also synthesized α-carotene, which was not observed in *E. carnea* or *P. naiadum*.

Table 10. *The general classification of the Bangioideae and Florideae (Rhodophyta)*

Subclass I, Bangioideae
- Order 1, Porphyridiales
  - *Rhodosorus*
  - *Porphyridium*
- Order 2, Goniotrichales
  - *Goniotrichum*
- Order 3, Bangiales
  - *Erythrotrichia*
  - *Porphyra*

Subclass II, Florideae
- Order Nemalionales
  - Fam. Chantransiaceae (*Rhodochorton*)
  - Fam. Helminthocladiaceae (*Nemalion, Cumagloea*)
  - Fam. Chaetangiaceae (*Gloiphloea, Galaxaura*)
- Order Ceramiales
  - Fam. Ceramiaceae (*Antithamnion*)
  - Fam. Rhodomelaceae (*Polysiphonia*)

Nemalion multifidum (order Nemalionales, family Helminthocladiaceae) differs from *Cumagloea andersonii* and *Galaxaura* sp. (same family; Strain, 1958) in that it does not synthesize zeaxanthin. Furthermore, *N. multifidum* synthesizes a pigment similar to neoxanthin; Strain (1958) never reported neoxanthin in any of a large number of Rhodophytes which he examined. *Polysiphonia fastigiata* (order Nemalionales, family Rhodomelaceae) is very similar to three other Polysiphonia species examined by Strain (1958), viz. *P. aquamara*, *P. californica* and *P. collinsii*. In all cases α-carotene was not detected and zeaxanthin preponderated over lutein; indeed rather surprisingly, no lutein was found by Strain in *P. collinsii*.

The observations recorded here on five Rhodophytes which had not previously been examined confirm the general conclusion of Strain (1958), who examined a very large number of red algae, that the main qualitative variations in the carotenoid pigment systems of the Rhodophytes, irrespective of the order to which they belong, are the presence or absence of zeaxanthin and the presence or absence of α-carotene. The further demonstration of the existence of α-carotene and β-carotene derivatives together in Rhodophytes adds weight to the conclusion that on evidence from caro-

tenoid studies the Rhodophytes occupy a central position in the evolutionary pattern of the algae (Goodwin, 1962).

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Type Cultures and Proposed Neotype Cultures of Some Species in the Genus *Bacillus*

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SUMMARY

Type cultures or proposed neotype cultures of 20 species in the genus *Bacillus* are listed, together with their histories and their accession numbers in the American Type Culture Collection, the National Collection of Industrial Bacteria and the National Collection of Type Cultures.

INTRODUCTION

The concepts of the type species and the type culture adopted by the *International Code of Nomenclature of Bacteria and Viruses* (1958) are exceedingly important in microbial taxonomy. If these concepts are to be followed in classification and identification, strains typifying the established species must be available for comparison with newly isolated strains and with strains used in new tests, observations and processes. In the present paper, cultures of 20 species in the genus *Bacillus* are arranged in two lists. The first is a list of type cultures. The second shows cultures which we now propose as being suitable for acceptance as neotype cultures. The lists are presented in the hope that they will assist future work; no attempt is made to include all the species of *Bacillus* which might deserve recognition.

The strains of the first list are those designated by the author of the species as the type culture, one designated as a neotype culture by international agreement (the Marburg strain of *Bacillus subtilis*), or, lacking such designations, those made available to other microbiologists by the author as representing his species. When more than one strain was distributed by the author, the first dispensed or the first of a numerical or alphabetical series is regarded as the type culture. The second list contains the proposed neotype cultures for the species whose type culture has, to the best of our knowledge, been lost. The strains selected are considered suitable representatives of their respective species; they have been available for a relatively long time from a type culture collection or the collection of an investigator interested

in the genus; and several are from the collection of the author of an accepted emendation of the species description.

The strains listed here are available from: the American Type Culture Collection, 2112 M Street, N.W., Washington 7, D.C., U.S.A. (ATCC); the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland (NCIB); the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9 (NCTC). The accession number of each strain in each of the three culture collections is given. In addition, its history is shown in parentheses by the names of the persons or collections responsible for propagating and transmitting it, starting with the final source of the culture and ending with the original. Pertinent references to the species are given by Smith & Gordon (1957) (referred to in the lists as *Manual*) and are not repeated here.

LIST OF TYPE CULTURES

Bacillus badius Batchelor *emend.* Saghafi & Appleman. Species description: *Manual*, p. 623. Type culture: ATCC 14574, NCIB 9364, NCTC 10333 (N. R. Smith 663; Henry 110; Batchelor).

Bacillus coagulans Hammer. Species description: *Manual*, p. 622. Type culture: ATCC 7050, NCIB 9365, NCTC 10334 (N. R. Smith 609; Porter; Hammer).

Bacillus firmus Werner. Species description: *Manual*, p. 624. Type culture: ATCC 14575, NCIB 9366, NCTC 10335 (N. R. Smith 613; Porter; Bredemann, original strain; Werner).

Bacillus laterosporus Laubach. Species description: *Manual*, p. 629. Type culture: ATCC 64, NCIB 9367, NCTC 6357 (American Museum of Natural History 797; Ford 6; Laubach).

Bacillus lentus Gibson. Species description: *Manual*, p. 624. Type culture: ATCC 10840, NCIB 8773, NCTC 4824 (Gibson 165).

Bacillus macerans Schardinger. Species description: *Manual*, p. 626. Type culture: ATCC 8244, NCIB 9368, NCTC 6355 (N. R. Smith 888; Porter; Pribram; Schardinger).

Bacillus pantothenticus Proom & Knight. Species description: *Manual*, p. 632. Type culture: ATCC 14576, NCIB 8775, NCTC 8162 (Proom CN 3028).

Bacillus pumilus Gottheil. Species description: *Manual*, p. 622. Type culture: ATCC 7061, NCIB 9369, NCTC 10337 (N. R. Smith 272; Löhnis; Král; Gottheil).

Bacillus sphaericus Neide. Species description: *Manual*, p. 633. Type culture: ATCC 14577, NCIB 9370, NCTC 10338 (Gibson 1013; Král, Marburg strain; Wund; Neide).

Bacillus stearothermophilus Donk. Species description: *Manual*, p. 627. Type culture: ATCC 12980, NCIB 8923, NCTC 10339 (N. R. Smith T 18; National Canners Association 26; Donk).

Bacillus subtilis Cohn *emend.* Prazmowski. Species description: *Manual*, p. 620. Type culture: ATCC 6051, NCIB 3610, NCTC 3610 (Conn, Marburg strain).

LIST OF PROPOSED NEOTYPE CULTURES

Bacillus alvei Cheshire & Cheyne. Species description: *Manual*, p. 626. Proposed neotype culture: ATCC 6344, NCIB 9371, NCTC 6352 (N. R. Smith 662; Lochhead

127). The original strains studied by Cheshire and Cheyne have not been found and may be presumed to have been lost. Lochhead isolated his strain 127 from a honeycomb with European foulbrood in 1925 and distributed it as a representative of the species. Because it is typical and has been available for some time, it is proposed as a neotype culture.

Bacillus anthracis Cohn *emend.* Koch. Species description: *Manual*, p. 618. Proposed neotype culture: ATCC 14578, NCIB 9388, NCTC 10340 (Microbiological Research Establishment, Porton, Wiltshire, England; Vollum; Dunkin). The anthrax bacillus (together with *Bacillus mycoides* and some insect pathogens such as *Bacillus thuringiensis*) overlaps with *Bacillus cereus* in many features. It is treated here as a separate species from the nomenclatural standpoint, without implying that we do or do not regard it taxonomically as a separate species. The original strains of Koch appear to have been lost in 1945, and none can now be traced. This strain, the Vollum strain, was isolated from bovine anthrax, has retained its virulence, and is typical culturally; it has been widely studied and is proposed as the neotype culture.

Bacillus brevis Migula *emend.* Ford. Species description: *Manual*, p. 630. Proposed neotype culture: ATCC 8246, NCIB 9372, NCTC 2611 (N. R. Smith 604; Porter; NCTC; Ford 27 B). The first definitive characterization of this species was provided by Ford. His strain 27B, which he deposited in culture collections, is believed to be the oldest available as a neotype of the species.

Bacillus cereus Frankland & Frankland. Species description: *Manual*, p. 617. Proposed neotype culture: ATCC 14579, NCIB 9373, NCTC 2599 (Gibson 971; NCTC; Ford 13). There appears to be no evidence that the original culture of *B. cereus* was preserved. An important and comparatively early contribution concerning this species was made by Lawrence & Ford (1916). Strain 13 which Ford sent to culture collections is still typical and is now proposed for acceptance as the neotype of the species.

Bacillus circulans Jordan *emend.* Ford. Species description: *Manual*, p. 628. Proposed neotype culture: ATCC 4513, NCIB 9374, NCTC 2610 (Ford 26). This species was adequately characterized for the first time by Ford. His strain 26 which he supplied to culture collections is therefore proposed as the neotype culture.

Bacillus licheniformis (Weigmann) Chester *emend.* Gibson. Species description: *Manual*, p. 619. Proposed neotype culture: ATCC 14580, NCIB 9375, NCTC 10341 (Gibson 46). No record has been found to indicate that Weigmann's original culture of this species was preserved. Gibson, who provided an amplified characterization of the species, distributed his strain 46 to culture collections, and it is proposed as the neotype of the species.

Bacillus megaterium De Bary. Species description: *Manual*, p. 616. Proposed neotype culture: ATCC 14581, NCIB 9376, NCTC 10342 (Gibson 1060; NCTC; Ford 19). De Bary's culture of this species does not appear to have been available to subsequent workers and may be presumed to have been lost. In view of the important contribution to the definition of the species made by Lawrence & Ford (1916), the strain that Ford supplied to culture collections (his strain 19) is proposed as the neotype.

Bacillus pasteurii (Miquel) Migula. Species description: *Manual*, p. 633. Proposed neotype culture: ATCC 11859, NCIB 8841, NCTC 4822 (Gibson 22). There appears

to be no reference in subsequent literature to the original culture of this species, thus indicating that Miquel did not propagate a specimen. The strain proposed as a neotype has been maintained in culture collections for a considerable time and, unlike some older cultures of the species, it has retained its original properties.

Bacillus polymyxa (Prazmowski) Migula. Species description: *Manual*, p. 625. Proposed neotype culture: ATCC 842, NCIB 8158, NCTC 10343 (Kluyver). Prazmowski's original culture of this species has not been mentioned by later writers, and there is a virtual certainty that it no longer exists. The proposed neotype culture was chosen for the reason that it has been a subject of investigation over a long period at Delft and elsewhere.

This study was supported in part by research grant G-5424 from the National Science Foundation, Washington, D.C., U.S.A.

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Clumping of Susceptible Bacteria by Bacteriophage Tail Fibres

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SUMMARY

Susceptible bacteria are clumped in the presence of purified chemically-separated T-even tail fibres. The clumping is partially host-specific, serologically specific, and dependent upon the ionic environment. The clumping principle is adsorbed by bacterial suspensions with coincident disappearance of tail fibres. Clumping is believed to be caused mainly by phage tail fibres and the evidence suggests that the mechanism is by the formation of bridges between bacteria. It is inferred that the tail fibre must have at least two adsorbing sites.

INTRODUCTION

The T-even series of bacteriophages are known to attach to susceptible bacteria by the tips of their tails (Anderson, 1953; Kellenberger & Arber, 1955; Williams & Fraser, 1956). The tails of these viruses are complex structures comprising several proteins different from that of the phage head (Levinthal & Fisher, 1952; Lanni & Lanni, 1953; De Mars, Luria, Levinthal & Fisher, 1953; Anderson, Rappaport & Muscatine, 1953). Electron micrographic studies show at least four structures: tail pins, sheaths, base plates and fibres (Williams & Fraser, 1956; Brenner *et al.* 1959). The finding that separated tail fibres adsorb to susceptible bacteria (Williams & Fraser, 1956) suggested that tail fibres might determine the attachment of bacteriophage. This idea is supported by a number of observations which will be discussed later, but it is probable from Franklin's (1961) results that specific attachment is not mediated solely by fibres. Brenner *et al.* (1959) have developed methods for separating and purifying bacteriophage components; hence it is now possible to study the properties of isolated fibres. We have studied some interactions of such preparations with bacterial suspensions and this paper records experiments on the bacterial clumping that may result.

METHODS

Bacterial suspensions. The following bacterial strains were used: *Escherichia coli* strain B (Luria), which is susceptible to infection by both bacteriophages T_2 and T_4, and two independently-arisen phage-resistant bacterial strains $B/2_1$ and $B/2_2$, which

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are resistant to bacteriophage T_2. All bacteria were grown in aerated nutrient broth to give concentrations of about 10^9/ml. Living cultures of bacteria were used in a few experiments, but unfortunately they were prone to spontaneous clumping and were therefore unsatisfactory. Accordingly standard suspensions of killed organisms were made. One volume of 40 % (w/v) formaldehyde was squirted rapidly into four volumes of a well-grown broth culture. After 5 min. at room temperature (about 30°) the formolized cultures were centrifuged at 3500 rev./min. (= 2000*g*) for 20 min. The deposited bacteria were washed twice in nutrient broth, resuspended in broth, and allowed to stand at 4° overnight. The supernatant fluid containing mostly individual bacteria was then carefully sucked off and stored in the refrigerator. This standard suspension of *E. coli* B was counted by using a Petroff Hauser chamber and had 2×10^9 organisms/ml. The other strains were adjusted to the same optical density. These suspensions which all contained about 96 % single unclumped organisms remained stable for the duration of the experiments (2 weeks).

Tail fibre preparations. We are indebted to Dr Naomi Franklin for preparations of tail fibres made from bacteriophages T_2 and $B \times T_4$ (Streisinger, 1956) by the method of Brenner *et al.* (1959). The latter phage was derived by crossing phages T_2 and T_4 and then serially backcrossing progeny possessing T_4 host range with phage T_2 (Streisinger, 1956). It was used because it is possible to obtain from it good yields of tail fibres with the host range and serological specificities of phage T_4. Concentrates of bacteriophage were treated in a solution containing 0·14 M-glycine and 0·1 M-NaCl at pH 2. This was followed by digestion with 10 mg./ml. DNase for 2 hr at room temperature and at about pH 7·4 with about 0·005 M-$MgSO_4$. The digest was then spun at 2000*g* for 30 min. The resulting pellet was suspended in 1·0 ml. 2 % (w/v) NaH_2CO_3 (pH 7·8) and digested with trypsin 50 mg./ml. and chymotrypsin 50 mg./ml. overnight at 23°. More enzyme was added and the tubes incubated at 37° for another 30 min. The now clear solution was centrifuged at 40,000 rev./min. (= 100,000*g*) in the SW 39 head of a Spinco model L centrifuge for 90 min. The resulting supernatant fluids had abundant tail fibres as seen by negative contrast electron microscopy.

Gamma globulins. Gamma globulins were prepared by ammonium sulphate treatment from rabbit sera made against bacteriophages T_2 and T_4. These were absorbed with bacteria before use. Organisms from a freshly grown broth culture of *Escherichia coli* B were sedimented by centrifuging at 3500 rev./min. (= 2000*g*) for 20 min. The bacteria were washed by resuspending in broth and sedimenting in 30×5 mm. tubes to give about 10^{10} organisms/tube. These were resuspended in the globulin preparation and allowed to react at 30° for 15 min. Most of the bacteria were then sedimented at 3500 rev./min. (= 2000*g*) for 20 min. and the supernatant fluid finally cleared of bacteria by centrifuging at 6000 rev./min. (= 5800*g*) for 20 min.

Experimental procedure. Owing to the small volume of material available all experiments were carried out on a micro-scale. Volumes were always measured with micropipettes delivering 0·015 ml. constructed from Pyrex glass. All reactions were carried out in test tubes having internal dimensions of 50×5 mm.

Except where otherwise specified, readings of all experiments on clumping were made after mixing equal volumes of an appropriate dilution of tail fibres (in broth) with the standard suspension of bacteria at 4°. After 1 hr at 4° the mixture was

diluted 1/5 in broth, mounted in an unruled chamber $\frac{1}{10}$ mm. deep, and random fields examined by dark ground microscopy. The proportions of bacteria that occurred in clumps were thus directly determined. At least 300 bacteria were counted for each determination.

In eighteen experiments, the average percentage of clumped bacteria in control suspensions was 3·82% with a standard deviation, $\sigma = 1{\cdot}66\%$. Thus, the probability of any values exceeding 8·8% by chance was $< 0{\cdot}001$. Because of the obvious difficulty in estimating the number of bacteria aggregated in large clumps, several estimates were always made of the proportion of bacteria clumped in the presence of tail fibres in each sample (e.g. four replicate determinations on one sample gave a mean of 38% clumped, with $\sigma = 5{\cdot}8\%$). However, the proportion of bacteria clumped by a particular dilution of tail fibres varied considerably in different experiments; this may be attributable to inaccuracies arising from handling small volumes and there were probably also other uncontrolled variables. We have therefore not attempted to develop an absolute titration procedure but have depended on the internal controls built into each experiment.

Electron microscopy. Negatively stained preparations of tail fibres were made by using potassium silicotungstate as described for potassium phosphotungstate (Anderson, 1962). They were examined in a Siemens Elmiskop I.

RESULTS

Clumping of susceptible bacteria by tail fibre preparations

It was soon found that suitable dilutions of tail fibres would clump formolized suspensions of bacteria. The first series of experiments was designed to show whether this agglutination was specifically caused by tail fibres or not.

Table 1. *Clumping of formolized and fresh suspensions of Escherichia coli strain* B *by phage* T_2 *tail-fibre preparations and absorption of clumping principle by bacteria*

Additions to bacterial suspensions	Percentage of bacteria clumped: Standard formolized suspension	Percentage of bacteria clumped: Fresh bacterial suspension
Fibres diluted 1/25 in broth (1)	52·0	46·0
Broth control	4·2	8·7
Supernatant fluids from (1) after reacting for 1 hr at 4°	4·3	3·8

Behaviour of fresh and formolized bacteria. In the first experiment the behaviour of fresh and formolized suspensions of *Escherichia coli* B were compared. Tail fibres diluted 1/25 in broth were mixed with equal volumes of each suspension under the standard conditions. After 1 hr at 4° samples were taken and the proportions of clumped bacteria determined. The remainder of the mixtures were centrifuged at 3500 rev./min. for 20 min., and the supernatant fluids tested for residual activity by using the standard formolized suspension of bacteria. The results are shown in Table 1. Both fresh and formolized suspensions were clumped in the presence of fibres and both evidently absorbed the clumping principle. Formolized and fresh

bacteria thus behaved similarly and all remaining experiments were made with the standard formolized suspension for reasons already given (Methods).

Coincident disappearance of tail fibres and of clumping principle. It would be desirable to show that tail fibres and clumping principle were absorbed in the same proportions when mixed with susceptible bacteria. Unfortunately tail fibres were uncountable. However, we were able to make the following comparisons. A 1/10 dilution of the tail fibre preparation in distilled water was examined in the electron

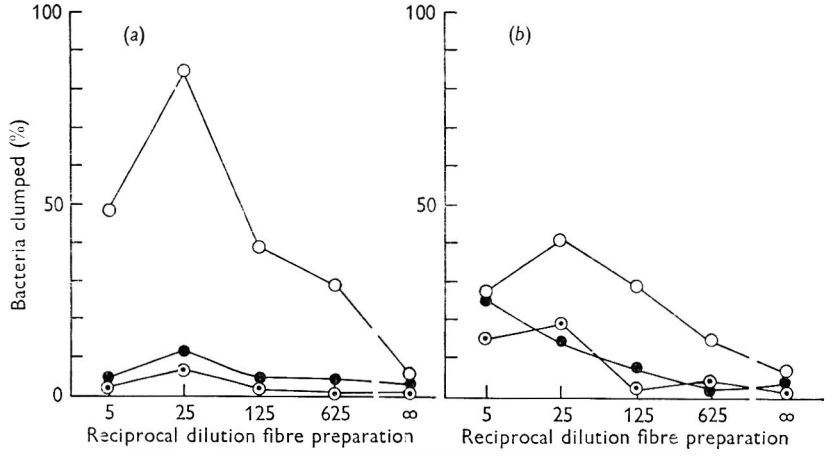

Fig. 1. Percentage bacteria clumped in suspension of *Escherichia coli* B, B/2$_1$ and B/2$_2$ by purified tail fibres of (*a*) phage T_2 and (*b*) phage B × T_4. ○, *E. coli* B; ●, *E. coli* B/2$_1$; ⊙, *E. coli* B/2$_2$.

microscope at a magnification of 40,000. Many tail fibres were seen in each field, with an occasional tail sheath. A pellet made from the standard suspension containing about $1{\cdot}2 \times 10^8$ formolized bacteria was resuspended in 0·06 ml. of the 1/10 fibre dilution and left at 4° for 1 hr, and then the bacteria sedimented. No fibres whatsoever were found in the supernatant fluid. The original tail fibre dilution clumped 44·0 % of the bacteria, but after absorption clumped only 7 %. This result shows that the loss of fibres was matched by a loss in clumping activity.

Host cell specificity. Bacteriophage T_2 will infect *Escherichia coli* B highly efficiently but will not infect the B/2 strains whose resistance is attributable to a lack of adsorption of the phage. To see whether T_2 tail fibres could clump the resistant bacteria, several fivefold dilutions of tail fibres were mixed with suspensions of the resistant strains, B/2$_1$ and B/2$_2$, in parallel with the standard suspension of strain B. The results are shown in Fig. 1*a*. Although significant clumping of the susceptible bacteria occurred in the presence of tail fibres diluted as much as 1/625, a prozone phenomenon was noted in which less clumping was produced by the 1/5 dilution than by the 1/25 dilution. Neither of the resistant strains was much clumped. It will, however, be noted that at a dilution of 1/25 there was an increase in the proportion of clumped B/2$_1$ and B/2$_2$ over the control. The probability of this having occurred by chance is very low ($P = < 0{\cdot}001$) since with each suspension the proportions of clumped bacteria exceeds the mean of controls by more than three standard deviations. The results therefore suggest that significant clumping occurred

with the two insusceptible strains at a dilution of 1/25 tail fibres, but to an extent that was small compared with the clumping that occurred with the standard suspension of susceptible bacteria.

Two questions arose from the above results. First, had the suspensions of *Escherichia coli* B/2 failed to clump because the organisms were deficient in specific T_2 receptors or were they inagglutinable for some other reason? Secondly, does the small amount of clumping observed with the resistant strains reflect poor but significant adsorption of tail fibres? The first question was answered by showing that the suspensions of *E. coli* B/2 were indeed clumped by tail fibres with T_4 host range. Several fivefold dilutions of $B \times T_4$ tail fibres were made in broth and were tested against the same formolized suspensions. The results are shown in Fig. 1*b*, and show that $B \times T_4$ tail fibres did agglutinate B/2 though they were more active in clumping the standard suspension of *E. coli* B.

Table 2. *Absorption of clumping principle from preparations of T_2 tail fibres by three strains of Escherichia coli*

1/25 T_2 tail-fibre preparation absorbed with	Percentage of *E. coli* B clumped by absorbed tail-fibre preparations
Broth (control)	31·0
E. coli B	3·8
E. coli $B/2_1$	9·0
E. coli $B/2_2$	15·0
Control with no tail fibres	4·2

The second question was answered by using freshly-prepared bacterial suspensions of the same three strains to absorb tail fibres. A 1/25 dilution of the T_2 tail fibres preparation was mixed with equal volumes of each bacterial suspension in broth containing 2×10^9 bacteria/ml. After 1 hr at 4° the tubes were centrifuged at 3500 rev./min. ($= 2000g$) and residual clumping activity in the supernatant fluid estimated with the standard formolized suspension of *Escherichia coli* B. The results are given in Table 2. No residual clumping activity was detected after absorption with *E. coli* B while diminished but significant clumping occurred after absorption with the resistant strains. The results suggest, therefore, that T_2 tail fibres are absorbed more efficiently by *E. coli* B than by its phage-resistant variants, and that clumping is therefore correspondingly increased. Neither effect is, however, absolutely specific.

Salt effect. Hershey, Kalmanson & Bronfenbrenner (1944) showed that T_2 bacteriophage requires cations for adsorption to its host, which suggested that clumping might also depend on the presence of cations. Accordingly, plain broth (to serve as a control) and a 1/17 dilution of the T_2 tail fibre preparation in broth were both dialysed for 3 hr against tap water at 20° and then against distilled water at 4° for a further 7 hr. A series of twofold dilutions of 1·0 M-NaCl were made in distilled water. Equal volumes of dialysed bacteria and dialysed fibres were mixed with two volumes of appropriate dilutions of sodium chloride. For controls, dialysed broth was substituted for the tail fibre preparation. The results are given in Fig. 2. Clearly in the controls without tail fibres, significant clumping was only found in the

presence of 0·4 M-NaCl. In the presence of the tail fibre preparation, however, significant clumping was found under all conditions, but it was plainly very much less in the absence of added salt and was maximal in the presence of 0·025 M-NaCl. The maximal and minimal values fall outside three standard deviations from the mean of the remaining values; the probability that these arose by chance is small ($P = < 0{\cdot}001$). The absorption of clumping activity by bacteria in the absence of a salt was not tested.

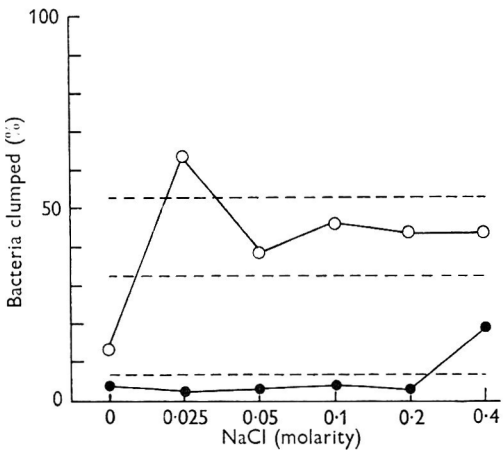

Fig. 2

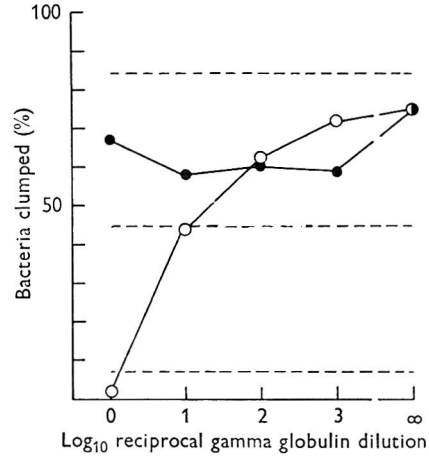

Fig. 3

Fig. 2. Effect of NaCl concentration on clumping of *Escherichia coli* B by phage T_2 tail fibre preparation. ○, Dialysed T_2 fibre preparation 1:17; ●, dialysed broth.

Fig. 3. Inhibition of clumping activity of T_2 tail fibres by previous treatment with anti-T_2 (○) or anti-T_4 (●) gamma globulin.

Neutralization of clumping activity by antibody. Tenfold dilutions of anti-T_2 and anti-T_4 gamma globulin were made in broth. Equal volumes of these and a 1/25 T_2 fibre preparation were incubated at 4° for 1 hr. The mixtures were then tested for ability to clump the standard bacterial suspension in the usual way. The results are given in Fig. 3. Treatment of T_2 fibres with undiluted anti-T_2 gamma globulin clearly neutralized clumping completely. Treatment with the 1/10 dilution of anti-T_2 gamma globulin gave a percentage of clumping about three standard deviations less than the mean of all the remaining points, so that the probability that this arose by chance is small ($P = < 0{\cdot}001$). Thus it is clear that antibody specific for T_2 phage neutralized the clumping activity by T_2 tail fibres. On the other hand, heterologous anti-T_4 gamma globulin had a negligible effect.

Enhancement of clumping by antibody. We have seen that T_2 tail fibres clumped susceptible bacteria, that the clumping factor was absorbed during the process, and that clumping was neutralized by homologous anti-phage antibody. It seemed possible that such antibody would enhance clumping of bacteria which had previously been treated with the tail-fibre preparations. Mixtures were therefore made of fivefold dilutions of the T_2 tail-fibre preparation and the standard formolized suspension of *Escherichia coli* B. After 1 hr at 4° each mixture was divided as follows: (1) one portion was diluted 1/5 in broth and the proportion of clumped

bacteria determined; (2) another portion was centrifuged at 3600 rev./min. (= 2000*g*) for 20 min. and the supernatant fluid tested for residual clumping activity; (3) other portions were mixed with equal volumes of undiluted anti-T_2 gamma globulin, 1/10 anti-T_2 gamma globulin, or (in one instance) undiluted anti-T_4 gamma globulin. These were left for 1 hr at 4° and then read in the usual way. The results are given in Fig. 4*a*. It will be seen: (1) that when broth was added as a control, direct clumping with a prozone was observed much as in Fig. 1, though to a lesser extent; (2) that no significant clumping was induced by the supernatant fluids added to fresh bacteria; (3) that both dilutions of anti-T_2 gamma globulin

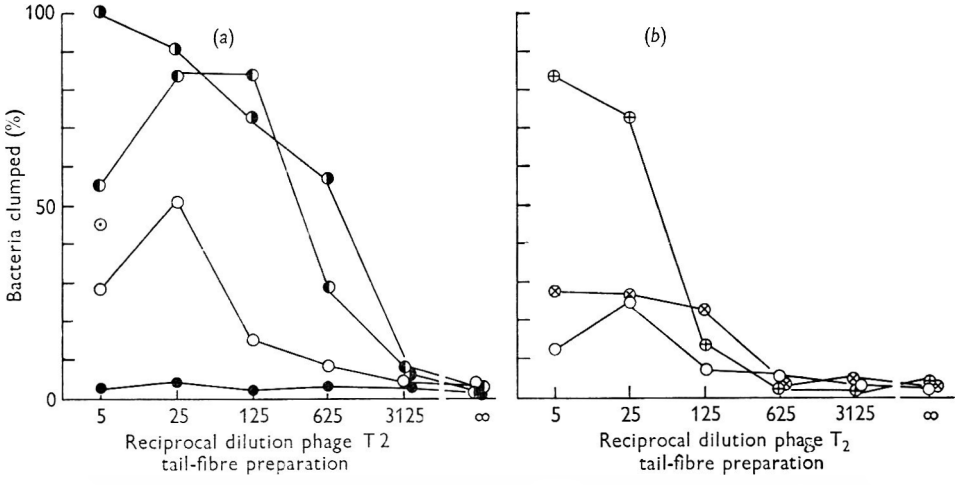

Fig. 4. The prozone phenomenon and its abolition. (*a*) Dilutions of tail fibres were mixed with *Escherichia coli* B in standard fashion and the degree of clumping measured (○). In the process clumping principle was absorbed from the fluid (●). Upon adding anti-T_2 gamma globulin (undiluted ◑ or diluted 1/10 ◐) the prozone is abolished or diminished. Undiluted anti-T_4 gamma globulin had a smaller effect (⊙). (*b*) The prozone may also be abolished by adding subsequently more *E. coli* B (⊕). A much smaller effect is seen when *E. coli* B/2_1 (⊗) was added. The standard curve is also shown (○) calculated from data in Fig. 4*a* assuming that no further clumping of bacteria would have occurred when more bacteria were added.

markedly increased the proportion of agglutinated bacteria but (as shown by additional controls) only when they had previously been exposed to the T_2 tail-fibre preparation; (4) that anti-T_4 gamma globulin produced a much smaller increase in the proportion of clumped bacteria; this will be discussed. A similar less elaborate experiment showed a similar enhancement of clumping by T_4 gamma globulin of bacteria coated with the B × T_4 tail-fibre preparation.

The nature of clumping

These results strongly suggested that clumping is specifically caused by some part of the bacteriophage, most probably by tail fibres which form bridges between bacteria. One feature which was constantly seen was a prozone (i.e. higher concentrations of the tail-fibre preparations clumped fewer bacteria than succeeding ones). This might be explained if it were assumed that, in the presence of excess tail fibres, most receptor sites on the bacteria became saturated before many bacterial collisions

had occurred. This view is supported by the results given in Fig. 4*a*, where it will be seen that the prozone was completely abolished by adding homologous antibody. A further experiment supports the hypothesis. When the mixtures of bacteria and dilutions of the T_2 tail-fibre preparation had been incubated at 4° for 1 hr, each was mixed with an equal volume of either the standard *Escherichia coli* B suspension or the standard *E. coli* $B/2_1$ suspension. After one further hour at 4° the proportions of clumped bacteria were counted; the results are given in Fig. 4*b*. The control curve shows the proportion of agglutinated bacteria to be expected if no further clumping occurred (based on the curve in Fig. 4*a*). In fact, however, the experimental curves show that the presence of new bacteria of the susceptible strain *E. coli* B enormously increased the proportion of clumped bacteria and, in particular, that the prozone was abolished. The addition of *E. coli* $B/2_1$ organisms also increased the proportion of agglutinated bacteria, though to a slight extent. The significance of this increase is not clear.

Abortive experiments

Two abortive experiments should be mentioned. The most satisfactory demonstration that clumping was caused by tail fibres bridging bacteria would be directly by electron microscopy. No attempt was made to show this with whole bacteria or whole bacterial ghosts owing to the thick welt of silicotungstate surrounding the bacteria which would probably obscure the delicate tail fibres. Attempts have, however, been made to show bridging by using fragments of bacterial cell walls, so far without success. It might be expected that tail fibres could be eluted from bacteria by lowering the salt concentration. We have been unable to demonstrate this either by washing clumped bacteria or by dialysing them. Though both procedures reduced the proportions of clumped bacteria, no clumping principle was found to have been eluted.

DISCUSSION

The results reported here clearly indicate that some bacteriophage-specific factor—probably tail fibres—can clump bacteria. Such a phenomenon would be dependent upon two sequential processes: the adsorption of tail fibres to bacteria, and collisions between bacteria. Some of our experiments distinguished between these two processes. For example, the prozone phenomenon shows that it is possible to saturate bacterial sites with tail fibres before all the bacteria have had a chance to collide and clump. The experiment of Fig. 4*a* shows that, even in the prozone region, all the detectable tail fibres were adsorbed to bacteria. This experiment also shows that such tail fibres have antigenic sites available for tail-fibre antibodies to link them together and thus form fibre-antibody-fibre bridges between saturated bacteria (Fig. 5). In addition, the experiment of Fig. 4*b* shows that tail fibres on saturated bacteria also have adsorption sites available to form bridges to receptor spots on more bacteria added later.

Our findings may be compared with previously published data on the adsorption of tail fibres and bacteriophage to bacteria. Hershey *et al.* (1944) found that the efficiency of plating of phage T_2 was maximal (1·0) in the presence of 0·2 M-NaCl, 0·1 with 0·03 M-NaCl and, in the absence of salt, was 0·001. Franklin (1961) reported that adsorption of serum-blocking power (which in her test was mostly a measure of tail-fibre antigens) was increased in low salt concentrations and was maximal at

concentrations where adsorption of bacteriophage was virtually abolished. We made no direct measurements of the adsorption of clumping principle in different ionic environments, but clumping itself was much decreased in the absence of added sodium chloride while, in accord with Franklin's finding, most clumping was observed at the lowest concentration tested (0·025 M). It seems therefore that the salt concentration optimal for clumping differs from that required for successful infection of bacteria.

It is not at all surprising that formolized suspensions of bacteria should absorb tail fibres as efficiently as fresh organisms because formalin does not inactivate the receptors of bacteriophage T_2 (Tolmach & Puck, 1952; Puck & Tolmach, 1954; Weidel, 1953).

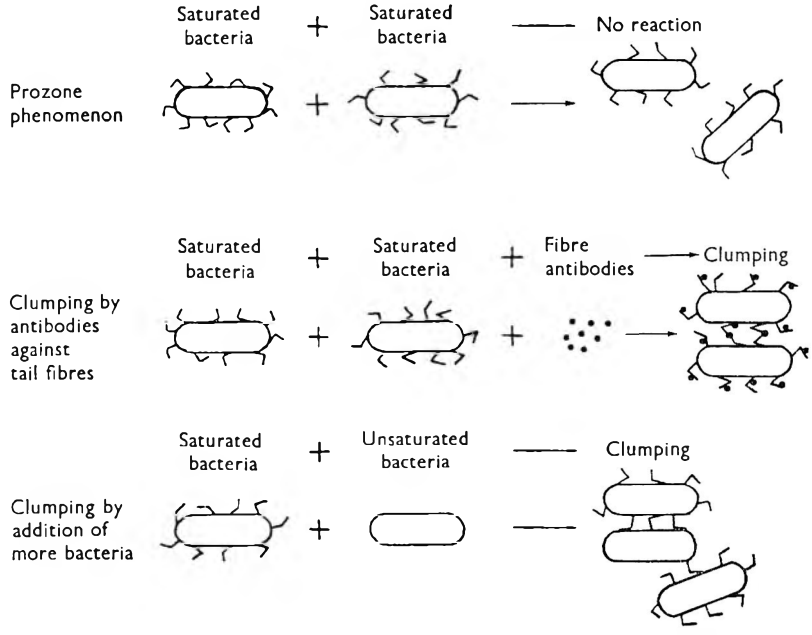

Fig. 5. Schematic diagram of the prozone phenomenon and its abolition by added tail-fibre antibodies and by adding more bacteria. The tail fibres and antibodies are drawn about five times too large relative to the bacteria and the antibody molecules are drawn as round balls for easy visibility in the diagram.

The question of how specifically tail fibres will adsorb to different host bacteria is of interest. Williams & Fraser (1956) found that tail fibres from frozen and thawed bacteriophage adsorbed specifically, and Franklin (1961) quotes Streisinger's result with chemically separated fibres that 95% T_2 serum-blocking power adsorbs to *Escherichia coli* B, whereas < 5% will adsorb to *E. coli* B/2 under similar conditions. In contrast, her own finding was that the serum-blocking power of chemically separated fibres seemed to adsorb equally well to *E. coli* B and B/2, but the results were erratic. Our findings suggest partial specificity of clumping and adsorption of clumping principle. As a crude estimate, about 50% of the clumping principle was taken up by *E. coli* B/2 organisms. Only slight clumping of the *E. coli* B/2 strains was observed in the presence of T_2 tail-fibre preparations. However, neither of the *E. coli* B/2 strains clumped as well as *E.* B *coli* in the presence of $B \times T_4$ tail fibres.

These results, together with our own, suggest that much of the specificity of T_2 bacteriophage infection may be attributable to the tail fibres, but that this is not the whole story is plain from Franklin's (1961) results which show that the sheath is also involved.

The structure of the tail fibres of T-even bacteriophage is known to be complex. Brenner *et al.* (1959) showed that 'fingerprints' of purified tail fibres were elaborate and indicated, if the fibres are built entirely from repeating sub-units, that each subunit must have a molecular weight of about 100,000. From morphological studies each tail fibre might be expected to have a particle weight of about 400,000, suggesting four such sub-units. On the other hand, the fibre might be composed of a large number of dissimilar subunits. Antigenically the tail fibres are complex. Franklin's (1961) results indicate at least two 'epitopes' or determinative groups on the fibres, both of which possess serum-blocking power; one is specific for homologous phage and the other is shared by both phage types. In view of these findings, it is surprising that the neutralizing activity of clumping principle by antibody appears so specific. Perhaps the heterologous component is a minor one and our experiments were too crude to detect it. Indeed in Fig. 4*a* some increase in clumping by addition of T_4 antibody is shown. Unfortunately, this has not been repeated and it is not known if this is a significant increase. Perhaps the most important fact suggested by the work reported here is that clumping probably occurs by tail fibres which form bridges between bacteria, suggesting that each fibre must have at least two adsorption sites. If this be so, the tail fibre is a multiple structure in the chemical sense, the serological sense, and in the functional sense.

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A Mycoplasma which Induces Acidity and Cytopathic Effect in Tissue Culture

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SUMMARY

An agent which induced acidity and cytopathic effects in HEp-2 tissue cultures was investigated. The agent grew well in certain other tissue culture systems. Typical mycoplasma colonies were isolated from the contaminated HEp-2 cultures and on re-inoculation into HEp-2 cultures produced effects indistinguishable from the original effects. There was no appreciable growth in tissue culture medium alone. The mycoplasma had biological properties similar to those of known mycoplasmas, including *Mycoplasma hominis* type 1 (the common tissue culture contaminant), but was serologically distinct from these. Fluorescent antibody and Giemsa-staining techniques showed extracellular forms. Other mycoplasmas were shown to grow in tissue culture; *M. gallisepticum* induced similar effects to the cytopathic agent but was distinct in serological and biological properties. The agent partially inhibited the growth of measles virus.

INTRODUCTION

Mycoplasmas are well-known contaminants of tissue culture. With few exceptions these contaminants have been identified as *Mycoplasma hominis* type 1. Kraemer, Defendi, Hayflick & Manson (1963) reported unclassified mycoplasmas which had lytic activity for murine lymphoma cells. Apart from this example, striking cytopathic effects have not been reported in mycoplasma-contaminated tissue cultures (Rothblat, 1960; Carski & Shepard, 1961), although certain experimentally infected tissue cultures showed some degeneration (Shepard, 1958; Nelson, 1960; Castrejon-Diez, Fisher & Fisher, 1963). We observed pronounced acidity and cytopathic effect in HEp-2 tissue cultures and this paper reports some of our observations and attempts to characterize the agent responsible for these effects.

METHODS

Organisms. *Mycoplasma hominis* type I, *M. hominis* type II, *M. salivarium*, *M. bovigenitalium*, *M. gallisepticum*, *M. laidlawii*, and *M. iners* were obtained from the stock culture collection held in these laboratories.

Tissue cultures. Monolayer primary tissue cultures of rabbit and monkey kidney (*Erythrocebus patas*, *Macaca mulatta* and *M. irus*) and HEp-2 and HeLa cells were prepared and maintained in the media described below. Cultures for experiments were grown in $6 \times \frac{5}{8}$ in. test tubes, each seeded with 200,000 cells in 2 ml. medium. The growth medium for HEp-2, HeLa and rabbit cultures was replaced by 1·5 ml.

of maintenance medium 3–4 days after initiation and for monkey cultures at 6–8 days.

Media. Mycoplasmas were cultivated in Edward medium, similar to that described by Butler & Knight (1960*b*) but with certain modifications. The horse serum component was not inactivated. Autolysed yeast extract was replaced by a 10% (v/v) solution of a boiled aqueous extract of bakers' yeast (Chanock, Hayflick & Barile, 1962). For semi-solid Edward medium 10% (v/v) of solid medium was mixed with liquid medium. For growth of HEp-2 and HeLa cultures, Eagle's medium (1959), containing 10% (v/v) calf serum, 0·1% (w/v) glucose, 0·22% (w/v) sodium bicarbonate, penicillin 200 units/ml., streptomycin 100 units/ml. was used; the maintenance medium differed only in that the concentration of serum was decreased to 1% (v/v). The growth medium for monkey kidney cultures was Earle's balanced salt solution with 2·5% (v/v) calf serum, 0·11% (w/v) sodium bicarbonate, 0·5% (w/v) lactalbumin hydrolysate and antibiotics as above. The maintenance medium consisted of Earle's balanced salt solution containing 0·5% (w/v) lactalbumin hydrolysate, 10% (v/v) liver digest (Smith, 1961), 0·14% (w/v) bovine albumin, 0·22% (w/v) sodium bicarbonate and antibiotics as above. For growth of young rabbit kidney cultures the medium differed from the monkey kidney medium in that the serum was present at a concentration of 5% (v/v). The maintenance medium was based on that of Carski (1960), and consisted of Eagle's medium (1959) containing 0·5% (w/v) lactalbumin hydrolysate, 1% (v/v)M arginine, 1% (v/v) 0·1M glycine, 0·22% (w/v) sodium bicarbonate and antibiotics as above.

Viable counts. The number of viable mycoplasma particles present in Edward medium was counted by the method of Butler & Knight (1960*a*). Titrations of tissue culture infective units were done by preparing serial decimal dilutions in Eagle's medium (1959) containing 0·22% (w/v) sodium bicarbonate, and inoculating groups of 3–5 tubes with 0·5 ml. of each dilution. The titre was calculated using the Kärber equation and expressed as $\log_{10}$ TCID 50/0·5 ml. The end-point in the HEp-2 cultures was determined by cytopathic effect and acidity. In other tissue cultures the end-point was determined primarily by cytopathic effect but was always confirmed by subculture of fluid from the test cultures into HEp-2 tissue in which characteristic cytopathic effects developed.

Serology. Mycoplasma antigens were grown in fluid Edward medium, harvested by centrifugation and resuspended appropriately. For agglutination saline suspensions at an opacity equivalent to Brown's tube No. 2 were used. For complement fixation the suspending fluid was veronal buffered saline and the opacity was equivalent to Brown's tube No. 5. For rabbit immunization phosphate-buffered saline suspensions (opacity equivalent to Brown's tube No. 2) were inoculated intravenously on alternate days in doses of 0·2, 0·4, 0·8 and 1·5 ml. Antigen for this purpose was grown in media containing rabbit serum instead of horse.

Growth inhibition and tube agglutination techniques were those of Edward & Fitzgerald (1951, 1954). Complement fixation tests were based on Le Bouvier's (1952) modification of the plate technique of Fulton & Dumbell (1949). Indirect fluorescent antibody tests were made with acetone-fixed colony impression films or infected tissue culture coverslip preparations. These were treated for 30–45 min. at 37° with hyperimmune rabbit serum followed by sheep anti-rabbit globulin coupled to fluorescein isothiocyanate (Burroughs Wellcome and Co.) for the fluorescing

component. Each stage was followed by washing in phosphate-buffered saline (pH 7·4) and the preparations were mounted in 10 % glycerol, phosphate-buffered at pH 7·4.

RESULTS

Recognition of the acid-inducing agent

Acidity and cytopathic effect were first observed in monolayer cultures prepared from a line of HEp-2 cells which had been grown in a chemostat for several months. This culture subsequently died quite suddenly. The monolayer cultures developed an unusual degree of acidity about 7–9 days after growth initiation (a change to maintenance medium having been made 3–4 days after initiation). The acidity was coincident with rounding of the cells, which shrank and became granular, leaving many spindle-shaped cells and cells with long fibroblastic extensions. The granular rounded cells tended to form clumps and clusters which were easily shaken off the glass surface; finally all the cells became rounded and granular and fell off the glass. Plate 1, fig. 1 *a*, illustrates cell degeneration about 4 days after acidity was observed. Degeneration of the cell sheet was usually complete between 7 and 10 days after acidity appeared. Similar cultures showed no appreciable acidity or cytopathic effect when the medium was replaced at regular 3- to 4-day intervals. Also, cultures renewed at weekly intervals by treatment of the cell sheet with ethylenediamine-tetra-acetate (EDTA) and seeding the cell suspension in growth medium into fresh bottles, remained apparently healthy; the medium in these cultures was replaced by maintenance medium 3–4 days after growth initiation.

Growth of the agent in different tissue cultures

The agent grew well in HeLa cell cultures in which it caused acidity and cytopathic effect very similar to that observed in HEp-2 cultures. In primary monkey kidney tissue there was only slight cytopathic effect and no acid formation. In *Erythrocebus patas* cultures there appeared at the edges of the culture swollen, granular, elongated and bizarre-shaped cells. This degeneration slowly spread to affect much of the cell sheet by about the second week after infection. The culture then showed signs of ageing and it became difficult to distinguish specific effects. In *Macaca mulatta* and *M. irus* kidney cell cultures little evidence of degeneration was seen apart from fine granulation and a tendency for the cells to elongate. Rabbit kidney cell cultures were severely affected by a granular round cell degeneration, associated with the formation of clumps of fibroblasts and swollen cells. The cell sheet was eventually destroyed altogether. Titration of the agent in these cultures showed that they were equally sensitive to the agent (Table 1). The extent of growth of the agent in these cultures was not measured. The addition, to the maintenance medium on monkey kidney tissue cultures, of calf serum or glucose at the concentration used in HEp-2 media (viz. 1 %) did not affect the quality of cytopathic effect or cause the development of acidity.

Other properties of the agent

Effect of freezing. The agent survived at $-20°$ to $-30°$ in infected fluids and on agar for several months, but with some loss of viability. Infected fluid (1 ml_2 sample) also survived three successive cycles of rapid freezing in ethanol + solid CO.

mixture followed by quick thawing in water at 25° with a loss of only one-tenth of the viability.

Effect of diethyl ether. One ml. infected fluid was mixed with 1 ml. ether and incubated for 1 hr at 37°, after which the ether was removed by bubbling air through the mixture. The agent was not detected in HEp-2 cultures inoculated with the ether-treated samples. Fluid from these cultures was removed after 15 days and subcultured in fresh HEp-2 cultures which also remained healthy.

Table 1. *Titration of the acid-inducing agent in various tissue cultures*

Expt. no.	Tissue culture	Titre at 7 days* ($\log_{10}$TCID 50/0·5 ml.)
1	HEp-2	$10^{7\cdot15}$
2	HEp-2	$10^{6\cdot5}$
3	HEp-2	$10^{6\cdot75}$
4	HEp-2	$10^{6\cdot5}$
	Patas	$10^{6\cdot5}$
5	HEp-2	$10^{6\cdot25}$
	Rhesus	$10^{6\cdot5}$
6	HEp-2	$10^{5\cdot5}$
	HeLa	$10^{5\cdot5}$
	Patas	$10^{5\cdot5}$
	Rabbit	$10^{5\cdot5}$

* Titre determined by presence of cytopathic effects.

Effect of tetracycline hydrochloride. The agent did not grow in HEp-2 cultures bathed in maintenance medium containing tetracycline 2·5 μg./ml. Samples from inoculated cultures were removed after 15 days and subcultured in fresh HEp-2 cultures which remained healthy. The agent had been isolated from cultures containing penicillin and streptomycin. All the work was carried out in the presence of these two antibiotics and no attempt was made to find out whether the antibiotics were inhibitory at higher concentrations; but the agent grew equally well in their absence.

Pathogenicity for mice. New-born mice inoculated intracerebrally (0·01 ml.) and intraperitoneally (0·1 ml.) remained healthy for 14 days. No cultures were made from these animals.

Growth and biological properties of the cytopathic agent in cell-free media

From HEp-2 cultures showing acid and cytopathic effects, mycoplasma-like colonies were grown on Edward solid medium directly or after initial passage in semi-solid medium. When aerobic conditions were used for primary culture relatively few colonies were obtained. However, incubation in 5% CO_2 in nitrogen usually resulted in profuse growth. Initially colonies were very granular with poorly defined centres, but after several subcultures they had the typical 'fried-egg' appearance of mycoplasmas. A single colony clone was obtained before further biological and antigenic properties of the agent were examined. Cultures of this organism inoculated into HEp-2 cultures produced characteristic acidity and cytopathic change

indistinguishable from the original effects. From such infected cultures the mycoplasma was re-isolated. The agent inoculated into HEp-2 maintenance medium showed a tenfold increase in titre during the 1st day, followed by a decline to zero within 14 days. No acidity was produced nor was any turbidity detected.

By using some of the criteria listed by Edward (1954, Table 1) for distinguishing between mycoplasmas the following characters were noted for the agent grown in Edward medium. It grew well on medium containing rabbit serum instead of horse serum but did not grow in the absence of serum. 'Film and spots' were produced on horse serum agar medium. Glucose was not fermented. Only a faint trace of α-type lysis was observed on horse blood agar medium. Colonial growth was unaffected by omission of penicillin and thallium acetate. In semi-solid medium growth was diffuse. In liquid medium a faint turbidity developed. On the basis of these properties alone the organism could not be distinguished from *Mycoplasma hominis* type I, the commonest mycoplasma contaminant of tissue culture cell lines, or from *M. hominis* type II, *M. salivarium* (Edward, 1954; Freundt, 1958) and *M. iners* (Edward & Kanarek, 1960).

Serological characters of the agent

Serological comparisons were made between the acid-forming mycoplasma and *Mycoplasma hominis* types I and II, *M. salivarium* and *M. iners*, which were very similar to the agent in biological tests. By complement fixation and agglutination tests the agent differed from the named mycoplasmas (Table 2). In growth inhibition

Table 2. *Serological comparison of acid-inducing* (A.I.) *mycoplasma with named species having similar biological properties*

Titres expressed as reciprocals of serum dilutions.

Antiserum ...	A.I. mycoplasma		*M. hominis* type I		*M. hominis* type II		*M. salivarium*		*M. iners*	
Antigen ...	C.F.*	Agg.†	C.F.	Agg.	C.F.	Agg.	C.F.	Agg.	C.F.	Agg.
A.I. mycoplasma	160–640	128–256	10	4	< 10	< 4	10	8	< 10	8
M. hominis type I	< 20	< 4	80	128–256	—	—	—	—	—	—
M. hominis type II	—	< 4	—	—	80–160	256	—	—	—	—
M. salivarium	—	—	—	—	—	—	640	256	—	—
M. iners	—	—	—	—	—	—	—	—	160	64

* C.F., Complement fixation.
† Agg., Agglutination.

tests (Edward & Fitzgerald, 1954) there was partial inhibition of the agent by homologous antiserum but no inhibition by antisera to the other mycoplasmas. In HEp-2 cultures in the presence of 1/20 antiserum acidity and cytopathic effects were delayed for several days and were not so pronounced as in normally infected cells. Results of the fluorescent antibody staining technique, described in the next section, confirmed the distinct serological character of the agent.

Staining the agent in tissue culture

Normal HEp-2 cells were grown on glass coverslips and samples were taken at various stages after infection with a strain of the agent which had always been grown in HEp-2 culture. The preparations were fixed in acetone and stained by the intensified Giemsa stain of Marmion & Goodburn (1961). When the cultures showed characteristic acid degeneration numerous faint-staining particles of less than 0·5 μ appeared, lying mainly outside but also around and on the tissue cells (Pl. 1, fig. 1*b*). There were similar particles in deposits of HEp-2 cells from severely degenerated cultures. Their shape and size indicated that these particles were probably the mycoplasma that could be isolated from such cultures. This was confirmed when apparently identical particles were stained specifically by the indirect fluorescent antibody by using hyperimmune rabbit serum prepared against Edward medium cultures of the agent. Such particles did not stain when treated with antisera prepared against *Mycoplasma hominis* types I and II. No such particles were detected either by Giemsa or by fluorescent antibody stains in healthy HEp-2 cultures.

Table 3. *Growth of various mycoplasma in HEp-2 tissue culture*

	First culture			Subculture of 14-day fluid	
	Colony count		Condition of culture.	Colony count.	Condition of culture.
Organisms	Zero	14 days	14 days	11 days	11 days
Acid-inducing agent	3·48*	5·48	cpe + a†	Not done	cpe + a
M. hominis type I	3·11	3·48	Healthy	4·48	Healthy
M. hominis type II	1·48	0	Healthy	0	Healthy
M. bovigenitalium	3·48	0	Healthy	0	Healthy
M. gallisepticum	3·48	3·48	Healthy	5·48	cpe + a
M. laidlawii	0	5·48	Healthy	5·48	Healthy

* Log colony count/ml.
† cpe, cytopathic effect; a, acid.

Comparison of the growth of the agent and other mycoplasmas in HEp-2 cultures

Cultures of the cytopathic agent, *Mycoplasma hominis* types I and II, *M. bovigenitalium*, *M. gallisepticum* and *M. laidlawii*, all grown in liquid Edward medium, deposited by centrifugation and resuspended in HEp-2 maintenance medium, were inoculated into cultures of HEp-2 cells. Samples from the inoculated cultures were removed, immediately and after 14 days of incubation, for colony counts. Subcultures (0·2 ml.) from the 14-day fluids were made into fresh HEp-2 cultures and colony counts made 11 days later. All cultures were examined periodically for cytopathic effect or acidity; Table 3 summarizes the results. The agent produced typical acidity and cytopathic effect and colonies were isolated after 14 days' incubation. *Mycoplasma hominis* type I and *M. laidlawii* appeared to grow quite well but had no detectable effect on the HEp-2 cells; *M. hominis* type II neither survived nor altered the cell sheet; *M. gallisepticum*, however, produced both cytopathic effect and acidity in the subcultures in HEp-2 cells and evidently grew

actively; *M. gallisepticum* differed from the acid-inducing mycoplasma in certain biological properties, e.g. production of large colonies and of acid from glucose, and was serologically distinct in slide-agglutination tests.

Effect of the agent on virus growth

Following preliminary observations that the growth of measles virus in HEp-2 cultures was affected by the agent, HEp-2 cultures infected with the agent were super-infected with measles virus 2 days later. After 9 days the measles virus grown in healthy HEp-2 tissue had a titre of $10^{5.5}$ TCID 50/0·5 ml. (the expected value) but in the presence of the acid-inducing agent, when the cultures were only slightly acid, the titre was $10^{4.5}$. However, at 14 days such cultures were completely degenerated. Measles virus growth was not affected by the presence of the laboratory strain of *Mycoplasma hominis* type I.

DISCUSSION

There is no doubt that the agent responsible for the acid-inducing and cytopathic changes in tissue culture was a mycoplasma. Typical mycoplasmas were demonstrated by staining and by cultivation from tissues contaminated with the agent, but were not present in healthy cultures. The characteristic acid production and cytopathic changes were reproduced by cultures of the mycoplasma following several serial passages in artificial medium. The properties of the agent, including susceptibility to ether and tetracycline hydrochloride and insensitivity to penicillin, were those of the mycoplasma group. Furthermore, experimental infection with the agent was partially inhibited by rabbit antiserum prepared against pure cultures of the isolated mycoplasma. The organism retained the typical colonial and cultural characteristics of a mycoplasma after several cycles of growth in tissue cultures without antibiotics.

As with most other reported instances of mycoplasma infection of tissue cultures the source of the contamination is unknown. Some workers (Rothblat & Morton, 1959) have suggested that such contaminants may be L-phases of bacteria. Barile, Malizia & Riggs (1962) reported a much lower frequency of contaminants in tissue cultures from laboratories where cells were maintained mainly in the absence of antibiotics. They interpreted this as supporting the hypothesis of the bacterial origin of the contamination. The strains they isolated were antigenically identical but unrelated to certain named species of mycoplasma, including *Mycoplasma hominis* type II. However, later findings (Dr M. F. Barile, personal communication) have shown that they were in fact *M. hominis* type I. It is more likely that the low frequency of mycoplasma contamination in tissue cultures without antibiotics reflects the use of a necessarily more careful aseptic technique. Although no objective criteria have been established for distinguishing absolutely between mycoplasmas and L-phase organisms, Dienes, Edward, Freundt & Klieneberger-Nobel (Discussion, 1960), have pointed out that there exist profound differences between the two groups of organisms. We are satisfied on their criteria that the colonial and cultural appearance of our agent are those of a mycoplasma.

In view of the striking cytopathic effects produced by our agent it is interesting that, on the basis of biological and antigenic properties, it is unrelated to any of the established Mycoplasma species including *Mycoplasma hominis* type I. To our

knowledge, apart from isolated instances involving *M. hominis* type II (Bailey *et al.* 1961) or *M. gallisepticum* (Edward, 1960) almost all fully investigated cases of mycoplasma contamination of cell lines were due to *M. hominis* type I (Collier, 1957; Coriell, Fabrizio & Wilson, 1960; Bailey *et al.* 1961) and did not involve the pronounced acid and cytopathic changes characteristic of the acid-inducing mycoplasma. Usually mycoplasma contaminations are only revealed by specific cytological or cultural investigations (Hayflick & Stinebring, 1960; Rothblat, 1960) and in this connexion it is noteworthy that *M. hominis* produced no detectable changes when growing in our HEp-2 cultures. Of two other mycoplasmas which grew very freely in HEp-2 cultures, *M. laidlawii* produced no obvious changes but *M. gallisepticum* caused acidity and cytopathic effect. Castrejon-Diez *et al.* (1963) observed similar effects with *M. gallisepticum* and suggested that the acidity was due to the production of acetate. It is improbable that the acid changes in HEp-2 and HeLa cultures caused by our agent were due simply to the effect of the agent on the sugar-containing cell culture medium, since the acid-inducing mycoplasma itself cannot ferment glucose in cell-free medium or produce acid in tissue culture media. As the agent caused observable cytopathic effects in both monkey and rabbit kidney tissue cultures without any acidification, the changes in HEp-2 cultures were probably due to its action on cell metabolism. Mycoplasmas are known to be able to influence the metabolism of animal cells in tissue culture (Powelson, 1961; Kenny & Pollock, 1963; Schimke & Barile, 1963).

Rouse, Bonifas & Schlesinger (1963) observed inhibition of adenovirus by mycoplasma. The ability of our agent to inhibit the growth of measles virus is another illustration of the hazards of mycoplasma contamination of tissue culture. Coriell, Tall & Gaskill (1958) pointed out that the use of such cultures for the production of virus antigen could also complicate the results of virus antibody-antigen studies. These observations of ours and others stress the importance of frequent testing of tissue cultures for the presence of mycoplasma. Without such checks, the present agent might easily have been assumed from its cytopathic effects to be a virus contaminant.

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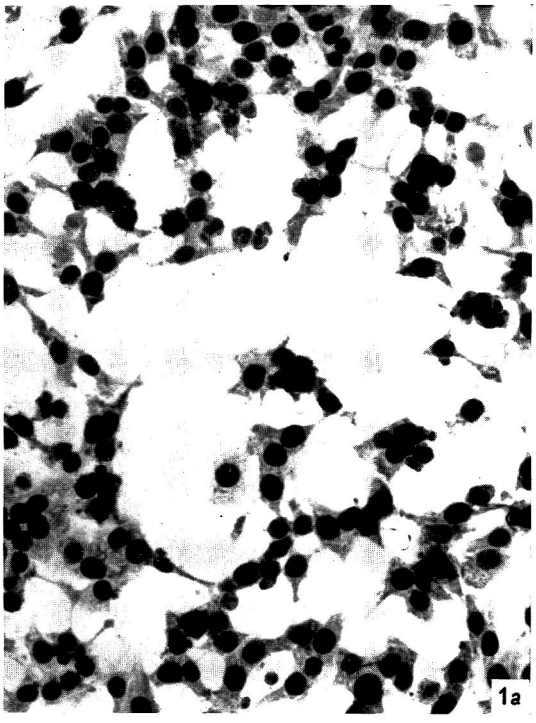
EXPLANATION OF PLATE

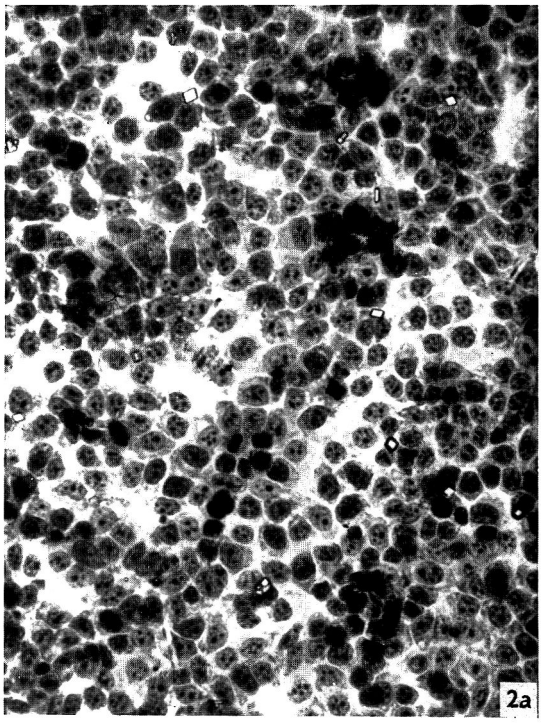
The effect of the acid-inducing agent on a HEp-2 monolayer.

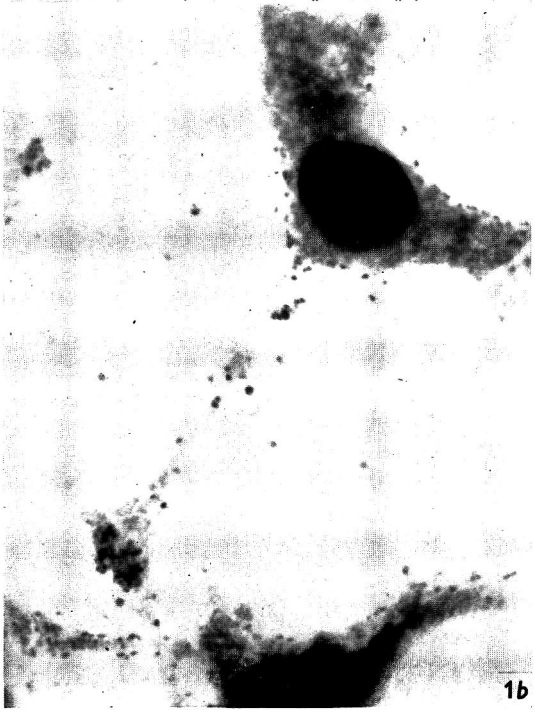
Intensified Giemsa stain.

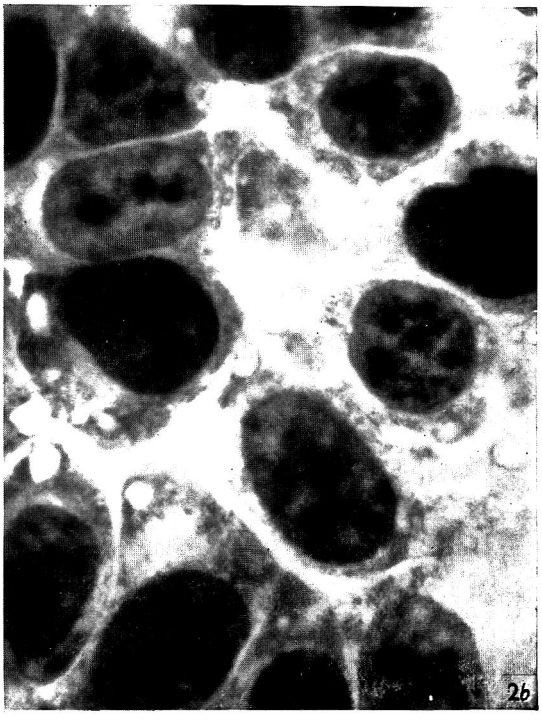
Fig. 1. About 4 days after acidity observed (*a*) × 200, (*b*) × 1250.

Fig. 2. Uninoculated culture of the same age (*a*) × 200, (*b*) × 1250.









M. BUTLER AND R. H. LEACH

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Immunological Studies of Amination Deficient Strains of *Neurospora crassa*

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SUMMARY

A survey has been made of the immunological relationships between glutamate dehydrogenase (GDH) and the related proteins of the amination deficient (*am*) mutants of *Neurospora crassa*. Serum against GDH was prepared in rabbits which had been injected with crude extracts of wild type *N. crassa*. The effect of this serum on the activity of normal GDH and the activity of activated mutant proteins was studied. The techniques of double diffusion in agar gel and of immunoelectrophoresis were used to study the relationships between the wild-type GDH and mutant forms of the protein. The identification of the relevant precipitation line on these gel plates was confirmed by the use of a specific stain for the enzyme antibody complex. The relationship between the antigenic and enzymic sites in the wild-type protein is discussed.

INTRODUCTION

Nineteen independent occurrences of a mutation at the *am* locus in *Neurospora crassa* are known. The *am* mutants require exogenous α-amino acids for normal growth and lack normal NADP (nicotinamide adenine dinucleotide phosphate)-linked glutamate dehydrogenase activity. Two previous immunological surveys of the relationships between the wild GDH and the proteins formed by the *am* mutants 1–8 have been carried out (Barratt, 1962; Sanwal & Lata, 1962). Some of the results of Sanwal & Lata (1962) differ from the results presented in this paper. This difference may have been due to techniques used; however, it now seems that the results in the present paper are the best description of the situation (Dr B. D. Sanwal, pers. commun.). The present survey repeats the previous surveys using different techniques and includes a further eleven mutants. Mutants 1–13, with the exception of 10, have recently been crossed with each other (Pateman, unpublished results). No mutant crossed with itself gave wild-type recombinants and with two exceptions all crosses between mutants gave detectable frequency of wild-type recombinants. From enzyme studies and the crossing data it was concluded that mutants 1–9, 11 and 13 are each the result of mutation at different genetic sites. No wild-type recombinants were recovered from the cross between *am*-3 and *am*-12 or from the cross between *am*-7 and *am*-13. Consequently in each case the pairs of mutants are considered to be independent occurrences of a mutational event at the same genetic site.

METHODS

Neurospora strains. The stock wild-type was basically St Lawrence 74A (Pateman, 1959); *am* mutants 1–11 were described by Fincham (1959). Mutants *am* 12 and 13 were induced by ultraviolet (u.v.) irradiation in the laboratory of Dr J. R. S. Fincham. The mutants *am* 14–19 were induced with nitrous acid by Dr D. Stadler.

Growth of cultures. For purification and preparation of antigen solutions for injection into rabbits, a culture of the strain was grown for one week on 300 ml. Vogel minimal medium (H. J. Vogel, *Microbial Genetics Bulletin*, no. 13) with 2% agar in a 1 l. culture flask at 25°. The conidia were harvested by washing the surface of the culture with 100 ml. sterile water. This suspension was poured into 15 l. Vogel minimal medium in a 20 l. flask and the culture incubated for 16–18 hr at 30° with vigorous aeration. The mycelium was harvested on muslin, washed thoroughly with distilled water, blotted dry (about 100–200 g. blotted mycelium was usually obtained), cut into small pellets and stored at −20° until required.

For testing, the strain was grown on 150 ml. liquid medium in a 1 l. culture flask for 40 hr at 25°. The mycelial pad was sucked dry on a small Buchner funnel after washing with distilled water, then blotted dry and kept at −20° until required. The medium for the wild-type fungus was Vogel minimal medium. The mutants were grown on Vogel minimal supplemented with either M/200 DL-alanine or M/400 L-glutamic acid sodium salt at 25°. In special cases the mutants were grown on minimal medium at 37°.

Preparation of extracts. Extracts for purification or injection were prepared in an MSE Atomix blender (Fincham, 1962). The frozen powder for purification was stirred into 0·05 M-sodium orthophosphate buffer (pH 8·0) with 0·001 M-ethylenediaminetetra-acetic acid (EDTA) added. The powder for injection was stirred into 0·02 M-sodium orthophosphate buffer (pH 7·2). Both extracts were centrifuged for 30 min. at 3,000*g*, then filtered under pressure through a 35 g. pad of kieselguhr on a 12 cm. Buchner funnel. These were crude extracts.

Extracts for testing were prepared by grinding with a pestle in a mortar with ground glass and buffer (0·05 M-sodium orthophosphate (pH 8·0) with 0·001 M-EDTA for extracts studied enzymatically and 0·02 M-buffer (pH 7·2) for extracts studied in gel plates). The resulting slurry was either filtered under pressure through a 4 g. pad of kieselguhr on a small Buchner funnel (extracts studied enzymatically) or centrifuged for 30 min. at about 37,000*g*, in an MSE 25 centrifuge (extracts studied in gel plates). In most cases the extracts were made of 1 g. blotted mycelium to 3 ml. buffer.

Purification. The crude extract was purified by the method of Fincham (1962).

Enzyme assays. Enzyme activities were measured by the methods of Fincham (1962), following either the reduction of nicotinamide adenine dinucleotide phosphate (NADP) or the oxidation of reduced NADP ($NADPH_2$) by optical density measurements at 340 mμ.

The reaction mixture for wild-type enzyme was 0·2 ml. 0·5 M-L-glutamic acid sodium salt, 0·2 ml. 0·2% NADP, enzyme, made up to 3 ml. with 0·05 M-tris HCl (pH 8·4) buffer. The enzyme assay was carried out in a 1 cm. silica cell in a Unicam S.P. 500 spectrophotometer with the cell housing maintained at 35° by circulating

water. Activities are expressed as the change in optical density at 340 mμ, ×1000. To assay the background, if any, a control run was made in which the reaction mixture was completed by the addition of glutamate; readings for the 2 min. before addition of glutamate gave the background activity. The standard assays were started by adding NADP, the background from the control run being subtracted. In the experiments to be discussed the serum was incubated with the reaction mixture without NADP: other experiments to be reported elsewhere showed that in the case of the wild-type enzyme neither the glutamate nor the NADP affected the inhibition of the enzyme activity by the treated serum. In the series of experiments with mutant *am*-2 0·2 ml. 2 M-L-glutamic acid sodium salt was used.

The reaction mixture used when studying the oxidation of $NADPH_2$ was: 0·1 ml. 0·2 M-disodium α-oxoglutarate in 0·4 N-NaOH; 0·1 ml. 0·2 M-NH_4Cl; 0·1 ml. 0·2% $NADPH_2$; enzyme; made up to 3 ml. with 0·1 M-sodium orthophosphate buffer (pH 8·0) and assayed at 20°. Background was assessed by taking readings before the reaction was started with the NH_4Cl. The serum was incubated with reaction mixture lacking $NADPH_2$ and NH_4Cl at 35° and then cooled to 20° before assay.

Protein estimations. The protein concentration of the purified preparations from the diethylaminoethyl cellulose (DEAE) columns was estimated by multiplying the optical density at 280 mμ by 1·10 (Fincham, 1962). All other protein concentrations were by the method of Lowry, Rosebrough, Farr & Randall (1951) with reference to a standard curve prepared with casein.

Preparation of antibodies against glutamate dehydrogenase. Each series of three rabbits (New Zealand Whites) was given an intraperitoneal injection of 10 ml. antigen preparation (crude wild-type extract in series B, purified GDH in series C) and an intramuscular injection of antigen mixed with an equal volume of Freund's adjuvant, giving a final volume of 5 ml. Each series of rabbits was given intramuscular injections 1 and 2 weeks later and bled 1 week after the last injection. Before each subsequent bleeding the series was given 2-weekly intramuscular injections and bled 1 week later. As far as possible this programme was always used. The series, rabbit and bleed are indicated thus: B–2–3 means the third bleed from the second rabbit in series B. In this paper all antibodies will be referred to as anti-am^+ antibodies, that is antibodies against the wild-type glutamate dehydrogenase. The blood was allowed to clot at room temperature overnight, the serum poured off, centrifuged, divided into samples which were stored at −20° until required.

Double-diffusion plates. The double-diffusion plates were a modification of the technique developed by Ouchterloney (1953), by using the method of preparation described by Coombs, Richards & Dodd (1963).

Immunoelectrophoretic plates. The immunoelectrophoretic plates were a modification of the technique of Grabar & Williams (1955). A sample (10 g.) of Noble special agar (Difco Laboratories, Detroit, Michigan, U.S.A.) was washed and made up to 500 ml. with 0·06 M-barbital buffer (pH 8·6; 1·84 g. barbituric acid, 10 g sodium barbitone in 1 l.). The agar was dissolved and after filtering divided into 5 ml. samples. To prepare the plates one vial of agar was melted and 5 ml. distilled water added, to give a 1% agar gel and altering the molarity of the buffer to 0·03 M. The plates were stored in plastic boxes containing wet lint, and holes in the agar cut with a cork borer; the slots were cut with a mapping pen. When the holes had been

filled with antigen the plate was placed in an electrophoresis tank (Shandon Scientific Co., London) and connected to the buffer compartment with lint wicks. The buffer used was 0·06 M-barbital buffer (pH 8·6). A potential difference of 150 V. was maintained on a Shandon power-pack (type 2540) for 45 min.; this gave a voltage drop across a single plate of 50–60 V. After electrophoresis the slots were cut and filled with serum. The plates were then stored as before for diffusion and precipitation to take place.

Staining. Clearing, drying and staining for all proteins was carried out as described by Coombs *et al.* (1963).

*Stain for GDH or ADH anti-*am$^+$ *serum complexes.* The staining technique used to locate the GDH and ADH anti-*am*$^+$ serum complex in double diffusion and immunoelectrophoresis plates was an adaptation of the general staining method for dehydrogenases used by Markert & Møller (1959). The staining technique can be used with any reaction which reduces NADP. Hydrogen from the $NADPH_2$ is carried to the neotetrazolium chloride which is reduced to an insoluble formazan dye in the region of the reaction.

(*a*) Reaction mixture for GDH anti-*am*$^+$ serum complex. After clearing, the slides were immersed in a reaction mixture of 5 ml. 0·05 M-tris buffer (pH 8·0), 0·4 ml. 0·05 M-hydrazine dihydrochloride (adjusted to pH 7–8 with N-NaOH), 1 mg. NADP, 1·5 mg. neotetrazolium chloride, 0·5 mg. phenazine methosulphate, 85 mg. Na-L-glutamate. The surface of the reaction mixture was covered with paraffin to exclude oxygen and the whole incubated at 37° for 5 min. The slide was washed in distilled water, dried and varnished with Ercalene varnish (Canning and Co. Ltd., Birmingham).

(*b*) Reaction mixture for ADH anti-*am*$^+$ serum complex. The reaction mixture for ADH was as above except that 0·05 M-tris buffer (pH 9·0) was used and 135 mg. L-alanine instead of glutamate. Since this reaction is much slower the reaction mixture was incubated for 50–60 min. at 37°. The slides were then treated as above.

RESULTS

Enzyme inhibition

In all the experiments discussed below with anti-*am*$^+$ serum, a control was done with serum from untreated rabbits. Under no condition tested did the presence of control serum affect the enzyme activity or the reaction mixture without enzyme.

To test the inhibition of GDH activity, serum was incubated at 35° for 30 min. with the enzyme and reaction mixture without NADP. The activities of constant amounts of enzyme incubated with differing amounts of anti-*am*$^+$ serum are expressed as the increase in optical density at 340 mμ × 1000 in the 1st min. of the reaction (Fig. 1).

An experiment was made to determine whether the 30 min. incubation of enzyme with serum was sufficient for the serum to have its full effect on the enzyme. Constant amounts of enzyme were incubated for various times at 35° with sufficient serum to decrease the enzyme activity to about 40%. It seems that the serum exhibited its full inhibitory effect within the first 25 min. (Fig. 2). Similar inhibition of enzyme activity was observed when the enzyme was incubated with the anti-*am*$^+$ serum and then assayed by following the oxidation of $NADPH_2$ (Fig. 3).

Some of the *am* mutant glutamate dehydrogenase proteins can be activated by substrate and subsequently catalyse the reduction of NADP (Fincham, 1962). Two of these mutant proteins were studied to see whether the *am*$^+$ serum would inhibit this activated catalytic activity. The enzyme from *am*-2 showed about a third of wild-type activity when four times the normal amount of glutamate was used and even more activity when it was incubated with glutamate before the reaction was started. Enzyme *am*-3 can be activated by substrate incubation and at high concentrations of substrate (four times normal) showed greater activity than did the wild-type GDH. The studies on *am*-2 are discussed below, *am*-3 showed a complicated

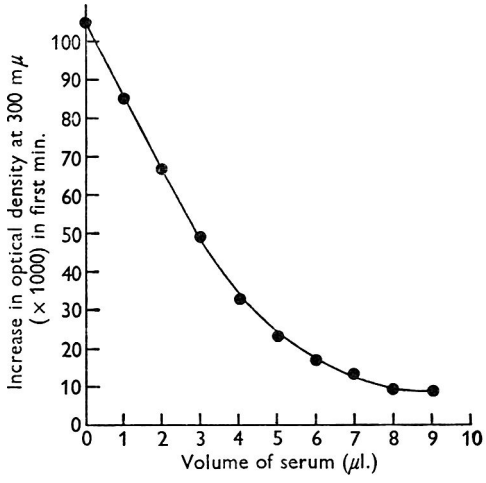

Fig. 1

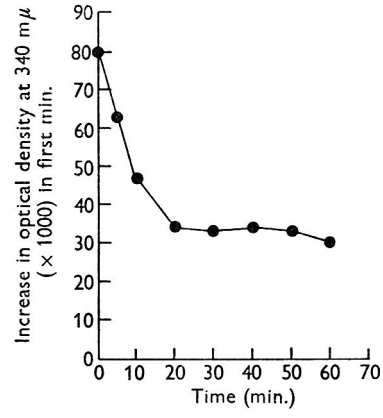

Fig. 2

Fig. 1. Effect of serum concentrations on the activity of wild-type GDH enzyme. Reaction mixture: 2·5 ml. 0·05 M-tris buffer (pH 8·4); 0·1 ml. crude wild-type extract; serum B-1-9 incubated for 30 min.; 0·2 ml. 0·2% NADP added. Readings taken on a spectrophotometer for background activity; 0·2 ml. 0·2 M-Na L-glutamate added. The background activity was subtracted from the activity in the 1st min. Incubation and assay at 35°. Wild-type extract protein concentration 1·2 mg./ml.

Fig. 2. Effect of incubation time on the inhibition of GDH enzyme activity by a constant amount of serum. Reaction mixture: 2·5 ml. 0·05 M-tris buffer (pH 8·4); 0·05 ml. crude wild-type extract; 0·05 ml. B-1-4 serum; 0·02 ml. 0·2 M-Na L-glutamate; incubated for different times at 35°. The assay was started by the addition of 0·2 ml. 0·2% NADP.

interaction with anti-*am*$^+$ serum (details to be published). However, the initial experiments showed it to possess a protein immunologically similar to wild-type GDH. A preliminary study of *am*-2 showed that its enzyme activity was inhibited by anti-*am*$^+$ serum (Table 1).

The majority of *am* mutants, however, do not produce any kind of glutamate dehydrogenase activity. Consequently any relationship between them and the anti-*am*$^+$ serum had to be studied indirectly. Experiments were done to see whether these *am* mutants produced any protein capable of inhibiting the inhibitory activity of the serum on wild-type GDH. Mutant extracts were incubated for 30 min. at 35° with serum in the reaction mixture less NADP. Enzyme was then added and the reaction started, after a further 30 min. incubation, with NADP. Controls were run with control serum and incubation of anti-*am*$^+$ serum with buffer for 30 min. before

Table 1. *A comparison of the effect of anti-am$^+$ serum on am$^+$ and am-2 extracts*

		Increase in optical density × 10^3 at 340 mμ in the 1st min.	
		am$^+$	*am*-2
Expt. 1	Control serum	80	30
	B-3-3	19	11
Expt. 2	Control serum	85	29
	B-3-3	17	9

0·05 ml. of serum; 0·025 ml. of *am*$^+$ and *am*-2 extracts; 0·2 ml. 2 M-Na-L-glutamate in these assays. *am*$^+$ extract 4·3 mg. protein/ml., *am*-2 extract 5·15 mg. protein/ml.

Table 2. *The effect of extracts of Neurospora crassa mutants am-4 and am-5 on the inhibitory activity of anti-am$^+$ serum*

The sera were incubated at 35° for 25 min. in 2·55 ml. of tris buffer and 0·2 ml. 0·5 M-Na-L-glutamate with buffer or *am*-4 or *am*-5, the final volume being about 2·8 ml. Crude wild-type extract (25 μl.) was then added and the mixture incubated for a further 25 min. The reaction was assayed at 35° and started by the addition of 0·2 ml. of 0·2 % NADP. The table shows that *am*-4 protected wild-type GDH activity from inhibition by the anti-*am*$^+$ serum while *am*-5 did not.

Protein concentrations: 5·0 mg. wild/ml., 5·5 mg. *am*-4/ml., 5·35 mg. *am*-5/ml.

Control serum (μl.)	B-3-4 anti-*am*$^+$ serum (μl.)	Crude *am*-4 (μl.)	Crude *am*-5 (μl.)	Activity. Increase in optical density × 10^3 at 340 mμ in 1st min.
2·5	—	—	—	100
2·5	—	25	—	98
—	2·5	—	—	46
—	2·5	25	—	91
6·25	—	—	—	106
6·25	—	25	—	98
—	6·25	—	—	33
—	6·25	25	—	86
5	—	—	—	108
5	—	—	25	106
—	5	—	—	24
—	5	—	25	27
3	—	—	—	118
3	—	—	25	112
—	3	—	—	35
—	3	—	25	36

incubation with wild-type enzyme. The results for two mutants are given in Table 2. It can be seen that the *am*-5 extract had no effect on the anti-*am*$^+$ serum, which inhibited wild-type enzyme activity as much in the presence of *am*-5 extract as in the presence of buffer only. The *am*-4 extract on the other hand protected the wild-type enzyme from inhibition by the anti-*am*$^+$ serum.

Similar results were obtained from experiments with the oxidation of $NADPH_2$ assay system. In these experiments mutant *am*-1 and *am*-11 were used. The mutant

extract was incubated at 35° for 30 min., with anti-*am*$^+$ serum, in the reaction mixture without $NADPH_2$ and NH_4Cl. The *am*$^+$ extract was then added and incubated for another 30 min. The mixture was cooled to 20° and $NADPH_2$ added; the reaction was started with NH_4Cl. The *am*-1 extract protected the wild-type enzyme by inhibiting the anti-*am*$^+$ serum (Fig. 3). The *am*-11 extract on the other hand did not protect the wild-type enzyme from the serum (Fig. 4).

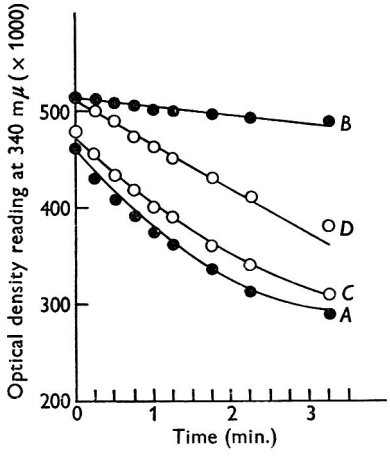

Fig. 3

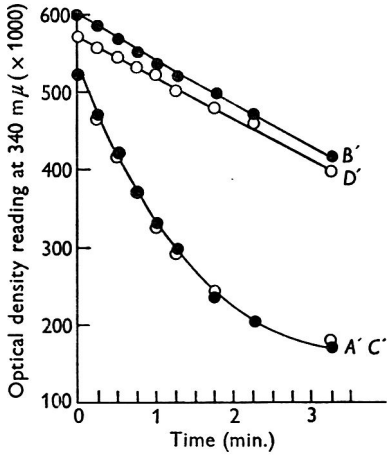

Fig. 4

The protection of wild-type enzyme activity from the inhibitory activity of the anti-*am*$^+$ serum by *am*-1 and *am*-11 extracts.

Fig. 3. Curve *A* shows the activity of 0·01 ml. of crude wild-type extract incubated for 30 min. with control serum after the serum had been incubated for 30 min. with 2·6 ml. tris buffer (pH 8·4) and 0·1 ml. of 0·2 M-α-oxoglutarate. The reaction was started by adding 0·1 ml. 0·2 % $NADPH_2$ followed by 0·1 ml. 0·2 M-NH_4Cl. Curve *B* shows the activity of crude wild-type extract as above in the presence of anti-*am*$^+$ serum B-2-3. Curve *C* shows the activity of crude wild-type extract where control serum was incubated for 30 min. with 0·01 ml. *am*-1 extract before the addition of wild-type extract. Curve *D* shows the activity of crude wild-type extract where the anti-*am*$^+$ serum was incubated with *am*-1 before addition of wild-type extract.

Fig. 4. Curves *A′*, *B′*, *C′*, *D′* as for *A*, *B*, *C*, *D*, in Fig. 3, but with *am*-11 extract instead of *am*-1 extract.

The *am*-3 protein, which shows a complicated interaction with the anti-*am*$^+$ serum in the NADP system, cannot be activated and assayed by studying the oxidation of $NADPH_2$. However it protected the wild-type enzyme from the activity of the anti-*am*$^+$ serum when the reactions were studied as above; that is it showed the same properties as the *am*-1 protein.

Six of the mutants have been studied by using these methods. The *am*-1, *am*-2, *am*-3 and *am*-4 proteins showed immunological similarity to the wild-type enzyme in that they were able to react with the anti-*am*$^+$ serum. It is not known whether *am*-5 or *am*-11 produced a mutant GDH protein or not. If they did this protein showed no immunological similarity to the wild-type enzyme. With the methods outlined above, they did not react in any detectable way with the anti-*am*$^+$ serum.

Double diffusion gel plates

Gel plates were set up to determine the immunospecificity of the wild-type enzyme and the proteins made by the mutants. Plate 1, figs. 1, 2, show the reactions between anti-*am*$^+$ serum and purified GDH, a crude extract of wild-type enzyme, and a crude extract of *am*-11. The reaction between purified GDH and antibody gave rise to two precipitation lines, the stronger one being presumed to be that due to GDH. This antigen was also present in the crude wild-type extract but not in the *am*-11 extract.

To remove any doubt as to the identity of the GDH anti-*am*$^+$ serum precipitation line it was stained for GDH activity. Fincham (1962) previously used this stain to identify the GDH band in starch gel after electrophoresis. Because the enzyme retained a certain degree of activity even with high quantities of serum (Fig. 1) it seemed possible that the antigen + antibody precipitates might show some activity. When washed plates with the GDH anti-*am*$^+$ serum line were immersed in the reaction mixture for 5–10 min. and incubated at 37°, one of the lines on the plate became stained. The stained line was the line that had been called the GDH anti-*am*$^+$ serum line on the basis of the previous experiment. This evidence identifies without doubt the GDH anti-*am*$^+$ serum line and shows that the line missing in the case of some of the mutants is the relevant line and not a line due to another system (Pl. 1, figs. 3 and 4).

Immunoelectrophoresis plates

Experiments were made with immunoelectrophoretic techniques; on these plates the antigens were placed in wells and a potential difference maintained across the plate for 45 min. This gave an electrophoretic separation of the proteins. Slots were cut in the agar parallel to the direction of electrophoresis and filled with serum; diffusion of antigens and antibodies took place, giving precipitation arcs in the gel. Again certain mutants lacked one line and this line was the prominent line in the purified GDH preparation. The plates were stained for glutamate dehydrogenase activity: the suspected GDH anti-*am*$^+$ serum line was the line stained. It was thus possible to identify the relevant line on the plates.

Alanine dehydrogenase

Bürk & Pateman (1962) showed that GDH also possessed alanine dehydrogenase (ADH) activity. Double-diffusion plates were used to see whether the GDH anti-*am*$^+$ serum line was also the ADH anti-*am*$^+$ serum line. Since ADH can react using NADP and alanine, the staining reaction mixture was changed in order to stain the ADH anti-*am*$^+$ serum complex on the plates. The ADH activity is about 0·1 % of the GDH activity under these conditions. Consequently the plates were incubated at 37° for 50–60 min. In spite of the quantity of background stain that could not be washed from the plates, one line was stained. This was the line previously determined as the GDH anti-*am*$^+$ line (Pl. 1, fig. 5).

Substrate inhibition

It was possible that alanine or glutamate in the medium used for growing the *am* mutants inhibited the production of mutant protein in certain of the mutants.

Wild-type and *am*-6 strains were used in experiments designed to test this possibility. These strains were grown up on medium containing M/200 DL-alanine and medium containing M/400 L-glutamate at 25° and on minimal medium at 37°. Extracts of the resulting mycelia were tested on double-diffusion plates for GDH anti-*am*$^+$ serum complex, against purified GDH and crude extracts of wild-type strain grown on Vogel minimal medium at 25°. Under all these conditions the wild-type strain possessed the GDH anti-*am*$^+$ serum line and under none of the conditions did the mutant *am*-6 produce an immunologically similar protein.

DISCUSSION

The sera from rabbits injected with crude extracts of the soluble proteins of *Neurospora crassa* inhibit the activity of glutamate dehydrogenase. It seems that incubation at 35° for 30 min. is sufficient time for the antibodies to have their full effect on the enzyme activity. In the reaction mixture studied with the spectrophotometer high concentrations of serum did not completely inhibit the enzyme activity. The enzyme anti-*am*$^+$ serum complex on double-diffusion plates still showed activity in that it was capable of reducing NADP to $NADPH_2$. That is, the complexes of GDH+antibody molecules still retained enzyme activity. The enzymatic site may not coincide with any antigenic site, in which case the inhibition

Table 3. *Summary of the immunological data on the am mutants*

Neurospora mutant	Enzyme studies	Double-diffusion plates	Immuno-electrophoretic plates
am-1	+	+	+
am-2	+	+	+
am-3	+	+	+
am-4	+	+	+
am-5	−	−	−
am-6	.	−	−
am-7	.	+	+
am-8	.	−	−
am-9	.	−	−
am-11	−	−	−
am-12	.	+	+
am-13	.	+	+
am-14	.	−	−
am-15	.	−	−
am-16	.	−	−
am-17	.	−	−
am-18	.	−	−
am-19	.	+	+

+, Protein immunologically similar to wild-type, i.e. cross-reacting material; −, no protein immunologically similar to wild-type, i.e. no cross-reacting material.

by the serum is probably due to steric hindrance. Alternatively an antigenic site and the enzymatic site may coincide. If this be the case the site antibody complex and the site substrate complex should each be in a state of dynamic equilibrium to account for the residual enzyme activity in the presence of excess serum. It is hoped that current studies with univalent antibodies may distinguish between these hypotheses.

The mutants have been studied by three techniques to test their immunological similarity with the wild-type protein. In all three methods there have been no contradictions to the conclusions drawn on the immunological relationship (Table 3). It is possible that some mutants which show cross-reacting material might show different types of cross-reaction on double-diffusion plates. The number of other lines on the plates may have obscured spur formation, except in three cases where purified *am*-1, *am*-2 and *am*-3 extracts were tested against purified wild-type extracts and against each other. In these cases the cross-reaction appears to be complete. These three mutants therefore all have the same types of antigenic sites as the wild-type protein.

The immunological similarity between *am*-3 and *am*-12, and *am*-7 and *am*-13 agrees with the conclusions from crossing experiments (J. A. Pateman, unpublished results), enzyme experiments (Fincham, 1962) and complementation studies (J. A. Pateman, unpublished results) that *am*-12 is a repeat occurrence of *am*-3 and *am*-13 a repeat of *am*-7. In only one instance, that of *am*-14, does a mutant which complements (D. R. Stadler, unpublished results) not show cross-reacting material (CRM). Dr J. R. S. Fincham has also failed to purify a mutant GDH protein from this mutant.

Specific stains for GDH and ADH show that the same anti-am^+ serum enzyme complex on the double-diffusion plates stains for both. This confirms the previous experiments of Bürk & Pateman (1962) which showed the same protein to possess both these activities. Since the ADH anti-am^+ serum complex on the plate can be stained, the ADH activity, like the GDH activity, is not completely inhibited by the serum.

When a wild-type strain and a non-cross-reacting mutant, *am*-6, were grown on different supplemented media and on minimal media at higher temperatures it was shown that they still had the same cross-reacting pattern. This tends to weigh against the possibility that the production of the mutant protein is suppressed by the substrates L-glutamate and L-alanine, at least in the case of *am*-6.

We wish to thank Mr C. B. Richards for his invaluable advice about immunological techniques. One of us (D. B. R.) wishes to acknowledge a grant from the Medical Research Council.

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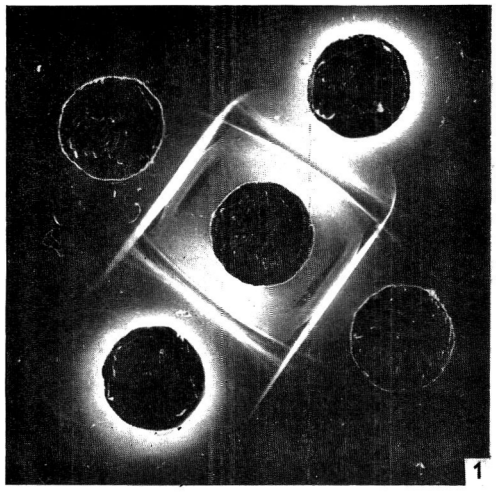

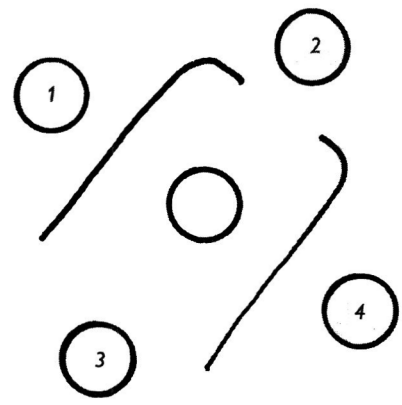

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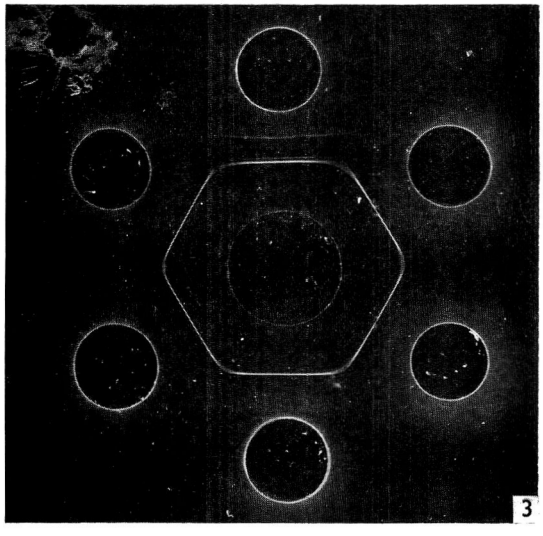

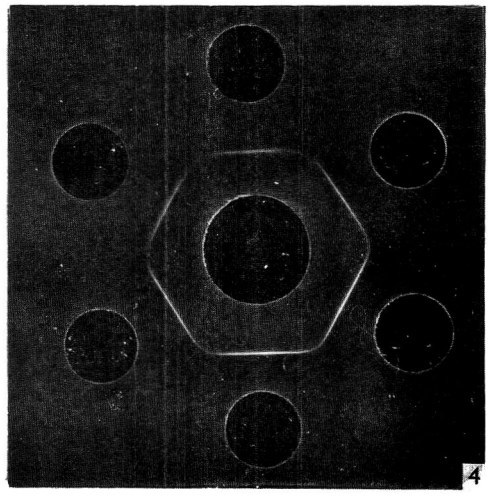

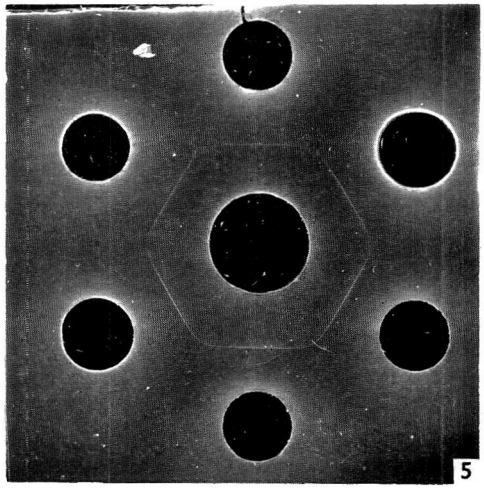

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EXPLANATION OF PLATE

Fig. 1. The centre well was filled with serum B-1-6. Wells 1 and 4 were filled with purified GDH, well 2 with crude extract of wild-type *Neurospora crassa* and well 3 with a crude extract of mutant *am*-11. The dominant line in the purified extracts is the GDH anti-am^+ serum line and is present in the wild-type but is absent in the mutant. $\times 1\frac{1}{2}$.

Fig. 2 is a schematic representation of Fig. 1 identifying the wells and illustrating the dominant purified GDH line. $\times 1\frac{1}{2}$.

Fig. 3. The centre well contains a purified extract of Neurospora GDH and the six outside wells anti-am^+ serum C-1-3. The plate was stained for all proteins with amido schwarz. $\times 1\frac{1}{2}$.

Fig. 4 was set up as Fig. 3 but stained for GDH activity. $\times 1\frac{1}{2}$.

Fig. 5 was set up as Fig. 3 but stained for ADH activity. $\times 1\frac{1}{2}$.

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The Effect of Uncoupling Agents on Carbon Dioxide Fixation by a Thiobacillus

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SUMMARY

A newly isolated strain (c) of *Thiobacillus thioparus* is described. The organism oxidizes thiosulphate, sulphide, tetrathionate or trithionate to sulphate; carbon dioxide fixation is coupled to the oxidation of each of these compounds. Concentrations of arsenate, 2,4-dinitrophenol, and other inhibitors of oxidative phosphorylation, inhibit carbon dioxide fixation without appreciably affecting oxygen uptake. Carbon dioxide fixation coupled to sulphide oxidation is more sensitive to arsenate and 2,4-dinitrophenol inhibition than that coupled to thiosulphate oxidation. This result is consistent with the suggestion that thiosulphate oxidation is linked to a substrate phosphorylation which is relatively insensitive to inhibition by 2,4-dinitrophenol and arsenate.

INTRODUCTION

Thiobacilli can grow autotrophically, obtaining energy from the oxidation of inorganic sulphur compounds, while carbon dioxide is fixed by the reactions of the Calvin cycle and by the carboxylation of phospho-enolpyruvate (Aubert, Milhaud & Millet, 1957; Suzuki & Werkman, 1958). Carbon dioxide fixation and subsequent biosynthetic processes require ATP and reduced nicotinamide adenine dinucleotide (Trudinger, 1956), which must be generated during sulphur compound oxidation. When thiosulphate is oxidized, NAD is reduced by extracts of *Thiobacillus thioparus* (Vishniac & Trudinger, 1962), and ATP is formed by whole *T. denitrificans* organisms (Milhaud, Aubert & Millet, 1957). ATP is probably formed at several stages in the oxidation of a substrate such as thiosulphate, and both substrate-level and electron transport phosphorylations may take place. Possibly thiosulphate and sulphide are oxidized through polythionate intermediates with oxidative phosphorylations coupled to the flow of electrons to the oxidant (Vishniac, 1952; Trudinger, 1959; Jones & Happold, 1961). Experiments of Peck (1960, 1962) indicate that at least part of the ATP formed during thiosulphate oxidation arises from substrate-level phosphorylation. In cell-free extracts of *T. thioparus* Peck demonstrated enzymes which (*a*) reductively split thiosulphate to sulphide and sulphite; (*b*) oxidized sulphite in the presence of adenosine monophosphate to form adenosine-5′-phosphosulphate (APS); (*c*) exchanged the sulphate of APS for phosphate to yield adenosine diphosphate and sulphate. ATP can be formed from ADP by adenylic kinase present in the extracts. This substrate phosphorylation is unaffected by 2,4-dinitrophenol, which uncouples oxidative phosphorylations (Peck &

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Fisher, 1962). We have briefly reported the effect of 2,4-dinitrophenol on CO_2-fixation by a newly isolated strain of *T. thioparus* and have suggested that both oxidative phosphorylations and the substrate phosphorylation studied by Peck may be coupled to thiosulphate oxidation by this organism (Kelly & Syrett, 1963). In the present paper these observations are extended and the effect of other uncoupling agents on CO_2-fixation described.

METHODS

The organism. A strain of Thiobacillus was isolated from canal water by enrichment culture in the medium for *Thiobacillus thioparus* described by Vishniac & Santer (1957). A pure culture was obtained by subculture of single colonies from medium solidified with 2% agar (Difco). Pure cultures were maintained on slopes of 1% thiosulphate medium, incubated at 30°, and subcultured every other day. The organism was a highly motile Gram-negative rod, about 2–3 $\mu \times 0{\cdot}5$ μ in size. Minute clear colonies were formed on agar; these developed an orange centre and deposited sulphur when allowed to age. The organism had a high cytochrome content, with absorption bands at 522 and 551 mμ. Washed organisms oxidized thiosulphate, tetrathionate, trithionate, sulphide and (very slowly) sulphur; sulphate was the end product. Nitrate or ammonium ion could be used for growth, which was the more rapid with ammonium as nitrogen source. The organism did not grow anaerobically with nitrate as oxidant, nor grow on thiocyanate. It did not grow on a wide range of heterotrophic nutrient media. The organism is regarded as a strain of *T. thioparus* (Vishniac & Santer, 1957), and is known as strain C.

When cultivated at 30° in liquid medium containing 1% (w/v) $Na_2S_2O_3.5H_2O$ and flushed vigorously with air, growth was exponential with a generation time of about 2 hr (Fig. 1). No elementary sulphur was deposited during logarithmic growth; growth ceased when thiosulphate was exhausted. Small quantities of trithionate and tetrathionate sometimes accumulated during growth (see Jones & Happold, 1961).

Growth of cultures. Cultures were grown in medium of initial pH 6·7–7·0, but the growth rate was unchanged as the pH value decreased to about 6 as a result of acid production. Growth ceased in unneutralized cultures when they approached pH 3. A final culture volume of 1 l. was made by adding 200 ml. inoculum to 800 ml. sterile medium. The inoculum was grown in the medium of Vishniac & Santer (1957), containing only 0·2% (w/v) $Na_2S_2O_3.5H_2O$, and shaken at 30° for 14–16 hr after inoculation from a 48 hr slope, and was added, aseptically, to 800 ml. of Pyrex glass-distilled water containing 10 g. $Na_2S_2O_3.5H_2O$; 0·8 g. $MgSO_4.7H_2O$; 0·4 g. NH_4Cl; 3·2 g. K_2HPO_4; 3·2 g. KH_2PO_4; 8·0 ml. trace element solution (Vishniac & Santer, 1957); 0·8 g. $KHCO_3$. The culture was forcibly aerated at 30° in a cylindrical culture vessel with a basal sintered-glass air inlet. After 7–8 hr the pH value began to decrease, and the culture was subsequently kept approximately neutral by adding saturated $NaHCO_3$ at intervals until all the thiosulphate was exhausted; this took 12–13 hr in all. Aeration for a further 12 hr provided the suspensions of starved organisms used in most experiments. The yield of bacteria was about 4·4 g. dry wt./mole thiosulphate oxidized.

Organisms were harvested by centrifugation, washed, and suspended in 0·1–0·13 M-phosphate buffer (pH 7·0). Suspensions were diluted to give either dilute sus-

pensions (equivalent to about 0·2 mg. dry wt./ml.) or dense suspensions (equiv. about 2·5 mg. dry wt./ml.). Dense suspensions oxidized thiosulphate too rapidly for the rate to be measured accurately by manometry; accurate measurements were possible with more dilute suspensions.

Gas exchange was measured by Warburg manometry at 30°.

Carbon dioxide fixation was estimated by sampling Warburg flasks in which $KH^{14}CO_3$ had been added to the suspension of organisms during the experiment. At the end of the experiment, a sample of suspension was pipetted into an equal volume of ethanol containing 5% (v/v) acetic acid. Portions of these mixtures were dried on ground-glass planchets as infinitesimally thin samples and ^{14}C counted with a scintillation counter or thin end-window G/M tube.

Sulphate was determined turbidimetrically as barium sulphate (Gleen & Quastel, 1953) after removal of organisms by centrifugation.

Thiosulphate was determined by volumetric iodine titration.

Chromatography of sulphur compounds. This was based on the methods of Pollard (1954) and Skarżyński & Szczepkowski (1959). With Whatman no. 4 filter paper, descending chromatograms were run at 22° with *n*-propanol + acetone + water (5 + 2 + 3, by vol.) containing 2 g. potassium acetate/100 ml. solvent, or acetone + butanol + water (2 + 2 + 1, by vol.) as solvents. Dried chromatograms were sprayed with 0·5% (w/v) $AgNO_3$ in ammonia solution (5 vol. aqueous ammonia solution, sp.gr. 0·88, to 95 vol. water), and spots developed by drying and heating the paper to about 100° for a few minutes to decompose the silver compounds to silver sulphide.

Chemicals. Analytical reagent grade sodium thiosulphate and sodium sulphide were used. Sodium sulphide solutions were always freshly prepared and kept in ice to minimize loss of sulphide. Dr F. H. Pollard (Bristol University) kindly gave specimens of potassium trithionate and potassium tetrathionate which were chromatographically pure.

RESULTS

Thiosulphate. This was rapidly and completely oxidized to sulphate by suspensions of washed organisms, usually at a constant rate. The Q_{O_2} was commonly 1600 μl. O_2/hr/mg. dry wt., and values up to 4000 were observed. With relatively dilute suspensions (equiv. 0·10–0·45 mg. dry wt. organism/flask), the rate of O_2 uptake was proportional to the mass of organisms, but with more dilute suspensions (equiv. 0·05–0·10 mg. dry wt. organism/flask) the rate of oxidation sometimes slowed down after 40–50 min. and the thiosulphate was incompletely oxidized (compare Vishniac & Trudinger, 1962). Up to the time at which the change in rate took place, chromatography showed that thiosulphate and trithionate were both present in the flasks; after the change to a slower rate of oxidation only trithionate was found. During thiosulphate oxidation, $^{14}CO_2$ was fixed. Fixation ceased when the thiosulphate had been completely oxidized, and there was no $^{14}CO_2$ fixation in the absence of thiosulphate (Fig. 2*a*). The amount of $^{14}CO_2$ fixed was proportional to the quantity of thiosulphate oxidized (Fig. 2*b*). Dense suspensions sometimes fixed a little more CO_2/unit thiosulphate than very dilute ones. Maximally, in these experiments, 7 mole CO_2 were fixed/100 mole thiosulphate oxidized.

Sulphide. Sulphide below 0·3 mM was oxidized rapidly by dilute suspensions, but higher concentrations were inhibitory. When an inhibitory amount of sulphide

was added to a dilute suspension, oxidation proceeded slowly until the sulphide concentration was sufficiently lowered; the remaining sulphide was then oxidized rapidly. The organisms did not adapt to sulphide since, after the oxidation of an initial quantity of sulphide, additional sulphide was still inhibitory and the kinetics of its oxidation were identical with those of the initial quantity. When dense suspensions of organisms were used, the decrease in sulphide concentration proceeded

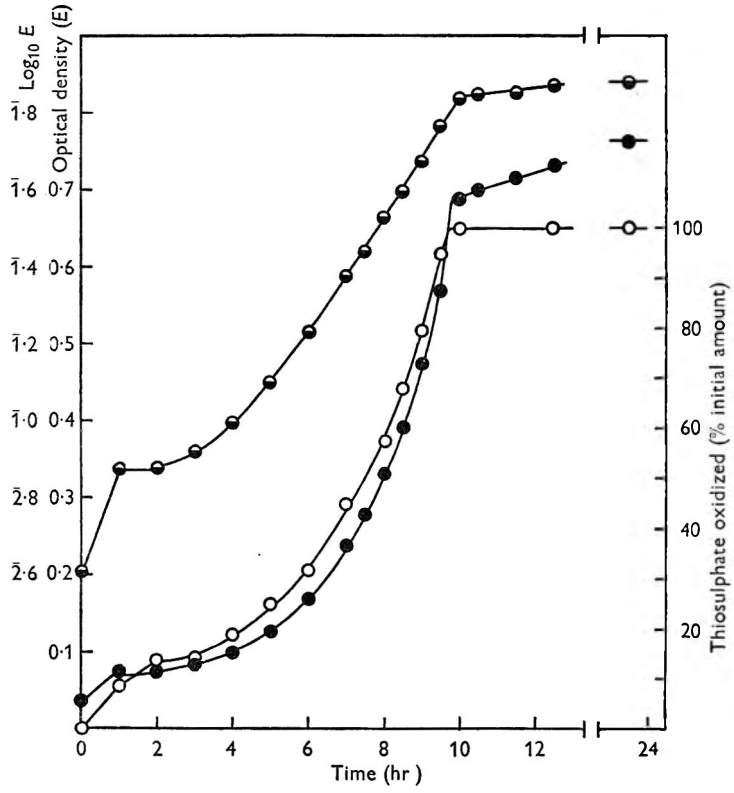

Fig. 1. Growth of Thiobacillus C. Optical density E (—●—●—) and $\log_{10}$ optical density (—◒—◒—) plotted against time. Note coincidence of growth and thiosulphate oxidation (—○—○—). Temperature, 30°. Initially at pH 7·0.

more rapidly; 1·3 mM-sulphide was oxidized rapidly and completely to the theoretical quantity of sulphate. Sulphide oxidation was also coupled to CO_2-fixation and, with dense bacterial suspensions, $^{14}CO_2$ fixation was proportional to the quantity of sulphide oxidized (Fig. 2*b*). From eleven experiments, the quantity of CO_2 fixed during the oxidation of one mole of sulphide was $66{\cdot}8 \pm 8{\cdot}6\%$ of that fixed when one mole of thiosulphate was oxidized.

Polythionates. Tetrathionate and trithionate were oxidized quantitatively to sulphate, but the course of oxidation was variable, particularly with trithionate. In some experiments, the oxidation proceeded at a constant rate to completion, but, in others, the rate increased during the experiment. The oxidation of trithionate was slower in the presence of 2,4-dinitrophenol (Fig. 3) so the entry of trithionate into the cells may be an energy-requiring process. $^{14}CO_2$ was fixed during polythionate

oxidation. With dense bacterial suspensions, $^{14}CO_2$ fixed per sulphur atom of each compound was estimated from three experiments to be: thiosulphate 100%, tetrathionate 98·8 ± 6·3%, trithionate 79·5 ± 4·6%. Fixation, per molecule oxidized, was thus, thiosulphate 100, tetrathionate 198, trithionate 119.

Sulphite and dithionate were apparently not oxidized by Thiobacillus strain c.

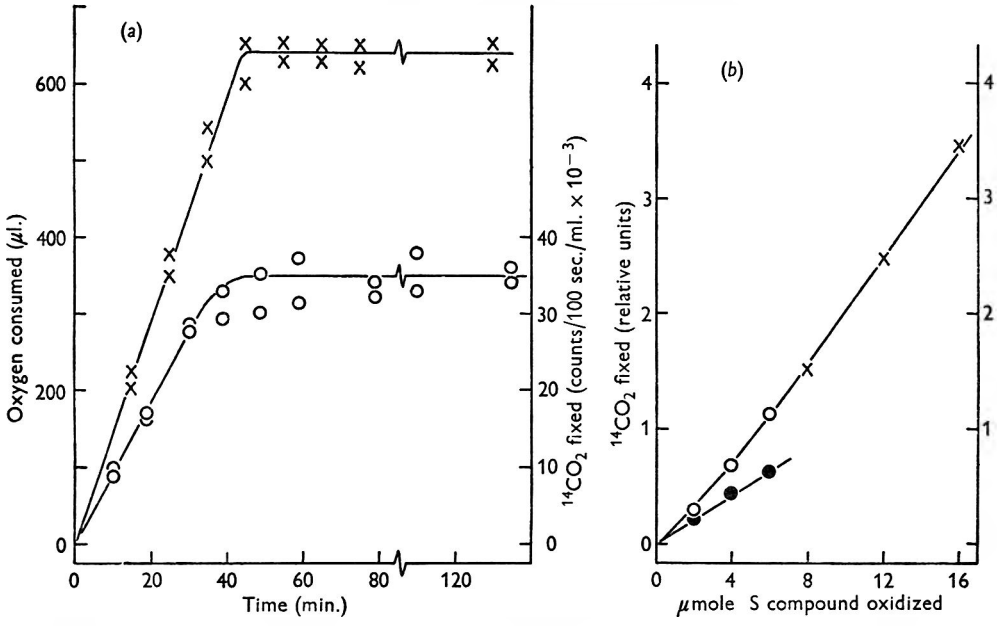

Fig. 2. (*a*) Oxygen uptake (— × — × —) and $^{14}CO_2$-fixation (—○—○—) accompanying thiosulphate oxidation. Results from replicate flasks are given. Warburg flasks contained in a final 2·0 ml.: 1·0 ml. 0·15 M-sodium phosphate (pH 7·25); 13 µmole $KH^{14}CO_3$ (0·88 µc ^{14}C); 15 µmole $Na_2S_2O_3$; equiv. 0·48 mg. dry wt. Thiobacillus strain c. Sampling flasks sealed with vaccine stoppers contained five times those quantities. $KH^{14}CO_3$ and $Na_2S_2O_3$ were tipped into the Warburg flasks, or injected into the sampling flasks at zero time. Samples were withdrawn at intervals from the latter, by using 1·0 ml. syringes, and the Warburg flasks sampled at the end of the experiment (135 min.). Temperature, 30°. Oxygen uptake for complete oxidation of added thiosulphate = 672 µl. 10^6 counts/100 sec. ≡ 1 µc ^{14}C. In the absence of thiosulphate there was no oxygen uptake and no $^{14}CO_2$-fixation.

(*b*) Proportionality of $^{14}CO_2$-fixation to quantity of thiosulphate (—○—○— and — × — × —) or sulphide (—●—●—) oxidized. The results of two separate experiments with thiosulphate are shown. ^{14}C-fixation was determined after added quantities of substrate had been completely oxidized. pH 7·0. Temperature, 30°.

The effect of uncoupling agents on $^{14}CO_2$ fixation

2,4-Dinitrophenol. CO_2-fixation was inhibited by 2,4-dinitrophenol when any one of the above sulphur compounds was oxidized. Thiosulphate-coupled fixation was inhibited by concentrations of 2,4-dinitrophenol which stimulated or did not affect the rate of oxidation (Table 4; also Kelley & Syrett, 1963). CO_2-fixation coupled to sulphide oxidation by dense bacterial suspensions was inhibited by a similar range of 2,4-dinitrophenol concentrations (Fig. 4) and the degree of inhibition was independent of the initial sulphide concentration (Table 1). However, sulphide-linked fixation was always inhibited more than that coupled to thiosulphate oxidation, whatever the dinitrophenol concentration (Fig. 4).

Fixation of $^{14}CO_2$, with tetrathionate as substrate, appeared to be about as sensitive to uncoupling by 2,4-dinitrophenol as that with thiosulphate as substrate (Table 2). The effect of dinitrophenol on the trithionate-linked fixation was somewhat inconsistent; this is possibly related to the variability of the time course of oxidation of this compound. Table 3 shows the results of an experiment in which trithionate was oxidized at a constant rate with a Q_{O_2} of 180. $^{14}CO_2$-fixation coupled to its

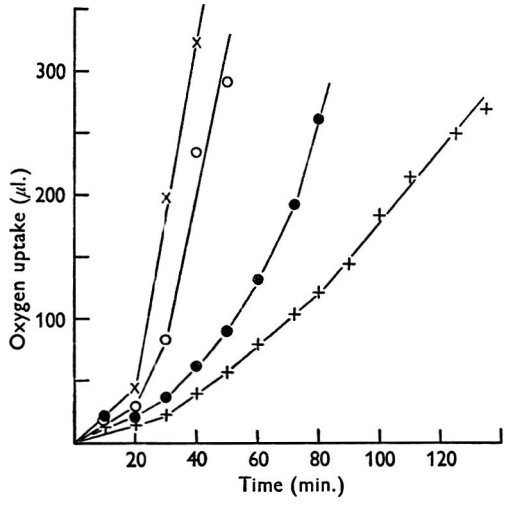

Fig. 3

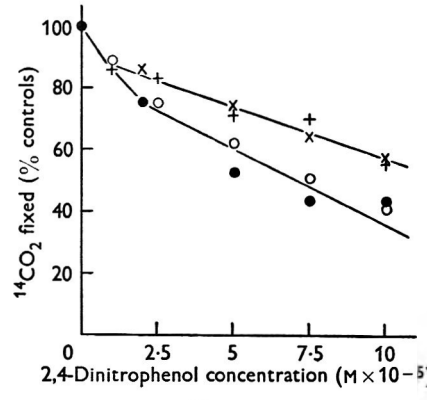

Fig. 4

Fig. 3. Inhibition of trithionate oxidation by 2,4-dinitrophenol. Equiv. 4·6 mg. dry wt. Thiobacillus strain c were incubated in 2·5 ml. phosphate buffer, containing 2,4-dinitrophenol, for 30 min.; 8 µmole potassium trithionate and 9 µmole $KHCO_3$ then added (zero time on graph). Temperature 30°; pH 7·0. 2,4-dinitrophenol concentrations: 0 (× — ×), 10^{-5} M (○—○), 5×10^{-5} M (●—●), 10^{-4} M (+ — +).

Fig. 4. Inhibition by 2,4-dinitrophenol of $^{14}CO_2$-fixation coupled to sulphide or thiosulphate oxidation. The results of two experiments are shown. Equiv. 6·3 mg. dry wt. Thiobacillus strain c in 2·5 ml. phosphate buffer (pH 7·0), oxidized 4·0 µmole Na_2S or $Na_2S_2O_3$ in the presence of 9 µmole $KH^{14}CO_3$ (1·5 µc ^{14}C). Sulphate recovery in experiments 1 and 2 was 3·1 and 4·5 µmole from sulphide, and 8·5 and 8·2 from thiosulphate, respectively. $^{14}CO_2$-fixation, as % of controls without 2,4-dinitrophenol, is shown for sulphide (Expt. 1, —○—○—; Expt. 2, —●—●—) and thiosulphate (Expt. 1, — + — + —; Expt. 2, — × — × —).

Table 1. *Relationship between quantity of sulphide oxidized and the effect of 2:4-dinitrophenol on coupled $^{14}CO_2$-fixation by Thiobacillus strain c*

The equivalent of 5·6 mg. dry wt. Thiobacillus strain c in 2·5 ml. phosphate buffer (pH 7·0) oxidized Na_2S in the presence of 9 µmole $KH^{14}CO_3$ (3 µc ^{14}C). $^{14}CO_2$-fixation was estimated when oxidation ceased. Temperature 30°.

Sulphide oxidized (µmole)	$^{14}CO_2$ fixed (counts/100 sec./flask)		Inhibition of CO_2-fixation (%)
	Control	$+5 \times 10^{-5}$ M-DNP*	
1·0	11,000	5,900	46
2·0	33,450	20,150	40
4·0	68,050	40,300	41

* DNP = 2,4-dinitrophenol.

oxidation was inhibited by dinitrophenol and, in this experiment, the inhibition was less than when thiosulphate was the substrate.

Other substituted phenols. Several aromatic compounds were tested for the ability to uncouple thiosulphate-dependent CO_2-fixation (Table 4). Phenol was ineffective; *o*-nitrophenol was ineffective as an uncoupling agent, and is known to have little

Table 2. *Effect of 2,4-dinitrophenol on $^{14}CO_2$-fixation coupled to thiosulphate and tetrathionate oxidation*

Dense suspensions of Thiobacillus strain c (equiv. 2·5 mg. dry wt./ml.) oxidized thiosulphate, or tetrathionate, completely to sulphate. $^{14}CO_2$-fixation and sulphate production were then measured. Sulphate recoveries in experiments 3 and 4, as % of expected amounts, were 107·5 and 115 for thiosulphate, and 109 and 108 for tetrathionate. Oxygen consumption always approximated to the theoretical values. Temperature 30°. pH 7·0.

Experiment	2,4-Dinitrophenol concentration (M)	Oxidation of: $S_2O_3^{2-}$ Inhibition of CO_2-fixation (%)	Oxidation of: $S_4O_6^{2-}$ Inhibition of CO_2-fixation (%)
1	$2·5 \times 10^{-4}$	69	80
2	$2·5 \times 10^{-4}$	76·5	71·5
3	2×10^{-4}	77·5	88
4	1×10^{-5}	9	2
	5×10^{-5}	32	36
	1×10^{-4}	44	55

Table 3. *Fixation of $^{14}CO_2$ during thiosulphate or trithionate oxidation by Thiobacillus sp. strain c and the effect of 2,4-dinitrophenol*

Thiobacillus c equiv. 6·3 mg. dry wt. oxidized $Na_2S_2O_3$ (9 μmole) or $K_2S_3O_6$ (6 μmole) in the presence of $KH^{14}CO_3$. The 2,4-dinitrophenol concentration was 5×10^{-5} M; pH 7·0; temperature, 30°.

Treatment	$^{14}CO_2$ fixed (counts/100 sec.)	$^{14}CO_2$ fixed (%/μatom sulphur)	Inhibition of fixation by 2,4-dinitrophenol (%)
$Na_2S_2O_3$	$7·12 \times 10^4$	100	—
$K_2S_3O_6$	$5·10 \times 10^4$	72	—
$Na_2S_2O_3$ + 2,4-dinitrophenol	$4·08 \times 10^4$	100	42·5
$K_2S_3O_6$ + 2,4-dinitrophenol	$3·66 \times 10^4$	89·5	28·5

effect on oxidative phosphorylation in other systems (Cross *et al.* 1949; Clowes *et al.* 1950). Picric acid inhibited fixation at high concentrations but its effect was probably complex; in other systems it is a poor uncoupling agent. Dichloro- and *p*-nitrophenol are well established as inhibitors of oxidative phosphorylation (Hackett, 1960; Clowes *et al.* 1950) and effectively uncoupled CO_2-fixation by our Thiobacillus. Low concentrations of 2,6-dichloro-4-nitrophenol probably have a similar effect (Krahl & Clowes, 1936) but high concentrations inhibit the oxidation of thiosulphate. 2,4-Dinitrophenol (2 mM) depressed the rate of oxygen uptake by more than 80%.

Arsenate. The effect of a range of arsenate concentrations on $^{14}CO_2$-fixation by

dense bacterial suspensions oxidizing thiosulphate or sulphide was measured (Table 5); in these experiments, the phosphate concentration remained constant. $^{14}CO_2$-fixation coupled to oxidation of thiosulphate or sulphide was inhibited by arsenate:phosphate ratios of the order of unity, but the fixation coupled to sulphide

Table 4. *Effect of substituted aromatic compounds on thiosulphate-linked $^{14}CO_2$-fixation by Thiobacillus strain c*

Compound	Concentration (M)	Inhibition of $^{14}CO_2$-fixation (% of control)	Initial oxidation rate (% difference from controls)
Experiment 1			
Phenol	2×10^{-4}	0	.
o-Nitrophenol		8·5	.
p-Nitrophenol		34	.
2,4-Dinitrophenol		48·5	+15
2,4,6-Trinitrophenol		10·5	.
p-Nitroaniline		14	.
p-Nitroanisole		0	.
p-Nitrobenzylalcohol		1	.
o-Nitrophenol	10^{-4}	14·5	−10
	5×10^{-4}	16	−13
p-Nitrophenol	10^{-5}	0	.
	5×10^{-5}	4	.
	10^{-4}	12	.
	5×10^{-4}	91	+20
Experiment 2			
2,4-Dinitrophenol	10^{-5}	3·5	.
	5×10^{-5}	14	.
	10^{-4}	49·5	.
	5×10^{-4}	74·5	.
2,4,6-Trinitrophenol	10^{-4}	7·5	− 7
	5×10^{-4}	38·5	−15
2,4-Dichlorophenol	10^{-5}	0	.
	5×10^{-5}	12·5	.
	10^{-4}	47	−21
	5×10^{-4}	100	−48
2,6-Dichloro-4-nitrophenol	10^{-5}	0	.
	5×10^{-5}	27	.
	10^{-4}	33	.
	5×10^{-4}	99	−50*

* 5×10^{-4} M 2,6-dichloro-4-nitrophenol blocked the oxidation after only 3·8 μmole of the theoretical 20 μmole of oxygen had been consumed.

oxidation was the more sensitive to the inhibitor. The rate of thiosulphate oxidation by dilute bacterial suspensions was unaffected by these arsenate:phosphate ratios.

Azide. Sodium azide inhibited both thiosulphate oxidation and $^{14}CO_2$ fixation. Both reactions were inhibited by 50 % by 10^{-4} M-azide and completely inhibited by 5×10^{-4} M. Azide, therefore, has no specific uncoupling effect on this system. Iwatsuka, Kuno & Maruyama (1962) obtained a similar result with *Thiobacillus thiooxidans*.

Table 5. *Inhibition of $^{14}CO_2$-fixation in Thiobacillus strain c by arsenate*

Dense suspensions of Thiobacillus strain c in 0·003 M-phosphate buffer (pH 7) were added to phosphate + arsenate buffer mixtures in Warburg flasks and incubated at 30°, pH 7·0, for 30–40 min. $KH^{14}CO_3$ and Na_2S or $Na_2S_2O_3$ were added. $^{14}CO_2$-fixation was measured when oxidation was complete. The final phosphate concentration was 0·05 M in each experiment.

Arsenate concentration (M)	Arsenate : phosphate ratio	Oxidation of: $S_2O_3^{2-}$ $^{14}CO_2$ fixed (%)	Oxidation of: S^{2-} $^{14}CO_2$ fixed (%)
Experiment 1			
0	0	100	100
0·02	0·4	115	77
0·05	1·0	102	61
Experiment 2			
0	0	100	100
0·001	0·02	105	96
0·01	0·2	80	89
0·05	1·0	81	33
0·1	2·0	80	22
Experiment 3			
0	0	100	100
0·01	0·2	112	87
0·025	0·5	102	82
0·05	1·0	72	56
0·1	2·0	21	17

DISCUSSION

CO_2-fixation in Thiobacillus takes place chiefly by the Calvin cycle (Aubert *et al.* 1957) and presumably these reactions require a supply of reduced NAD or NADP and ATP (Racker, 1955). Since, in our experiments, CO_2-fixation is inhibited by low concentrations of 2,4-dinitrophenol, which uncouples phosphorylation in other systems, one can assume that the amount of CO_2-fixation indicates the quantity of high-energy phosphate available to the organisms. Taking the value with thiosulphate as substrate as 100, CO_2-fixation/mole substrate oxidized was 119 units with trithionate, 198 with tetrathionate and 67 with sulphide. Since, however, these compounds contain different numbers of sulphur atoms, a better comparison is CO_2-fixation/sulphur atom oxidized. The figures then are, with thiosulphate as 100, trithionate 80, tetrathionate 99 and sulphide 134.

The complete oxidation to sulphate of one molecule of sulphide or thiosulphate requires two molecules of oxygen. Nevertheless, only about two-thirds as much CO_2-fixation is coupled to the oxidation of sulphide. If the amount of CO_2 fixed does indicate the quantity of high-energy phosphate made available during oxidation, this difference suggests that the oxidation of a molecule of thiosulphate is linked to more phosphorylating steps than that of one of sulphide. An additional phosphorylation would occur during thiosulphate oxidation if the substrate-level phosphorylation studied by Peck (1962) in cell-free extracts also takes place in intact organisms.

The inhibition of CO_2-fixation by 2,4-dinitrophenol, and other phenolic compounds

which are known to uncouple oxidative phosphorylations in mitochondrial systems, indicates that oxidative phosphorylation plays a part in the coupling between substrate oxidation and CO_2-fixation in Thiobacillus. However, CO_2-fixation coupled to sulphide oxidation is always more sensitive to 2,4-dinitrophenol inhibition than is the CO_2-fixation coupled to thiosulphate oxidation. Such a difference in sensitivity would be expected if the substrate phosphorylation, studied by Peck, is relatively more important in supplying energy for CO_2-fixation during the oxidation of thiosulphate than it is when sulphide is oxidized. This view is supported by the observation that the ATP content of intact Thiobacillus organisms increases rapidly after the addition of thiosulphate or sulphide (Kelly & Syrett, unpublished); but, whereas, when thiosulphate is added, this increase is unaffected by 2,4-dinitrophenol, it is strongly inhibited when sulphide is the substrate. If the only phosphorylations coupled to sulphide oxidation are oxidative ones, sensitive to 2,4-dinitrophenol, then our data indicate that two-thirds of the phosphorylations coupled to thiosulphate oxidation are of this type and one-third are unaffected by 2,4-dinitrophenol (Kelly & Syrett, 1963). As yet, the pathway of sulphide oxidation is uncertain and the possibility of some substrate phosphorylation during sulphide oxidation cannot be excluded.

Arsenate can replace phosphate in many enzyme reactions and, with it, ADP-sulphurylase catalyses the arsenolysis of adenosine-5′-phosphosulphate (Robbins & Lipmann, 1958). Both this enzyme and adenosine phosphosulphate are reactants in the phosphorylation system studied by Peck & Fisher (1962) and, in their cell-free extracts, arsenate could replace for phosphate in catalysing the oxidation of thiosulphate to sulphate. However, when phosphate and arsenate were both present, in equimolar quantities, phosphate esterification was unaffected by arsenate. Thus arsenate competes poorly with phosphate in the reaction with adenosine phosphosulphate. On the other hand, an arsenate:phosphate ratio of unity uncouples oxidative phosphorylation in a mitochondrial system (Crane & Lipmann, 1953). Our results with whole organisms of Thiobacillus show that arsenate resembles 2,4-dinitrophenol in that a given concentration inhibits $^{14}CO_2$-fixation more when sulphide is the substrate than with thiosulphate. The difference in sensitivity again suggests that thiosulphate oxidation is linked to a type of phosphorylation which is relatively less important in the oxidation of sulphide.

When tetrathionate is added, CO_2-fixation/sulphur atom oxidized is very similar to fixation with thiosulphate as substrate and the sensitivity to 2,4-dinitrophenol inhibition is much the same. Such similarity is to be expected if the first step in the metabolism of a molecule of tetrathionate is its conversion to two molecules of thiosulphate (Peck & Fisher, 1962). The present results with trithionate suggest less CO_2-fixation/sulphur atom oxidized than with the other substrates and a lower sensitivity to 2,4-dinitrophenol. It is attractive to think that the trithionate ion, $S_3O_6^{2-}$, might be reduced to two sulphite ions and one of sulphide. If this sulphite were then oxidized with a coupled substrate phosphorylation as suggested by Peck, phosphorylations insensitive to 2,4-dinitrophenol might be quantitatively more important in the oxidation of trithionate in Thiobacillus than in the oxidation of thiosulphate, $S_2O_3^{2-}$, where only one sulphite ion, together with one of sulphide, is formed on reduction.

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The Intracellular Location of the Agent of Mouse Scrapie

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SUMMARY

Homogenates of brains from mice clinically affected with scrapie have been fractionated by differential centrifugation and equilibration in sucrose density gradients. Most of the infectivity was found in the heavy particulate fraction containing mitochondria and possibly lysosomes.

INTRODUCTION

Scrapie is a chronic, progressive, degenerative disease of the central nervous system occurring naturally in sheep, and possibly in goats, and almost invariably terminating fatally. It can be transmitted from sheep to goats, and most of the experimental work carried out on the disease over the last 30 years has made use of these two large domestic animals (see reviews by Gordon, 1957, and Stamp, 1962). Recently a form of encephalopathy (termed mouse scrapie throughout this paper) has been produced in mice by inoculating them with brain material from scrapie-affected goats (Chandler, 1961, 1963), and it is now possible to make quantitative studies of the disease (Hunter, Millson & Chandler, 1963) by carrying out titrations in large numbers of animals. Chandler's (1961) initial experiments have been successfully repeated in other laboratories (Morris & Gajdusek, 1963; Eklund, Hadlow & Kennedy, 1963; Zlotnik & Rennie, 1963). Two groups of workers have further shown that it is possible to transmit the disease directly to mice from sheep (Morris & Gajdusek, 1963; Zlotnik & Rennie, 1963).

Although the scrapie agent has been characterized for some time as a filter-passing agent, a fact confirmed recently in the case of mouse scrapie (Chandler, 1963; Eklund *et al.* 1963), its exceptional resistance to chemical and physical agents has aroused much speculation as to its precise nature. Our earlier results (Hunter *et al.* 1963; Hunter, 1963) demonstrated the association of the agent of mouse scrapie with cytoplasmic particles. More refined methods of fractionation have now been applied to homogenates of brain from scrapie-affected mice and the bulk of the infectivity has been found to reside in the purified mitochondrial fraction of the cell. This fractionation may also be rich in lysosomes. Some of these results have been reported previously in a preliminary form (Hunter & Millson, 1963).

METHODS

Mice. B.S.V.S. (bacteria susceptible, virus susceptible, Schneider, 1959) white mice were used. Cases of scrapie were diagnosed as described previously (Hunter *et al.* 1963), following the criteria laid down by Chandler (1963) and by Pattison & Smith (1963).

Cellular fractionation of mouse-scrapie brain. Four scrapie-affected mice were decapitated, their brains removed immediately and homogenized in a Potter-type homogenizer in 0·25 M-sucrose (10·8 ml.) containing 5 mM-$CaCl_2$. A portion of the homogenate (0·5 ml.) was set aside and used for the preparation of the saline dilutions of control material. The remaining material (R) was maintained between 0° and 4° in all subsequent manipulations.

One half of the remaining material (R) was transferred to a 3 × 1 in. lusteroid tube containing 0·88 M-sucrose (10 ml.) covered with a separate layer of 0·32 M-sucrose (6 ml.). After centrifugation at 53,500*g* in a Spinco Model L SW. 25 rotor for 1 hr the tube was placed in a dry ice-acetone mixture and the rapidly frozen 'top layer' sawn off with a small hack-saw. This fraction was allowed to thaw slowly in a small beaker, and the whole process repeated before saline dilutions were prepared from it for titration. As the material tended to adhere to the surface of glassware, saline dilutions for titration were prepared by using as far as was conveniently possible the whole of the material, and a pipette (10 ml.) was used only when the volume to be handled exceeded 1 l.

The other half of the homogenate (R) was carefully pipetted on to the surface of 0·88 M-sucrose (15 ml.) and centrifuged as above. The clear supernatant layer (soluble fraction S) was removed, transferred to a sterile tube and left at 2° until required. The myelin layer (M) at the interface between the 0·88 and 0·25 M-sucrose layers was removed as completely as possible and also transferred to a sterile tube at 2°. The bulk of the 0·88 M-sucrose solution (88 S) was now removed with minimal contamination with particulate matter, leaving a deposit containing nuclei, cell debris, mitochondria, etc. Distilled water (5·25 ml.) and 0·32 M-sucrose (3 ml.) were added to this deposit which was then gently rehomogenized before adding to 2 M-sucrose (1 ml.) in a 3 × 1 in. lusteroid tube. The homogenizer was rinsed with 0·32 M-sucrose (5 ml.) and the tube then centrifuged at 1000*g* for 10 min. The supernatant solution (NS 1) was removed carefully and the sedimented nuclear fraction resuspended in water (5·25 ml.) and 0·32 M-sucrose (3 ml.) and treated as before. After centrifuging the supernatant solution (NS 2) was combined with the previously obtained solution (NS 1). The nuclear deposit was resuspended in water (5·25 ml.) and spread carefully on the surface of 0·88 M-sucrose (15 ml.) in a 3 × 1 in. lusteroid tube. The nuclear fraction was collected after centrifuging at 17,000*g* for 55 min. to remove most of the residual myelin. Dilutions in saline were prepared for titration in the usual way (Hunter *et al.* 1963) after a final sedimentation of the nuclei from 0·32 M-sucrose at 1000*g* for 10 min.

The combined supernatant solutions (NS 1 and NS 2) removed from above the nuclear sediments were centrifuged at 17,000*g* for 55 min. and the supernatant solution now obtained (MS) retained at 2° for the preparation of microsomal fractions. The deposit was resuspended in 0·32 M-sucrose and lightly homogenized before layering on to a discontinuous sucrose gradient constructed from 1·5 M-sucrose

(0·5 ml.), 1·2 M-sucrose (10 ml.) and 0·88 M-sucrose (10 ml.). After centrifuging at 53,500 *g* for 2 hr in a Spinco SW. 25 rotor, mitochondria were removed carefully from the 1·5–1·2 M-sucrose interface, and nerve-ending particles from the 1·2–0·88 M interface. The process was repeated on the separated fractions in order to effect further purification and they were finally sedimented into firm pellets (105,000 *g* for 1 hr) from 0·32 M-sucrose preparatory to the making of saline dilutions for titration as above.

The myelin fraction (M) obtained as above was layered on to the surface of 0·88 M-sucrose (20 ml.) and centrifuged at 53,500 *g* in a Spinco SW. 25 rotor for 2 hr. The myelin from the interface was collected in the minimum volume of liquid (approx. 2 ml.) and the suspension diluted with 0·32 M-sucrose (10 ml.). It was finally resedimented at 105,000 *g* for 1 hr. Dilutions in saline for titration were prepared as above.

The clear supernatant layer (S) obtained from the first centrifugal run was recentrifuged at 105,000 *g* for 90 min. before freezing rapidly in acetone-solid CO_2. The frozen upper and lower portions of the solution were removed with a hack-saw, and the central portion (soluble fraction or cell sap) collected and thawed out before preparing saline dilutions for titration in the usual way. In this instance, all dilutions from 10^{-3} downwards were prepared directly from the sucrose solution isolated as above, but the 10^{-2} dilution was dialysed against saline before inoculating the mice as the sucrose concentration was rather high for intracerebral injection.

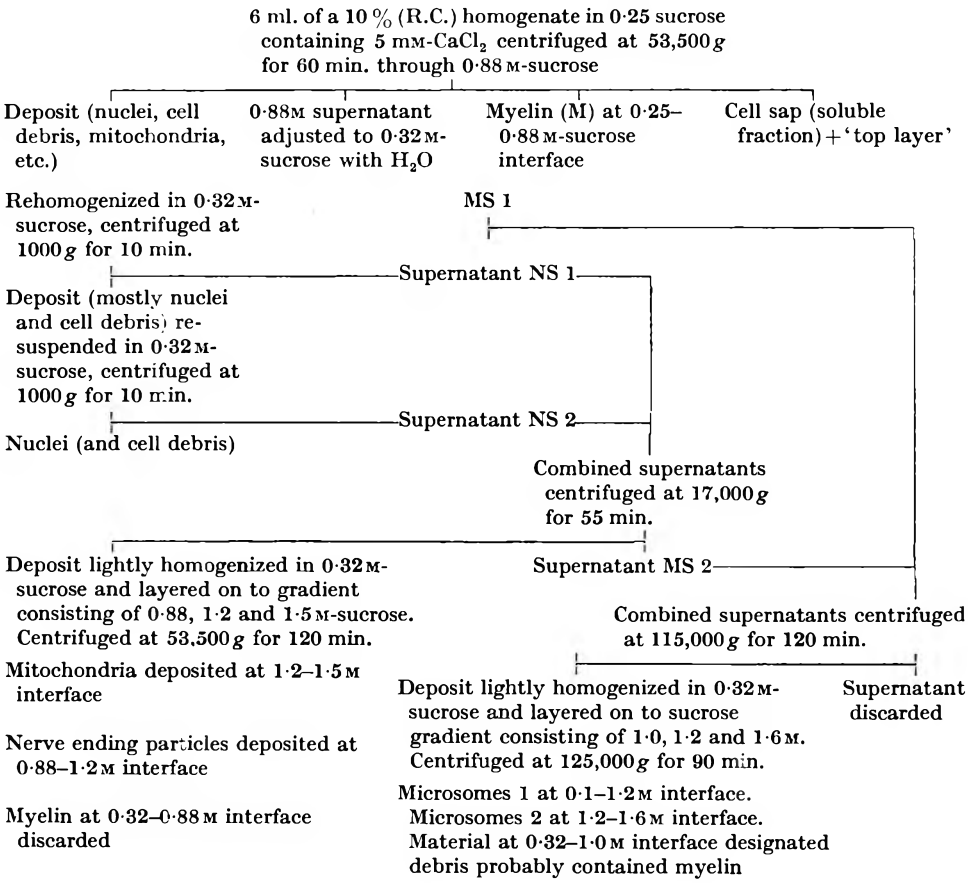
For the preparation of the microsomal fractions, the original 0·88 M-sucrose solution (88 S) obtained above was first diluted to 0·32 M-sucrose and centrifuged at 10,000 *g* for 10 min. The supernatant solution was combined with mitochondrial supernatant solution (MS) and centrifuged at 105,000 *g* for 90 min. The supernatant solution was decanted and the microsomal pellet resuspended in 0·32 M-sucrose (1 ml.) and lightly rehomogenized. It was then layered on to a discontinuous sucrose gradient made up from 1·6 M-sucrose (1 ml.), 1·2 M-sucrose (1 ml.) and 1·0 M-sucrose (1 ml.). After centrifuging at 125,000 *g* for 1 hr in a Spinco SW. 39 rotor three fractions were collected: (1) a 'debris' fraction, partly myelin, resting on the 0·32–1·0 M interface; (2) microsomes 1 at the 1·0–1·2 M-sucrose interface; and (3) microsomes 2 at the 1·2–1·6 M-sucrose interface. The fractions were carefully removed, diluted to approximately 0·32 M-sucrose and sedimented at 105,000 *g* for 90 min. Saline dilutions of each fraction were prepared for titration in the usual way. In every case, allowance was made for operational losses before preparing the dilutions.

The whole procedure adopted for the cellular fractionation is summarized in Scheme 1.

Electron microscopy

Samples of control and infected mitochondria and of infected microsomes were prepared as described above and were fixed in buffered osmium tetroxide solution (Palade, 1952) for 45 min. The material was then centrifuged into pellets, dehydrated in a conventional ethyl alcohol series followed by propylene oxide and embedded in British Ciba Araldite. Sections were cut at about 60 mμ on a Huxley ultramicrotome and examined in a Siemens Elmiskop I electron microscope. Micrographs were made at plate magnifications of around 20,000 using the double condenser system at 60 kV.

Scheme 1. *Subcellular fractionation of scrapie mouse brain by differential centrifugation and equilibration on discontinuous sucrose density gradients*



RESULTS

Structure of cellular fractions

The composition of the various cellular fractions was checked by electron microscopy. An electron micrograph of myelin prepared as described here has been shown previously (Hunter *et al.* 1963). Plate 1, figs. 1 and 2, show that the microsome fractions obtained here are fairly typical, and that only the microsome fraction 2 seems to contain appreciable numbers of free ribosomes. Figure 3 shows the mitochondrial fraction, which in addition to large numbers of mitochondria also contains other bodies of comparable size, possibly lysosomes. No particles resembling a large virus have been seen in any of the sections examined so far. Figure 4 shows a normal mouse brain mitochondrial preparation.

The only unusual feature arising from the cellular fractionation of the scrapie material is the appearance of a thin 'top layer' that floats on the top of the original homogenate after high-speed centrifugation. Electron microscopy showed that this fraction, which constituted only a very small proportion of the original homo-

genate, consisted largely of myelin fragments and large osmiophilic granules that were almost certainly globules of fat.

Potencies of the various cellular fractions

The only material to approach the infected whole homogenate in potency was the mitochondrial fraction (Tables 1 and 2). In view of the large numbers of mice required, several of the fractions were assayed using a limited titration only (Hunter *et al.* 1963), and it is gratifying to see (Table 2) that the calculated infectivity of the mitochondrial fraction is practically the same using either method of assay. The myelin, nuclei and nerve ending particles also contained some scrapie agent, and the microsome fractions possibly slightly less. However, the 'top layer' and soluble fractions of the cell had very low infectivity.

Table 1. *Percentage deaths from scrapie in groups of mice inoculated with various dilutions of purified cellular fractions*

B.S.V.S. mice (groups of 8) were inoculated intracerebrally with materials prepared as described in the text. Clinical diagnosis and histopathological confirmation of the presence of mouse scrapie were carried out as described in Methods. The experiment was terminated 7 months after the inoculation.

	Dilution of the inoculum related to whole brain								
Inoculum	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	ID 50
Control	100	100	100	100	87	0	0	0	6·4
Mitochondria	100	100	100	100	71	0	0	0	6·2
Microsomes 1	100	100	100	43	37	0	0	0	5·3
Microsomes 2	100	100	75	25	12	0	0	0	4·6
'Top layer'	75	25	0	0	0	0	0	0	2·5
Soluble	100	100	0	0	0	0	0	0	3·5

Table 2. *Incubation periods of scrapie in groups of mice inoculated with various dilutions of purified cellular fractions*

The data refer to the same experiment as that described in Table 1. The calculations of infectivity based on length of the incubation period are made according to Hunter *et al.* (1963), who defined ST 50 for each group as the time after inoculation by which 50 % of the mice in the group had been killed in the final clinical stage of the disease.

	ST 50 in days of dilution group			% of control infectivity based on length of incubation periods	% of control infectivity based on complete titration
Inoculum	10^{-2}	10^{-3}	10^{-4}		
Control	136	146	146	100	100
Myelin	154	154	171	13	—
Nuclei	146	154	164	23	—
Mitochondria	136	136	168	62	63
Nerve ending particles	136	146	182	23	—
Microsomes 1	139	157	189	10	7·9
Microsomes 2	146	154	182	11	1·6
'Debris'	164	202	—	—	—
'Top layer'	189	—	—	—	0·013
Soluble	168	182	—	—	0·13

DISCUSSION

The method of cellular fractionation used here is based to some extent on that used by Gray & Whittaker (1962) who were mainly concerned with the preparation of nerve-ending particles from guinea-pig brain. The only unusual feature, the obtaining of a 'top layer' when centrifuging scrapie homogenates, does not appear to be of any fundamental importance. The 'top layer' contains very little of the infective agent and probably consists of myelin fragments and cytoplasmic fat globules arising from degenerate cells. Otherwise the electron micrographs show that all the fractions examined closely resembled those obtainable from normal brain homogenates. The mitochondrial fraction contained a number of bodies that could possibly be lysosomes.

The high infectivity found in the mitochondrial fraction, incidentally representing a five- to tenfold concentration of the agent, would at first sight suggest that the scrapie agent is a large particle with a size and density somewhat similar to that of brain mitochondria. The finding of a considerable scrapie potency in the fractions most similar in size and density to the mitochondria (i.e. nuclei and nerve ending particles) would be consistent with this suggestion, and these two fractions would contain some contaminating mitochondria. However, we have been unable to find any object resembling a large virus in any of the electron micrographs, and it is also somewhat surprising to find an appreciable, if low, titre in the soluble fraction if a large virus is concerned.

A second possibility may be that the agent is a small or medium-sized virus which is for some reason specifically adsorbed to mitochondria or lysosomes, or, perhaps, multiplies within these larger organelles. If the lysosomes were the organelles concerned, the lytic action of scrapie upon brain cells would be readily understandable. Eklund *et al.* (1963) consider that the scrapie agent is a medium-sized virus largely on the basis of filtration evidence. It is not clear, however, what proportion of the infectivity actually passed through a 100 mμ filter. The filtered material in their hands was no longer resistant to boiling and it seems possible that their filter removed all the mitochondrial-bound agent.

In either case, an experimental attack involving disruption of the large mitochondrial organelles by physical and chemical agents should provide valuable information. Transfer of the bulk of the residual infectivity to the microsomal fractions would then indicate the presence of a small mitochondrial- or lysosomal-bound agent. On the other hand, if the bulk of the residual infectivity could be transferred to the soluble fraction of the cell, it would appear that scrapie involves a derangement in protein metabolism, the presence of a colicin-type factor, or the introduction of a toxic protein capable of stimulating its own reproduction.

Myelin, nuclear and mitochondrial fractions obtained here retained higher absolute potencies than the corresponding fractions prepared previously from scrapie brain (Hunter *et al.* 1963) by a rather less elaborate density gradient procedure. The scrapie agent appears to be stable in strong solutions of sucrose (Hunter & Millson, 1963), but it is clear that in certain circumstances, at present not precisely understood, cellular fractionation can lead to a loss of scrapie infectivity.

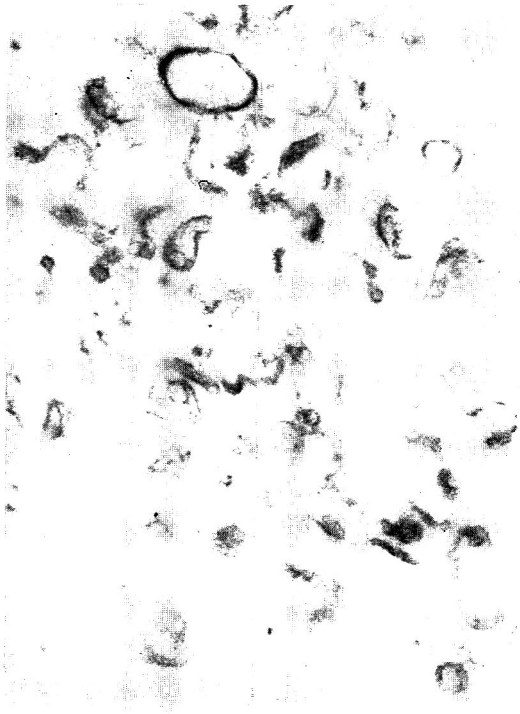


Fig. 1

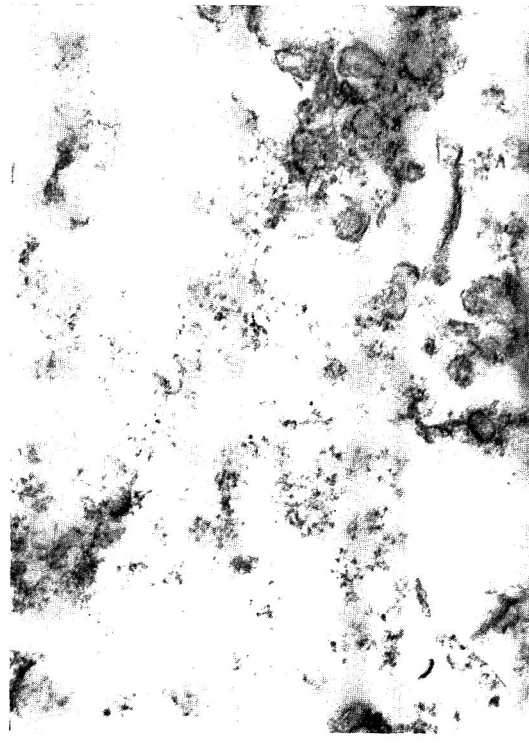


Fig. 2

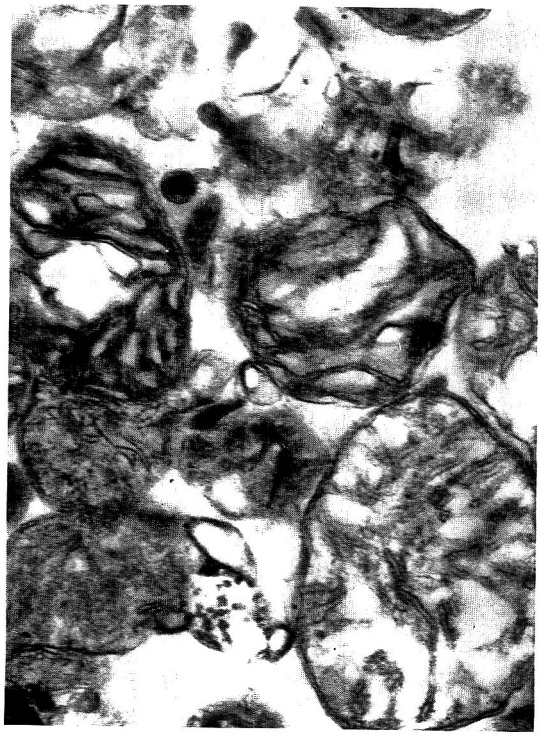


Fig. 3

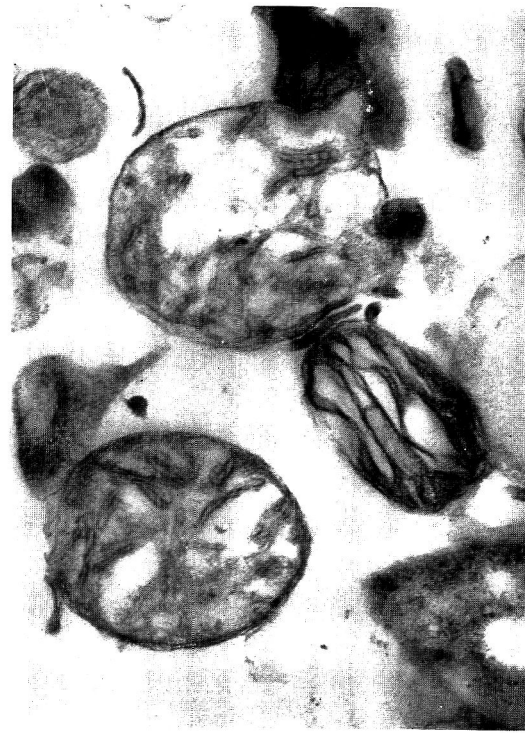


Fig. 4

(*Facing p.* 325)

The authors are grateful to Dr W. S. Gordon, C.B.E., for his continued interest and advice, and to Dr A. Mackenzie and Mr P. Dennis for preparing the histological specimens. They also wish to acknowledge the skilled technical assistance of Mr M. Vockins, and the excellent work carried out by the staff of the animal house in the breeding and handling of mice. This work was supported in part by grants made to the A.R.C. from U.S. Government funds administered under U.S. Public Law 480.

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EXPLANATION OF PLATE

Fig. 1. Electron micrograph of microsomes 1 fraction prepared as described in the text (magnification × 53,000).

Fig. 2. Electron micrograph of microsomes 2 fraction prepared as described in the text (magnification × 53,000).

Fig. 3. Electron micrograph of mitochondria prepared from scrapie mouse brain as described in the text (magnification × 53,000).

Fig. 4. Electron micrograph of normal mouse brain mitochondria (magnification × 53,000).

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The Effect of Pyruvate and Acetate on the Rate of Decrease in Optical Density of Suspensions of *Pseudomonas aeruginosa* in Sodium, Potassium or Sodium-Potassium Phosphate Buffers

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SUMMARY

The rate of decrease in optical density (increase in size of organisms) was determined in washed suspensions of a strain of *Pseudomonas aeruginosa* after they were added to potassium, sodium, or potassium + sodium phosphate buffers. The rate was increased by the addition of pyruvate or acetate. The maximal effect of pyruvate occurred in potassium + sodium buffer; of acetate in potassium buffer. Rate changes were also dependent on pH value and osmotic pressure.

INTRODUCTION

Mager, Kuczynski, Schatzberg & Avi-Dor (1956) were the first to show that changes in osmotic pressure of the medium caused changes in size of several Gram-negative bacteria and these changes could be measured by a light-scattering method. Packer & Perry (1961) found that light-scattering changes in suspensions of *Escherichia coli* were dependent on energy supplied by oxidizable substrates, and Bernheim (1963) showed that such changes in *Pseudomonas aeruginosa* reflected the size and density of the organisms and that the rate of change was affected by the cation present and by the integrity of the membrane. The following is a further study of cation and substrate effects.

METHODS

A strain of *Pseudomonas aeruginosa* which has been maintained in the laboratory for 15 years, was grown at 34° for 24 hr in Difco nutrient broth. The organisms were centrifuged down and washed twice with distilled water; in this process they lost potassium (Bernheim, 1963). They were finally suspended in distilled water and the concentration adjusted for estimation of light absorption in a Coleman junior spectrophotometer at 490 mμ so that the initial optical density varied no more than 10% between experiments. However, the results obtained were similar when the number of organisms was doubled or halved. It was not possible to free the organisms of all sodium and potassium, small amounts of which, estimated in a flame photometer, were present in the suspensions. To 2·5 ml. of either a salt or sucrose solution. 0·5 ml. of suspension was added and the optical density read immediately. After 1 min. either NaCl, sodium acetate, or sodium pyruvate in 0·1 ml. was added, mixed, and the change in optical density determined every

30 sec. for the next 5 min. This strain of *P. aeruginosa* oxidizes acetate and pyruvate with a minimum latent period and they are thus suitable substrates for short-term experiments. The amount of sodium (0·2–0·28 mg.) added with the substrate did not apparently affect the results. For instance, when potassium phosphate was used as the osmotic agent, the rate of change in optical density with sodium acetate was the same as that with potassium acetate. All experiments were done at 23°. The optical density is expressed in terms of the scale reading, $-\log T \times 1000$. In the range of suspension concentrations used, transmission plotted against number of organisms gave a straight line. It was therefore assumed that the same relationship held between transmission and size of organism.

RESULTS

The effect of added substrate on the optical density change is rapid. When 0·035 M-Na+K phosphate buffer (pH 7·7) was used as the osmotic agent, the addition of 0·1 ml. water caused a 6-unit decrease in the next 30 sec. When 1·0, 0·1, or 0·01 mg. of sodium pyruvate was added, each concentration in 0·1 ml., a 10-unit decrease occurred, but 0·001 mg. was no better than water. Table 1 compares the effect of several concentrations of acetate and pyruvate on the change in optical density 5 min. after their addition to organisms in 0·035 M-Na+K phosphate buffer (pH 7·7). Maximal rate was obtained with 1·0 mg. pyruvate but had not been obtained with 4·44 mg. acetate, 6 times the molar concentration. Table 1 also shows that as the osmotic pressure of the medium increased, pyruvate was more effective in increasing the size of organism than was equimolar acetate.

Table 1

The effect of several concentrations of sodium pyruvate and sodium acetate on the change in optical density (OD) 5 min. after their addition to organisms suspended in 0·035 M-Na+K phosphate buffer (pH 7·7).

Compound	Mg added	Decrease in OD
NaCl	1·1	10
Na pyruvate	0·5	75
	1·0	87
	2·0	86
Na acetate	0·74	40
	1·48	49
	2·96	63
	4·44	77

The effect of 1·0 mg. sodium pyruvate and 0·74 mg. sodium acetate on the change in optical density (OD) 5 min. after their addition to organisms suspended in buffer (pH 7·0).

Compound	Decrease in OD in 0·035 M-buffer	Decrease in OD in 0·053 M-buffer
Na pyruvate	60	66
Na acetate	59	33

Table 2 and Fig. 1 show the effect of cations and pH value on the activity of acetate and pyruvate. In the presence of equimolar amounts of sodium and potassium ions, the acetate effect at pH 6·2 was better than at pH 7·7, but the reverse was true for pyruvate. The acetate effect was maximal when potassium phosphate was used as the osmotic agent; it was virtually absent when sodium phosphate was used, i.e. it was little better than NaCl. The pyruvate effect was maximal when sodium and potassium phosphates were used and was minimal but still appreciable when only sodium ion was present. Thus acetate required potassium

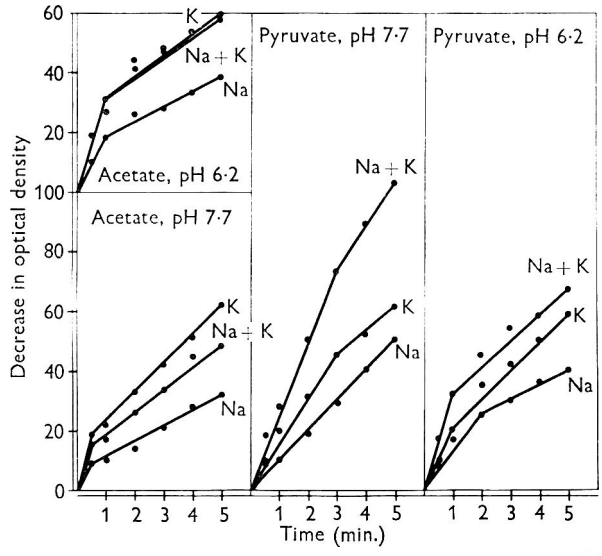

Fig. 1. The rate of decrease in optical density after the addition of 1·0 mg. sodium pyruvate or 0·74 mg. sodium acetate to organisms in potassium, sodium, or sodium + potassium buffers, 0·035 M, at pH 6·2 or 7·7. When 0·6 mg. NaCl was added instead of the substrates the curve obtained was identical with that of acetate in sodium buffer at pH 7·7.

Table 2. *The effect of* 1·0 *mg. sodium pyruvate or* 0·74 *mg. sodium acetate at pH* 7·7 *or* 6·2 *on the decrease of optical density (OD) after the organisms were suspended in* 0·035 M*-potassium, sodium or sodium + potassium buffers. The figures represent the decrease* 5 *min. after the addition of the substrates*

		Decrease in OD		
Buffer cation	pH value	NaCl	Acetate	Pyruvate
K	7·7	33	62	61
K	6·2	30	60	59
Na	7·7	31	32	50
Na	6·2	32	38	40
Na + K	7·7	35	48	103
Na + K	6·2	32	58	67

ions whereas pyruvate acted in the presence of either sodium or potassium but was more efficient when both were present. These differences may be correlated with the rates of oxidation of the two compounds under the different conditions. Fig. 2 shows the relative oxidation rates. There is a correlation but it is not perfect. At pH 6·2 and 7·7 acetate was oxidized more rapidly in potassium than in sodium buffer and this correlates with its effect on the optical density. But at both pH values it was oxidized most rapidly in the sodium + potassium mixture, but this was not reflected in the rate of change of optical density. At both pH values pyruvate was oxidized most rapidly in the sodium + potassium mixture and this correlates with its effect on the optical density. Pyruvate was oxidized less rapidly in sodium than in potassium buffer and this also correlates with the optical density

changes. Finally, at pH 6·2, acetate in potassium buffer was oxidized more slowly than pyruvate, yet the effect of both on the rate of change of optical density at this pH value was the same. Relative oxidation rates are therefore important but may not be the only factor involved.

It has been shown by DeTurk & Bernheim (1960) that chelators of ferrous iron inhibit the oxidation of a number of substrates by the *Pseudomonas aeruginosa* used here. Washed organisms were incubated for 60 min. with 50 μg./ml. of 2,2′-dipyridyl before addition to 0·035 M-Na+K phosphate (pH 7·7) and the decrease in optical density compared with the control. When acetate or pyruvate was added, the 5-min. values showed a 28 % inhibition. But when NaCl was added instead of these substrates, the slow decrease in optical density was not inhibited. The endogenous mechanism responsible for this slow decrease was not affected by the chelator.

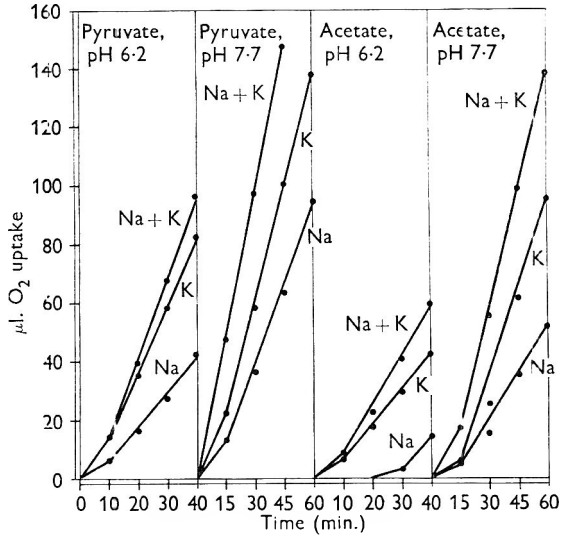

Fig. 2. The oxidation of 1·0 mg. sodium pyruvate and 0·74 mg. sodium acetate in potassium, sodium, or sodium + potassium buffers, 0·035 M, at pH 6·2 and 7·7.

DISCUSSION

Increase in organism size after osmotic shrinking with inorganic salts must be the result of water intake with or without ions. There is no feasible way of measuring ion penetration into the organism after the addition of large salt concentrations. Loss from the liquid is too small to determine and washing causes progressive loss of intracellular ions so that no definitive value can be obtained. Ions, however, accelerate the swelling. As shown previously (Bernheim, 1963) osmolar sucrose shrinks the organisms to the same extent as inorganic salts, but the subsequent swelling is at a much slower rate unless a salt is added. A potassium salt increases the rate more than sodium which in turn is more effective than lithium, caesium, or rubidium. The cations thus show a relative specificity in affecting water transport or in being themselves transported with their water of hydration.

It is generally agreed that energy is required for swelling after osmotic shrinking.

It is therefore not surprising that oxidizable substrates increase the rate. (In the previous paper it was shown that when salts of organic acids, whether substrates or not, were used as osmotic agents, the subsequent swelling was more rapid than when osmolar concentrations of salts of inorganic acids were used. The reason for this is not clear but damage to the membrane by high concentrations of organic anions is a possibility.) In the present experiments the organic acids were used in substrate amounts, their effect on the rate of swelling is, under most conditions, correlated with their oxidation rates, and cations affect the two substrates differently. Moreover, substrates such as succinate and other Krebs cycle compounds which show a latent period before being oxidized have little effect on swelling rate during the 5 min. observation period.

Neither pyruvate nor acetate is oxidized in the absence of added cation, i.e. in Tris buffer and Tris-neutralized compounds. Thus oxidation is linked with the presence of cations, specifically sodium and potassium (lithium and caesium are much less effective) and swelling is also linked with their presence. It is as yet impossible to decide whether cations are necessary for the transport of pyruvate, acetate, and water or whether pyruvate and acetate accelerate the transport of cations and water. Finally, pyruvate may be metabolized to acetate and the greater effectiveness of pyruvate on swelling rate may be the result of the presence of both anions.

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Teichoic Acids and Group Antigens in Lactobacilli

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SUMMARY

Purified teichoic acids and extracts with group-specific activity have been examined with specific antisera for a number of lactobacilli. The wall teichoic acids from groups D and E lactobacilli have been identified as the group-specific substances, whereas in group A the intracellular teichoic acid showed group-specificity. It is possible that group-specificity is associated with intracellular teichoic acid in group F.

INTRODUCTION

The teichoic acids are widely distributed in Gram-positive bacteria (Armstrong *et al.* 1958, 1959; Baddiley, 1961). Investigation of these polymers in *Lactobacillus* spp. and *Staphylococcus* spp. indicated that their presence within the cell wall of an organism was of taxonomic significance and could be correlated with serological behaviour (Baddiley & Davison, 1961; Davison & Baddiley, 1963). In staphylococci, ribitol teichoic acid containing β-glucosaminyl residues from walls of *Staphylococcus aureus* (Baddiley, Buchanan, RajBhandary & Sanderson, 1962*a*; Baddiley, Buchanan, Martin & RajBhandary, 1962*b*) is serologically indistinguishable from the group-specific precipitinogen, polysaccharide A, of this organism (Haukenes, Ellwood, Baddiley & Oeding, 1961; Haukenes, 1962), and glycerol teichoic acid containing glucosyl residues from walls of *S. saprophyticus* (Davison & Baddiley, 1963) is the group-specific precipitinogen, polysaccharide B, of *S. albus* (Morse, 1963). Moreover, the intracellular glycerol teichoic acid of group D streptococci is the group antigen, and slight differences in behaviour of antigen preparations from different strains in group D have been related to differences in chemical structure (Wicken, Elliott & Baddiley, 1963).

In *Lactobacillus* spp. ribitol teichoic acid from walls of a strain of *L. plantarum* 17-5 contains both unsubstituted ribitol phosphate units and some to which one or two α-glucosyl residues are attached (Archibald, Baddiley & Buchanan, 1961), whereas in the intracellular teichoic acid from this organism and from a strain of *L. casei* var. *rhamnosus* only a small proportion of the glycerol phosphate moieties are substituted with glucose (Kelemen & Baddiley, 1961; Critchley, Archibald & Baddiley, 1962).

Although walls of many species of lactobacilli possess teichoic acids (Ikawa & Snell, 1960; Baddiley & Davison, 1961), those of groups B, C, F and G contain no teichoic acid, and it is of interest that Knox (1963) has recently shown that the

specific antigens of groups B and C are serologically distinct cell-wall polysaccharides. However, the presence of teichoic acids in walls of group A, D and E indicates that the antigenic specific determinants of these groups could be their wall teichoic acids. The present investigation was undertaken in an attempt to relate directly the work on the composition of teichoic acids from *Lactobacillus* spp. with the serological classification of this genus (Sharpe, 1955*b*; Sharpe & Wheater, 1957; Rogosa & Sharpe, 1959), and to show that the immunological group-specificity of some of these organisms is related to the presence of teichoic acids.

METHODS

Preparation of teichoic acids. Lactobacilli were grown in batch culture (15 l.) in a liquid medium of the following composition: tryptone (Oxoid), 20 g.; yeast extract (Difco), 3 g.; sodium acetate, 10 g.; glucose, 20 g.; potassium dihydrogen phosphate, 4·5 g.; inorganic salts B (Barton-Wright, 1946), 5 ml.; oleic acid, 0·01 ml.; Tween 40, 1 ml.; demineralized water, 1000 ml. *Lactobacillus bulgaricus* strain B 9, *L. helveticus* NCIB 8025, *L. jugurti* NCIB 2889 and *L. lactis* NCIB 7278 were grown at 37° for 16 hr, *L. plantarum* NCIB 7220 at 28° for 16 hr, and *L. brevis* NCIB 8169 and *L. buchneri* NCIB 8007 at 28° for 45 hr.

Bacteria were harvested in a refrigerated Sharples centrifuge, washed with cold 0·85% sodium chloride solution, the cell-wall and cytoplasmic-gel fractions isolated as previously described (Davison & Baddiley, 1963) and residual cell cytoplasm from the group A species (*Lactobacillus helveticus* and *L. jugurti*) was preserved by freeze-drying.

The chemical composition of teichoic acids isolated by extraction with cold 10% (w/v) trichloroacetic acid solution at 4° was determined by hydrolysis with 2 N-HCl or 2 N-NaOH solutions for 3 hr at 100° and by examination of hydrolysates by paper chromatography as described in detail by Armstrong *et al.* (1958) and Archibald *et al.* (1961). Sugars and amino sugars were separated in aqueous propan-2-ol (Smith, 1960) and in a mixture of pyridine + ethyl acetate + acetic acid + water (Fischer & Nebel, 1956).

Serological methods

Preparation of antisera. Cultures for immunization were grown for 18 hr in 40 ml. of MRS broth (de Man, Rogosa & Sharpe, 1960) from which the meat extract had been omitted as being a substance likely to be adsorbed on to the organisms to be inoculated and to lead to the formation of antibodies against its components (Sharpe, 1955*a*). Temperatures of incubation and procedures for the preparation of antisera were those described by Sharpe (1955*b*). After the addition of thiomersalate (0·01%) antisera were stored in 2·0 ml. portions at −20°, apart from small samples for current work, which were kept at 4°. Antiserum for group A was prepared against *Lactobacillus jugurti* NCIB 2889, for group D against *L. plantarum* NIRD A 164, and for group E against *L. lactis* NCIB 7278.

Preparation of extracts. Organisms for extraction were grown for 48 hr in 40 ml. of MRS broth from which the yeast extract had been omitted to prevent a non-specific precipitin reaction with antibodies formed against it when preparing the group antisera. The extracts were prepared as described by Sharpe (1955*b*). Strains

were representative from the collection of lactobacilli of one of the authors (M.E.S.); wherever possible, strains belonging to different serological types were used.

Ring precipitin tests. The method of Jones & Shattock (1960) was used.

Gel-diffusion tests. The agar gel double-diffusion test of Ouchterlony (1953) was used.

Teichoic acid solutions. Concentrations of 1·0, 0·1 and 0·01 mg./ml. were used for the precipitin tests and 0·1 mg./ml. for the gel-diffusion tests. They were examined by these precipitin methods and their reactions compared with those of antigens which had been extracted by using HCl. Samples from the same and additional strains belonging to the different serological groups of lactobacilli were compared.

RESULTS

Intracellular glycerol teichoic acid in the cytoplasmic-gel fraction of lactobacilli in groups D and E sedimented at 100,000 *g*, but with the species in group A (*Lactobacillus helveticus*, *L. jugurti*) the teichoic acid was not isolated by ultracentrifugation, but remained in the residual cell cytoplasm. The composition of teichoic acids extracted from walls and cell cytoplasm is given in Table 1. Walls of *L. plantarum*

Table 1. *Origin, location and chemical nature of teichoic acids from different lactobacilli and staphylococcus*

	Location	Type	Sugar	Alkali hydrolysis product
Group A lactobacilli				
L. helveticus NCIB 8025	Wall	G	—	—
	Intracellular	G	Glucose (tr.) Ribose (tr.)	Glucosylglycerol —
L. jugurti NCIB 2889	Wall	G	—	—
	Intracellular	G	Glucose Ribose (tr.)	Glucosylglycerol —
Group D lactobacilli				
L. plantarum NCIB 7220	Wall	R	Glucose	Mono- and di-glucosyl-ribitol phosphates
Group E lactobacilli				
L. buchneri NCIB 8007	Wall	G	Glucose	Glucosylglycerol
L. bulgaricus B 9	Wall	G	Glucose Galactose	Glucosylglycerol
L. brevis NCIB 8169	Wall	G	Glucose	—
L. lactis NCIB 7278	Wall	G	Glucose	Glucosylglycerol
Staphylococcus epidermidis	Wall	G	Glucose	Glucosylglycerol

G = glycerol teichoic acid; *R* = ribitol teichoic acid.

(group D) contained a ribitol teichoic acid with mono- and di-glucosyl units. Strains of the different species in group E all possessed a chemically similar glycerol wall teichoic acid with glucosyl residues, whereas glycerol wall teichoic acid of the species in group A contained no sugar moieties.

Ring precipitin tests. Table 2 shows that the wall teichoic acid of *Lactobacillus plantarum* 7220 reacted with the antiserum for group D (prepared against a different strain, NIRD A 164) and that wall teichoic acids of the three strains in group E all reacted with the group E antiserum; but wall teichoic acids of the two strains in group A did not react with the specific group A antiserum. Cross-reactions with other group antisera were not observed, except for a weak cross-reaction between

the teichoic acid from *L. bulgaricus* B 9 and group D antiserum. Specific reactions occurred with concentrations of 0·1–0·01 mg./ml. teichoic acid.

With the intracellular teichoic acid preparations, material from both the strains of lactobacilli in group A reacted with the group A antiserum, and in addition a weak cross-reaction occurred between material from *Lactobacillus jugurti* 2889 and group D and E antisera. Preparations from *L. lactis* 7278 and *L. buchneri* 8007 also reacted with group D antiserum in addition to reacting to their own group E antiserum.

Table 2. *Ring precipitin reactions of teichoic acid preparations with specific group antisera of lactobacilli*

Teichoic acid preparation		Group antiserum A	B	C	D	E	F	G
Cell wall								
Group A	*L. helveticus* 8025	–	–	–	–	–	–	–
	L. jugurti 2889	–	–	–	–	–	–	–
Group D	*L. plantarum* 7220	–	–	–	+ – +	–	–	–
Group E	*L. lactis* 7278	–	–	–	–	+ +	–	–
	L. buchneri 8007	–	–	–	–	+ + +	–	–
	L. bulgaricus B 9	–	–	–	±	+ +	–	–
Intracellular								
Group A	*L. helveticus* 8025	+	–	–	–	–	–	–
	L. jugurti 2889	+ +	–	–	±	±	–	–
Group E	*L. lactis* 7278	–	–	–	+	+ +	–	–
	L. buchneri 8007	–	–	–	+ +	+ + +	–	–

Positive reaction with teichoic acid concentration of 0·01 mg./ml., + + +; 0·1 mg./ml., + +; 1·0 mg./ml., +; 1·0 mg./ml. (weak), ±.

Gel-diffusion tests. Agar gel-diffusion tests confirmed and extended the results obtained with the ring precipitin tests. Group D antiserum, prepared against *Lactobacillus plantarum* A 164, gave precipitin lines of identity in agar with ribitol teichoic acid from the walls of *L. plantarum* NCIB 7220, and with antigen extracted with HCl from these and additional strains in group D. Group E antiserum prepared against *L. lactis* NCIB 7278 reacted in a similar manner with glycerol teichoic acids from walls of this strain of *L. lactis*, *L. buchneri* NCIB 8007, *L. bulgaricus* B 9 and with HCl extracts of these and additional strains of *L. lactis*, *L. bulgaricus* and *L. brevis* from group E; lines of identity occurred between all these preparations. The reactivity of material from strains of *L. bulgaricus* with group E antiserum was somewhat weaker than that observed with teichoic acid from other organisms in group E, but the lines of identity between preparations from *L. buchneri*, *L. bulgaricus* and *L. lactis* indicated that the group antigen was the same.

Teichoic acid from walls of the organisms in group A (*Lactobacillus helveticus* NCIB 8025 and *L. jugurti* NCIB 2889) showed no reaction with group A antiserum prepared against *L. jugurti* NCIB 2889. However, intracellular teichoic acid from the cell cytoplasm of those two strains, and acid extracts of these and other strains in group A, gave lines of identity in agar with group A antiserum. The specific substance for group A was therefore the intracellular teichoic acid and not the

Table 3. *Immunological reactions of teichoic acid preparations of cell walls and cell contents of lactobacilli, and of HCl extracts of whole cells of lactobacilli, using slide gel-diffusion tests*

Serological group	Strains of lactobacilli used for preparing antigen (HCl-extracts)	Nature of antigen (teichoic acid preparation dilution 0·1 mg./ml.)	Agar-gel precipitin reaction. Specific antiserum: Group A	Group D Reaction	Group E
A	*L. helveticus* NCIB 8025, NIRD E8, H19; *L. jugurti* NCIB 2889, 8115, NCDO 999		+ Reactions of identity	−	−
		Cell contents NCIB 8025, NCIB 2889	+	−	−
		Cell wall NCIB 8025, NCIB 2889	−	−	−
D	*L. plantarum* NCIB 7220, 6376, NIRD A164, P12		−	+ Reaction of identity	+ weak
		Cell wall NCIB 7220	−	+	−
E	*L. lactis* NCIB 7278, NIRD L2, L10, AH7		−	+ weak	+ Reactions of identity
	L. bulgaricus NIRD, B8, B17		−	−	+
	L. buchneri NCIB 8007		−	−	+
	L. brevis NCIB 6107, NIRD X10, T6		−	+ weak	+
		Cell wall *L. lactis* NCIB 7278	−	+ weak	+
		Cell wall *L. buchneri* NCIB 8007	−	−	+
		Cell wall *L. bulgaricus* NIRD B9	−	−	+
	Staphylococcus epidermidis	Cell wall *S. epidermidis*	−	−	−

None of the teichoic acid preparations showed any reaction with antisera for groups B, C, F or G.

wall teichoic acid. Results are given in Table 3; reactions were specific in that teichoic acids from groups A, D and E showed no reactivity with antiserum for groups B, C, F, or G, and all sera were negative when examined with a chemically similar teichoic acid from walls of *Staphylococcus epidermidis*.

DISCUSSION

Knowledge of the serological composition of lactobacilli enables their division in broad groups (Sharpe, 1955*b*; Sharpe & Wheater, 1957; Rogosa & Sharpe, 1959). Satisfactory grouping sera are not, however, available for all species of lactobacilli and certain widely divergent species such as *Lactobacillus lactis*, *L. bulgaricus* on the one hand and *L. brevis* and *L. buchneri* on the other share the group E antigen. More detailed investigation of the chemical composition of teichoic acids in the walls of some species of lactobacilli (Baddiley & Davison, 1961) has shown that walls of the homofermentative species, *L. lactis* and *L. bulgaricus*, and the heterofermentative species, *L. brevis* and *L. buchneri*, of group E have a chemically similar glycerol teichoic acid containing glucosyl residues. Moreover, the gel-diffusion reaction of identity of teichoic acid from these strains and group-specific material extracted with hydrochloric acid (Sharpe, 1955*b*) from whole organisms of these and additional strains in group E clearly indicates that the wall teichoic acid is the group E precipitinogen.

Similarly, ribitol teichoic acid from the single group D species *Lactobacillus plantarum* is the group substance. Teichoic acid from *L. plantarum* NCIB 7220 is structurally similar to that previously isolated from the walls of strain 17-5 by Archibald *et al.* (1961). Walls of this latter strain, however, contain a polysaccharide which was not found in strain 7220, and therefore presumably could be a type-specific component. Investigation of other strains in group D is in progress. Some cross-reactions occurred between wall teichoic acid preparations from group E and antiserum for group D. Cross-reactions have previously been observed between group E antiserum and HCl extracts of organisms in group D, and between group D antiserum and HCl extracts of organisms in group E. The possibility that in these cases cross-reactions may be due to the intracellular teichoic acids is under investigation.

By contrast, the group A precipitinogen is the intracellular glycerol teichoic acid and not the wall teichoic acid. Intracellular teichoic acid from the group A species *Lactobacillus helveticus* and *L. jugurti* was atypical in its behaviour during isolation: it was not sedimented by centrifugation at 100,000 *g* as is the case with all other intracellular teichoic acids investigated (Davison & Baddiley, 1963; RajBhandary & Baddiley, 1963; Wicken, Elliott & Baddiley, 1963; Critchley *et al.* 1962). The term 'intracellular' is used to indicate that these teichoic acids are not in the wall but are isolated from the cell contents fraction of disrupted organisms. In at least two cases these compounds are believed to be located between the wall and the protoplast membrane (Hay, Wicken & Baddiley, 1963).

The absence of serological reactivity and the low sugar content of glycerol teichoic acid from walls of lactobacilli in group A indicate that appreciable amounts of sugar must be attached to a teichoic acid in order to confer immunological activity; moreover, with *Lactobacillus* spp. specificity must be partly dependent on

glycosidic configuration, since a chemically similar glycerol teichoic acid possessing glucosyl residues from the walls of *Staphylococcus epidermidis* gave no reaction with group E or any other group antisera. Lactobacilli in groups B, C, F and G contain no wall teichoic acid and, whereas the group B and C precipitinogens are serologically distinct polysaccharides (Knox, 1963), preliminary evidence suggests that in group F intracellular teichoic acid could be the group-specific substance. It seems unlikely that purified teichoic acids would themselves engender an immunological response *in vivo*. They probably behave rather as haptenes and therefore are in that respect similar to the group-specific polysaccharides of streptococci of groups A and C.

The serological, and possibly chemical, identity of teichoic acid preparations from the walls of lactobacilli in group E supports the suggestion (Davis, 1936) that the biochemically dissimilar members of the group may have had a common origin, and the differentiation of homofermentative and heterofermentative species into separate genera might be misleading (Rogosa *et al.* 1953). Thus, knowledge of the structure of wall and intracellular teichoic acids provides a useful method for the classification of these organisms and may assist in establishing relationships of groups which are not yet fully understood.

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The Mycococcus Form of Mycobacteria

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SUMMARY

Cultures of form 2 mycobacteria, which had been recently isolated from form 1 of strains of *Mycobacterium tuberculosis* and atypical mycobacteria, were cultivated on nutrient agar in screw-capped bottles and kept unopened for 6 weeks at room temperature. Gram-positive granules developed in organisms which had penetrated into the medium. When these organisms were incubated in digest broth at 37° with intermittent aeration, the granules were liberated into the medium and gradually increased in size to that of a small coccus. After a resting period of about 2 weeks, the 'cocci' started to multiply independently, forming diplococci and tetrads. At this stage the cocci yielded colonies on subculture on nutrient agar. The cocci were identified as members of the genus *Mycococcus*, family *Mycobacteriaceae*, order *Mycobacteriales* (Krassilnikov, 1959). Mycococci were not obtained in broth cultures which were either not aerated or were aerated frequently or continuously. Prolonged subcultivation of the parent form 2 strains on nutrient agar prevented the development of mycococci in broth. Evidence, such as the failure to isolate mycococci from uninoculated but aerated media, is submitted that the mycococci were not contaminants. Previous work on isolation of similar cocci from different members of the order *Actinomycetales*, including human and bovine strains of *M. tuberculosis* is reviewed.

INTRODUCTION

It has been previously reported (Csillag, 1961, 1962) that strains of *Mycobacterium tuberculosis* grown for 14–27 weeks on inspissated or autoclaved Löwenstein–Jensen medium with intermittent aeration yielded, on subculture onto nutrient agar, rapidly growing not acid-fast, endospore-forming organisms (form 2 mycobacteria). In primary isolation the majority of the form 2 mycobacteria were bacilli, but some were cocci (Csillag, 1961). When grown for a short period on nutrient agar, the morphology of the form 2 mycobacteria resembled that of certain Bacillus species (Csillag, 1961), but when cultivated for sufficiently long in slide-culture, so that the bacillary morphology could be examined undisturbed, the life-cycle of these organisms was found to be complex, and resembled that of certain species of the *Actinomycetaceae* (Csillag, 1963*a*). In slide-culture, septate pseudo-branching filaments were first formed; these fragmented first into bacillary elements and later a few of the bacilli fragmented into coccoid elements. In anaerobic culture, the coccoid elements continued to multiply by binary fission, which occurred in any plane, to form diplococci, tetrads and sarcina-like configurations, in which the individual cocci varied considerably in size. The formation of coccoid elements could

not easily be studied in the slide cultures, since it occurred rather infrequently. A further method for producing cocci regularly and in large numbers was therefore elaborated. This method, described here, and referred to elsewhere (Csillag, 1962, 1963*b*, *c*), has been used for the identification of 131 strains of form 2 mycobacteria during the past 2 years.

METHODS

Organisms

Mycobacterium tuberculosis. Most of the work was done with a form 2 strain isolated (Csillag, 1962, method no. 2) from a drug-sensitive nicotinamide-positive form 1 strain (I 1413) obtained from the sputum of a British patient with pulmonary tuberculosis. The morphology in slide culture and certain other characteristics of this form 2 strain have been described (Csillag, 1963*a*, *b*). A further 23 strains were obtained from newly diagnosed untreated British patients with pulmonary tuberculosis, and 18 strains from corresponding South Indian patients participating in chemotherapy studies at the Tuberculosis Chemotherapy Centre, Madras. A BCG strain (Statens Seruminstitut, Copenhagen) and strain H 37 RV were included.

Other mycobacteria. Group I (Runyon, 1959) anonymous mycobacteria, 3 strains; group II, 2 strains; group III, 3 strains; group IV, 5 strains, obtained from Dr E. H. Runyon, Salt Lake City, Utah, or described by Selkon & Mitchison (1959).

Bacillus licheniformis (NCTC 1158) and Bacillus subtilis (NCTC 2591)

Media. (1) Hartley's digest broth (*Mackie & McCartney's Handbook*, 1960) was prepared with the following modifications: (*a*) distilled water was used instead of tap water; (*b*) a double amount of pancreatic extract was used. The medium was dispensed in 8 ml. amounts in $\frac{1}{2}$-oz. bottles with screw-caps, which were checked for tight fitting. (2) Nutrient agar was prepared by addition of 1·4% (w/v) agar to meat extract + peptone-broth (Oxoid No. 2, Oxo Ltd., London), was dispensed in 4 ml. amounts in $\frac{1}{2}$-oz. screw-capped bottles. (3) Peptone broth was prepared by adding 1% peptone (Evans Medical Ltd., Liverpool) to the digest broth and was dispensed in 10 ml. amounts in $6 \times \frac{5}{8}$ in. test tubes plugged with cottonwool. (4) Peptone agar plates were made with nutrient agar containing an additional 1% peptone. All media were autoclaved at 120° for 15 min. (5) Human blood medium as described by Tarshis *et al.* (1955), but without penicillin or agar, was dispensed in 8 ml. amounts in $\frac{1}{2}$-oz. screw-capped bottles. (6) Undiluted horse serum, sterilized by filtration, was dispensed in 8 ml. amounts in $\frac{1}{2}$-oz. screw-capped bottles. All media were incubated before use at 37° for 4 days and at room temperature for another 3 days, as a test of sterility.

Manipulation and cultivation. Inoculation and aeration of the cultures was done in an arm-hole cabinet, previously irradiated with ultraviolet (u.v.) radiation. Plates were sealed in polythene bags during incubation. All cultures were incubated at 37° unless otherwise stated.

Staining methods. Smears were allowed to dry under u.v. lamp and were fixed in methanol. Jensen's method for Gram staining (*Mackie & McCartney's Handbook*, 1960) was modified by decolorizing rapidly with acetone and counterstaining with

dilute (1/15) carbol fuchsin. All dye solutions were filtered through filter paper (Whatman, No. 2) immediately before use.

Isolation of cocci from form 2 mycobacteria. Cocci were obtained from form 2 mycobacteria as follows. Soon after its isolation from the form 1 strain, the form 2 culture was purified twice by single colony selection on nutrient agar. After incubation for 2 days, a colony from the second plate ('initial culture') was inoculated on to a nutrient agar slope, which was incubated for 3 days with the cap loose until sporulation had occurred. The cap was tightened and the slope left unopened at room temperature for 6 weeks ('old culture'). About 2 mg. (moist weight) of the growth, including portions of the colonies that ramified below the surface of the medium, was added to bottles containing 0·4 ml. sterile distilled water and glass beads. After the bottles had been shaken mechanically for 1 min., about 0·1 ml. of the suspension was inoculated into several screw-capped bottles of digest broth ('test cultures'). The test cultures were then incubated, and were aerated, first at the 7th day after inoculation and therafter once a week. Aeration was performed by flaming the cap, removing the cap for about 20 sec. while holding the opening of the bottle over the flame, and then replacing the cap firmly. The bottles were then swung gently by hand to settle the pellicle of growth while leaving the sediment undisturbed and were incubated, with particular care to avoid accidental shaking. At each aeration, one of the test cultures was investigated by removing, with a Pasteur pipette, macroscopically visible floccules of the undisturbed sediment, some of which were stained, and the remainder inoculated on two nutrient agar plates. These plates were incubated for 1 day. Each test culture was discarded after sampling.

The following controls were always included. (1) Inoculum controls: all cultures used for inoculating other media (initial culture, shaken suspension) were each spread on 8–10 nutrient agar plates, which were incubated for 2 days. Colonies were examined and smears made from 5–6 isolated colonies and from the pool of each plate to exclude the presence of cocci. (2) Contamination controls: the same number of bottles of digest broth as were used for the test cultures were 'inoculated' with a sterile loop and were subjected to all subsequent procedures (including aeration) in the same manner.

RESULTS

Origin of cocci from form 2 mycobacteria

The colonial and bacillary morphology of the cultures of form 2 (strain I 1413) which preceded the test cultures were as follows:

(1) *Initial culture.* The colonies were 3–6 mm. diam. at 48 hr with a finely granular, 'beaten-copper' surface (Pl. 1, fig. 1). The growth was easily emulsified, but the colonies ramified under the surface of the medium and could not be removed without disrupting it. The colonies were composed of Gram-negative rods which sometimes contained endospores, but never contained granules (Pl. 1, fig. 2).

(2) *Old cultures.* After incubation at room temperature for 6 weeks, the old culture consisted of free endospores and a few Gram-variable vegetative organisms, some of which contained Gram-positive granules (0·1–0·3μ diam.).

(3) *Shaken suspension.* After shaking, the suspension of the old culture contained extra-cellular Gram-positive granules (0·1–0·3μ diam.). Cocci were never obtained from cultures in which liberated granules were not present. Deviation from the

procedure described above often resulted in a failure to yield granules or in their disappearance from the culture.

(4) *Test cultures.* After incubation for 24 hr, the medium of the test cultures was unchanged except for a slight flocculent deposit. At 7 days, and after each subsequent aeration, a thin surface pellicle was formed, which submerged during the shaking. After 4 weeks, no new pellicle was formed and the medium became turbid.

In the serial smears from the test cultures, particles of the sedimented pellicle stained slightly pink. The pink patches from the 7-day test cultures contained uniformly stained Gram-variable rods, often long (Pl. 1, fig. 3), and occasional endospores. At 14 days, the filamentous rods had fragmented into short rods, some of which contained Gram-positive granules, most often in the poles (Pl. 1, fig. 4). Free endospores were still present. At 21 days, more granules were present in the rods and some were extracellular (Pl. 1, fig. 5). At about 28 days, a greater proportion of the granules had been liberated and these varied in size from just visible to small circular cocci (0·7 μ diam.; Pl. 1, fig. 6). At about 35 days, many of the rods began to disintegrate, leaving chains of distinctly stained granules surrounded by an indistinct pink cell wall (Pl. 1, fig. 7). At the same time, some of the free cocci elongated (Pl. 1, fig. 8). At 42 days, some of the cocci divided to form diplococci (Pl. 1, fig. 9), tetrads or irregular clumps. Gram-variable rods, which stained weakly, were also present, but no endospores. At 60 days, when the experiment was terminated, most of the cultures consisted entirely of cocci; a few still contained rods undergoing lysis.

Subculture of the test cultures on to nutrient agar plates yielded the following results. When the test cultures had been incubated for less than 42 days and contained intracellular or extracellular granules which had not started independent division, the colonies on nutrient agar were similar to those described for the initial culture (Pl. 1, fig. 1). However, when the large free-lying coccal elements had begun to multiply to form diplococci or tetrads (42 days), subculture yielded an additional type of colony. These colonies were variable in size (0·3–1·0 mm. diam.), circular with an entire edge, raised, white and smooth (Pl. 1, fig. 10). Smears from the colonies showed Gram-positive cocci of uneven shape (circular, ovoid, irregular) and size (0·3–0·7 μ diam.), lying singly, in pairs or in irregular clusters (Pl. 1, fig. 11). No cocci were seen on the inoculum-control plates or in the smears made from them. The contamination-control plates remained sterile.

Description of the coccal strain

Soon after its isolation the coccal strain was purified three times by single colony selection on nutrient agar. After incubation for 2 days, a colony was inoculated on to 10 peptone agar plates and into 10 peptone broth tubes. The plates were incubated at 37° for 1 day and then kept at room temperature for a further 13 days; the tubes were incubated at 37° for 14 days. Half of the tubes and plates were examined at 1 day and the other half at 14 days.

The colonies on the plates at 1 day were similar to those obtained in the first isolate, but they were more irregular in size (0·3–1·3 mm. diam.; Pl. 2, fig. 1). At 14 days, the colonies had increased in size (1·0–5·0 mm. diam.), their edges were slightly irregular and the surface was differentiated into a central dense area, an intermediate, less opaque zone and a translucent periphery (Pl. 2, fig. 2); daughter

colonies were often present on the surface. Smears from the 1-day colonies showed circular or slightly elongated Gram-positive cocci, arranged singly, in pairs or in clusters; their size was variable (0·4–1·0μ diam.; Pl. 2, fig. 3). The cocci on the 14-day plates were still Gram-positive; their shape was more irregular than before, with thick (Pl. 2, fig. 4) or thin (Pl. 2, fig. 5) elongated forms present. Some of the cocci appeared to be dividing by binary fission in successive planes at right angles to each other (Pl. 2, fig. 6).

The peptone broth cultures were, at 1 day, slightly turbid with a powdery sediment; at 14 days, the turbidity had increased and the sediment was very mucoid. The morphology of the cocci at 1 day was similar to that on the solid medium, but at 14 days the shape of the organisms was more variable. Crescent-shaped (Pl. 2, figs. 7 and 8), triangular (Pl. 2, fig. 9), pinched or nearly rectangular organisms were observed. Small numbers of Gram-variable fine short rods were also present (Pl. 2, fig. 10). Some of the cocci appeared ring-shaped, being unstained at the centre; some cocci were more deeply stained than the rest and a few of these produced short germination tubes (Pl. 2, fig. 7). In a few organisms, multiplication by lateral budding was observed (Pl. 2, fig. 10). A broth culture was sterilized by autoclaving for 5 min. at 15 lb./sq.in. After incubation for 14 days the peptone broth cultures and 4 colonies from each plate were plated out on nutrient agar. Only colonies of cocci, similar to those described above, were obtained. On the basis of the above description the coccal strain was identified as belonging to the genus *Mycococcus*, family *Mycobacteriaceae*, order *Mycobacteriales* (Krassilnikov, 1959).

Influence of aeration on the isolation of mycococci

The influence of aeration on the isolation of mycococci from the form 2 of strain I 1413 was studied in three experiments. (1) Cottonwool plugs were inserted in the neck of 12 test cultures, below the screw caps. These cultures were aerated intermittently (in the rhythm previously described) by loosening the caps and leaving them loose for 3 hr. Mycococci were isolated on nutrient agar from 9 of the 12 test cultures within 28 days. The cottonwool plugs in the necks of the remaining 3 cultures became soaked with broth and mycococci were not isolated from these within 40 days. (2) A further group of 12 test cultures was divided into 6 cultures which were aerated intermittently, but without cottonwool plugs, and 6 which were not aerated. The cultures that were aerated intermittently all yielded mycococci within 41 days, while the cultures that were not aerated did not develop pellicles and did not yield mycococci within 90 days. (3) A further group of 18 test cultures was divided into three groups of 6 each, which were either (i) aerated intermittently (as described above) without plugs, (ii) were aerated three times a day without plugs, or (iii) were exposed in a moist chamber to continuous aeration through plugs (the bottles not being capped). Mycococci were isolated from all of the first group of cultures (aerated intermittently) within 32 days, but were not isolated from any of the remaining cultures within 50 days.

Multiplication of mycococci in digest broth in the absence of form 2 mycobacteria

A 5-hr shaken, digest broth culture of the mycococcus of strain I 1413 was serially diluted in tenfold steps. From each dilution, 0·2 ml. was added to each of two bottles of digest broth, which were incubated for 1 day or 4 days, with their

caps closed. After incubation, 0·2 ml. from each bottle was plated on nutrient agar. The bottles seeded from the 10^{-6} and the 10^{-7} dilutions yielded innumerable colonies on nutrient agar when incubated for 1 day. Of the bottles seeded with the 10^{-8} dilution, the one incubated for 1 day yielded 5 colonies, and the one incubated for 4 days yielded 142 colonies. The bottles seeded with the 10^{-9} and 10^{-10} dilutions remained sterile. Mycococci in pure culture were also shown to grow freely in plugged test tubes in an aerobic and in an anaerobic atmosphere.

Stabilization of form 2 mycobacteria by frequent subcultivation

In all experiments described so far, mycococci were obtained from the form 2 strains at 6 months or less after their isolation from the form 1 cultures of strain I 1413. However, in further experiments, the form 2 strain was maintained, as described previously (Csillag, 1963*a*) for longer periods, by successive subcultivation on nutrient agar at intervals of 2 months. The mean period of incubation of the test cultures until mycococci were first isolated was 42 days for form 2 strain which had been maintained for 6 months, 51 days for a strain maintained for 11 months, 63 days for a strain maintained for 20 months, and mycococci were not isolated within 85 days when the form 2 strain had been maintained for 28 months.

Isolation of mycococci from further form 2 strains

Mycococci were isolated from the form 2 of 10 strains of mycobacteria (3 British and 3 Indian strains of *Mycobacterium tuberculosis*, strain BCG, 2 strains of group I and 1 strain of group III anonymous mycobacteria), first at 2 months after their isolation from the form 1 cultures and again after the form 2 strains had been maintained for 12 months and for 19 months. Strains of *Bacillus subtilis* and *B. licheniformis* were included in the first two experiments. In the first experiment, mycococci were isolated from the BCG strain after incubating the test cultures for 7 days, from the Indian and the anonymous strain, at between 13 and 24 days, and from the British strains after 33 days. In the second experiment, mycococci emerged more slowly, but were obtained first from strain BCG and last from the British strains. In the third experiment, only strain BCG yielded mycococci within 42 days. The Bacillus species did not yield mycococci.

Further experiments with the form 2 of 131 different strains of mycobacteria showed that most British strains of *Mycobacterium tuberculosis* yielded mycococci for the first time at 6 to 8 weeks, but a few yielded mycococci within 4 weeks or after 12 weeks. Mycococci were obtained for the first time at 2–4 weeks with Indian strains. The speed of emergence of mycococci was different in different test cultures of the same strain in the same experiment. It seemed probable that mycococci were obtained more rapidly when the 'old culture' had been left for long periods at room temperature.

Production of mycococci in blood and serum media. In further experiments similar to those described above, mycococci were obtained from test cultures of 57 form 2 strains in which human blood medium or undiluted horse serum was used in place of digest broth.

DISCUSSION

The evidence that the mycococci were derived from the form 2 mycobacteria and did not arise from contaminants present in the inoculum, the culture medium or gaining access during aeration, is as follows. Mycococci were obtained only from test cultures which were aerated once a week and not from those aerated more frequently; contaminants would be expected to grow at least as frequently in the latter cultures as in the former. Further, the development of the cocci was followed in smears; free cocci were visible in the cultures at about the 28th day of incubation but were not viable on subculture until after another fortnight; contaminating organisms would be unlikely to show this 'resting period'. Finally, the procedure described has resulted in the isolation of mycococci from all of the numerous form 2 cultures studied. No other organisms have been isolated from these cultures and it would be remarkable if mycococci were the only type of contaminants to be found.

There are certain other reasons for the specific exclusion of the inoculum used for the test cultures as being contaminated with cocci. The cultures used for preparation of the suspension had been purified by single colony selection. The suspension itself was plated out on nutrient agar for the presence of cocci and these were never found. If contaminants had been present, growth of cocci would have been expected in the first few days of incubation of the test cultures, but they were isolated only at about 42 days. Finally, cocci were never obtained from cultures which had not been aerated, even though a small inoculum of a pure culture of mycococci grew freely under these conditions.

Contamination of the medium at the start of the experiments or during aeration is also unlikely since about 800 control cultures (in 20 separate experiments) which were 'inoculated' with a sterile loop and were aerated in the same manner as the test cultures failed to yield cocci. Furthermore, if the cocci were contaminants of this type, they would have been obtained from the form 2 cultures that had become stabilized. Finally, it might be argued that the cocci gained access to the cultures as contaminants, but were only able to grow in association with the form 2 organisms. However, the cocci grew freely from small inocula in the absence of the form 2 bacilli.

The serial examination of smears indicates that mycococci might have developed from form 2 mycobacteria by the following process. In the 'old cultures' some of the vegetative cells, mainly those which penetrated into the medium, developed intracellular granules, which were liberated during the shaking with glass beads. In the test cultures these granules, together with those which developed later in the sedimented pellicle, were incubated under microaerophilic conditions at the bottom of the tubes, where they gradually increased in size. After a long resting period, the cocci started to multiply independently and at this stage were capable of forming colonies on subculture to nutrient agar. Microaerophilic conditions appeared necessary, since mycococci were not isolated when the test cultures were aerated either continuously or three times daily, nor were they isolated when the test cultures were incubated with their caps closed throughout.

Frequent subcultivation of the parent form 2 strains resulted in the loss of the ability to produce mycococci, that is, in stabilization of the strain in the form of a spore-bearing rod. The same phenomenon was reported earlier (Csillag, 1963*b*),

when it was also observed that stabilized cultures failed to yield pseudobranching filaments in slide culture. It is not yet known whether stabilization is a reversible process. If it were irreversible, some form 2 mycobacteria might be misclassified as bacillaceae. On the other hand, if it were reversible, organisms classified as bacillaceae might occasionally be found to produce mycococci. This could be the explanation of the findings of Appleby (1939) who isolated cocci which were morphologically similar to those described in the present paper, from aerated broth cultures of a Bacillus species. Moustardier, Dulong de Rosnay, Pasquier & Latrille (1962) also observed cocci in cultures of *Bacillus mesentericus*, but these cocci did not multiply independently.

The cocci isolated from the form 2 mycobacteria were identified as mycococci on the basis of the description of Krassilnikov (1959) who regards mycococci as 'degraded' mycobacteria. In this description mycococci are defined as Gram-positive cocci, whose most characteristic feature is the great variability in the shape and size of the cells within the same culture. They multiply by fission, lateral budding and by germination tubes which are produced by the resting cells in old cultures. Some species produce saltants which are indistinguishable from mycobacteria. On ordinary media mycococci are so closely similar to micrococci that 'it is easily possible that a number of the so-called micrococci belong in fact to the mycococci'. Krassilnikov (1934) found that only prolonged cultivation can reveal the true nature of mycococci and enable them to be distinguished from micrococci. According to Demikhovskii (1961) bacilliform cells can be found in all cultures of mycococci and these remain present even after serial purification. According to Nellis (1955) the germination of the resting cells of mycococci should be considered as analogous to the germination of the conidiospores of the Actinomycetes. Waksman (1961) also included the genus *Mycococcus* within the *Actinomycetales*, but did not regard them as 'true' Actinomycetes. In a tentative phylogeny of *Actinomycetales*, Jensen (1953) suggested that the starting point might be a coccoid organism leading via mycococci and coryneform bacteria to the acid-fast mycobacteria and to the potentially acid-fast nocardia.

Similar observations to those described in the present paper have been reported earlier with different members of the families *Actinomycetaceae* and *Mycobacteriaceae*. In cultures of some *Actinomycetaceae* cocci appear regularly as a result of fragmentation of the vegetative mycelium (Henrici, 1947; Waksman, 1961). With some strains, cocci are produced in such a large number that, after prolonged incubation, nothing but cocci are seen in smears (Topping, 1937). The cocci are similar in shape and size to the spherical bacteria (Waksman, 1959) and, after being produced, continue to divide by binary fission (Henrici, 1947). When subcultivated into suitable medium the cocci usually develop into fresh mycelium either by forming germination tubes or by lateral budding (Waksman, 1961), but under exceptional circumstances they can be maintained in subcultures for several generations as cocci and even 'stabilization in this form is not improbable' (Lieske, 1921). Coccal strains were isolated from 16 strains by Koelz (1933) and from further strains by Luksch (1930) and Novak & Henrici (1933). Colien (1935) obtained a coccus from a human throat. This coccus then yielded a filamentous organism which reverted later to the original coccal form. The coccus was reinvestigated by Gillespie & Bowen (1936) and was identified as the final form of an Actinomyces-like organism. Some

authors (for instance, Waksman, 1959) considered that cocci, which had been isolated from *Actinomycetaceae*, were mutants or variants, but others (Krassilnikov, cit. Waksman, 1959) regarded these cocci as a stage in the normal development of nocardia, rather than as mutants.

The appearance of cocci in cultures of mycobacteria had also been reported by several authors (Sweany, 1928; Kahn, 1929; Karwacki, 1929; Fontes, 1931; Pla Y Armengol, 1931; Vaudremer, 1931; Lindegren & Mellon, 1933; Mellon, Richardson & Fisher, 1933; Weissfeiler & Dwolaitskaja-Berischew, 1935; Kölbel, 1951; Xalabarder, 1954, 1958; Juhasz, 1962; Mazet, 1962). In most instances these cocci were described as being uneven in size and appearing in pairs or in tetrads. Since tetrads were observed, the organisms must have been capable of dividing in different planes, a characteristic regarded as essential in the identification of a coccus (Bisset, 1962) and differentiating the organism from short rods, which divide only in a single plane. Most authors regarded the appearance of cocci either as a part of the life-cycle of mycobacteria or as mutants. Some authors suggested that the cocci originate from the liberation of the Gram-positive granules which were first described in cells of mycobacteria by Much (1907, 1931). Sweany (1926, 1928) isolated granules with a micromanipulator and followed their development into cocci in hanging drop cultures. Vaudremer (1931) confirmed the results of Sweany. On the basis of investigations with the electron microscope, Xalabarder (1954, 1958) regarded the cocci as disjoint conidia, liberated from the micromycelium of mycobacteria. There are some reports on the isolation of cocci from cultures of mycobacteria and their subsequent maintenance in coccal form (Pla Y Armengol, 1931; Vaudremer, 1923; Krassilnikov, 1934; Kirchner, 1928; Møllgaard, 1931). Møllgaard isolated cocci from mycobacteria in two steps, closely similar to those described by the present author. He cultured mycobacteria (human and bovine strains) in yeast extract and obtained rapidly growing, not acid-fast, Gram-negative rods which later developed Gram-positive granules. The granular rods were later subcultivated in broth, where the granules developed into diplococci. The cocci which were isolated by these authors were not, however, described in sufficient detail to identify them with certainty as mycococci.

Further experiments to be described in due course have shown that the mycococci can be reverted to form 1 (acid-fast) and form 2 (spore-bearing) mycobacteria.

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EXPLANATION OF PLATES

PLATE 1

Figs. 1–2. Morphology of a form 2 strain of *M. tuberculosis* (I 1413) on nutrient agar, after incubation for 2 days ('initial culture').

Fig. 1. Colonial morphology, × 3·4.

Fig. 2. Cellular morphology, Gram stain, × 950. Gram negative rods with or without endospores. Intracellular granules are not present.

Figs. 3–9. Various stages of the development of mycococci in the intermittently aerated digest broth cultures of the form 2 strain, Gram stain, × 950 ('test cultures').

Fig. 3. 7 days. Uniformly stained Gram-variable rods, often long.

Fig. 4. 14 days. Some of the cells contain intracellular, Gram-positive granules.

Fig. 5. 21 days. Some of the granules are extracellular.

Fig. 6. 28 days. Some of the extracellular granules reach the size of 0·7 μ diam.

Fig. 7. 35 days. Chains of granules within disintegrating rods.

Fig. 8. 35 days. Some free cocci are elongated.

Fig. 9. 42 days. Cocci have divided to form diplococci.

Figs. 10–11. Morphology of the mycococcus isolated from the 42 days old test culture, after incubation for 2 days on nutrient agar.

Fig. 10. Colonial morphology, × 3·4.

Fig. 11. Cellular morphology, Gram stain, × 950. Gram-positive cocci with irregular shape and size.

PLATE 2

Figs. 1–10. Morphology of the mycococcus, isolated from the test cultures.

Figs. 1–2. Colonial morphology on peptone agar plates, × 3·4.

Fig. 1. 1 day. Size of colonies ranges between 0·3–1·3 mm. diam.

Fig. 2. 14 days. Size of colonies ranges between 1·5–5·0 mm. diam. Colonies differentiated by concentric rings into three sectors.

Figs. 3–6. Cellular morphology on peptone-agar plates. Figs. 3–5. Gram stain. Fig. 6. Ziehl–Neelsen stain, × 2000.

Fig. 3. 1 day. Size of cocci variable (0·4–1·0 μ diam.).

Fig. 4. 14 days. Some thick, elongated forms.

Fig. 5. 14 days. Some fine, thin elongated forms.

Fig. 6. 14 days. Division by binary fission in successive planes.

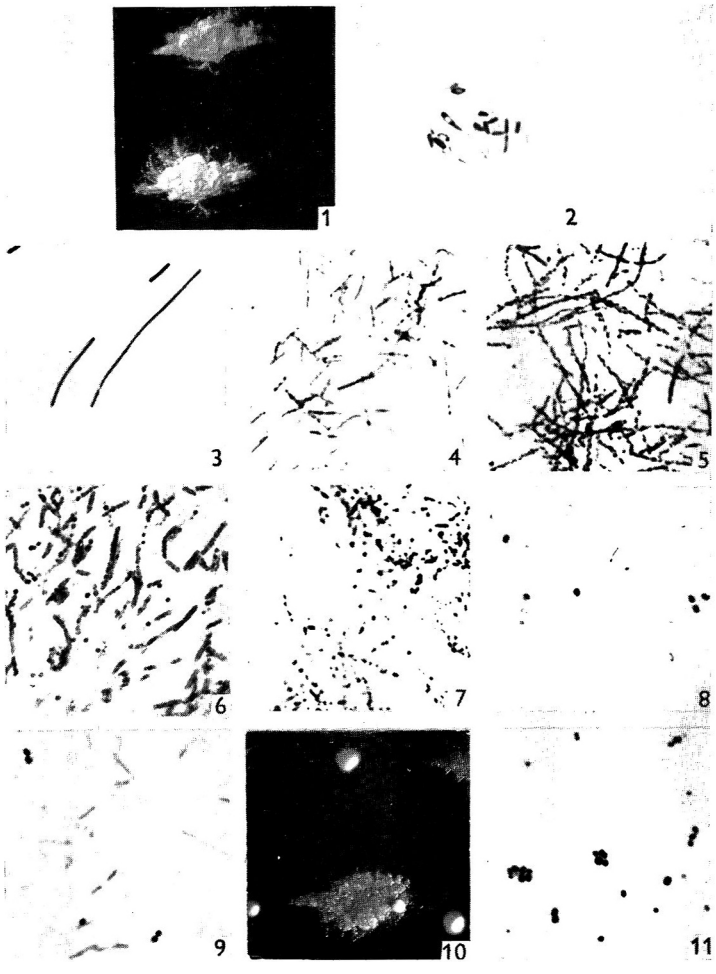
Figs. 7–10. Cellular morphology in peptone-broth. 14 days, Gram stain, × 2000.

Fig. 7. Crescent-shaped form; one coccus has a short germination tube.

Fig. 8. Crescent-shaped form.

Fig. 9. Triangular form.

Fig. 10. Fine, short rods. Multiplication by lateral budding.



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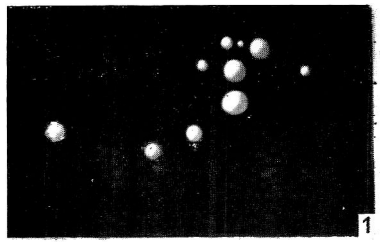
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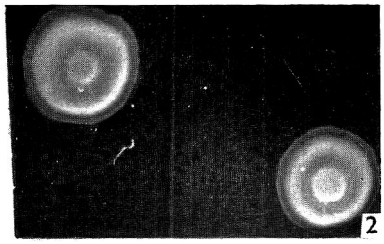
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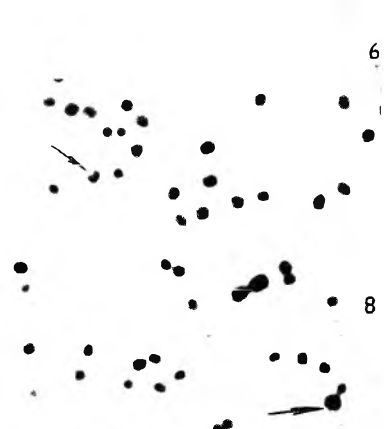
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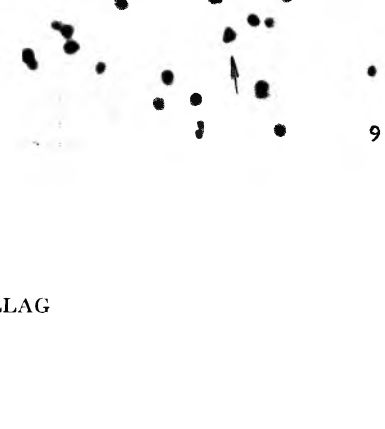
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