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The Amino Acid Requirements of a Single Strain of *Actinomyces israelii* Growing in a Chemically Defined Medium

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SUMMARY

The Wills strain of *Actinomyces israelii* will survive repeated subculture in the medium HP 9 described by Christie & Porteous (1962*b*) but modified to contain ammonium sulphate and only seven amino acids (serine, cysteine, glutamic acid, lysine, leucine, isoleucine and tryptophan); the total–N content of this new medium was 240 μg./ml. On the evidence available this new medium probably represents the minimal medium in respect of amino acids (and growth factors) for this strain of *Actinomyces israelii*. The yield of organism from this new medium was comparable with that reported by Christie & Porteous (1962*a*) for more complex media, namely approximately 70 μg. cell total-N/ml. medium. Some observations are presented on inhibition of growth of *Actinomyces israelii* by 'growth factors' and by purine and pyrimidine bases.

INTRODUCTION

Christie & Porteous (1962*a*) described a medium HP6/B, referred to hereafter as medium CP1 (Table 1). This medium contained 613 μg. total-N/ml. and sustained the Wills strain of *Actinomyces israelii* in repeated anaerobic subculture with a yield of organism equivalent to 70 μg. total-N/ml. medium. L-Cysteine and L-tryptophan were shown to be essential nutrients of this single strain of *A. israelii* when grown in the presence of all sixteen growth factors of medium CP1 (Table 1). In semi-quantitative experiments it was shown (Christie & Porteous, 1962*b*) that of the sixteen growth factors present in medium CP1, only five were essential for the growth of this strain of *A. israelii*. Medium CP1 modified to contain these five growth factors is referred to hereafter as medium CP 2 (Table 1). These semi-quantitative experiments have now been put on to a quantitative basis. It has also been shown that only seven of the amino acids of medium CP 2 are required to sustain the Wills strain through at least ten serial cultures, when the medium is supplemented with ammonium sulphate.

METHODS

With the following exceptions and additions, the materials and techniques used in the present work were those described by Christie & Porteous (1960, 1962*a*, *b*).

Organism. The Wills strain of *Actinomyces israelii* (Christie & Porteous, 1962*a*) was used.

Media and analytical solutions. These were made up in de-ionized water; all amino acids were the L-isomers.

Preparation of inoculum suspensions of Actinomyces israelii, *and quantitative inoculation of experimental media.* The technique described by Christie & Porteous (1960) was used with slight modifications: (i) a chemically defined medium was used in both stages of the stirred culture method of preparing inoculum suspensions; (ii) the colonies were harvested from the first stage culture when the medium fell to pH 5·3, i.e. at the end of the rapid growth phase (Christie & Porteous, 1962*a*); (iii) these harvested colonies were washed aseptically with 3 × 10 ml. volumes of sterile distilled water before being transferred to the second stage medium; (iv) when the pH of the second stage medium fell to a value of 5·3 colonies were again harvested and washed as before. Quantitative inoculation of this final suspension of colonies into experimental media was carried out as described by Christie & Porteous (1960).

α-Amino-nitrogen determinations. These were carried out by the method of Yemm & Cocking (1955).

Amino acid chromatography. Zeo-Karb 225 ion-exchange columns (5·0 cm. × 0·8 cm.) were freshly converted to the H-ion form and washed with de-ionized water in the usual way; a measured volume of medium (3–5 ml.) was added to the column and inorganic salts eluted with de-ionized water. The amino acids were eluted with N-ammonia; elution was continued until tests with microlitre volumes of eluate spotted on to filter paper and dried in a current of warm air showed that no more ninhydrin-positive material was leaving the column. The complete ammonia eluate was shell-frozen in solid carbon dioxide + ethanol mixture and dried from the frozen state *in vacuo* (0·1 mm. Hg) over solid KOH and conc. H_2SO_4. The solid residue was dissolved in 500 μl. de-ionized water. Control experiments showed that this procedure removed ammonia from the eluate thus permitting determination of the recovery of α-amino-N in the eluate (Kornberg & Patey, 1957). The shell-freezing and drying technique permitted convenient concentration of amino acid solutions before application of a known volume (usually 15 μl.) containing 50–75 μ-g. total α-amino-N to paper chromatograms.

The solvents used for descending two-dimensional chromatography by the technique of Levvy & Chung (1953) were: *n*-butanol + acetic acid + water (4 + 1 + 5 vol.) followed by phenol + *m*-cresol + 0·06 M-borate buffer (pH 9·3) (25 g. + 25 g. + 7 ml.). This solvent pair gave excellent separation, on 46 cm. × 56 cm. sheets of Whatman no. 1 chromatography paper, of all amino acids (Table 1) except methionine, valine, leucine, isoleucine, phenylalanine and tryptophan which formed an overlapping group of three large ninhydrin-positive spots in the corner of the chromatograms diametrically opposite the starting point. The area of paper containing these six amino acids was dried at 40°, cut into strips and the amino acids eluted according to the method of Dent (1947). The combined eluates were ether extracted to remove traces of phenol and *m*-cresol, desalted to remove any borate eluted from the previous chromatograms, then shell-frozen as described. The dry residue was dissolved in 50 μl. de-ionized water before application of the whole solution to a further sheet of Whatman no. 1 chromatography paper, 23 cm. × 28 cm. The solvents used for ascending two-dimensional chromatography were: *tert*-butanol + methylethylketone + water (4 + 4 + 2 vol.) followed by *tert*-butanol + methanol + water (4 + 5 + 1 vol.) as described by Boissonas (1950).

For qualitative detection of amino acids the chromatograms were sprayed with

0·1% (w/v) ninhydrin in water-saturated *n*-butanol (Consden, Gordon & Martin, 1944) to which 1% (v/v) 2:4:6-collidine had been added; the sprayed chromatograms were heated at 80° for 5–10 min. For quantitative work the amino acids were located by the ultraviolet irradiation technique of Fowden (1951), then eluted according to the method of Kornberg & Patey (1957) and determined by the method of Yemm & Cocking (1955).

RESULTS

Amino acid assimilation by a culture of Actinomyces israelii. A rapidly growing culture of the Wills strain in medium CP1 was harvested and washed aseptically with sterile distilled water (3 × 10 ml.). The suspension of organisms was used to prepare an inoculum in medium CP1 by the culture technique described under Methods. The final suspension provided a massive inoculum for an experimental flask containing about 100 ml. medium CP1 sealed with liquid paraffin. This flask, together with a similar flask of uninoculated medium, was incubated at 37°.

Initially the medium was at pH 7·2. The pH value of the medium was measured

Table 1. *The composition of medium CP*1 *and of medium CP*2

		Per litre (g.)	Per litre (mg.)
1. Major salts:	KH_2PO_4	15·0	—
	$MgSO_4.7H_2O$	—	200·0
	$CaCl_2.2H_2O$	—	20·0
	$CH_3COONa.2H_2O$	—	300·0
2. Minor salts:	$FeSO_4.7H_2O$	—	4·0
	$MnSO_4.2H_2O$	—	0·15
	$Na_2MoO_4.2H_2O$	—	0·15
3. Glucose		10·0	—
4. Glutathione		—	100·0
5. Amino acids: Glycine and the L-amino acids: alanine, serine, cysteine.HCl, threonine, leucine, isoleucine, valine, aspartic acid, asparagine, lysine, arginine, histidine, proline, hydroxyproline, phenylalanine, tyrosine, methionine	each	—	200·0
	L-Glutamic acid	—	1000·0
	L-Tryptophan	—	40·0
6. Growth factors: Thiamine.HCl, Ca pantothenate, *p*-aminobenzoic acid, inositol,* riboflavin*	each	—	2·0
Pyridoxamine.HCl, pyridoxal.HCl,* oleic acid, nicotinamide, nicotinic acid*	each	—	1·0
			(μg.)
DL-Thioctic acid, biotin,* pimelic acid	each	—	100·0
Haemin		—	200·0
Folic acid		—	500·0
Vitamin B_{12}		—	1·0

The above components, dissolved in de-ionized water, were brought to pH 7·2 at the pH meter with KOH solution (20%, w/v), made to volume with de-ionized water, the pH checked and the solution immediately sterilized by filtration. Thiol compounds (cysteine, glutathione and thioctic acid) were made up as fresh stock solutions and added to the solution of other components immediately before adjusting the pH; after sterilization the media were immediately dispensed and inoculated, or dispensed and stored under hydrogen.

* Medium CP1 contained all the above components; medium CP2 differed from medium CP1 in that it contained only those growth factors (item 6) marked with an asterisk.

twice daily and the colonies harvested when the pH of the medium reached a value of 5·3, at which point the culture was about to enter the stationary phase. A sample of the early stationary phase medium was filtered through Whatman no. 1 filter paper to remove traces of liquid paraffin, sterilized by filtration through a cellulose acetate membrane (Christie & Porteous, 1962*a*) and subjected, along with a similarly treated sample of uninoculated medium, to chromatographic analysis for amino acids. The results obtained are shown in Table 2; 101 μg. α-amino-N were removed from each millimetre of medium during 6 days of growth. Most of this net decrease in the α-amino-N was accounted for by a decrease in the concentrations of serine, cysteine, glutamic acid, lysine and (leucine + isoleucine), which together accounted for a decrease of 86 μg. α-amino-N/ml. medium. Tryptophan, which was

Table 2. *Changes in the amino acid composition of medium CP*1 (*Table* 1) *after* 6 *days of growth of the Wills strain of* Actinomyces israelii *at* 37° *under liquid paraffin seal*

	μg. α-NH_2-N/ml. medium			μg. amino acid/ml. medium			
		Determined		Determined			
Amino acid	Calcd. 0 (days)	0 (days)	6 (days)	0 (days)	6 (days)	Increase or decrease	% increase or decrease
Glycine	37·3	34·8	39·2	186	210	+ 24	+12·9
Alanine	31·4	29·9	23·6	190	150	− 40	−21·1
Serine	26·6	22·3	11·1	167	83	− 84	−50·3
Cysteine.HCl	17·9	25·9	11·1	292	125	−167	−57·2
Threonine	23·5	23·2	28·1	197	238	+ 41	+20·8
Methionine	18·8	20·1	15·6	213	166	− 47	−22·1
Aspartic acid	21·0	20·1	17·8	190	169	− 21	−11·1
Asparagine	21·2	22·3	22·3	210	210	0	0
Glutamic acid	95·2	85·0	49·0	897	516	−381	−42·4
Lysine	19·2	22·8	11·1	238	116	−122	−51·3
Arginine	16·0	17·3	14·8	215	184	− 31	−14·4
Histidine	18·1	19·8	19·4	219	214	− 5	− 2·3
Valine	23·9	22·3	25·9	186	216	+ 30	+16·1
Leucine/isoleucine	42·6	40·6	28·1	379	262	−117	−30·9
Proline	24·3	22·3	22·3	183	183	0	0
Hydroxyproline	21·4	23·6	17·8	221	167	− 54	−24·4
Phenylalanine	16·9	13·4	9·8	157	115	− 42	−26·7
Tyrosine	15·5	13·4	10·7	173	138	− 35	−20·2
Tryptophan	2·7	—	—	—	—	—	—
Totals	493·5	479·1	377·7	—	—	—	—
% calculated value	—	97	—	—	—	—	—
Net decrease	—	—	101·4	—	—	—	—

present initially at a much lower concentration than the other amino acids, was not estimated in this experiment, but Christie & Porteous (1962*a*) showed that tryptophan (and cysteine) were essential nutrients for this strain of *Actinomyces israelii*. Christie (unpublished) obtained some growth of the Wills strain of *A. israelii* in medium CP1, modified to contain only the seven L-amino acids serine, cysteine, glutamic acid, lysine, leucine, isoleucine and tryptophan, but the organism did not survive more than a few subcultures in this medium. The results shown in Table 2, together with the work of Christie & Porteous (1962*a*), nevertheless suggested that

these seven amino acids belonged to a group which was preferentially removed by the Wills strain from a complete amino acid medium. It was therefore decided to determine the growth curves for the Wills strain in six different media based on medium CP1 and on medium CP2. These six media are listed in Table 3.

Table 3. *The composition of six test media based on medium CP1 and on medium CP2 (Table 1)*

$(NH_4)_2SO_4$ added at a concentration of 200 μg./ml. and the purines and pyrimidines (adenine sulphate, guanine hydrochloride dihydrate, uracil, xanthine and thymine) each at a concentration of 20 μg./ml. (Christie & Porteous, 1962*a*).

Medium	Components
A	Medium CP1 + $(NH_4)_2SO_4$
B	Medium CP1 + $(NH_4)_2SO_4$ + purines and pyrimidines
C	Medium CP2 + $(NH_4)_2SO_4$
D	Medium CP2/7aa* + $(NH_4)_2SO_4$
E	Medium CP1/7aa* + $(NH_4)_2SO_4$
F	Medium CP2/7aa* + $(NH_4)_2SO_4$ + purines and pyrimidines

* Medium CP1 or medium CP2 modified to contain only seven L-amino acids (serine, cysteine, leucine, isoleucine, lysine, glutamic acid and tryptophan) at the concentrations given in Table 1.

Table 4. *The yield of organism obtained from the stationary phase of cultures of the Wills strain of* Actinomyces israelii *in seven defined media (Table 3)*

All cultures entered the stationary phase after about 200 hr. incubation; the approximate lag periods were: media B and C, 60 hr.; media A and D, 70 hr.; E and F, 100 hr. Conditions of incubation are given in the text.

Medium	Yield of organism in stationary phase (μg. total-N/ml. medium)
A	80
B	80
C	70
D	70
E	40
F	42
CP2	70

Growth of the Wills strain of Actinomyces israelii *in modified media.* Medium B was used in the first and second stages of the modified culture technique of Christie & Porteous (1960) described under Methods. The final washed suspension was used for uniform quantitative inoculation of replicate paraffin-sealed tubes of each of seven test media. The results obtained are shown in Table 4.

Media A, B, C, D and CP2 (Tables 1 and 3) gave good yields of *Actinomyces israelii*, comparable with those reported by Christie & Porteous (1962*a*), and growth rates in the four media were very similar. Medium D was the simplest medium, containing only seven amino acids and five growth factors (Tables 1 and 3). Poor yields and growth rates were obtained in media E and F.

At the conclusion of this experiment colonies were subcultured from medium D into tubes of fresh medium D; subcultures were made at 5-day intervals thereafter until the growing colonies had passed through seven serial cultures in this medium. Colonies from twelve tubes each containing 2 ml. medium D were then harvested,

washed as previously described and taken through the first and second stages of the culture technique of Christie & Porteous (1960), using medium D and the slight modifications to the technique already described under Methods, except that the pH value of the second-stage medium was allowed to fall to pH 5·5. The two stages of this culture technique constituted the eighth and ninth serial passage of the growing colonies through medium D. The harvested and washed colonies from the ninth culture were used to provide uniform inocula (Christie & Porteous, 1960) for replicate tubes each containing 2 ml. medium D. The growth curve obtained in this tenth serial culture in medium D is shown in Fig. 1.

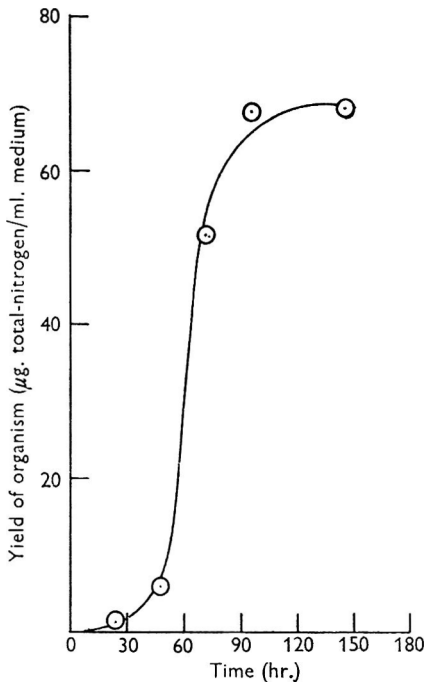

Fig. 1. Growth during the tenth serial subculture of the Wills strain of *Actinomyces israelii* at 37° under liquid paraffin seals in 2 ml. volumes of medium D (Table 3).

The possibility remained that the amino acid complement of medium D might be still further decreased, or that ammonium sulphate could be eliminated as shown by Christie & Porteous (1962*a*) for media containing twenty amino acids. Since chromatographic analysis of medium CP 1 did not distinguish between assimilation of leucine and of isoleucine by *Actinomyces israelii* it was decided to test the requirement for these two amino acids and for ammonium sulphate. Eight media were prepared (Table 5); the basal medium in each case consisted of items 1, 2, 3 and 4 of Table 1 together with the L-amino acids serine, cysteine, lysine, glutamic acid and tryptophan and the growth factors inositol, riboflavin, nicotinic acid, pyridoxal and biotin, at the concentrations given in Table 1. The inoculum suspension of colonies was prepared in medium D (Table 3) in the manner described under Methods. Replicate tubes of each of the media D–M inclusive (Table 5) were uniformly inoculated and growth followed by visual scoring and by determining the

changes in titratable acidity and changes in the pH value of the media during growth. Some growth was obtained in the first culture in all eight media. Very little growth occurred in a second serial culture in media G, L and M and virtually none in media H, I, J, and K; only in medium D was good growth obtained in this subculture. In a third serial culture very little growth was obtained in any medium except medium D.

Table 5. *The composition of eight test media*

For composition of the basal medium, see the text.

	Additions to basal medium		
Medium	Leucine (200 mg./l.)	Isoleucine (200 mg./l.)	$(NH_4)_2SO_4$ (200 mg./l.)
D (Table 3)	+	+	+
G	+	+	−
H	−	+	+
I	−	+	−
J	+	−	+
K	+	−	−
L	−	−	+
M	−	−	−

DISCUSSION

Experiments culminating in the results shown in Fig. 1 demonstrated that medium CP 2 (Table 1) containing twenty amino acids and five growth factors could be further simplified in respect of its amino acid content to give medium D (Table 3) containing only seven amino acids (serine, cysteine, lysine, glutamic acid, leucine, isoleucine, tryptophan) while still sustaining vigorous growth of the Wills strain of *Actinomyces israelii* in serial subculture. Further experiments showed that the leucine, isoleucine and ammonium sulphate were all essential components of medium D (Tables 3 and 5). Christie & Porteous (1962*a*) demonstrated that cysteine and tryptophan were essential nutrients for this organism. It remains to be shown that serine, lysine and glutamic acid were also essential components of medium D; experiments to elucidate this point are planned, but these three amino acids were amongst the group of amino acids, including the essential amino acid cysteine which was assimilated to the greatest extent during growth of *A. israelii* in a complete amino acid medium (Table 2). It seems probable therefore that the seven amino acids of medium D (Table 3) constitute the minimal amino acid requirement of the Wills strain of *A. israelii*, provided an inorganic source of nitrogen (ammonium sulphate) is also present in the medium; medium D also contained the minimal growth factor requirements for this organism (Christie & Porteous, 1962*b*) and therefore represents the simplest chemically defined medium yet devised for the growth of *A. israelii*.

In comparative experiments, media A, B, C and D (Table 3) gave similar yield of *Actinomyces israelii* and the organism grew at similar rates in the four media. The growth obtained (Table 4) in media A and B confirmed the observation of Christie & Porteous (1962*a*) that purines and pyrimidines were not required by the Wills strain of *A. israelii* when grown in a complete amino acid medium containin

sixteen growth factors (Tables 1 and 3); comparison of the growth of *A. israelii* in media B and C (Tables 3 and 4) showed that the purines and pyrimidines were not required for good growth even when the growth factor complement of the complete amino acid medium was decreased from sixteen to five components.

It will be noted, however, that purine and pyrimidine bases inhibited growth of the Wills strain of *Actinomyces israelii* in certain circumstances, namely when the amino acid complement and the growth factor complement of medium CP1 were simultaneously decreased in a specified manner (Table 3). Thus medium F gave poor growth while medium B and medium D gave good growth of *A. israelii* (Table 4); medium F and medium D both contained the same seven amino acids and the same five growth factors but medium F contained purine and pyrimidine bases in addition; medium F and medium B both contained the same purine and pyrimidine bases but differed in that the former contained seven amino acids and five growth factors while the latter contained twenty amino acids and sixteen growth factors (Table 3). Christie & Porteous (unpublished) found that addition of adenine and thymine to medium CP2 (Table 1) resulted in less vigorous growth of the same strain of *A. israelii*; this last observation, together with the present results, suggests that it was the absence of one or more of a group of certain growth factors, rather than the absence of one or more of a group of certain amino acids from medium F (Table 3) which elicited the inhibitory effect of the bases on growth of the organism. If this deduction is correct, addition of purine and pyrimidine bases to medium C (Table 3) should inhibit growth of the Wills strain of *A. israelii*; this is to be tested in experiments in progress.

Medium E differed from medium A only in that the latter contained twenty instead of seven amino acids (Table 3); since good growth was obtained in Medium A and poor growth in medium E (Table 4) it might seem that medium E was deficient in amino acids. On the other hand, medium E differed from medium D only in that the latter contained five instead of sixteen growth factors (Table 3); since medium D gave good growth of the organism, comparable with that obtained in medium A, it is apparent that the poor cell yields and growth rates obtained in medium E (Table 4) were in fact due to some antagonistic effect on growth of one or more of the 'growth factors' present in medium E but absent from medium D (Tables 1 and 3). This antagonistic effect only became manifest when the twenty amino acids of medium A were decreased in number to the seven present in medium E (Tables 1 and 3).

Christie (unpublished) failed to obtain consistent growth of the Wills strain of *A. israelii* in medium CP1 (Table 1) modified to contain only the seven amino acids listed in Table 3 instead of the original complement of twenty amino acids; the present results suggest that this failure was due to the presence of inhibitory 'growth factors' in the simplified medium and/or to the absence of ammonium sulphate from the simplified medium.

These observed inhibitions of growth of *Actinomyces israelii* by 'growth factors' and by certain purines and pyrimidines in specified circumstances lend support to the supposition of Erikson & Porteous (1953) that reported failures to obtain consistent growth of *A. israelii*, particularly in complex and undefined media, may be due as much to the presence of growth inhibitors in the medium as to the absence of required nutrients. It is apparent (see also Christie & Porteous, 1962*a*, *b*) that

Actinomyces israelii is a fastidious organism because it, like many other organisms, requires a carefully balanced medium rather than one containing excessive quantities or a wide variety of nutrients.

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The Participation of Filament Anastomosis in the Developmental Cycle of *Nostoc muscorum*, a Blue-Green Alga

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SUMMARY

During the resumption of development in cultures of *Nostoc muscorum* A, grown in the dark, and then exposed to light, cellular attachment occurs. This process is believed to be prerequisite for the formation of heterocystous filaments. The significance of anastomosis in the developmental cycle of Nostoc is discussed and a tentative scheme for an alternation of sporogenous and heterocystous generations is presented.

INTRODUCTION

During a study of the growth of the blue-green alga *Nostoc muscorum* A in the dark, it had been noted that inhibition of growth, in darkness, is accompanied by an interruption of the normal sequence of morphological development which can be observed in cultures grown in the light. Exposing dark grown cultures to light of low intensity allowed resumption of growth and synchronous differentiation of the various stages in the development of this organism. The details of the complex cycle of development, its relationship to light, as well as the techniques for cultivating *N. muscorum* A have been described in another paper (Lazaroff & Vishniac, 1961).

METHODS

The basal medium consisted of the following substances dissolved in 1 l. of distilled water: K_2HPO_4, 0·15 g.; $MgSO_4.7H_2O$, 0·20 g.; $CaCl_2.2H_2O$, 0·025 g.; Na_2SiO_3, 0·025 g.; $FeCl_3.6H_2O$, 2·0 mg.; $MnCl_2.4H_2O$, 0·4 mg.; $Na_2MoO_4.2H_2O$, 0·4 mg.; H_3BO_3, 0·6 mg.; $CuSO_4.H_2O$, 0·04 mg.; $ZnSO_4.7H_2O$, 0·04 mg. The pH was adjusted to 7·2 before autoclaving.

The sequence of developmental changes was most conveniently studied by periodic observation of micro-colonies growing in a thin layer of medium solidified with 0·9% agar. The cooled but still molten agar was seeded with a Nostoc suspension prepared by homogenization for 30 sec. in a sterile Waring micro-blendor cup. Four ml. of seeded medium were pipetted into inverted Carrel flasks so that a thin layer adhered to the upper plane surface of the flask after solidification. The flasks were generally placed in the dark for 2 weeks, then exposed to continuous illumina-

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tion of 50–100 ft.c. from a cool white fluorescent source. The developmental phenomena occurring at or just below the interface of the agar with the wall of the Carrel flask could be observed and photographed at high magnification.

RESULTS

Although growth in complete darkness in culture media containing glucose or sucrose fails to display normal development, the stage of interrupted development may differ according to the outline given in Table 1. If inocula are grown in the dark, subsequent transfer to media containing glucose or sucrose followed by incubation in complete darkness yields growth which is maintained at the aseriate stage of development. Plate 1, fig. 1, shows *Nostoc muscorum* grown in this manner which results in the formation of clumps of large cells, each clump derived from a single intercalary cell of the parent hormogonia. Similar development is obtained

Table 1. *Effect of origin of inoculum on the morphology of* Nostoc muscorum *in the dark*

Inoculum	Inoculated into basal medium supplemented with	Incubation period (days)	Dark development
Unwashed pellet from 30-day-old shake culture, basal medium, 250 ft.c.	1 % glucose	24	Aseriate, some filamentation
	1 % sucrose	24	Opening clusters and motile trichomes
Washed suspension from 14-day-old shake culture, basal medium, 250 ft.c.	1 % glucose	24	Aseriate
	1 % sucrose	24	Aseriate, some filamentation
Washed suspension from 20-day-old culture, basal medium + 1 % sucrose, 150 ft.c.	1 % glucose	35	Aseriate
	1 % sucrose	35	Aseriate, some filamentation
Washed suspension from 30-day-old culture, basal medium + 1 % glucose, 150 ft.c.	1 % glucose	33	Aseriate
	1 % sucrose	33	Aseriate
Loopful from dark grown culture, basal medium + 1 % glucose	1 % glucose	33	Aseriate
	1 % sucrose	33	Aseriate
Loopful from dark grown culture, basal medium + 1 % sucrose	1 % glucose	33	Aseriate
	1 % sucrose	33	Aseriate

if a small amount of a homogenized suspension of filaments, grown in the light, is used to inoculate culture media containing glucose, which are then incubated in complete darkness. However, if the inoculum has been prepared from Nostoc grown photo-autotrophically or in media containing sucrose in the light, then transferred to media containing sucrose and incubated in the dark, short filaments are formed (Pl. 1, figs. 2–4). These filaments, which are capable of motility, are derived from the aseriate stage of development but without the previous differentiation of heterocysts. This mode of development contrasts with the formation of hormogonia which are derived from heterocystous filaments by fragmentation at the heterocyst (Pl. 1, figs. 5, 6).

When the short motile filaments, produced in the dark, are exposed to continuous illumination at low intensity (20–50 ft.c.), synchronous development does not take

place. Instead, the short filaments swarm and anastomose, occasionally producing spore-like structures at the point of attachment (Pl. 2, figs. 7–13). Heterocystous filaments are produced in the light following filament fusion. The drawings in Figs. 1 and 2 compare the developmental sequences observed when aseriate packets are exposed to light with the phenomena which occur after the dark-grown short filament forms are similarly exposed.

Continuous observation of the spore-like structures produced after the attachment of filaments has indicated that germination can take place to produce a motile filament. It should be emphasized that in most cases the attachment of filaments occurs without the formation of a specialized structure at the point of attachment. The factors which govern whether the attachment of cells will lead to these spore-like structures or whether directly to filaments which eventually produce heterocysts are unknown.

Whether the enlarged structures are normal formations or teratological does not detract from the significance of their occurrence. Although it is difficult to ascertain whether a physiological connexion occurs between the anastomosing cells during the process of normal heterocystous filament formation, there can be little doubt that the structures which form only after the attachment of filaments proceed from the interaction of the joining cells.

When dark-grown aseriate packets are crushed between slide and coverslip or are dissected with a micro-needle, single cells or chains 2 or 3 cells long are extruded from each packet. If the dark-grown aseriate cultures are incubated at a light intensity of approximately 50 ft.c., for periods up to 3 days, fewer short chains and single cells are to be found. After 3 days' illumination, the contents of each packet are converted into a single trichome containing developing heterocysts. In a number of such observations the maturing heterocystous filament occurred in the form of a closed loop. These observations are summarized in Fig. 3.

DISCUSSION

The synchrony of development which results when aseriate cultures are exposed to light apparently depends on a high incidence of filament fusion taking place among the cells derived from the aseriate stage, thus yielding a homogeneous progeny of heterocystous filaments. In cultures, in which filamentation has taken place in the dark, swarming of the short filaments as well as anastomosis takes place on exposure to light. Therefore the development lacks synchrony, i.e. the population quickly becomes heterogeneous with respect to the stage of development. Not only are heterocystous filaments produced, but also filaments which are capable of fragmentation, spore formation, and anastomosis (Pl. 2, fig. 13).

The relationship of these two modes of development are summarized in Fig. 4 which presents a tentative scheme for the alternation of heterocystous and sporogenous generations.

The short motile trichomes which result from the fragmentation of sporogenous filaments may be regarded as spore germlings produced *in situ* on their parent filaments. These clearly differ from hormogonia in their mode of subsequent development. It may be noted that Harder (1917*b*) had described a positive phototaxis for the spore germlings (primary hormogonia) of *Nostoc punctiforme* but

apparent photo-indifference of the motile trichomes (secondary hormogonia) produced from heterocystous filaments.

The concept of an alternation of heterocystous and sporogenous generations helps to clarify a long-standing inconsistency in descriptions of the life cycle of the fila-

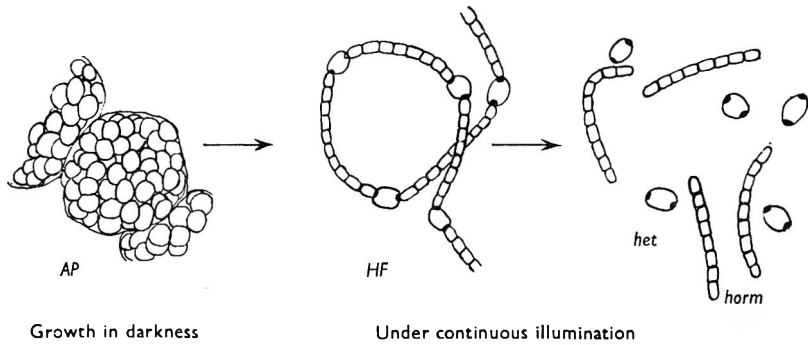


Fig. 1

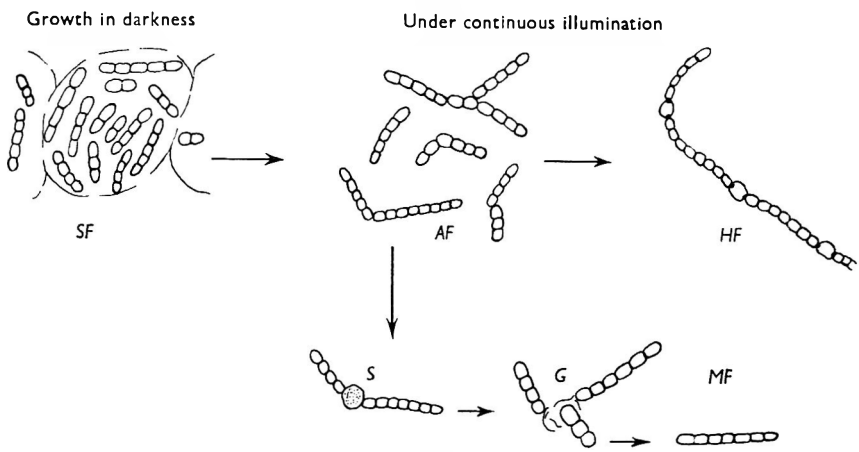


Fig. 2

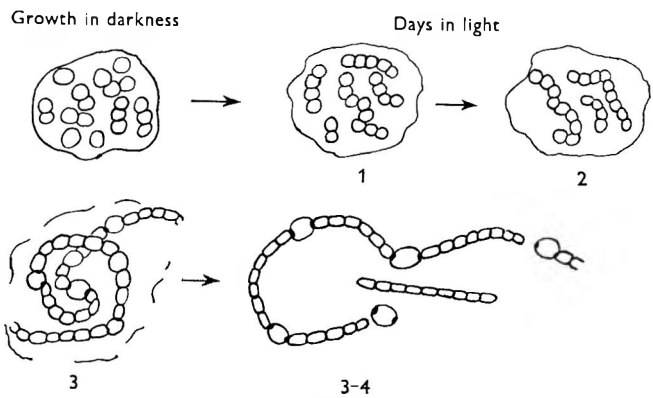


Fig. 3

For legend see facing page.

mentous blue-green algae. It had been frequently noted that motile trichomes could form by breakage of parent filaments at heterocysts or from the fragmentation of non-heterocystous filaments. In those cases where the further development of hormogonia, derived from a mature heterocystous thallus, was studied, an aseriate stage was produced which eventually differentiated to become a new heterocystous colony (Thuret, 1844; Sauvageau, 1897). Investigators who observed the further development of motile trichomes derived from germinating akinetes reported a different phenomenon (Janczewski, 1874; Harder, 1917*a*). The sequence generally involved the elongation of the parent trichome with the formation of heterocysts only from the terminal cells. Eventually the contorted filament fragmented without the participation of heterocysts to produce short filament segments, again capable of motility. It was realized that a heterocystous filament could eventually be produced from a spore, although the developmental sequence was obscure.

The observations of Fogg (1949), that the production of heterocysts in cultures of *Anabaena cylindrica* is influenced by the presence of certain metabolizable substances, are not inconsistent with the concept of a nostocacean developmental cycle. It has been pointed out in a previous paper (Lazaroff & Vishniac, 1961) that environmental factors, such as light intensity and the presence of glucose, influence the maintenance of the heterocystous phase of development. At high light intensities, in the presence of glucose, fragmentation of the heterocystous filaments to yield hormogonia ceases, yielding extremely long filaments with abundant intercalary heterocysts.

The results of our investigation indicate that the two types of motile filaments produced by *Nostoc muscorum* A may not be equivalent structures. The first type, for which the term 'hormogone' should be reserved, produces heterocystous colonies.

Figs. 1–3. Comparison of the developmental sequence, after light exposure, of dark-grown aseriate forms with that of short filament forms.

Fig. 1. Each aseriate packet (*AP*) is enclosed in a firm gelatinous envelope and, when exposed to light, forms a single heterocystous filament (*HF*). The heterocystous filament fragments at the heterocysts (*het*), yielding free intercalary heterocysts and gliding hormogonia (*horm*).

Fig. 2. The short filaments (*SF*), produced in the dark on media containing sucrose, are held within a diffuse gelatinous membrane. After illumination these short filaments glide out of their gelatinous matrix and anastomose (anastomosing filaments *AF*). The filaments resulting from anastomosis most frequently elongate by cell division and form heterocysts (heterocystous filaments *HF*). In some instances one or more spore-like structures (*S*) are produced from the anastomosing cells. These structures may germinate (*G*), and yield a short filament which elongates by cell division and differentiates into a motile filament (*MF*).

Fig. 3. Development within aseriate packets after exposure to light. Material from dark-brown aseriate cultures in liquid media was examined immediately after removal from darkness and at daily intervals during development. The contents of the developing packets were observed by gently crushing the packets between slide and coverslip or by dissection with a micro-needle. Immediately after removal from darkness the packets contained single cells or very short chains, 2–3 cells long. In the light each packet with time contained a decreasing number of chains, each of increased length. After 3–4 days the enclosing membranes of the original packets dissolved. At that time the cells in each packet formed a single heterocystous filament, occasionally in the shape of a closed loop. These filaments then underwent fragmentation with the liberation of heterocysts and hormogonia.

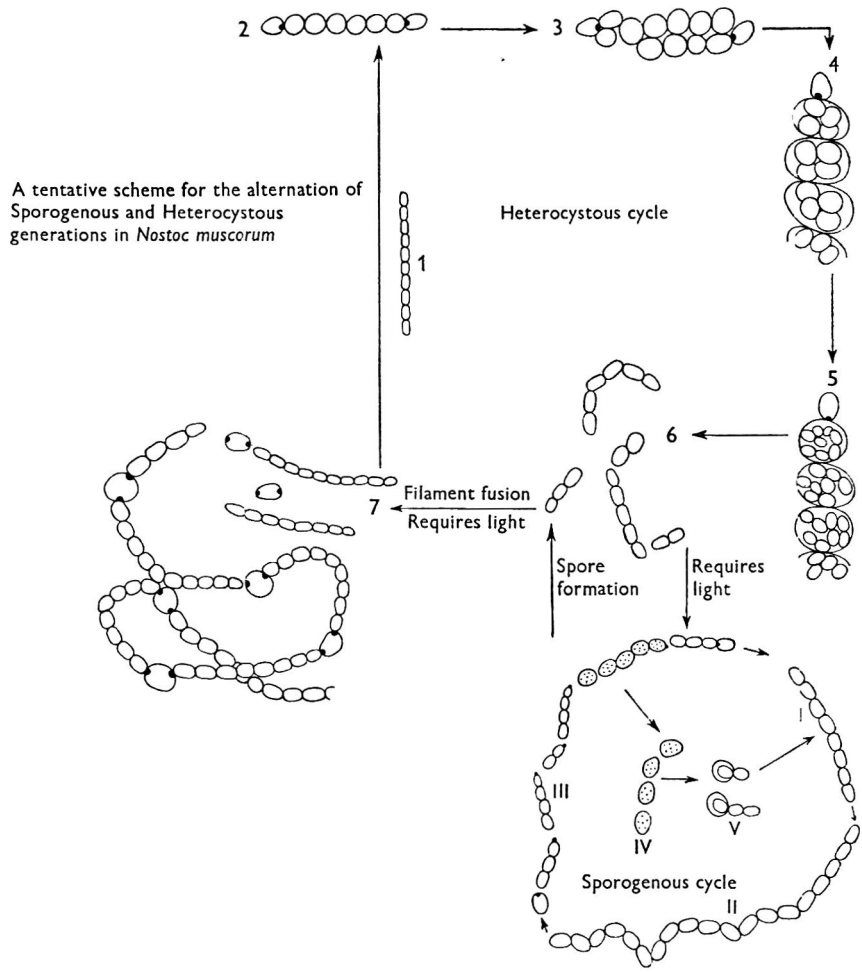
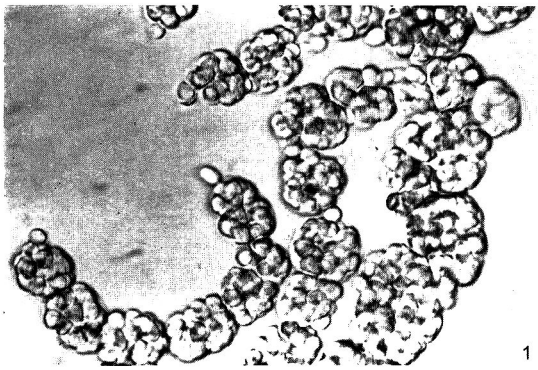


Fig. 4. For legend see facing page.

The second type, which develops in a manner similar to 'spore germlings' may form a heterocystous colony after a process of filament anastomosis has taken place, but alternatively may swarm, grow in length, fragment, or sporulate, maintaining a developmental sequence independent of the heterocystous cycle.

Supported by grants from the National Science Foundation and the National Institutes of Health. A portion of this work was carried out by one of us (N.L.) with the support of pre-doctoral fellowships from the National Science Foundation and the U.S. Public Health Service. This work forms part of a dissertation submitted by N.L. to the Graduate School of Yale University as requirement for the degree of Doctor of Philosophy.



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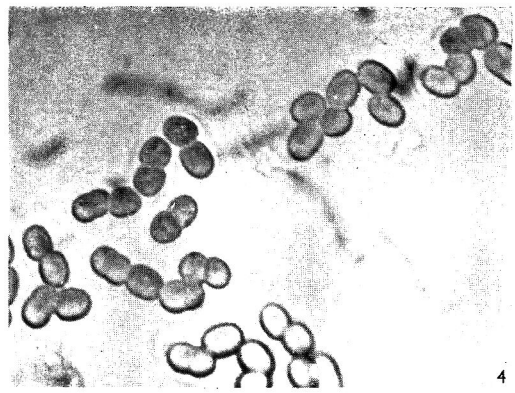

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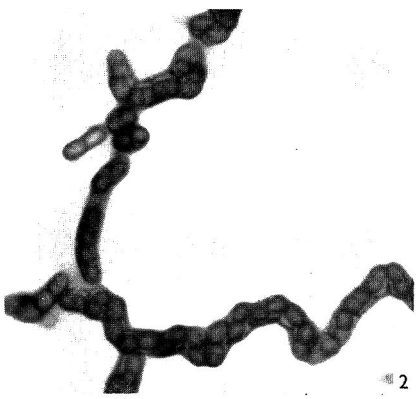

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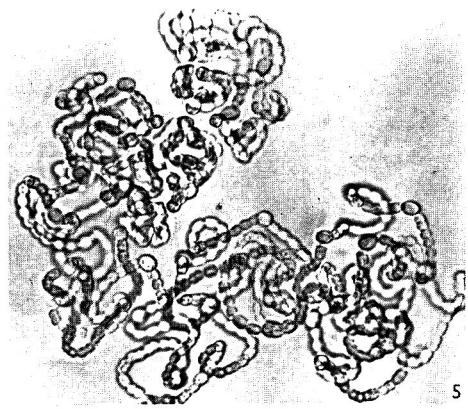

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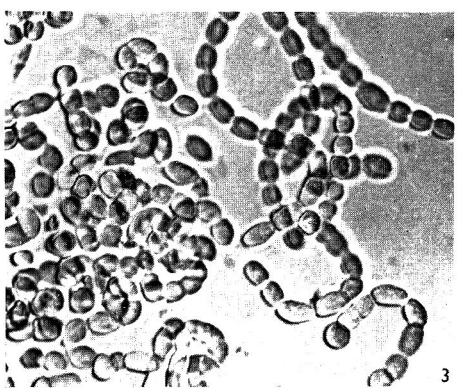

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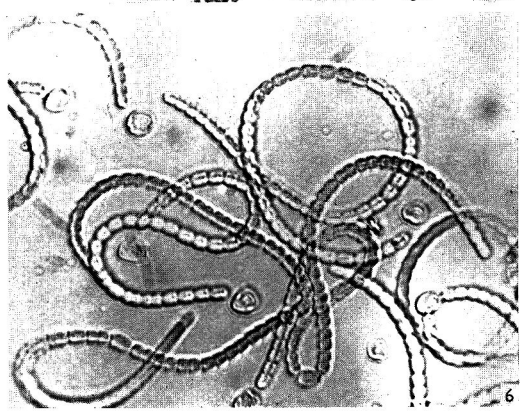

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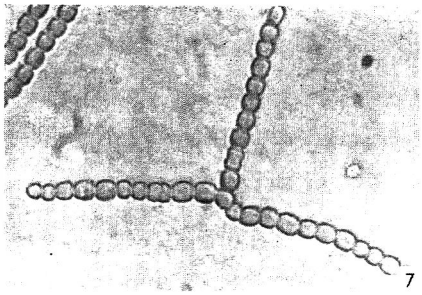

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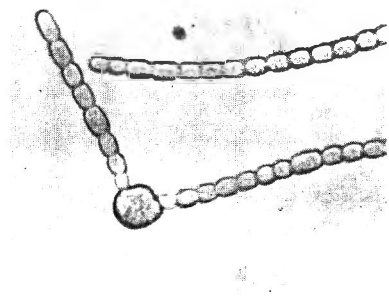

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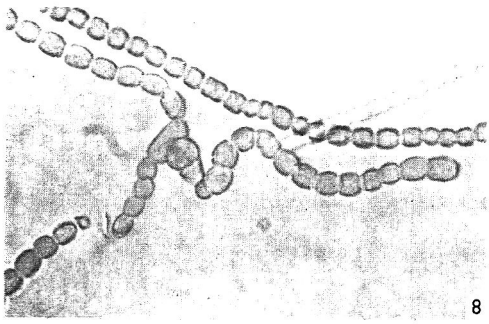

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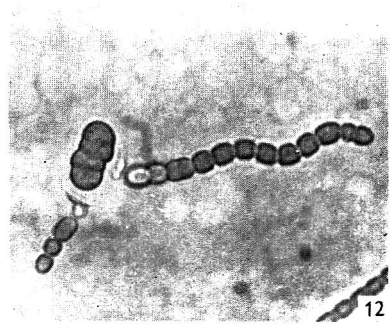

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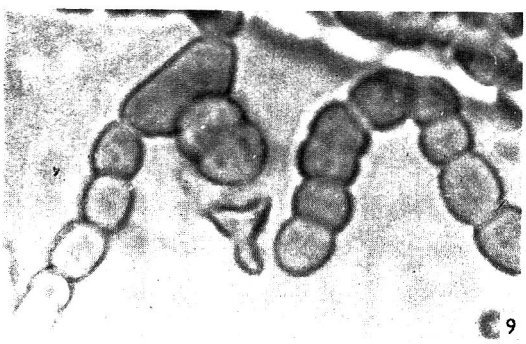

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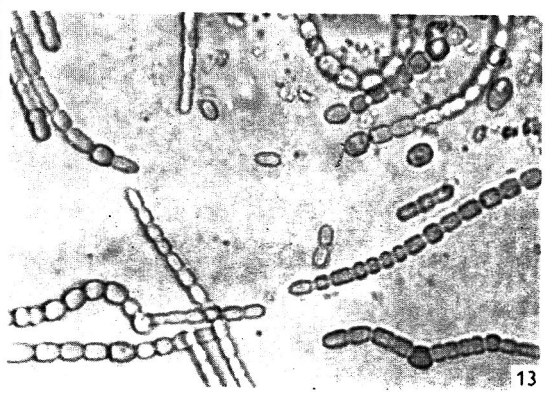

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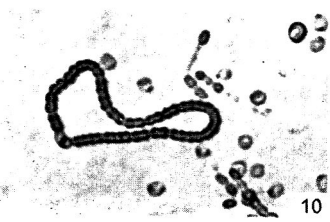

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Observations on Ascospore Initiation in the Discomycete *Dasyscyphus* sp.

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(*Received* 6 *July* 1961)

SUMMARY

Electron microscopy of apothecia of a species of a discomycete, *Dasyscyphus* sp., provided the opportunity to observe various aspects of ascospore formation. This report presents a series of micrographs arranged to represent what are interpreted to be successive stages in the process.

INTRODUCTION

Fungal cytology, unlike that of most plants and animals, lies near the limits of resolution of the light microscope. Consequently the nature of the various cell inclusions and organelles in fungal cells is poorly understood. Within recent years, however, the electron microscope has provided one means for gaining a clearer insight into the micromorphology of the somatic and reproductive structures of these organisms. The purpose of this paper is to show some of the changes in fine structure that occur in the ascus.

METHODS

Fruiting bodies (apothecia) of a species of a discomycete, *Dasyscyphus* sp., were collected in the field and prefixed in aqueous unbuffered 2% (w/v) $KMnO_4$ for 9 min. and after briefly rinsing in distilled water were fixed for 5 hr. at 4° in buffered 2% (w/v) OsO_4. Subsequently they were rinsed in buffer alone, dehydrated in an ethanol series and embedded in a methacrylate mixture. (Details of this protocol appear in Moore, 1962.) Sections were cut on a Servall Porter–Blum ultramicrotome with a Fernández–Morán diamond knife and examined in a Siemens Elmiskope I electron microscope.

RESULTS

Within the ascus during ascospore formation the fusion of opposing haploid nuclei is followed immediately by meiosis. Typically in the Ascomycetes, including Dasyscyphus, there is a post-meiotic division to produce a total of eight nuclei. Subsequently these nuclei become encapsulated by wall material to become ascospores. Plate 1, fig. 1, is interpreted as a stage in karyogamy and shows a fusion nucleus (*N*) composed of typical light (electron transparent), coarse granular, and

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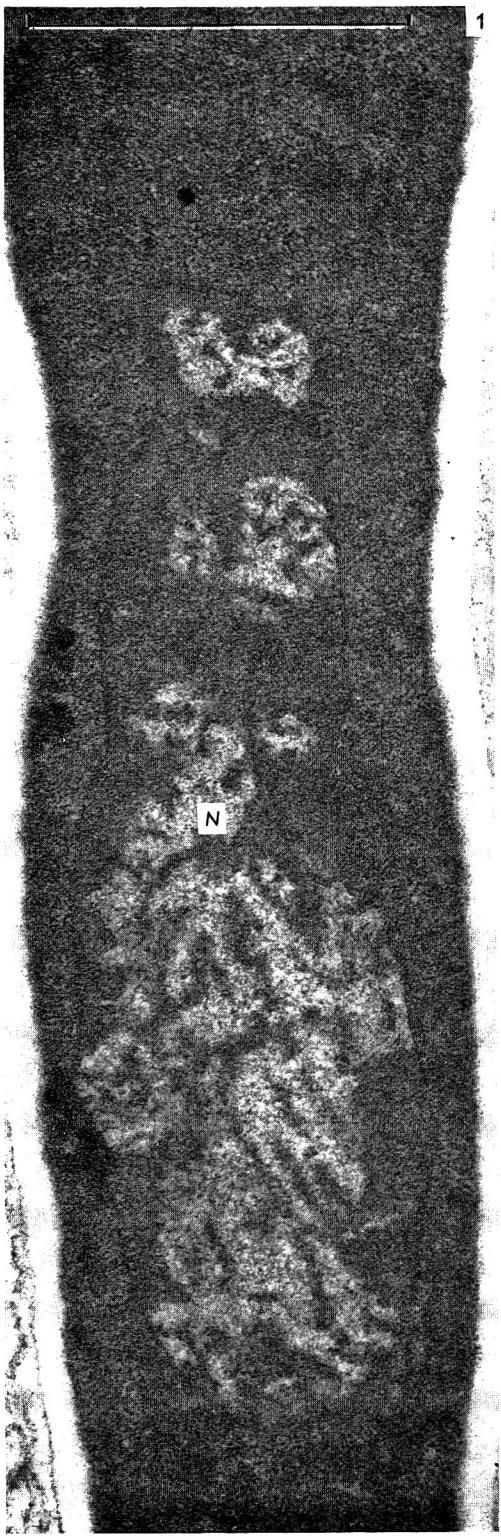
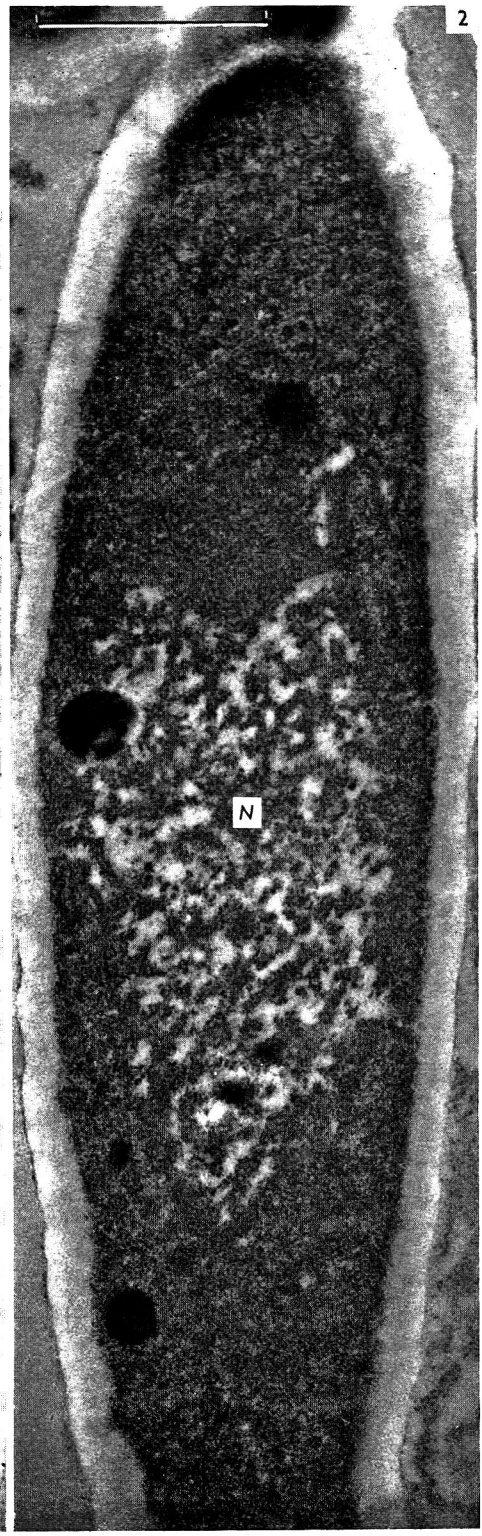
dark (electron dense), fine granular regions (see McAlear & Edwards, 1959; Moore, 1962; Moore & McAlear, 1961). Plate 1, fig. 2, is interpreted as representing an early stage in meiosis. The nuclear envelope has broken down and is not discernible; the nucleophase, however, is still prominent but merges indistinguishably into the cytoplasm. Plate 2, fig. 3, shows two masses of material (*N*), each composed of a coarse granular electron-transparent discrete phase, and an electron-dense fine granular phase that merges with the ascus cytoplasm. These are interpreted to be two units of nucleoplasm and to represent a stage of meiosis subsequent to that shown in the previous figure. A membrane fragment (wedges), observable near the lower moiety, may be a segment of the dissociated nuclear envelope. Plate 2, fig. 4, represents a stage in the reformation of endomembranes. Elements of the endoplasmic reticulum (*ER*) are prominent and the reconstitution of the nuclear envelopes is well advanced. Plate 3, fig. 5, is interpreted as a stage of late telophase. Three of the presumed four nuclei (*N*) are evident. The nuclear envelopes are nearly complete but nuclear separation has not yet occurred as evidenced by the opposing gaps (arrows) in the respective nuclear envelopes. Plate 4, fig. 6, is a much later stage and shows a nearly mature ascus and five of the final eight ascospores. Within the ascospores may be seen typical nuclei (*N*), mitochondria (*M*) and the endoplasmic reticulum (*ER*). This last (in the upper right spore) appears to form continuities between the nuclear envelope and the plasma membrane. Such continuities are not rare in fungi and have been reported in *Blastomyces dermatitidis* (Edwards & Edwards, 1960), *Stilbum zacalloxanthum* (McAlear & Edwards, 1959), *Mollisia* sp. (Moore & McAlear, 1961) and *Ascodesmis sphaerospora* (Moore, 1962). The cytoplasmic material left outside the spore, epiplasm (*E*), contains no nuclei and breaks down during spore maturation. The dark material bounding the spores is apparently a separable layer (wedges). It may be a residue of the epiplasm or material that has passed out through the spore wall in a manner similar to the formation of the opaque secondary spore coat in Ascodesmis (Moore, 1962).

DISCUSSION

The sequence of events in ascospore initiation in Dasyscyphus appears to be quite complementary to those reported for another discomycete (Moore, 1962). In that report micrographs showed the fusion nucleus, the terminal eight nucleate stage at the end of free nuclear division before wall initiation, and ascospore maturation. The present observations show the stages believed to occur between karyogamy and the formation of the final octad of nuclei, i.e. during free nuclear division. We have other micrographs which show initial wall formation in two other discomycetes (*Coryne* sp. and *Neobulgaria pura*); in these the new wall material first appears as a thin electron-transparent shell encompassing each of the final nuclei and a portion of the adjoining cytoplasm. Subsequently, the wall becomes much thickened. In Dasyscyphus this is the only spore wall and it appears to be quite similar to the primary wall in Ascodesmis. By both light and electron microscopy it appears smooth and transparent. By collating observations from several allied fungi we are able to reconstruct nearly the whole sequence of ascospore formation. However, the over-all rarity of the micrographs that we have been able to obtain of the various steps strongly suggests that the ontogeny occurs quite rapidly. In a number of Ascomycetes spore maturation involves further modifications such as the formation





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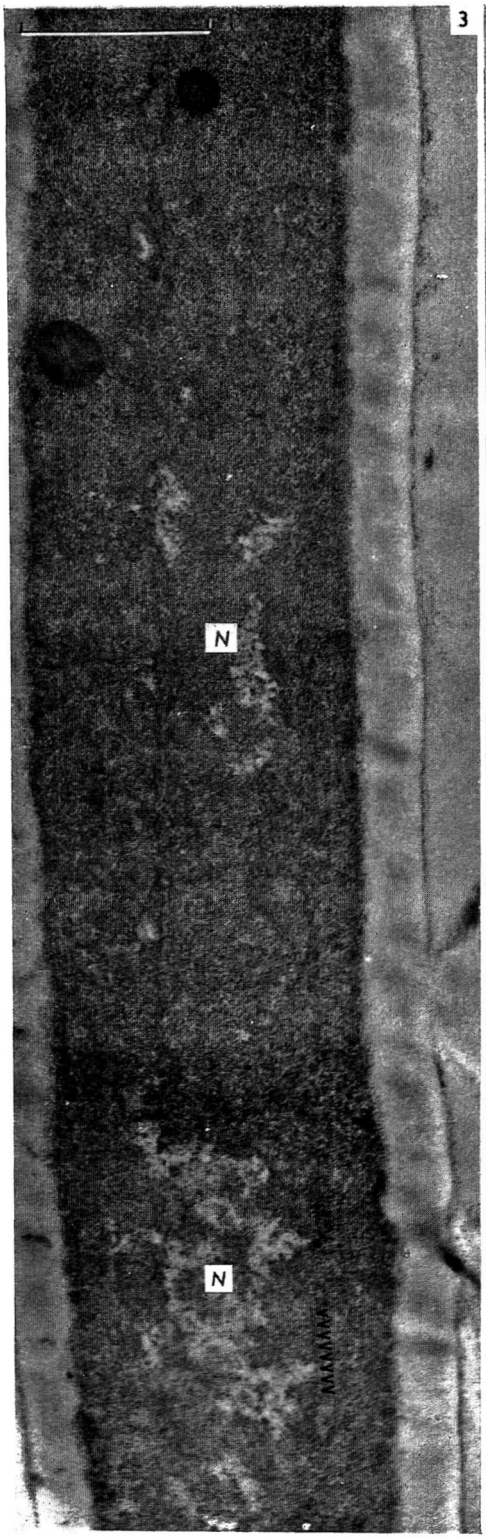

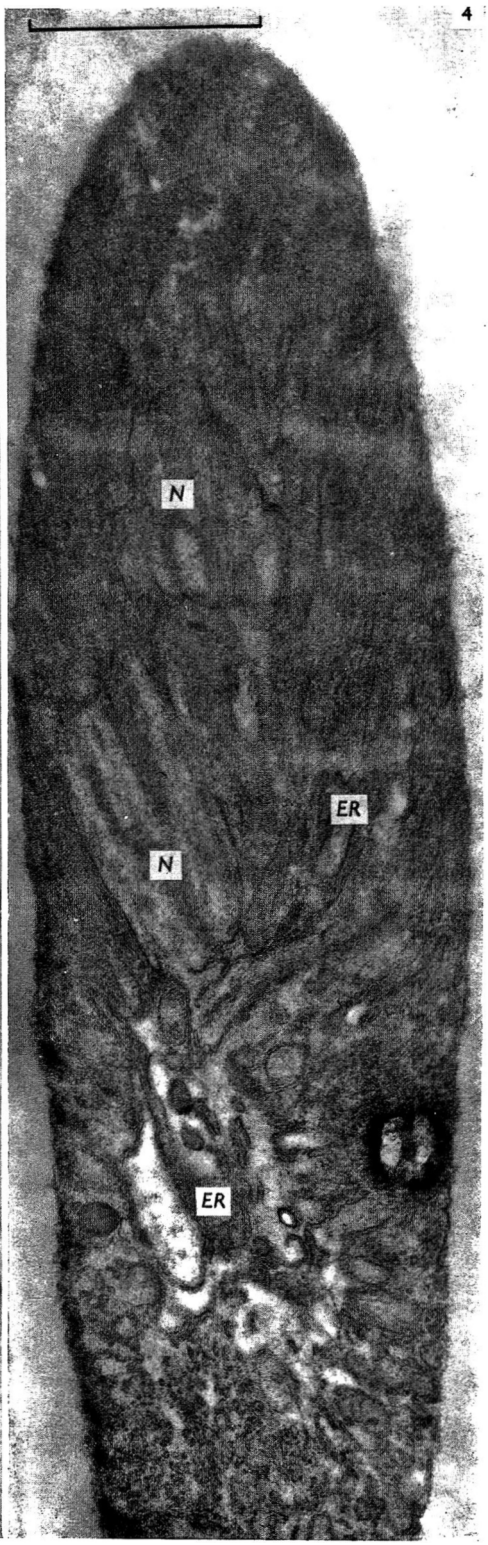

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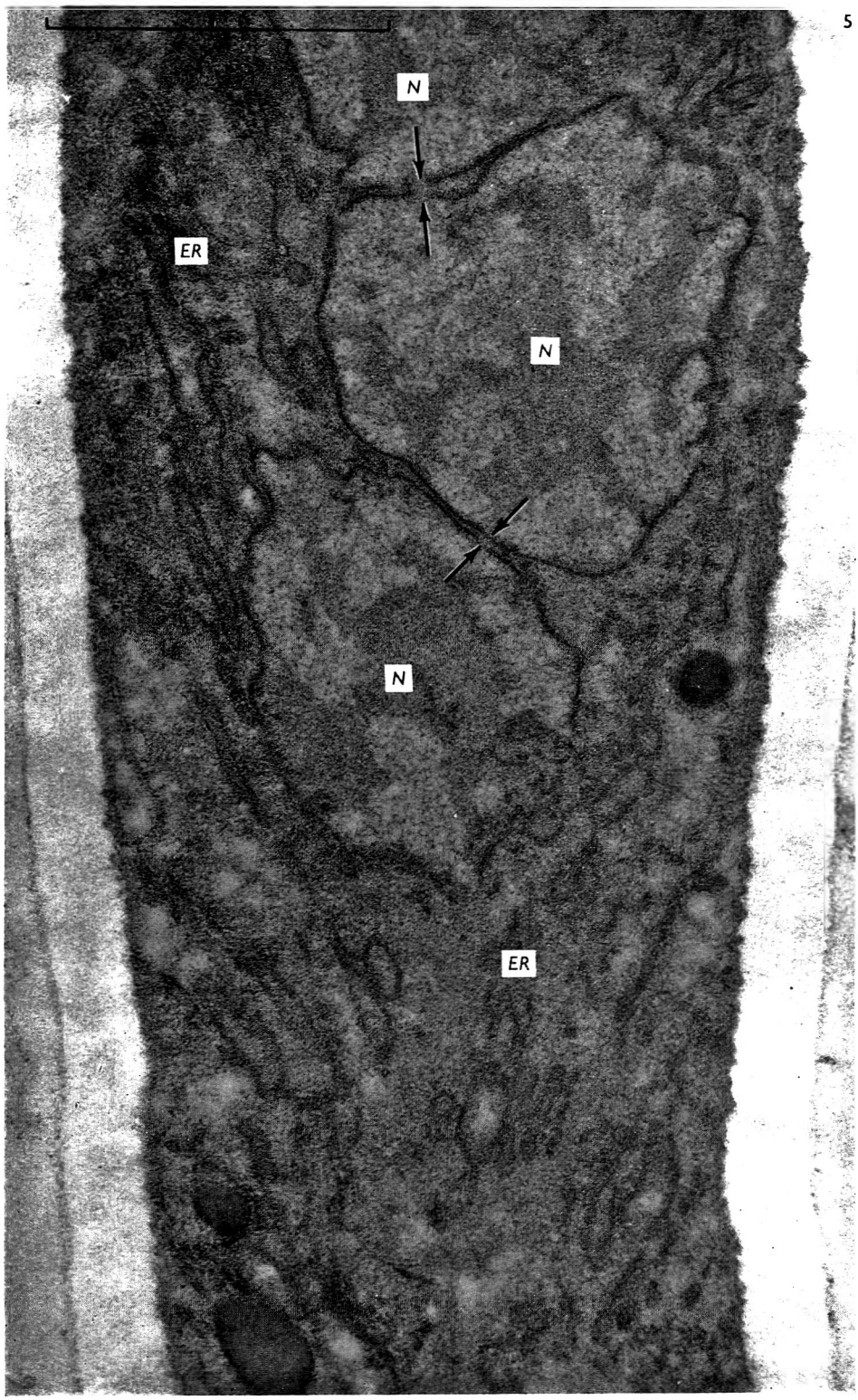

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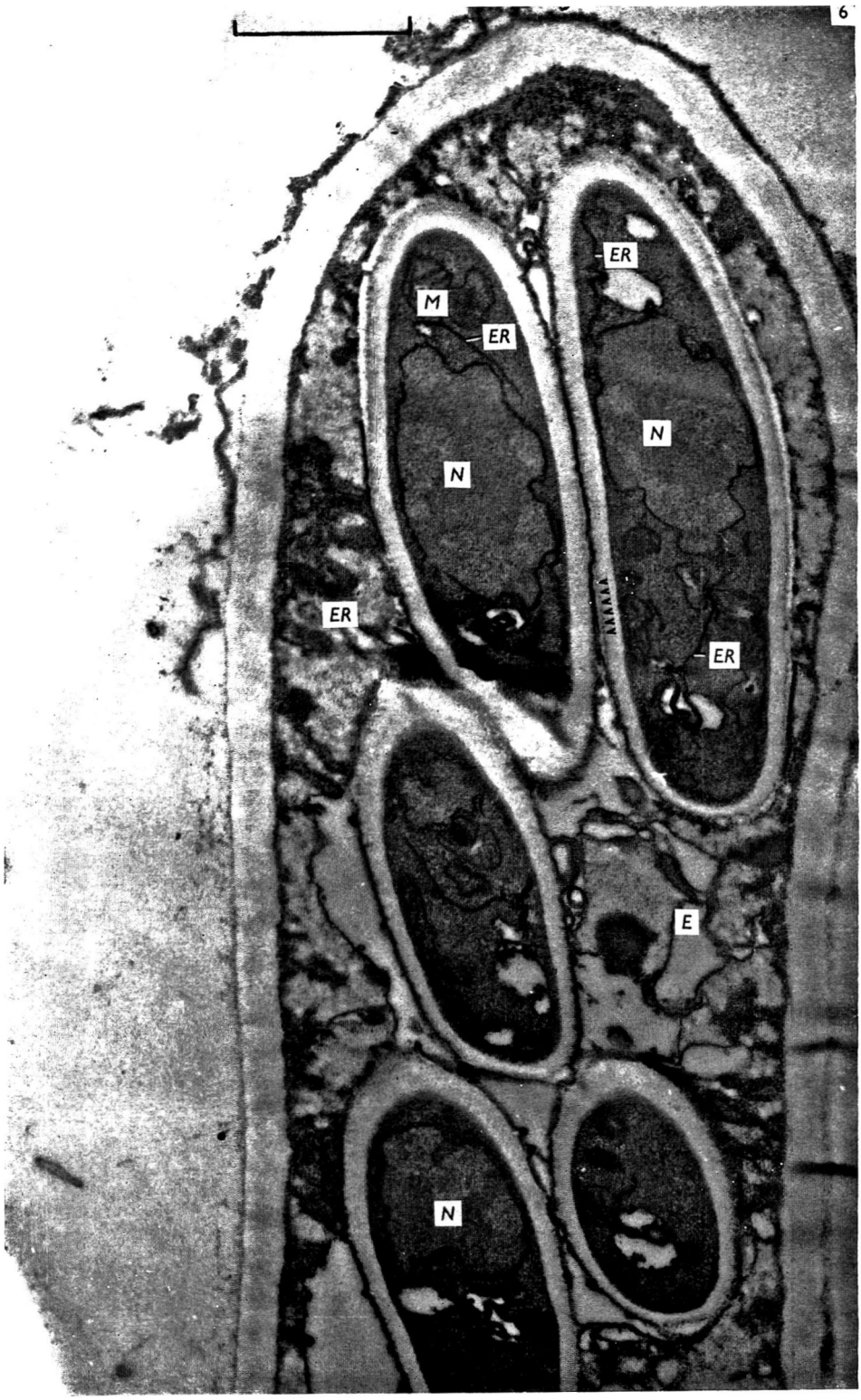

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of a secondary spore coat, production of septa, nuclear multiplication, budding and fragmentation, and spore elongation; but we feel that the observations made and referred to here are typical and fundamental in ascospore formation.

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This paper is No. 6 in a series on the Fine Structure of Mycota.

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EXPLANATION OF PLATES

Asci of *Dasyscyphus* sp. showing progressive steps in ascospore initiation and maturation. *E*, epiplasm; *ER*, endoplasmic reticulum; *M*, mitochondria; *N*, nucleus. Scale lines equal 1 μ.

PLATE 1

Fig. 1. Fusion nucleus. The nuclear membrane is still present and the nucleoplasm shows typical light, coarse granular, and dark, fine granular regions. Approx. × 50,000.

Fig. 2. An early stage in meiosis. The nuclear envelope has dissociated and the nucleophase merges into the cytoplasm. Approx. × 30,000.

PLATE 2

Fig. 3. A stage considered to be later than fig. 2. The two masses composed of light and dark moieties are interpreted to be nuclei; a membrane fragment near the lower aggregate (wedges) may be a segment of the dissociated nuclear envelope. Approx. × 25,000.

Fig. 4. A later stage showing re-formation of nuclear and endoplasmic reticulum endomembranes. Approx. × 30,000.

PLATE 3

Fig. 5. A still later stage believed to represent late telophase. Re-formation of the nuclear membranes is nearly complete, but that nuclear separation has not yet begun is suggested by the opposing gaps in the respective nuclear envelopes (arrows). The endoplasmic reticulum is prominent and has become more highly organized. Approx. × 50,000.

PLATE 4

Fig. 6. A nearly mature ascus. Walls have encapsulated the nuclei and portions of the ascus cytoplasm; the epiplasm, cytoplasm left outside the ascospores, is partially broken down. The spores contain what may be interpreted as typical nuclei, mitochondria and endoplasmic reticulum; the last mentioned appears to form continuities between the nucleus and the plasma membrane in the upper right spore. Each spore is bounded by a dark layer of material that appears to be separable from the spore wall (wedges). Approx. × 25,000.

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Genes Influencing the Conversion of Citrulline to Argininosuccinate in *Neurospora crassa*

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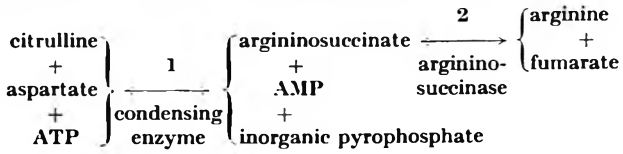
(*Received* 8 *August* 1961)

SUMMARY

The enzymic conversion of citrulline → argininosuccinate → arginine in wild-type *Neurospora crassa* has been shown to be essentially like that in mammalian tissues in all respects tested. The enzymes responsible for the two reactions (condensing enzyme and argininosuccinase) have been partially separated. Five mutants at the *arg-1* locus have normal argininosuccinase and little or no condensing activity. The lack of condensing activity appears to be due to a simple absence of enzyme, alternatives such as inhibitor production or increased ATPase competition having been ruled out. Small amounts of apparent condensing activity, detected in the substrate-disappearance assay, were shown to be due to side reactions. When grown at the usual high arginine concentrations, three mutants at the *arg-10* locus, which are known to lack argininosuccinase, have normal condensing activity. However, when *arg-10* strains are grown at low arginine concentrations, the resulting extracts have very little condensing activity. This also appears to be due to a simple loss of condensing enzyme. It has not been determined whether the low activity is a specific secondary effect of *arg-10* on the condensing enzyme, or whether it is merely a non-specific effect of the inadequate supplementation, which might cause reductions in many enzyme activities. Despite its very low condensing activity *in vitro*, *arg-10* grown at low arginine concentrations must be active at some time *in vivo*, since its mycelium accumulates argininosuccinate but not citrulline. In contrast, an *arg-1 arg-10* double mutant, grown at low arginine concentrations, must be inactive *in vivo*, since it accumulates citrulline but not argininosuccinate. It is concluded that *arg-1* is probably the primary locus controlling the synthesis of condensing enzyme.

INTRODUCTION

Numerous studies of gene–enzyme relationships suggest that a single genetic locus determines the basic ability to form a given enzyme, but that the quantity of the enzyme produced can be modified by other genes and by varying environmental conditions (see review by Fincham, 1958; Yanofsky & St Lawrence, 1960). The present paper is an analysis of similar gene–enzyme relationships concerned with the conversion of citrulline to arginine in *Neurospora crassa*. The mechanism of this conversion in mammalian tissues has been studied extensively by Ratner and co-workers (Ratner & Petrack 1951, 1953*a*, *b*, 1956; Ratner, Petrack & Rochovansky, 1953). They found that two reactions were involved:



The condensation of citrulline and aspartate to form argininosuccinate (reaction 1) is greatly accelerated by the presence of inorganic pyrophosphatase (Ratner & Petrack, 1956).

Evidence is presented here that citrulline is converted to arginine in wild-type *Neurospora crassa* in essentially the same way as in mammalian tissues. Independently Boylen & Fincham (personal communication) have obtained preliminary evidence for the same reaction sequence in wild-type Neurospora, and have studied the argininosuccinase reaction in detail (Fincham & Boylen, 1957). Nine mutant strains blocked between citrulline and arginine have been classified genetically into two types, representing mutations at two unlinked loci (Newmeyer, 1957); these are called *arg-1* and *arg-10*. Fincham & Boylen (1957) have shown that *arg-10* mutants lack argininosuccinase. The present paper is concerned with the effects of the two loci on the condensation reaction.

METHODS

Strains. The origin and genetic analysis of the mutants were described previously (Newmeyer, 1957). The following stocks were used:

arg-1 (Linkage group I; uses arginine but not citrulline): alleles 36703T, 46004, H4250, B312, and B369.

arg-3 (Linkage group I; uses citrulline or arginine): allele 30300.

arg-10 (Linkage group VII; uses arginine but not citrulline): alleles B317, B368, and B370.

wild-type: ST-74A.

All mutants were inbred to the wild type.

The *arg-1 arg-10* double mutant was isolated from a non-parental ditype ascus from a cross of inbred lines of 36703T and B317. Its constitution was verified by crossing to wild type and recovering both single mutants from the same ascus.

Enzyme preparations. Cultures were grown in Erlenmeyer flasks at 30° with moderate shaking. Each flask contained 650 ml. Fries minimal medium (Beadle & Tatum, 1945) containing 20 g. sucrose per l., with or without arginine, as indicated in Results. The mycelia were harvested by filtration, washed with distilled water, sucked dry, and ground in a cold mortar with an equal weight of alumina powder (Buchler's Levigated, no. 1557AB). The mixture was extracted with cold 0·01 M-phosphate buffer (pH 7·4), centrifuged at 21,600 *g* for 30 min. at 5°, and the extract stored at −15°. Dialyses were carried out against 0·01 M-phosphate buffer (pH 7·4) at 5° overnight. Crude extracts containing about 15 mg. protein/ml. were fractionated with solid ammonium sulphate at 0° as shown in Table 1. A sample of the 25–50% fraction was diluted to about 9 mg. protein/ml. and refractionated with ammonium sulphate as shown in Table 1. Precipitates were dissolved in 0·1 M 2-amino-2-hydroxymethylpropane-1:3diol (tris buffer; pH 7·4).

Chemicals and analytical methods. The colorimetric methods used for determining arginine, citrulline, urea and inorganic phosphate, as well as the preparation of reagents, substrates and supplementary enzymes, were all as described by Ratner (1955) and Ratner & Petrack (1951, 1953*b*) except as noted. Extracts of urease (Nutritional Biochemicals Corporation) were made fresh each day, 50 mg. being extracted with 5 ml. water or 0·1 M-phosphate buffer (pH 7·4); these extracts did

not interfere in any of the assay procedures. Protein was estimated by the biuret method (Gornall, Bardawill & David, 1949) on samples clarified by repeated centrifugation.

Measurement of condensing activity. The complete assay system contained (in μmole per 2 ml.): 15, L-citrulline; 15 L-aspartate; 13·2 $MgSO_4$; 100 tris or phosphate buffer (pH 7·4); 2·5 ATP; 25 to 75, D-3-phosphoglycerate (PGA). (The PGA was used to regenerate ATP. In Neurospora extracts, ATPase activity is much greater than condensing activity, so that 60 or more μmole PGA were often necessary in order to achieve linear reaction rates. It was found unnecessary to add an outside source of the enzymes needed to regenerate ATP from PGA, even when fractions were being assayed.) The mixtures were incubated at 33° and the reactions stopped with trichloroacetic acid (TCA).

Table 1. *Separation of condensing and argininosuccinase activities in wild type* Neurospora crassa

			Condensing activity		Argininosuccinase	
Fraction no.	$(NH_4)_2SO_4$ saturation (%)	Protein (mg.)	Specific activity*	Total units†	Approximate specific activity*	Approximate total units†
Crude extract	—	3090	0·59	1823	0·76	2350
I	0–25	103	0	0	⩽0·20	0–21
II	25–50	1740	0·86	1498	0·86	1498
III	50–85	842	⩽ 0·035	0–29	⩽ 0·03	0–25
II‡	25–50	739	0·86	635	0·86	635
II*a*	0–30	214	⩽ 0·05	0–11	0·13	28
II*b*	30–44	284	1·01	287	2·03	577
II*c*	44–70	187	1·68	314	0·01	2

* Units/mg. protein.

† One unit caused the disappearance of 1 μmole citrulline or formation of 1 μmole arginine in 1 hr. at 33°.

‡ The sample used for refractionation.

Condensation was assayed by measuring citrulline disappearance colorimetrically (Ratner, 1955). (Neurospora extracts contain too much urease to permit the assay of condensing activity by measuring urea production.) With the wild type, the citrulline disappearance method is reliable, since no side-reactions have been detected at the protein concentrations used for assay. With the mutants, however, this assay presents two difficulties.

(1) Citrulline disappearance might be due to side-reactions, because much larger amounts of protein must be tested in order to detect small amounts of condensing activity. Furthermore, citrulline accumulation resulting from a genetic block might induce the synthesis of enzymes catalysing such side-reactions. Therefore, the apparent small activity found in *arg-1* extracts was tested for its relevance to condensation by dependency tests, and by testing for argininosuccinate production by two-dimensional chromatography.

(2) With *arg-1* extracts it is hard to obtain accurate citrulline disappearance measurements without increasing the time and/or amount of enzyme so much that the linear range is exceeded; the enormous ATPase competition under these conditions often makes it impossible to add an excess of PGA. The *arg-1* 'specific

activity' values given under Results are therefore only best estimates, and are shown bracketed when the only assays were definitely in the non-linear range. In order to show that the difference between *arg-1*$^-$ and *arg-1*$^+$ is nevertheless real, typical raw data are also given which compare *arg-1*$^-$ and *arg-1*$^+$ extracts when both are assayed under similar conditions, at high time and enzyme levels.

All assays of citrulline disappearance were made on dialysed extracts, because undialysed extracts, particularly of *arg-1* mutants, contain material which gives colour in the citrulline assay; only part of this can be citrulline itself. Urea also interferes in the citrulline determination, but in crude extracts it is destroyed as fast as formed. In at least one assay on each extract, the enzymic reaction was stopped by heat and the mixture re-incubated with excess urease before citrulline was determined. This procedure always gave the same results as when urease was omitted.

Arginine desimidase (Oginsky & Gehrig, 1952) was not expected to interfere seriously because of its sensitivity to Mg^{++} and its low activity at pH 7·4; desimidase activity was specifically shown to be absent from wild-type fraction II under the conditions used here.

Measurement of argininosuccinase. An approximate assay was obtained by determining the amount of CO_2 formed in the presence of excess arginase and urease. Double-armed Warburg vessels were used; the main compartment contained 0·10 ml. arginase solution, 0·20 ml. urease solution (buffer extract), 0·20 ml. of 1·0 M-phosphate buffer (pH 7·4), and dialysed Neurospora extract in a total volume of 0·70 ml. The reaction was started by tipping in 0·30 ml. of a solution containing 6 μmole argininosuccinate. Standard manometric procedures were used, with air as gas phase. Endogenous respiration was negligible. CO_2 production was linear with time so long as 25% of the substrate remained.

Measurement of ATPase. For estimating ATPase competition, crude assays were made by measuring the total inorganic phosphate liberation in the TCA filtrates used for measuring citrulline disappearance; the complete system was used except that ATP (20 μmole/2 ml.) was used as sole phosphate donor. Most of the phosphate liberation was due to ATPase; approximate correction for the small amount that could be due to condensation (0–30% of the total) was based on the citrulline disappearance measurements. For measuring the ratio of phosphate liberation to citrulline disappearance, exact correction was made for ATPase, and also for a trace reaction involving aspartate, by subtracting the phosphate liberated in the absence of citrulline from the amount liberated when both amino acids were present.

Chromatography. Ascending paper chromatography was used to detect the formation of argininosuccinate and other products by the Neurospora extracts. Only dialysed extracts were used. Incubation mixtures were made with phosphate buffer since tris buffer altered the R_F values of several compounds. After incubation the mixtures were deproteinized by heating and chromatographed either with 4+1+2 by vol. butanol+acetic acid+water (run twice in the same direction) or with 3+1 by vol. phenol+water with or without ammonia atmosphere. For two-dimensional chromatograms, phenol without ammonia was used as the first solvent and butanol+acetic acid as the second; in this case it was not essential to develop twice with butanol+acetic acid. With the incubation mixtures used here, one-

dimensional phenol chromatograms rarely gave adequate separation of argininosuccinate from glutamate, and one-way butanol + acetic acid chromatograms failed to separate argininosuccinate from aspartate. Argininosuccinate was therefore identified by two-dimensional chromatography, and by elution and re-development, with similarly eluted authentic argininosuccinate as a standard. All critical identifications were checked by addition of known amino acids.

RESULTS

Neurospora crassa *wild type*

Extracts of wild-type Neurospora readily catalysed the disappearance of citrulline and the appearance of arginine in the presence of the complete system. The enzymes involved are quite stable to dialysis and storage. The rate of citrulline disappearance for various wild-type extracts is given in Table 2; this rate was affected very little by the presence or absence of arginine in the culture medium. The wild-type Neurospora system resembles the mammalian one in all respects tested. Specifically:

(1) In dialysed fractionated material, both citrulline disappearance and arginine formation were completely dependent on the presence of aspartate, citrulline, $MgSO_4$ and ATP. Aspartate could not be replaced by ammonium ion or glutamate.

(2) The rate of citrulline disappearance was greatest when a combination of D-3-phosphoglycerate + a catalytic amount of ATP was used as the source of high-energy phosphate, since high ATP concentrations inhibited the reaction. In the absence of D-3-phosphoglycerate, activity was decreased at ATP concentrations greater than 20 μmole/2 ml.; this concentration gave only 50–80 % of the activity obtained with 2·5 μmole ATP + optimal concentrations of D-3-phosphoglycerate (Table 2).

(3) The reaction is accompanied by a liberation of inorganic phosphate which depends on the simultaneous presence of citrulline and aspartate. The ratio of phosphate liberation to citrulline disappearance was determined using fraction II with 2·5 μmole ATP + 45 μmole D-3-phosphoglycerate; after correcting for phosphate liberated by ATPase, values of 1·53, 1·53, and 1·49 were obtained. In liver, the Δphosphate/Δcitrulline ratio is typically between 1·0 and 2·0, the exact value depending on the concentration of inorganic pyrophosphatase and on the source of ATP. When ATP was supplied from D-3-phosphoglycerate, values of about 1·5 were obtained only when the pyrophosphatase concentration was nearly optimal (Ratner & Petrack 1953*a*, 1956). The Neurospora results therefore suggest that pyrophosphatase is similarly involved in its condensing system, and is probably not seriously limiting. Pyrophosphatase is evidently not removed by the partial purification used here, since there is little loss of condensing activity during fractionation (Table 1).

(4) The reaction occurs in two steps with argininosuccinate as intermediate. The evidence for this is as follows. Citrulline disappearance always exceeded arginine formation. (No attempt was made to achieve equilibrium.) Partial purification gave two active fractions (Table 2). One (II*b*) caused a rapid disappearance of citrulline, of which 55 % was found as arginine; this fraction contained most of the argininosuccinase. The other active fraction (II*c*) caused a more rapid disappearance

Table 2. *Condensing activities of mutant and wild-type extracts*

Specific activities are based on measurements of citrulline disappearance in dialysed crude extracts. 'PGA system' indicates complete incubation mixture with 2·5 μmole ATP + optimal concentrations of D-3-phosphoglycerate (PGA) as described in Methods. 'ATP system' indicates complete incubation mixture with 20 μmole ATP and no PGA. For *arg-1*$^+$ strains, incubation was for 1–4 hr., with 1–4 mg. protein. For *arg-1*$^-$ it was necessary to use more protein. Where this resulted in non-linearity, the specific activity values are enclosed in brackets; in such cases the raw data indicate decidedly less activity than is given by *arg-1*$^+$ under similar conditions. See Methods and Table 3.

			Growth conditions		Specific activity	
Genotype	Allele	Extract no.	No. days	Arginine (mg./ml.)	PGA system	ATP system
Wild type	ST-74A	5	3	0	0·59	—
	ST-74A	11	3	0·2	0·46	0·30
	ST-74A	13	3	0·2	0·63	—
	ST-74A	15	5	0	0·57	0·46
	ST-74A	17	5	0·2	0·79	0·39
	ST-74A	19	1·8	0·07	0·62	—
arg-3	30300a	26	5	0·2	0·71	—
arg-10	B317a	24	5	0·2	0·41	0·29
	B370a	30	5	0·2	0·46–0·53	0·16–0·18
	B368a	32	5	0·2	0·35–0·43	0·17–0·21
	B317a	38	3	0·5	0·48	0·28
	B317a	60	3	0·5	0·51	—
	B370a	64	3	0·5	0·60	—
arg-1	36703T-a	4	5	0·2	0·06*	—
	36703T-A	12C	5	0·2	—	0·07
	36703T-A	12D	5	0·2	—	0·11
	36703T-A	16C	5	0·2	0·08	0·10
	36703T-A	16D	5	0·2	—	0·04
	46004A	14	5	0·2	0·06	[0·03]
	46004a	44	5	0·2	[0·03]	—
	B369a	20	5	0·2	0·08	0·09
	H4250A	22C	5	0·2	0·10	0·13
	H4250A	22D	5	0·2	0·12	0·14
	H4250a	46	5	0·2	[0·06]	—
	B312a	10	4	0·2	0·04	[0·02]
arg-1 arg-10 (double mutant)	36703T-B317	18	5	0·2	—	0·02*
	36703T-B317	42	3	0·5	[0·02]	0·01*

* Not significantly greater than zero.

of citrulline, of which not more than 5% was found as arginine; this fraction contained very little argininosuccinase and negligible arginase. It was shown by sequential incubations that fraction II*c* converted the missing citrulline to an intermediate compound, which could be converted to arginine by an argininosuccinase preparation from yeast. (This preparation, the 35–50% ammonium sulphate fraction from a plasmolysed extract of baker's yeast, had negligible condensing activity.) The intermediate was identified as argininosuccinate by paper chromatography. When incubated with the complete system, the dialysed crude extract and fraction II each produced both arginine and argininosuccinate, while fraction II*c* and a dialysed crude extract of *arg-10*(*B317*) each produced a strong argininosuccinate spot but never more than a dubious arginine spot. (Arginine was

detectable because, in the presence of the filtrates, it was displaced upward on phenol chromatograms, in the absence of ammonia, to R_F 0·70, and was thus separated from citrulline. Under the same conditions, ornithine was displaced downward, so as to be superimposed on slow-moving material present in all mixtures before incubation; the expected traces of ornithine were therefore not detectable.) No other products were found. By using fraction II, detectable argininosuccinate production was found to be completely dependent on the presence of citrulline, aspartate, $MgSO_4$ and ATP.

Neurospora crassa *mutants*

Condensing activity at standard growth conditions. Extracts of the *arg-1* and *arg-10* mutants (and also of the citrullineless mutant, *arg-3*) were ordinarily prepared from mycelia grown for 5 days on minimal medium + 0·2 mg. arginine/ml.; a few strains were also grown for 3 days at 0·5 mg./ml. The condensing activities of these preparations are summarized in Table 2 as specific activities, and typical raw data comparing *arg-1*$^+$ with *arg-1*$^-$ are given in Table 3. The *arg-1* extracts invariably caused much less citrulline disappearance than the wild-type controls, although they were not completely inactive. Extracts of *arg-10* and *arg-3*, on the other hand, were essentially as active as wild type. As expected, the *arg-1 arg-10* double mutant showed the reduced activity characteristic of *arg-1*.

The low activities of the *arg-1* extracts were not due merely to a deficiency in the enzymes which regenerate ATP from D-3-phosphoglycerate. This is shown, first, by the results with ATP as sole phosphate donor (Tables 2 and 3). The difference between *arg-1*$^-$ and *arg-1*$^+$ activities was smaller here than with the D-3-phosphoglycerate system, because the *arg-1*$^-$ extracts did not show the ATP inhibition characteristic of liver and wild-type Neurospora. Even so, there was still no overlap in the activities of *arg-1*$^+$ and *arg-1*$^-$. Secondly, when extracts from three different *arg-1* mutants were assayed with the D-3-phosphoglycerate system, addition of a muscle preparation to supply the enzymes needed for regenerating ATP (Ratner, 1955) caused no increase in condensing activity.

Mixing crude extracts of *arg-1* with active control extracts caused no significant stimulation or inhibition (Table 4). As a further test, *arg-1* extract no. 4 was put through a single ammonium sulphate fractionation. The resulting fraction II, which should have contained all the condensing activity, was still quite inactive, and caused no stimulation or inhibition when combined with wild-type fraction II (Table 4). The mutant fraction II also caused no stimulation when combined with the inactive wild-type fractions I, II*a*, and III. (A small amount of activity was found in mutant fraction III, however. The residual *arg-1* activity thus fractionated abnormally, suggesting that it might be due to a different enzyme.)

The low activity of the *arg-1* extracts does not appear to be ascribable merely to increased ATPase activity. ATPase assays on various extracts of the wild type, *arg-1*(*36703T*), *arg-10*(*B317*), and the *arg-1 arg-10* double mutant, showed that while this activity varied considerably, depending on the growth conditions, it was unaffected by the presence or absence of *arg-1*$^-$. Excessive ATPase competition was further excluded by the lack of inhibition when *arg-1* extracts were combined with wild type (Table 4). The lack of inhibition also makes it unlikely that the low activity of the *arg-1* extracts was due to a large increase in arginine desimidase.

Table 3. *Typical raw data comparing* arg-1$^+$ *and* arg-1$^-$ *extracts of* Neurospora crassa *at high time and enzyme levels*

See Methods for rationale. 'PGA system' indicates complete incubation mixture with 2·5 μmole ATP + D-3-phosphoglycerate (PGA) as indicated. 'ATP system' indicates complete incubation mixture with 20 μmole ATP and no PGA. Tests done under similar conditions are grouped together.

		Assay conditions			μmole citrulline disappearance			
					arg-1$^+$		arg-1$^-$	
Strain	Extract no.	PGA (μmole)	Protein (mg.)	Incubation time (hr.)	Wild type	*arg-10*	*arg-1*	*arg-1 arg-10* double mutant
PGA system								
ST-74A	15	60	2·5	4·3	4·17	.	.	.
B370a	64	35	2·4	4·2	.	4·45	.	.
36703T-A	16C	50	2·9	4	.	.	0·95	.
ST-74A	13	45	5·5	3	9·06	.	.	.
B369a	20	45	5·6	3	.	.	1·35	.
B317a	60	35	4·9	4·3	.	5·54	.	.
46004a	44	35	6·3	4·5	.	.	0·97	.
H4250a	46	35	5·6	4·5	.	.	1·57	.
B370a	64	35	11·8	3·8	.	5·30	.	.
36703T-B317	42	35	11·8	4·4	.	.	.	0·87
ATP system								
B317a	38	—	8·6	1	.	2·42	.	.
36703T-B317	42	—	8·6	1	.	.	.	0·10
B317a	24	—	4·7	2	.	2·80	.	.
36703T-A	12C	—	4·5	2	.	.	0·65	.
36703T-A	16D	—	4·5	2	.	.	0·38	.
ST-74A	11	—	2·5	2·5	1·84	.	.	.
B369a	20	—	3·2	3	.	.	0·95	.
H4250A	22C	—	3·2	3	.	.	1·27	.

Table 4. *Mixtures of* arg-1$^+$ *and* arg-1$^-$ *extracts of* Neurospora crassa

Complete incubation mixture with 2·5 μmole ATP and the indicated amounts of D-3-phosphoglycerate (PGA). Differences of less than 0·35 μmole citrulline are within experimental error.

	arg-1$^+$		*arg-1*$^-$		Assay conditions		μmole citrulline disappearance caused by		
	Strain	Protein (mg.)	Strain	Protein (mg.)	Incubation time (hr.)	PGA (μmole)	*arg-1*$^+$	*arg-1*$^-$	Both together
Crude extracts	Wild type	0·60	H4250	0·62	4·25	85	1·32	0·16–0·32	1·82
	Wild type	0·60	46004	0·63	4·25	85	1·32	0·09–0·16	1·40
	Wild type	0·60	36703T-B317 (double mutant)	0·59	4·25	85	1·32	0·04–0·07	1·37
	Wild type	2·6	36703T	*c.* 3·1	2	45	2·43	0	2·60*
	30300 (*arg-2*)	1·06	B369	4·0	3	45	2·28	1·07	3·15*
Fractions	Wild type fraction II	3·57	36703T fraction II	3·43	1	50	3·04	0	2·85

* In these two cases, it was impossible to add enough PGA to insure excess high energy phosphate when the two extracts were mixed; therefore a slight synergism might go undetected. All other tests contained at least twice the amount of PGA required by the *arg-1*$^+$ component alone.

This possibility was ruled out completely for the *arg-1 arg-10* double mutant, since the *arg-10* block prevents arginine formation.

Mixing extracts from two different *arg-1* mutants was tested in two cases (Table 5); neither mixture showed synergism. Furthermore, the five *arg-1* mutants do not complement in heterocaryons in any combination (Newmeyer, 1957). It is concluded that all five *arg-1* mutants involve a simple loss of the same enzyme.

The residual activity of the *arg-1* extracts cannot reasonably be ascribed to bacterial action during the rather long incubation periods because controls incubated without enzyme never showed any citrulline disappearance, and because of the reproducibility of the activity when any strain was grown repeatedly under the same conditions. The question remained whether the residual citrulline disappearance was due to condensation or to irrelevant side reactions, as discussed in Methods. To facilitate answering this question, growth conditions were found which gave increased residual activity. But the *arg-10* controls grown under these conditions gave unexpected results which should first be discussed.

Anomalous behaviour of arg-10 mutants. The results reported so far indicate that *arg-10* strains, grown as in Table 2, gave extracts with completely normal condensing activity. However, when grown for 3 days at low arginine concentrations (0·035 or 0·07 mg./ml.) both *arg-10* alleles tested gave extracts with very little condensing activity. Four extracts from mycelia grown at 0·07 mg./ml. had specific activities in the D-3-phosphoglycerate system of only 0·05–0·06; the one extract from mycelium grown at 0·035 mg./ml. had a specific activity of 0·15.

It was shown that the low activity of these extracts was not due to variations in the amount of grinding, or to inadequate regeneration of ATP, or to inhibitor production (Table 6, line 1), and that only a small part of the reduction in activity could be explained by increased ATPase. Since the defect thus seemed to be a simple loss, it was of interest to assay the defective *arg-10* extracts in admixture with *arg-1*, to test the possibility that one type lacked the condensing enzyme while the other had insufficient pyrophosphatase. Since no extract of an *arg-1* single mutant was available for these tests, an extract of the double mutant was used, which was made from mycelia grown under conditions which gave normal condensing activity for *arg-10* alone. No stimulation was found (Table 6, lines 2, 3, and 4); it appears therefore that both *arg-1* and defective *arg-10* extracts lack the same enzyme.

However, accumulation tests indicated that the low activity of the defective *arg-10* extracts did not accurately reflect the situation during growth of the cultures. These tests were made by chromatographing undialysed samples of extracts prepared as for enzyme assay. They showed clearly that the defective *arg-10* cultures accumulated large amounts of argininosuccinate and no or negligible amounts of citrulline. Therefore, even at low arginine concentrations, *arg-10* must have had condensing activity *in vivo*; furthermore, if this activity was subnormal, citrulline synthesis must have been subnormal also. In contrast, the *arg-1 arg-10* double mutant, grown under the same conditions, accumulated citrulline but not argininosuccinate; this would indicate that it had no condensing activity *in vivo*. A wild-type culture accumulated neither compound, as expected.

Since at these arginine concentrations *arg-10* gave only 0·35 and 0·60 times as much growth as comparable wild-type cultures, it is quite possible that its extracts

Table 5. *Mixtures of two* Neurospora crassa arg-1$^-$ *extracts*

Tested as in Table 4 (except as noted) with crude extracts in all cases.

1st component		2nd component		Assay conditions			μmole citrulline disappearance caused by		
Allele	Protein (mg.)	Allele	Protein (mg.)	Incubation time (hr.)	PGA (μmole)	ATP (μmole)	1st component	2nd component	Both together
45004	2·5	B312	3·9	2	50	2·5	0·30	0·18–0·39	0·25
B369	3·2	H4250	2·4	3	0	20	0·95	1·05	1·40

Table 6. *Mixtures involving defective* arg-10 *extracts of* Neurospora crassa

Tests on dialysed crude extracts as in Table 4. Incubated 4·1 or 4·2 hr. with 71–75 μmole D-3-phosphoglycerate (PGA), conditions being constant throughout a given experiment. Half the amount of PGA used was adequate for the active *arg-10* component of line 1. All extracts were made from mycelia grown for 3 days at the arginine concentrations indicated in Table.

Defective *arg-10*			Other component			μmole citrulline disappearance caused by		
Allele	Arginine concentration of growth medium (mg./ml.)	Protein (mg.)	Type of extract	Arginine concentration of growth medium (mg./ml.)	Protein (mg.)	Defective *arg-10*	Other component	Both together
B317	0·07	1·30	active *arg-10*	0·5	2·36	0·36	4·29	4·05
B317	0·07	1·30	*arg-1 arg-10* double	0·5	1·07	0·35	0·12	0·12
B370	0·07	1·55	*arg-1 arg-10* double	0·5	1·07	0·35	0·10	0·35
B317	0·035	1·60	*arg-1 arg-10* double	0·5	1·07	1·07	0·10	1·27

had low activity simply because the cultures were growing slowly, or had even stopped growing, at the time of harvesting. If so, many enzyme activities might be diminished, both inside and outside the arginine pathway; the effect would then be unspecific and unimportant. A specific effect has not been excluded however. Although it was not feasible in this case to run curves of growth *v.* time, such as were run for all other experimental conditions, the available data make it quite unlikely that the 0·07 mg./ml. cultures had actually stopped growing before they were harvested. Furthermore it appears that the poor growth was not simply due to arginine limitation, since at this same concentration the inbred *arg-1 arg-10* double mutant grew nearly as well as the wild type. It is therefore possible that the excessive accumulation of argininosuccinate may interfere with growth; it might also prevent further synthesis of condensing enzyme. It has been shown by accumulation tests that these effects could not occur through depletion of the free amino acid pool; but the effect on condensing enzyme could happen through a repression mechanism (Vogel, 1957), which could affect citrulline synthesis as well.

Nature of residual arg-1 *activity.* Both the abnormal fractionation behaviour and the lack of ATP inhibition suggested that the residual activity of the *arg-1* extracts was due to loss of citrulline through irrelevant side-reactions (e.g. via citrullinase). Furthermore, the *arg-1* activity, at least in two alleles, was found to be largely independent of aspartate, Mg^{++} and ATP, in contrast to the strong dependence of the controls (Table 7). Additional tests on extracts 16 and 40 indicated that no single constituent of the system was required, although multiple omissions decreased the activity somewhat. Adding acetyl glutamate, substituting ADP for ATP, and lowering the pH to 6·1, all gave slight increases in activity.

Direct evidence for side-reactions was sought by chromatographically testing whether the citrulline disappearance caused by *arg-1(36703T)* was accompanied by argininosuccinate formation (Table 8). In some cases the *arg-1 arg-10* double mutant was used (the *arg-10* block being included to prevent the removal, through argininosuccinase and arginase, of any argininosuccinate that might be formed). Of the double mutant extracts used, no. 78 was made from mycelium grown under the same conditions as the active *arg-10* control extract, so that the lack of argininosuccinate production could be ascribed to the *arg-1* block. The others (nos. 40 and 48) were prepared under the conditions which gave the defective *arg-10* extracts. They could therefore not be used as proof that *arg-1* prevents argininosuccinate formation, but, because of their higher activity, they provided the best evidence for extensive side-reactions, and facilitated identification of the side-products.

All the wild-type and active *arg-10* control filtrates gave the expected strong argininosuccinate spots. None of the *arg-1* or double mutant filtrates showed any argininosuccinate, although the sample sizes were such that if all the missing citrulline had been converted to argininosuccinate, it should have given more than 2·5 times the minimum detectable amount.

Instead of argininosuccinate, the *arg-1* and *arg-1 arg-10* filtrates each gave four spots detected in the *arg-1*$^+$ controls; these were tentatively identified as glutamate, alanine, isoleucine and valine. In addition, a trace spot tentatively identified as carbamyl aspartate (detected by Ehrlich's reagent) was sometimes found, as well as dubious traces of ornithine. No arginine was detected, but it would not always have been detectable on these chromatograms. Its detection should be unnecessary,

Table 7. *Dependency tests on* arg-1$^+$ *and* arg-1$^-$ *extracts of* Neurospora crassa

All tests were made on dialysed crude extracts. 'Complete system' is as in Methods, with 2·5 μmole ATP and the indicated amounts of D-3-phosphoglycerate (PGA). 'Minimal system' contains citrulline and buffer only, with no aspartate, Mg, PGA or ATP.

			Assay conditions			Δ citrulline (μmole)		
	Strain	Extract no.	PGA (μmole)	Protein (mg.)	Incubation time (hr.)	Complete system	Complete minus aspartate	Minimal system
High activity	Wild type	5	25	3·2	1	−1·88	+0·20	.
	Wild type	15	60	2·5	4·3	−4·17	.	.
	Wild type	15	0	10·0	4·7	.	.	+0·12
	Wild type	17	35	6·9	1·0	−4·55	.	.
	Wild type	17	0	9·2	4·7	.	.	−0·03
	arg-10 (B317)	38 (active)	55	1·1	4·6	−2·51	.	.
	arg-10 (B317)	38 (active)	35	8·6	1·0	−2·70	.	.
	arg-10 (B317)	38 (active)	0	11·4	4·7	.	.	−0·03
Low activity	*arg-10* (B317)	34* (defective)	35	4·1	4·4	−0·70	.	.
	arg-10 (B317)	34* (defective)	0	4·1	4·7	.	.	+0·22
	arg-1 (36703T)	16C	40	4·2	4·0	−1·15	−1·15	.
	arg-1 (36703T)	16C	0	c. 4·4	4·3	.	.	−0·77
	arg-1 (36703T)	16C	0	c. 8·8	4·3	.	.	−1·07
	arg-1 (46004)	44	35	7·0	4·4	−1·05	.	.
	arg-1 (46004)	44	0	7·0	4·7	.	.	−0·30
	arg-1 (H4250)	46	35	6·2	4·4	−1·50	.	.
	arg-1 (H4250)	46	0	6·2	4·7	.	.	−1·08
	arg-1 arg-10 (double mutant)	40*	20	10·1	4·6	−1·90	−1·75	.
		40*	0	10·1	4·6	.	.	−1·20

* From mycelia grown for 3 days at 0·07 mg. arginine/ml. Growth conditions for all other extracts are given in Table 2.

Table 8. *Chromatographic tests for argininosuccinate formation by* arg-1 *mutants of* Neurospora crassa

'Complete' indicates complete system, as described in Methods, with 35 μmole PGA but only 10 μmole aspartate, unless otherwise noted. 'Minimal system 'indicates citrulline and buffer only. The incubation mixtures were deproteinized and developed on two-dimensional chromatograms. The estimated citrulline disappearance is based on assays performed under similar incubation conditions. For *arg-10* and the double mutant, the amount of citrulline disappearance per sample used on chromatograms should equal the amount of argininosuccinate found, if all activity is due to condensation. Under the conditions used, 0·14 μmole argininosuccinate is readily detectable, as determined by adding authentic argininosuccinate to unincubated control filtrates on two-dimensional chromatograms. Results are reported only from runs in which all spots were well-separated, except for occasional superimposition of arginine and citrulline.

	Genotype	Extract no.*	Incubation conditions: Protein (mg.)	Incubation conditions: Time (hr.)	Incubation conditions: Remarks	Estimated amount of citrulline disappearance per chromatogram sample (μmole)	Compounds detected†: Argininosuccinate	Compounds detected†: Glutamate
arg-1⁻ strains	*arg-1*	16C	*c.* 5·2	*c.* 5	Complete	*c.* 0·05	–	+
	arg-1	16C	*c.* 5·2	*c.* 5	Minimal system	0·045	–	+
	arg-1 arg-10 (double mutant)	78	10·7	4	Complete (50 μM-PGA)	0·035‡	–	+
	arg-1 arg-10 (double mutant)	40	10·2	5	Complete	–§	–	+
	arg-1 arg-10 (double mutant)	48	7·7	4	Complete	0·105	–	+
arg-1⁺ controls	*arg-10*	60 (active)	1·94	4¼	Complete	0·065	+ + +	–
	Wild type	15	1·24	4¼	Complete	0·07–0·09	+ + +	–
	Wild type	15	1·24	4¼	Complete (60 μM-PGA)	0·057	+ +	–

* Extract 48 is from mycelium grown 3 days at 0·035 mg. arginine/ml.; extract 78, from mycelium grown 3 days at 0·5 mg./ml. Growth conditions for all other extracts are given in Tables 2 and 7.

† See text for additional products. Neither argininosuccinate nor any of the other compounds was found in unincubated controls, except for part of the glutamate.

‡ This extract not assayed under these conditions. Estimate based on activity of extract 42C, which was prepared from the same strain under the same conditions.

§ Not run on two-dimensional chromatograms. Tested by elution of the argininosuccinate glutamate region from a one-dimensional phenol chromatogram followed by development of the eluate with butanol + acetic acid + water.

however, because of the inclusion of the *arg-10* block. Actually, much of the glutamate and alanine was also formed in the absence of citrulline. The spots tentatively identified as isoleucine and valine, however, were strongly citrulline-dependent. These two spots were identified only by their failure to separate from the added authentic compounds; several other amino acids with similar R_F values were not excluded. How citrulline could assist in the formation of isoleucine, valine, or any of these alternative amino acids is not known.

The separation of argininosuccinate and glutamate on two-dimensional chromatograms was unequivocal. On such chromatograms the product in the *arg-1*$^-$ and double mutant filtrates behaved exactly like glutamate. The possibility that *arg-1*$^-$ extracts did form argininosuccinate, but immediately destroyed it, e.g. by cyclization (Ratner *et al.* 1953), was excluded by adding equal amounts of argininosuccinate to samples of a double mutant incubation mixture, before and after incubation. Chromatograms of these two were identical. It is concluded that most or all of the citrulline disappearance caused by *arg-1*(*36703T*) extracts was unrelated to condensation. The results of the dependency tests and the lack of ATP inhibition indicated that this was probably true also for alleles B369 and H4250. A trace of true condensing activity in mutant 46004 was not excluded.

Nature of residual activity in defective arg-10 extracts. A dependency test on a defective *arg-10* extract suggested that the remaining activity was really due to condensation, since it vanished completely in the absence of aspartate + Mg^{++} + ATP (Table 7). The low protein concentration of these extracts precluded the conversion of enough citrulline to argininosuccinate to test this chromatographically.

Argininosuccinase. Fincham & Boylen (1957) showed that the four *arg-10* mutants lacked detectable argininosuccinase; they mentioned that the *arg-1* allele 36703T had about the wild-type concentration of this enzyme. Results obtained here, with the extracts recorded in Table 2 confirmed their results and showed that the other four *arg-1* alleles were also highly active in this respect.

DISCUSSION

The enzyme missing from the *arg-1* extracts is almost certainly the condensing enzyme itself, rather than the stimulatory enzyme, inorganic pyrophosphatase. Inasmuch as inorganic pyrophosphate is involved in several essential reactions and inhibits several others, it seems most unlikely that a deficiency of pyrophosphatase would result in viable mutants with an essentially complete requirement for a single growth factor. This conclusion is strengthened by the essentially complete absence of condensing activity in the *arg-1* mutants. Since the *arg-1* mutants lacked activity under all conditions tested, and since insertion of this block into the double mutant prevented argininosuccinate accumulation *in vivo*, it seems likely that *arg-1* is the primary locus determining ability to synthesize the condensing enzyme. The defective *arg-10* extracts also appear to lack condensing enzyme, but the inadequate supplement used here may well cause drastic reductions in many enzyme activities. Therefore it is doubtful whether *arg-10* has any specific effect on the condensing enzyme. If there is such an effect, it must be a secondary one, since *arg-10* strains have normal activity when grown at high arginine concentrations.

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On the Nature of Cytoplasmic Inclusions of *Nocardia rubra*

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SUMMARY

A cytological investigation of cell walls, lipid granules, metachromatic granules and chromatinic bodies of *Nocardia rubra* revealed that each of these structures existed as separable entities. Multicellularity was not observed, as indicated by the lack of septa in non-fragmenting organisms, but lipid inclusions were frequently found in close association with cell walls. Metachromatic granules were separable from lipid inclusions and were found to be homologous with electron opaque granules. No evidence for the origin of metachromatic granules as cytoplasmic condensations upon cell septa was found. Acid hydrolysis or ribonuclease treatment revealed Giemsa-staining chromatinic structures which were removed by deoxyribonuclease digestion. Metachromatic granules were not removed by ribonuclease, deoxyribonuclease hydrolysis or a combination of both, but these granules were removed by acid hydrolysis.

INTRODUCTION

In recent years several investigators have studied the cytology of members of the genus *Nocardia* (Bisset & Moore, 1949; McClung, 1950; Morris, 1951; Webb, Clark & Chance, 1954; Webb & Clark, 1957; Hagedorn, 1959*a*, *b*). Although their studies have been extensive, frequently the findings of these authors have been conflicting. The present investigations were initiated to augment knowledge of this group and to attempt to consolidate the different hypotheses which have been advanced about the cytology of the genus.

METHODS

Nocardia rubra received as *Proactinomyces ruber* (Casabó) Bald, from the Centraalbureau voor Schimmel cultures) *Bacillus megaterium*, and *B. subtilis* from the stock culture collection of the University of Georgia, were used in these studies. The organisms were routinely maintained on Difco nutrient agar slopes, and transferred at appropriate intervals prior to the manipulations which will be described below.

Cell-wall staining. The cell-wall staining techniques of Robinow (1945), Welshimer & Robinow (1949) and that of Webb (1954) as used by Adams & McClung (1960) were compared. Both conventional and heat-fixed impression smears of *Nocardia rubra* and *Bacillus megaterium* were made on microscope slides, and stained according to the above methods.

Chromatin staining. Several chromatin-staining methods, involving many modifications of the techniques as originally described, were examined in order to compare the results of other workers who studied the genus *Nocardia*. The crystal violet nuclear staining method developed by Chance (1952) and used by Webb, Clark &

Chance (1954) and by Webb & Clark (1957) for *Nocardia corallina*, was tested with heat-fixed impressions and those fixed over the vapours of 2 % (w/v) OsO_4, of *N. rubra* and *Bacillus subtilis*. Modifications of Chance's method were attempted in which Hucker's crystal violet (Society of American Bacteriologists, 1957), 0·5 % (w/v) aqueous crystal violet or 0·5 % (w/v) aqueous Azure A was substituted for the 1 % (w/v) aqueous crystal violet used in the unmodified technique.

The staining method devised by Smith (1950), eliminating formaldehyde mordanting, in which 0·3 % (w/v) aqueous basic fuchsin served as the chromatin stain was used in preliminary experiments with osmium-fixed impression smears of *Nocardia rubra*. The effects of different pH values of staining reagent were studied by preparing 0·3 % (w/v) basic fuchsin in 0·1 N-$Na_2HPO_4 + KH_2PO_4$ at pH 5·5, 5·9, 6·8 and 7·9 and substituting these dye solutions for the aqueous solution originally recommended. Periods of hydrolysis in N-HCl at 60° were from 2 to 10 min. and the effects of these variations were observed after staining with 0·3 % (w/v) aqueous basic fuchsin.

The following modification of Robinow's (1945) Giemsa chromatin staining technique was used as a basis for study. Impression smears of *Nocardia rubra*, fixed through the agar with osmium tetroxide vapours, were prepared, the organisms treated for 10 min. in N-HCl at 60°, washed in tap water, then distilled water. The smear was then stained in a solution of 1 part Giemsa stock solution to 10 parts tap water for 30 min. at room temperature, mounted in distilled water under a coverslip, sealed with paraffin and examined.

Modifications of Robinow's technique were attempted and determinations of optimum staining times were carried out by varying the periods of staining at room temperatures from 5 to 30 min. Smears which were stained and mounted under a coverslip in distilled water were compared to those which were stained and observed directly. The effects of treatment with HCl for 2 to 10 min. were determined as described above. The effects of ribonuclease digestion were determined according to the methods of Tulasne & Vendrely (1947): osmium-fixed impression smears were digested *in situ* with 0·02 % (w/v) ribonuclease in 0·1 M-$Na_2HPO_4 + KH_2PO_4$ (pH 7·0) at 55° for 15 min. and stained in a 1/10 dilution of Giemsa's stock solution in tap water for 5 min. The preparation was then washed in distilled water, blotted, and observed directly. The effects of deoxyribonuclease were determined by digesting an osmium tetroxide-fixed preparation in 0·02 % (w/v) deoxyribonuclease dissolved in the buffer described above, at 55° for 15 min. These were stained and examined in the manner already described.

Metachromatic granule staining. Heat-fixed impressions of *Nocardia rubra* were stained for metachromatic granules with Loeffler's methylene blue (Society of American Bacteriologists, 1957) for 1·5 min. The preparation was then washed with distilled water, blotted and examined. A modification of this technique was used in which 0·25 % (w/v) methylene blue adjusted to pH 3·1, 4·9, 7·1 or 8·3 with 0·04 M-Na_2HPO_4 was substituted for Loeffler's methylene blue. Mudd's (1953) technique was also used for the demonstration of these granules.

The effects of hydrolysis in N-HCl at 60° for 6 min. on the appearance of metachromatic granules in *Nocardia rubra* were observed by staining hydrolyzed organisms with methylene blue at pH 3·1 or with 0·5 % (w/v) aqueous Azure A substituted in the method described above. The effects of ribonuclease and deoxy-

ribonuclease digestion on the appearance of metachromatic granules in *N. rubra* were observed by digesting organisms as already described and staining with methylene blue at pH 3·1.

Lipid staining. Heat-fixed impression smears of *Nocardia rubra* were stained according to Burdon's (1946) Sudan Black B method; this technique was modified by omitting the counterstain. The lipid-staining method of Clark & Aldridge (1960) was also used.

The effects of lipase digestion of heat-fixed impression preparations were also studied. These experiments were conducted by subjecting *Nocardia rubra* to digestion with a 0·01 % (w/v) solution of lipase in 0·1 M-$Na_2HPO_4 + KH_2PO_4$ (pH 7·0) at room temperatures for 15 and 30 min. periods. The preparations were then stained by the modified Burdon technique and examined for the presence of lipid inclusions.

Successive staining. Two successive staining techniques similar to those of Tronnier (1953) and Knaysi (1955*a*, *b*, 1959), were used in the present work. The first method may be more readily described as counterstaining rather than successive staining; this method for the differentiation of lipid inclusions from metachromatic granules was carried out as follows: a heat-fixed impression smear of *Nocardia rubra* was stained by the modified Burdon technique. A counterstain was then used by treating with Azure A to reveal metachromatic granules. After treating the smear with both staining reagents, the slide was washed in distilled water, blotted and examined. This method allowed simultaneous demonstration of lipid inclusions and metachromatic granules. A second more complex method for successive staining was done as follows: an osmium-fixed impression was prepared in the usual way and stained with one of the previously described stains (e.g. Azure A for metachromatic granules). The slide was observed under oil, representative fields located and mechanical stage settings recorded; sketches of the field under observation also aided a return to the same field. Such fields were then photographed. The immersion oil was then removed with xylene, which also partially decolorized the preparation. Further decolorization was carried out with 95 % (v/v) ethanol in water until no more colour was removed. The slide was then washed in copious quantities of water and, after drying, restained by one of the methods described above (e.g. Burdon's modified lipid-staining technique); the fields which had previously been photographed were again located. After re-examination and re-photographing, the preparation could again be decolorized, after which staining for a third or fourth time was possible. Although the sequence of staining techniques applied to a single preparation was theoretically unlimited, practical considerations led to definite sequences which had to be used. Acid hydrolysis, as used in the chromatin-staining procedures, partially destroyed cellular integrity, and consequently was limited to the last place in a successively-stained sequence. Likewise, mordanting in 10 % (w/v) tannic acid for 30 min., as used in cell-wall staining techniques, affected the staining affinity of the organism, hence cell-wall staining was usually relegated to the last or next to last position of a sequence. However, sometimes cell-wall staining had to be done first in a sequence.

Microphotography. A Bausch and Lomb Dynoptic Research Microscope equipped with a 2 mm., N.A. 1·30 apochromatic objective with a ×12·5 compensating ocular and a variable focus condenser (N.A. 1·40) was used with a Bausch and Lomb

35 mm. microphotographic camera. A green filter was used to increase photographic contrast with Kodak High Contrast Copy 35 mm. film as the negative material. Magnification at the negative plane was ×900.

Electron microscopy. Nutrient agar slope cultures of *Nocardia rubra* were incubated for 5 hr. The organisms were removed from the slope, suspended in distilled water and a drop of suspension placed on a copper-grid supported collodion membrane. After the drop had dried the organisms on the surface of the membrane were washed with distilled water, re-dried and shadow-cast with platinum + palladium. Some preparations were treated for 2·5 min. in N-HCl at 60° and washed three times in distilled water before placing them on the collodion membrane for observation with the RCA EMU-2 electron microscope.

RESULTS

The cell-wall staining methods of Robinow (1945), Welshimer & Robinow (1949) and Webb (1954) were all found to be suitable for the demonstration of the cell walls of *Bacillus megaterium*, but only Webb's technique resulted in the consistent demonstration of cell walls and septa in young (Pl. 1, fig. 1) and old (Pl. 1, fig. 2) *Nocardia rubra* preparations. In the present investigations several methods were used to locate and differentiate metachromatic granules from other inclusions of *N. rubra*. Successive staining techniques were most fruitful in these respects. The location of septa and metachromatic granules was found to differ when a comparison was made of organisms stained for metachromatic granules with Azure A (Pl. 1, fig. 3), and decolorized and restained for cell walls (Pl. 1, fig. 4). The 'septa' seen at the arrows in Pl. 1, fig. 4, are metachromatic granules which stained bright-red and were incompletely decolorized with the xylene + ethanol treatment; in contrast, septa (Pl. 1, fig. 2) always stained a bright purple colour. In organisms successively stained for metachromatic granules and lipid inclusions (Pl. 1, figs. 5, 6) it was evident that metachromatic granules were not composed of lipids but that structures stained by these two methods could be closely associated. The counterstaining of Sudan Black B stained cells with Azure A confirmed their close, but separable, association.

Metachromatic granules, stained brightly with methylene blue at pH 3·1, against a contrasting lightly-stained cytoplasm are shown in Pl. 2 fig. 9. When using methylene blue reagent at various pH values, it was found that, as the pH value increased the cytoplasm stained more intensely, until at pH 8·3 (Pl. 2, fig. 10) metachromatic granules were undifferentiated or differentiated only slightly from the cytoplasm. Thus the pH value effects accounted for the poor differentiation of metachromatic granules observed when staining with Loeffler's methylene blue. The granules which stained with methylene blue at pH 3·1 were not removed by digestion with ribonuclease (Pl. 2, fig. 11) or by deoxyribonuclease (Pl. 2, fig. 12); but acid treatment removed them completely.

Electron opaque granules (Pl. 1, fig. 7) observed in young organisms were located in similar positions and were of the same form as the granules which were seen when metachromatic granules were stained in young organisms. Further evidence for the homology of electron opaque granules with metachromatic ones resulted from the removal of electron opaque granules by hydrolysis with N-HCl for 2·5 min. (Pl. 1, fig. 8).

Chance's (1952), Smith's (1950) and Robinow's (1945) methods for chromatin staining and modifications of these were compared to determine the relationship, if any, of metachromatic granules to nuclear materials. Organisms stained by Chance's method, without modification are shown in Pl. 3, figs. 15, 16 and 18. Five-hr. germinating organisms of *Nocardia rubra* (Pl. 3, fig. 15) contained single chromatinic structures in cell portions from which germ tubes originated but organisms from an 18 hr. culture (Pl. 3, fig. 16) contained chromatinic bodies throughout the filament length. Similar results were obtained when Hucker's crystal violet or 0·5 % (w/v) aqueous crystal violet were used. When Azure A was substituted for crystal violet in Chance's (1952) procedure, distinct bright-red staining granules (Pl. 3, fig. 17) were distributed at some distance from each other along the filament length, suggesting that Azure A did not stain chromatinic bodies but metachromatic granules by this procedure. Evidence for homology of chromatinic bodies stained by the unmodified procedure of Chance with metachromatic granules demonstrated by means of Azure A staining can be seen in organisms stained successively for these structures in Pl. 3, figs. 18 and 19, respectively. From these figures it is obvious that crystal violet non-differentially stained metachromatic granules which were then not distinguishable from similarly stained chromatinic structures. Additional evidence for the non-specificity of the crystal violet nuclear staining technique for chromatinic bodies of *N. rubra* is shown in the following observations. Chromatinic structures were revealed in germ tube segments of young organisms (Pl. 3, fig. 20) stained by Smith's (1950) or Robinow's (1945) techniques (Pl. 3, fig. 21). Chromatinic structures of germ tubes of germinating cells were not visible, however, in preparations stained by Chance's method (Pl. 3, fig. 15).

The modified technique of Smith (1950) was effective for the demonstration of distinct chromatinic structures in old non-germinating cells of *Nocardia rubra*. Chromatinic structures in organisms from cultures older than 3 days stained without appreciable differences when subjected to periods of acid treatment ranging from 2 to 7 min. However, both the pH value of the staining solution and the duration of hydrolysis preceding application of the stain affected the affinity for the stain of germ-tube chromatinic structures. The affinity of the chromatinic structures of the germ tube portions of the organisms varied inversely with the time of acid treatment and with decrease of pH value. As the hydrolytic period increased from 2 to 10 min. the affinity of the germ tube chromatinic bodies decreased. As the pH value changed discontinuously (see methods) from 5·5 to 6·7, their staining affinity increased; it remained constant when tested at pH 6·7 and 7·5. Optimum staining of the germ tube bodies was obtained when organisms were heated for 5 min. and stained at pH 6·7 (Pl. 3, fig. 20). However, as the age of the cultures increased to 15–20 hr., the staining affinity of chromatinic structures changed in relation to the duration of acid treatment; 2·5 min. was about optimum. These findings lend further support to the observed differences between staining affinities of germ-tube chromatinic structures and chromatinic structures in mature hyphae when stained by Chance's technique (compare Pl. 3, figs. 15–20).

The method of Robinow (1945) was found to be only applicable to *Nocardia rubra* when osmium tetroxide-fixed impressions were observed directly. Mounting in water sealed under a coverslip decreased the definition of chromatinic structures of Giemsa-stained preparation as compared to unmounted preparations, thus con-

firming similar observations on several organisms by Cassel (1951). Nevertheless, water mounting was found to be quite suitable for *Bacillus subtilis*.

The length of the periods of treatment with N-HCl was found to affect only slightly the definition and affinity of chromatinic structures of *Nocardia rubra* cells for Giemsa's solution. Periods of treatment from 2 to 10 min. were satisfactory for demonstrating these structures; 6 min. was optimum. Duration of the periods of immersion of the preparations in dilute Giemsa's solution also affected the definition of chromatinic structures. We found that a staining period of 5 min. at room temperature was optimum for *N. rubra*, while Robinow's (1945) original procedure was satisfactory with *Bacillus subtilis*.

While the acid-hydrolysis Giemsa technique is one generally accepted for the demonstration of chromatinic structures of bacteria (Robinow, 1956) conflicting reports about the nuclear elements of members of the genus *Nocardia* (Morris, 1951; McClung, 1950, 1955, 1956; Webb *et al.* 1954; Webb & Clark, 1957; Clark & Frady, 1957; Hagedorn, 1959*a*, *b*) dictated a re-evaluation of the specificity of this method as applied to *Nocardia rubra*. Osmium tetroxide-fixed impression smears of this organism, stained without previous acid or other treatment with Giemsa's solution, were found to have a medium to dark-blue stained cytoplasm interspersed with bright-red stained granules. Such untreated organisms appeared much like those stained for metachromatic granules with Azure A. Treatment with ribonuclease before staining with Giemsa's solution resulted in organisms like those shown in Pl. 2, fig. 13. Although dark-blue stained chromatinic bodies became visible as a result of ribonuclease digestion, the bright-red stained metachromatic granules were not affected. Deoxyribonuclease treated organisms contained lightly staining bands such as those seen at *A* in Pl. 2, fig. 14, but also contained bright-red staining bodies such as those seen at *B*. When treated with ribonuclease and deoxyribonuclease, the organisms stained with a mottled appearance, with no obvious separation of distinct granules other than the metachromatic granules which stained bright red. Organisms stained successively for metachromatic granules with Azure A (Pl. 3, fig. 23) and chromatinic bodies (Pl. 3, fig. 24) by Robinow's technique, offered further evidence that the metachromatic granules and chromatinic bodies were not homologous. Bright-red metachromatic granules visible after Azure A staining, as shown at the arrows in Pl. 3, fig. 23, were not observed at identical sites in Giemsa-stained preparations of the same organisms; see arrows in Pl. 3, fig. 24. Furthermore, bright-red granules were not visible after the application of Giemsa's solution; only chromatinic bodies which stained dark blue against very light blue cytoplasm were seen in acid-treated Giemsa-stained preparations.

Lipid inclusions in *Nocardia rubra* were readily observed by means of the modified Burdon technique (Pl. 1, fig. 6) but the counterstain used in the unmodified technique sometimes masked small lipid granules. Preparations stained successively for metachromatic granules and lipid inclusions (Pl. 1, figs. 5, 6) indicated that, although these two kinds of structures may be closely associated, they were separable entities. The apparent diameter of organisms stained for lipid inclusions compared with those stained for cell walls suggested that these structures were closely associated. In preparations stained by the method of Clark & Aldridge (1960), lipid inclusions were contrasted sharply against the nigrosin background (Pl. 1, fig. 6*a*) but when a comparison was made between nigrosin-decolorized organisms and non-decolorized

organisms in the same field, it was evident that many lipid granules were completely or partially decolorized as a result of the action of nigrosin. Lipase digestion under the conditions used in these experiments did not affect the affinity of these granules for Sudan Black B.

DISCUSSION

As early as 1898 Lachner-Sandoval described fragmentation of actinomycetes following septation. It was later reported by Lieske (1921) that fragmentation occurred after separation of the protoplasm, without prior septation, after which individuals separated. More recently (Bisset & Moore, 1949; McClung, 1950) the problem of the existence of septa in the actinomycetes and the genus *Nocardia* in particular, has been re-examined. Bisset & Moore (1949) reported that multicellularity was a characteristic of the genus *Nocardia*, but McClung (1950) was unable to demonstrate septa in *N. rubra* by means of Dyar's (1947) cell-wall staining technique. He later observed, by phase microscopy, that septum formation preceded fragmentation of *N. rubra* (McClung, 1955). Cell walls and septa of *N. corallina* were demonstrated by Webb *et al.* (1954) who suggested that anomalous multicellularity of this organism, time of fragmentation, and non-septate filament length, were influenced by environmental factors such as medium composition. By ultrathin sectioning, Hagedorn (1959*a*, *b*) did not observe multicellularity of *N. corallina*, except as a result of fragmentation. Under the conditions of the present experiments, non-fragmenting organisms of *N. rubra* were not multicellular, thus confirming the reports of previous investigators and of the authors (Adams & McClung, 1960).

Many investigators have discussed the deeply-staining granules of an unknown nature present in actinomycetes. Neukirch (1902) reported that strongly refractive granules could be stained with dilute solutions of methylene blue. Gilbert (1904), however, did not confirm the presence of such granules. Drechsler (1919) and Lieske (1921) observed in actinomycetes refractive granules which the former described as occluded wastes and the latter as nuclei. Lieske (1921) speculated whether there might be two types of methylene-blue staining granules, the first nuclear and the second, which appeared frequently at the apical tips of growing hyphae, as reserve materials such as volutin. Von Plotho (1948) was unable to demonstrate methylene-blue staining granules before acid hydrolysis in 5 day coccoids of *Nocardia rubra*, but McClung (1950) clearly established their presence by using different basic aniline dyes. The present studies have indicated a possible solution to the questions raised by the foregoing authors about the nature of these granules. We have shown that the manifestation of metachromatic granules with methylene blue is dependent upon the pH value of the solution used. Earlier workers did not indicate that the pH value of the staining reagent was taken into consideration and it seems likely that this factor was responsible for the conflicting results which have been previously reported.

Electron-opaque granules, designated type 'A' by McClung (1956), were observed in the present studies. Winkler (1953) who used a technique in which the same organisms were observed successively in the light-microscope and in the electron-microscope, found that electron-opaque granules of a bacillus were homologous with the metachromatic granules. By using a similar technique, Glauert & Brieger

(1955) found that the electron opaque granules of *Mycobacterium phlei* were identical with the metachromatic granules observed by light microscopy, but were not analogous with nuclei seen when the HCl-Giemsa technique was used. Oiwa (1960) reported that the acid-fast granules and electron opaque granules of *M. avium* appeared identical when studied by this technique. The location and hydrolyzable nature of the electron-opaque bodies of *Nocardia rubra* indicated that they were not nuclear in nature but similar to the electron-opaque inclusions described by the above authors and as volutin by Grula, Weaver & Edwards (1954) in Caulobacter. Lieske (1921) suggested that some of the deeply-staining granules present in actinomycetes were the result of cytoplasmic condensations; this idea was promulgated by Bisset (1952, 1953) who suggested that septa are sites of such condensations. The present successive-staining studies have negated the plausibility of such an explanation at least for *N. rubra*. The close proximity of cell walls and electron-opaque granules in this organism was shown in electron micrographs previously published by the authors (Adams & McClung, 1960).

The problem of the nucleus of actinomycetes has received attention through the years. Grigorakis (1931) reported amitotically dividing nuclei in *Actinomyces bovis*. The observations of Schaede (1939), however, were not in accord with such findings and he reported that the Feulgen technique resulted only in a diffuse uptake of the stain by the organism in question. Newcomer & Kenknight (1939) and von Plotho (1940) reported the presence of Feulgen-positive bodies in actinomycetes. McClung (1950) was unable to demonstrate a Feulgen-positive reaction in *Nocardia rubra* examined by conventional techniques; but when staining was carried out at 45° (McClung, 1953, unpublished data) Feulgen-positive bodies were observed. Hagedorn (1959*a*) reported that Feulgen-positive bodies could be found in *Nocardia corallina*.

The results of workers who used techniques other than the Feulgen reaction have not been so contradictory in demonstrating the presence of nuclear materials in organisms of the genus *Nocardia*. With the electron microscope McClung (1950) observed two types of granules present in *N. rubra* grown on nitrogen-deficient medium. He postulated that one of these structures was nuclear in nature, but he was unable to differentiate chromatinic bodies from metachromatic granules on the basis of concurrent light microscope observations of stained organisms. However, he did find that the solubilities of certain of these granules observed in stained preparations precluded the deduction of the homology of these granules with volutin. Webb *et al.* (1954) observed discrete nuclei in *N. corallina*, and in further studies (Webb & Clark, 1957) indicated that a complex developmental cycle existed, involving fragmentation and rearrangement of the nuclear apparatus of the organism. Hagedorn (1959*a*) objected to certain of their interpretations and suggested that inadequacies inherent in the crystal-violet nuclear-staining technique which they used accounted for faulty conclusions. In contrast, Hagedorn (1959*a*, *b*) described amitotically-dividing spherical to ovoid nuclei which were observed in ultrathin sections of *N. corallina*. Since we have shown that the crystal-violet staining technique may non-differentially stain metachomatic granules and may not stain chromatinic bodies demonstrable by other more specific means (e.g. Smith's or Robinow's techniques) our studies tend to confirm the suggestions of Hagedorn (1959*a*, *b*). The interpretations of Webb *et al.* (1954), and of Webb & Clark (1957) about ploidy changes suggested by microscopic observations with the crystal-violet

nuclear-staining technique would, as a result of the studies of Hagedorn (1959*a*, *b*) and ourselves, appear to be somewhat uncertain. However, Morris (1951) described a complex life cycle for members of the genus *Nocardia* in which complex nuclear reorganizations were observed by the acid Giemsa technique. Although we have not attempted to remove inclusion bodies of *N. rubra* stained by this technique and subject them to chemical analysis, the specificity of enzyme digestion and the resultant appearance of organisms after ribonuclease and deoxyribonuclease digestion strongly suggests that they are, in fact, composed primarily of deoxyribonucleic acid. Since genetic analysis of ploidy of members of the genus *Nocardia* has not yet been undertaken, it would appear advisable that further work should be done to determine more fully the role of the chromatinic structures during the developmental cycles of members of the genus *Nocardia*.

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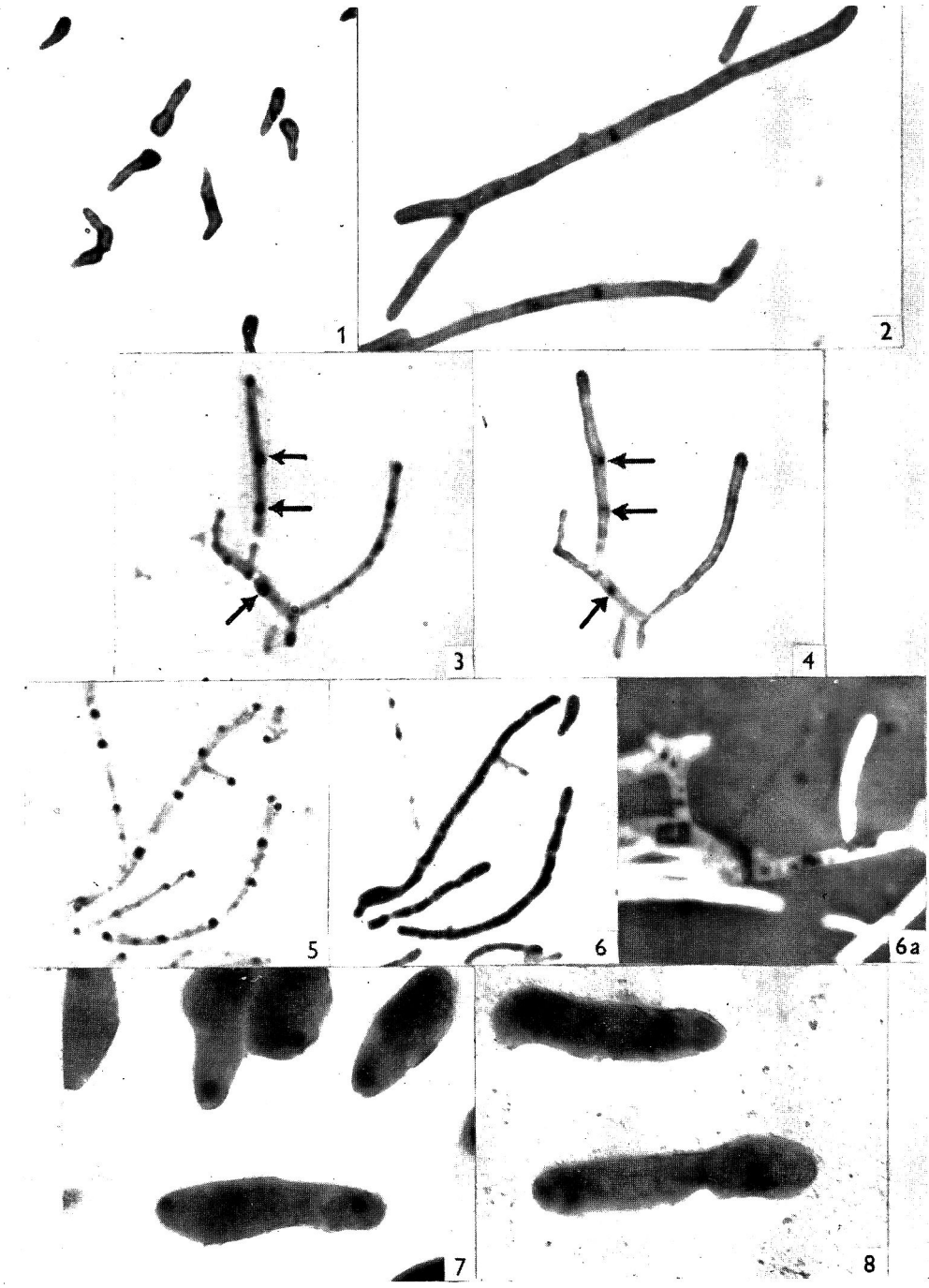
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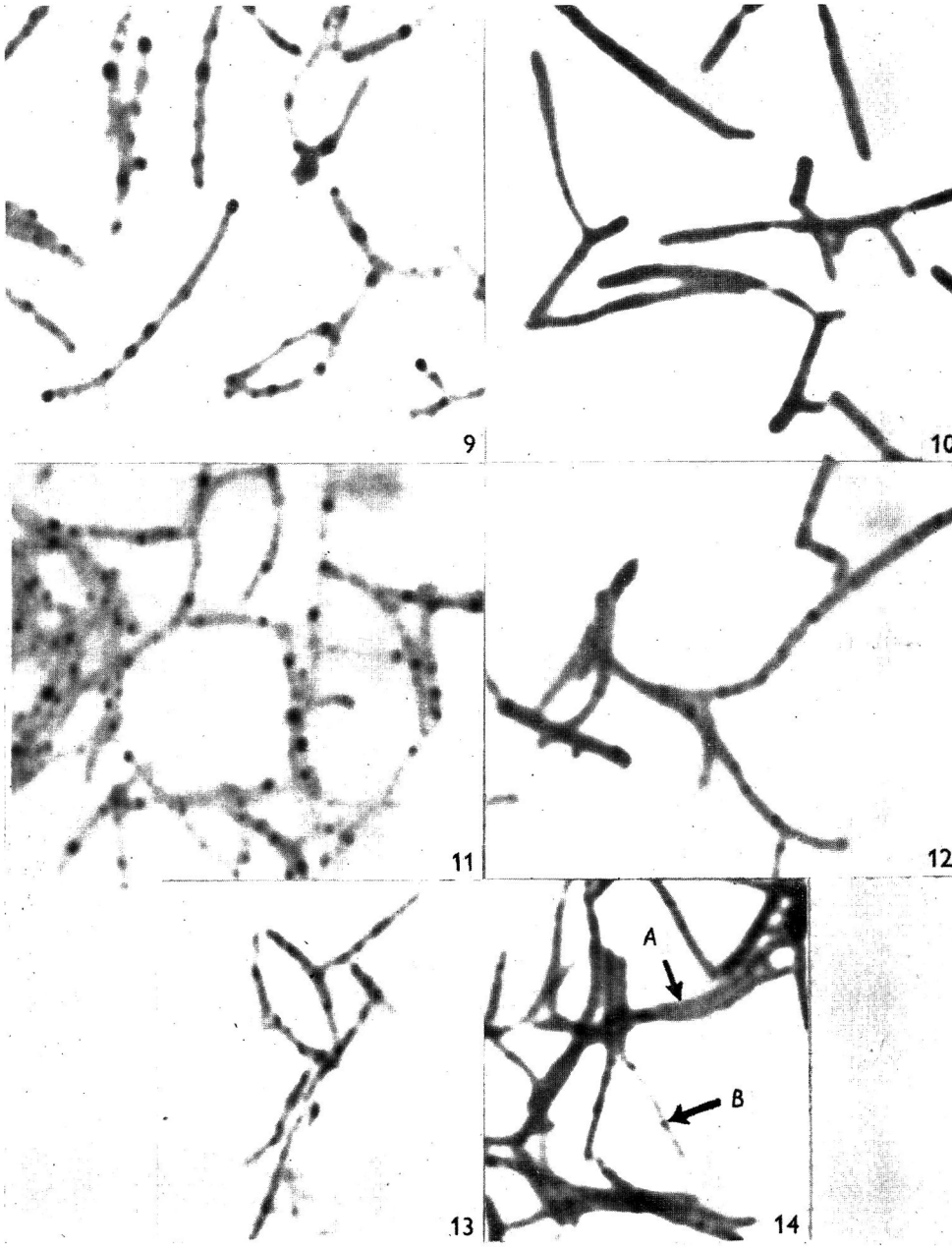
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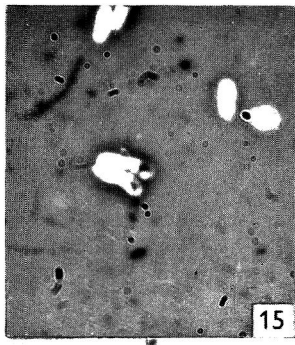

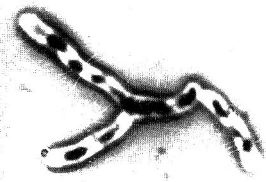

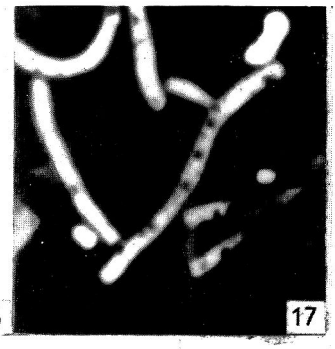

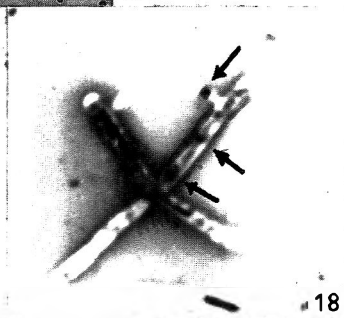

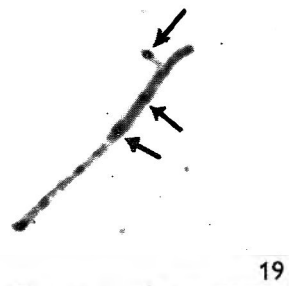

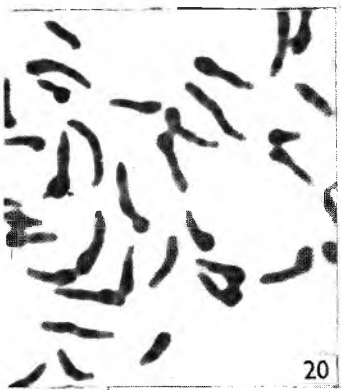

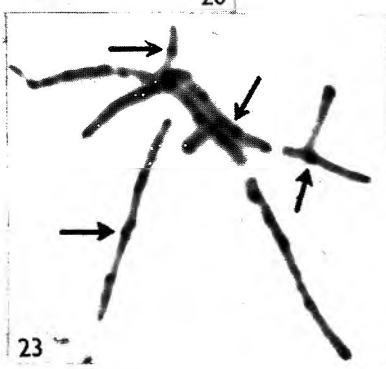

J. N. ADAMS AND N. M. McCLUNG

EXPLANATION OF PLATES

Figs. 1–6*a*, 9–24. Light micrographs of *Nocardia rubra*. ×2500.

Figs. 7, 8. Electron micrographs of *Nocardia rubra*. ×13,300.

Plate 1

Fig. 1. Germinating organism, 8 hr. culture. Webb's cell-wall stain.

Fig. 2. Filamentous organisms, 18 hr. culture. Webb's cell-wall stain.

Figs. 3, 4. Branching organisms, 18 hr. culture, stained successively for metachromatic granules and cell walls. Arrows indicate identical locations in each figure. Fig. 3. Azure A stain. Fig. 4. Organism shown in Fig. 3, Webb's cell-wall stain.

Figs. 5, 6. Organisms from 18 hr. culture stained successively for metachromatic granules and lipid inclusions. Fig. 5. Azure A stain. Fig. 6. Same organism as shown in Fig. 5, stained for lipid inclusions. Modified Burdon technique.

Fig. 6*a*. Organisms from 18 hr. culture, Clark and Aldridge lipid stain.

Fig. 7. Direct electron microscope preparation, 5 hr. culture. Shadowed with platinum + palladium.

Fig. 8. Organisms from 5 hr. culture hydrolysed with N-HCl for 2·5 min. before direct electron-microscope preparation. Shadowed with platinum + palladium.

Plate 2

Figs. 9–14. Organisms from 18 hr. cultures.

Fig. 9. Stained with methylene blue (pH 3·1).

Fig. 10. Stained with methylene blue (pH 8·3).

Fig. 11. Organisms digested with ribonuclease, stained with methylene blue (pH 3·1).

Fig. 12. Organisms digested with deoxyribonuclease, stained with methylene blue (pH 3·1).

Fig. 13. Organisms digested with ribonuclease, stained with dilute Giemsa's solution.

Fig. 14. Organisms digested with deoxyribonuclease, stained with dilute Giemsa's solution. Banding effects of digestion indicated by arrow at *A*; metachromatic granule indicated by arrow at *B*.

Plate 3

Fig. 15. Germinating organisms, from 5 hr. culture. Unmodified Chance's nuclear stain.

Fig. 16. Branching organism from 18 hr. culture. Unmodified Chance's nuclear stain.

Fig. 17. Organisms from 18 hr. culture. Chance's nuclear stain modified by substituting Azure A for crystal violet.

Figs. 18, 19. Organisms from 18 hr. culture, stained successively for chromatinic structures and metachromatic granules. Arrows indicate identical locations in each figure. Fig. 18. Unmodified Chance's nuclear stain. Fig. 19. Same organism as shown in Fig. 18, stained for metachromatic granules, Azure A stain.

Fig. 20. Germinating organisms from 5 hr. culture. Modified Smith's basic fuchsin nuclear stain.

Figs. 21, 22. Organisms stained using Robinow's acid Giemsa technique. Fig. 21. Germinating organisms from 5 hr. culture.

Fig. 22. Organisms from 18 hr. culture.

Figs. 23, 24. Organisms from 18 hr. culture stained successively for metachromatic granules and chromatinic structures. Arrows indicate identical locations in each figure. Fig. 23. Azure A stain.

Fig. 24. Same organisms as shown in Fig. 23, Robinow's acid Giemsa technique.

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Nucleic Acid Precursor Requirements of *Mycoplasma laidlawii*

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SUMMARY

The nucleic acid precursor requirements of *Mycoplasma laidlawii* strain A were determined by using a partially defined medium. Adenine, guanosine and cytidine were found to be the minimal growth requirements. However, best growth was obtained with undegraded RNA or oligoribonucleotides. Thymidine was not essential for growth when folinic acid was present in the medium; folic acid was completely inactive. *M. laidlawii* utilized the 2′- and 3′-ribonucleotides most poorly. The dephosphorylation of these mononucleotides to nucleosides rendered them growth promoting.

INTRODUCTION

The effects of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) on the growth of several Mycoplasma strains in a partially defined medium were described by Razin & Knight (1960*b*). The saprophytic *Mycoplasma laidlawii* strain A did not grow in the basal medium alone (Razin & Knight, 1960*a*) but grew on addition of suitable concentrations of RNA and DNA. Thymidine was found to be the moiety of DNA essential for growth. The fragments of RNA molecule needed for growth were not defined. Chemical or enzymic degradations of RNA to fragments smaller than oligonucleotides abolished its growth-promoting activity. It was therefore suggested that an oligoribonucleotide is required for growth of *M. laidlawii* strain A. The main purpose of the present work was to define the fragments of RNA molecule essential for growth of *M. laidlawii* strain A. Improvement of Razin & Knight's partially defined medium enhanced growth and permitted a more correct analysis of nucleic acid precursor requirements. In the latter medium, RNA could be replaced by adenine + guanosine + cytidine. However, growth of the test organism was best with undegraded RNA or with the oligonucleotides derived from it.

METHODS

Organism. *Mycoplasma laidlawii* strain A (PG 8) was received through the courtesy of Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent).

Media. A modified Edward medium (Razin & Oliver, 1961) was used for keeping stock cultures and for growing the organisms used as inoculum in the nutrition experiments.

The nutrition experiments were carried out in a modified partially defined medium

(Razin & Knight, 1960*a*). In this medium, consisting of inorganic salts, Casamino acids, vitamins, nucleic acids and glucose, 10% (v/v) pooled inactivated human serum was added instead of the horse serum used previously. This modification improved growth of the test organism without altering its requirements for nucleic acid precursors. The modified medium without added RNA and DNA will be referred to as basal medium.

Pyrex glassware was used in all experiments. Before use it was cleaned in chromic + sulphuric acid mixture and thoroughly rinsed in distilled water. The basal medium was dispensed in 9 ml. quantities into 6 in. × $\frac{5}{8}$ in. sterile screw-cap test tubes. The solutions of nucleic acids or their degradation products to be tested were added to give a final volume of 10 ml. Sterilization of nucleic acids and degradation product solutions was carried out by passage through sintered glass filters or by steaming for 20 min. (Merrifield & Dunn, 1950).

Conditions of growth. Five ml. liquid Edward medium were inoculated with 0·1 ml. stock culture and incubated for 24 hr. at 37°. The organisms were harvested and washed once with 0·01 M-K_2HPO_4 buffer, pH 7·0 (Butler & Knight, 1960). Each tube of experimental medium received 0·1 ml. of a 1/10 dilution of the washed suspension. Viable counts showed the initial inoculum to contain about 10^5 to 10^6 viable particles/ml. medium. Inoculated test tubes were incubated statically in air at 37°. Growth was usually estimated after incubation for 96 hr.

Assessment of growth. The extent of growth was measured by titration of the acid formed by the organisms during growth (Razin & Knight, 1960*a*).

Chemicals. Ribonucleic acid (Na salt from yeast; Na-RNA), deoxyribonucleic acid (Na salt from thymus gland; Na-DNA), purines, pyrimidines, nucleosides, nucleosides-2′- and 3′-phosphates, nucleosides-5′-phosphates and ribonuclease (× 4 crystallized, bovine pancreas) were the products of L. Light and Co. Ltd. (Colnbrook, Bucks). Folinic acid (Citrovorum factor) and aminopterin (4-aminopteroylglutamic acid) were obtained from the American Cyanamid Co. (Lederle Laboratories Division, New York, N. J., U.S.A.). Folic acid was the product of Hoffmann La Roche & Co. Ltd. (Basel, Switzerland). Acid phosphatase (wheat germ) was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Viper venom (*Vipera palestinae*) was obtained from the Zoology Department of the Hebrew University, Jerusalem, through Dr D. Nelken. The venom was freeze-dried over P_2O_5 at 0·3 mm. Hg. (Centrifugal freeze-drier, W. Edwards and Co., London).

Degradation of ribonucleic acid. Acid hydrolysis of RNA to oligonucleotides was done by the method of Merrifield & Woolley (1952) as described by Razin & Knight (1960*b*). Degradation of RNA to oligonucleotides and pyrimidine mononucleotides by ribonuclease and to nucleosides by crude viper venom was carried out as described previously (Razin & Knight, 1960*b*). Hydrolysis of RNA to nucleoside-2′- and -3′-phosphates was done by dissolving 300 mg. Na-RNA in 20 ml. 0·5 N-NaOH. The solution was incubated at 25° for 24 hr., neutralized with N-HCl and made up to volume for bioassay. The nucleoside-2′- and -3′-phosphates obtained by the alkaline hydrolysis of RNA were dephosphorylated to give nucleosides by acid phosphatase. Five mg. of this enzyme were added to 6·5 ml. of the mononucleotides solution, which was brought to pH 5·6 and incubated at 37° for 22 hr. Inorganic phosphorus determination (Umbreit, Burris & Stauffer, 1957) at the end of the

incubation period showed the degradation of 90 % of the mononucleotides to nucleosides.

Dialysed human serum was prepared by the method described previously (Razin & Knight, 1960*a*).

RESULTS

Requirement of Mycoplasma laidlawii *for thymidine*

Previous work has shown that the requirement of *Mycoplasma laidlawii* for DNA might be completely replaced by thymidine and to a lesser extent by thymine (Razin & Knight, 1960*b*). Further study has now shown that *M. laidlawii* can grow

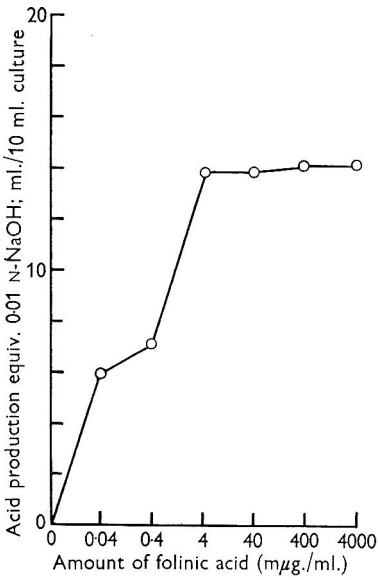

Fig. 1

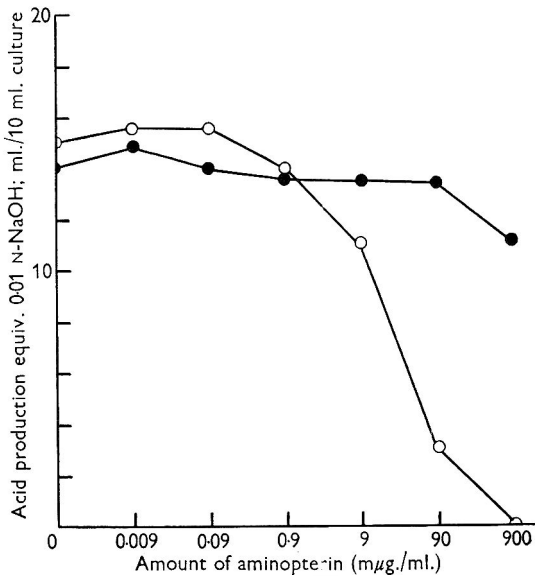

Fig. 2

Fig. 1. The growth response of *Mycoplasma laidlawii* to folinic acid. Test tubes contained 10 ml. basal medium supplemented with 50 μg. Na-RNA/ml. and various concentrations of folinic acid. Initial inoculum 2×10^5 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

Fig. 2. Growth inhibition of *Mycoplasma laidlawii* by aminopterin and its annulment by folinic acid. Test tubes contained 10 ml. basal medium supplemented with 50 μg. Na-RNA/ml. and various concentrations of aminopterin. One series of test tubes contained 4 mμg. folinic acid/ml. (○); the other, 400 mμg. folinic acid/ml. (●). Initial inoculum 5×10^5 viable particles/ml. Acid production determined after incubation at 37° for 114 hr.

in the absence of added DNA or thymidine when folinic acid is added to the medium. The growth response of *M. laidlawii* to various concentrations of folinic acid is presented in Fig. 1. A concentration of 4 mμg. folinic acid/ml. sufficed for optimal growth and 0·4 mμg./ml. enabled about 50 % of optimal growth.

Folic acid was completely inactive in replacing thymidine, even at the highest concentration tested (50 μg./ml.).

Aminopterin inhibited growth promoted by folinic acid. This growth inhibition was overcome by raising the concentration of folinic acid or by incorporating thymidine (50 μg./ml.) into the medium (Fig. 2).

Requirement of Mycoplasma laidlawii *for RNA components*

The medium used for these experiments contained thymidine or folinic acid in amounts sufficient for optimal growth. Degradation of RNA to oligonucleotides by treatment with 6 N-HCl for 1–3 min. at 25° (Merrifield & Woolley, 1952) did not significantly affect its growth-promoting activity. Further hydrolysis of the oligonucleotides by longer treatment with acid rapidly abolished their growth-promoting activity (Fig. 3).

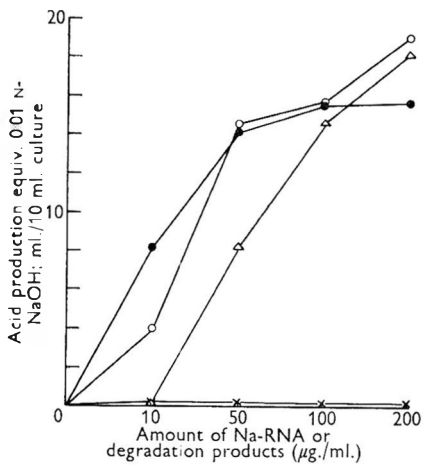

Fig. 3

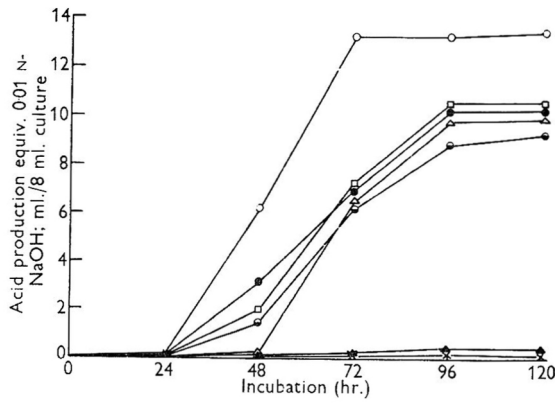

Fig. 4

Fig. 3. The effect of acid hydrolysis of RNA to oligonucleotides on its growth-promoting activity for *Mycoplasma laidlawii*. Test tubes contained 10 ml. basal medium supplemented with 50 μg. thymidine/ml. and different concentrations of: undegraded RNA (●); RNA hydrolysed by 6 N-HCl at 25° for 1 min. (○); 3 min. (△); 7 min. (×). Initial inoculum 10^5 viable particles/ml. Acid production determined after incubation at 37° for 96 hr.

Fig. 4. The effect of RNA and its degradation products on the growth of *Mycoplasma laidlawii*. Erlenmeyer flasks contained 40 ml. basal medium supplemented with 50 μg. thymidine/ml. and: Na-RNA, 50 μg./ml. (○); degradation products of RNA by ribonuclease, 50 μg./ml. (●); nucleoside mixture (adenosine, guanosine, cytidine, uridine, 15 μg./ml. of each) (□); 5′-ribonucleotide mixture (adenylic acid, guanylic acid, cytidylic acid, uridylic acid, 15 μg./ml. of each (◒); alkaline hydrolysate of RNA, 500 μg./ml. (△) or 50 μg./ml. (▲); 2′- and -3′-ribonucleotide mixture (adenylic acid, guanylic acid, cytidylic acid, uridylic acid, 15 μg./ml. of each (×). Initial inoculum 4×10^5 viable particles/ml. Acid production determined in 8 ml. samples of cultures taken after various incubation periods at 37°.

Figure 4 represents growth curves of *Mycoplasma laidlawii* inoculated into the basal medium, supplemented with 50 μg. thymidine and Na-RNA or its degradation products/ml. Best growth was obtained with undegraded RNA. Degradation of RNA to a mixture of oligonucleotides and -3′-pyrimidine mononucleotides by pancreatic ribonuclease decreased its growth-promoting activity, and alkaline hydrolysis of RNA to nucleosides-2′- and -3′-phosphates nearly abolished it. Fifty μg. of the alkaline degradation products/ml. medium were unable to support growth, while 500 μg./ml. of these degradation products enabled slow and limited growth (Fig. 4). Similar results were obtained with a mixture of the four nucleoside-2′- and -3′-phosphates obtained from commercial sources (adenylic, guanylic,

cytidylic, uridylic acids). Mixtures of the -5′-phosphate isomers of the above mentioned mononucleotides were active in growth promotion. A mixture of the four nucleosides, adenosine, guanosine, cytidine, uridine (15 μg./ml. each) enabled growth to take place. Growth promoted by nucleosides was inferior to that promoted by undegraded RNA (Fig. 4). Degradation of RNA to nucleosides by viper venom decreased, but did not abolish, its growth-promoting activity (Fig. 5). Hydrolysis of RNA to purine bases and pyrimidine nucleotides by 1·5 N-H_2SO_4 at 100° abolished its growth-promoting activity completely. Purines and pyrimidines, in several mixtures and concentrations, were inactive in growth promotion. Addition of D-ribose to the purines and pyrimidines mixture was without any effect.

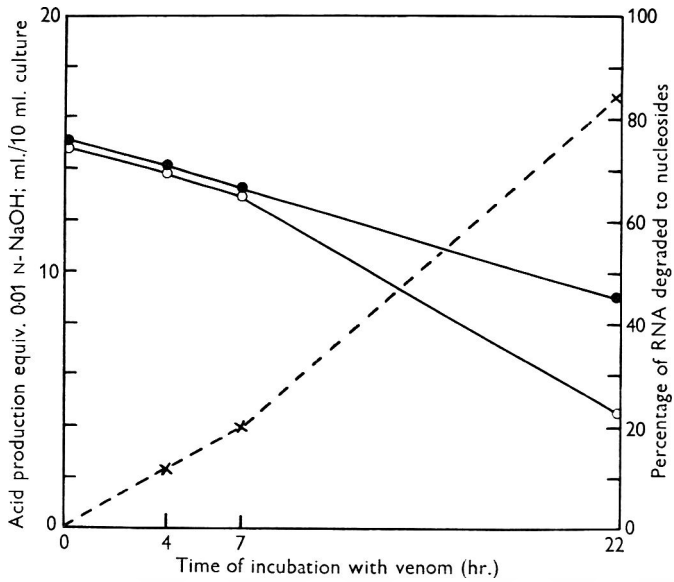

Fig. 5. The effect of degradation of RNA to nucleosides on its growth-promoting activity for *Mycoplasma laidlawii*. Twenty mg. of dried viper venom were added to 10 ml. of Na-RNA solution (5 mg./ml.) in 0·01 M-$MgCl_2$. The mixture was brought to pH 8·6 by N-NaOH and incubated at 37°. Samples for bioassay and inorganic phosphorus determination (Umbreit, Burris & Stauffer, 1957) were taken at the time intervals indicated and placed for 5 min. in a boiling water bath to stop the reaction. Test tubes contained 10 ml. basal medium supplemented with 50 μg. thymidine/ml. and 250 μg./ml. (●) or 50 μg./ml. (○) of RNA degradation products. Percentage of RNA degraded to nucleosides (×) was calculated from inorganic phosphorus determinations. Initial inoculum 10^5 viable particles/ml. Acid production determined after incubation at 37° for 87 hr.

On the basis of these results experiments were carried out to determine the nucleosides essential for the nutrition of *Mycoplasma laidlawii*. By the single omission method it was found that adenosine, guanosine and cytidine were required, whereas uridine could be omitted without affecting growth. Best growth was obtained when the concentration of the three nucleosides in the medium was rather high (100 μg./ml. of each); at lower concentrations growth was markedly diminished (see Figs. 4, 5).

The possibility of replacing the nucleosides by their corresponding purine and pyrimidine bases was tested. Adenosine could be replaced by adenine; while

5 μg./ml. was found to be optimal, higher concentrations showed some growth-inhibitory activity. Hypoxanthine could replace the requirement for adenosine, but was less effective than adenine. Guanosine could not be replaced by guanine. Nor could cytosine replace cytidine. The pyrimidine ring precursors ureidosuccinic acid and orotic acid were inactive. Thus the smallest fragments of RNA still capable of promoting growth of *Mycoplasma laidlawii* are adenine (5 μg./ml.), guanosine (100 μg./ml.) and cytidine (100 μg./ml.).

Experiments were also performed in order to replace each of the three nucleosides, adenosine, guanosine or cytidine, by its corresponding mononucleotide, either the -2′-, -3′- or the -5′-isomer. Adenosine could be replaced by any of the three isomers, whereas guanosine and cytidine could be replaced only by the -5′-isomers. These results are in agreement with the observation that the mixture of -2′- and -3′-mononucleotides obtained by alkaline hydrolysis of RNA was practically inactive in growth promotion. The alkaline hydrolysate of RNA became active when its constituent mononucleotides were dephosphorylated to nucleosides by acid phosphatase.

All the above results were obtained with the partially defined medium which contained 10 % (v/v) whole human serum. In order to exclude the possibility that small amounts of nucleic acid components present in the whole serum were masking the true nutritional requirements of *Mycoplasma laidlawii* to a certain degree, the serum was exhaustively dialysed. Although growth of the test organism in the medium containing the dialysis residue of the serum was somewhat inferior to that obtained in whole serum medium, it sufficed for the analysis of nucleic acid precursor requirements. Adenine, guanosine, cytidine and thymidine were found essential for growth in the dialysed-serum medium.

DISCUSSION

The results of the present work show that *Mycoplasma laidlawii* strain A is capable of synthesizing thymidine when folinic acid is added to the medium. Folic acid is inactive, probably due to inability of the Mycoplasma to reduce it to folinic acid, the derivatives of which are the biologically active forms of this vitamin (Rabinowitz, 1960). The amount of folinic acid required for growth of *M. laidlawii* is of the same order as that required by *Pediococcus cerevisiae*. The latter micro-organism, however, is able to grow when a high concentration of folic acid (2 μg./ml.) is present in the medium (Nichol, 1959; Grossowicz & Mandelbaum, 1961), whereas even with 50 μg. folic acid/ml. no growth of *M. laidlawii* took place. Aminopterin inhibited growth of *M. laidlawii* only when its concentration was several times higher than that of folinic acid. Aminopterin is particularly effective as an inhibitor of the enzymic reduction of folic acid to the tetrahydro form. Therefore its antagonistic activity towards organisms which are unable to reduce folic acid to folinic acid is lower (Nichol, 1959; Handschumacher & Welch, 1960). The complete reversal by thymidine of growth inhibition caused by aminopterin indicates that *M. laidlawii* requires folinic acid for the biosynthesis of thymidine only, when growing in the partially defined medium of Razin & Knight (1960*a*).

The minimal growth requirements of *Mycoplasma laidlawii* for nucleic acid precursors were found to be the nucleosides adenosine, guanosine and cytidine.

Of these, only adenosine could be replaced by its purine base adenine. The better utilization of nucleosides for growth of various micro-organisms, as compared with their purine or pyrimidine bases, has been described (Loring & Pierce, 1944; Nakamura, 1957; MacLeod, Hogenkamp & Onofrey, 1958; Chakraborty & Loring, 1960).

The findings of the nutrition studies indicate the presence in *Mycoplasma laidlawii* of various enzymic activities concerned with nucleic acid metabolism. This organism is capable of transforming ribonucleosides to deoxyribonucleosides. Vitamin B_{12} is usually involved in this transformation (Wacker, Kirschfeld & Traeger, 1959; Manson, 1960). The question whether *M. laidlawii* requires vitamin B_{12} for growth must remain open until a completely defined medium is devised for it. *M. laidlawii* is capable of deaminating cytidine to uridine, apparently by the action of a cytidine deaminase described in a variety of microbial and mammalian cells (Crosbie, 1960). The enzymic methylation of deoxycytidine or deoxyuridine, with folinic acid as cofactor, apparently supplies *M. laidlawii* with the required thymidine. Another enzymic ability of this organism is the amination of hypoxanthine to adenine.

The very significant decrease in the growth-promoting activity of RNA after its hydrolysis to 2′- and 3′-mononucleotides might be explained by the well-known observations that bacteria and various animal tissues incorporate mononucleotides only after dephosphorylation to nucleosides (Leibman & Heidelberger, 1955; Lesley & Graham, 1956; Lichtenstein, Barner & Cohen, 1960). *M. laidlawii* apparently lacks the phosphatases which attack the 2′- and 3′-mononucleotides. Dephosphorylation of these mononucleotides to nucleosides by commercial acid phosphatase enabled good growth of *M. laidlawii*. The decrease in the growth-promoting activity of RNA after its degradation by pancreatic ribonuclease might be similarly explained by the appearance of the biologically inactive 3′-pyrimidine mononucleotides as part of the degradation products (Davidson, 1960).

A problem which as yet remains unsolved is why growth of *Mycoplasma laidlawii* should be much faster and better with undegraded RNA or oligoribonucleotides than with smaller fragments of the molecule. Are undegraded RNA or oligonucleotides capable of penetration into the Mycoplasma cell? Undegraded DNA is known to penetrate into bacterial cells (Fox & Hotchkiss, 1960) and mammalian cells (Gartler, 1960). Lately RNA was also found to be capable of penetrating into tissue cells (Amos, 1961). The thin and plastic cell envelope of the Mycoplasma (Edwards & Fogh, 1960; van Iterson & Ruys, 1960) is apt to facilitate such penetration. Oligoribonucleotides were found to serve as primers for RNA synthesis by polynucleotide phosphorylase of *Azotobacter agile*. The oligonucleotides abolished the lag period usually observed on testing the activity of this enzyme (Singer, Heppel & Hilmoe, 1960). Utilization of undegraded oligonucleotides by Mycoplasma might similarly enhance RNA synthesis and consequently protein synthesis, thus explaining the faster growth rate observed in the presence of undegraded RNA or oligoribonucleotides.

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Oxidation of Homogentisic Acid by Cell-free Extracts of a Vibrio

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SUMMARY

Acetoacetic acid is a product of degradation of homogentisic acid by cell-free extracts of a vibrio grown with phenylacetic acid as sole carbon source. 4-Maleylacetoacetate was identified as a reaction intermediate; reduced glutathione was a cofactor for this enzymic degradation. These reactions are similar to those catalysed by enzymes of rat liver.

INTRODUCTION

Homogentisic acid is an intermediate in the bacterial oxidation of tyrosine (Suda & Takeda, 1950; Jones, Smith & Evans, 1952), phenylalanine (Dagley, Fewster & Happold, 1953) and phenylacetic acid (Dagley *et al.* 1953; Kunita, 1955*a*). In animal liver 4-maleylacetoacetate is first formed from homogentisate by the single-step addition of O_2, catalysed by homogentisate oxidase; it then isomerizes to 4-fumarylacetoacetate. This reaction, which requires reduced glutathione (GSH), is followed by hydrolysis of 4-fumarylacetoacetate to fumarate and acetoacetate (Knox & Edwards, 1955; Knox, 1960). Many alternative routes exist for the oxidation of aromatic compounds by bacteria (Dagley, Evans & Ribbons, 1960) and further evidence was therefore required before it could be assumed that the same reactions occur when bacteria oxidized homogentisic acid or its precursors. This evidence is now presented.

METHODS

The organism used was the vibrio O 1 (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen; NCIB 8250) originally isolated by Happold & Key (1932). Batches of organisms were grown at 30° with forced aeration in a medium adjusted with NaOH to pH 7·2 and containing (g./l.): KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0·4; phenylacetic acid, 0·7. Organisms were transferred from a slope to 25 ml. of this medium aerated at 30° in a boiling tube (6 in. × 1 in.) and after 24 hr. the whole of the fully-grown culture was used to inoculate 1 l. of medium aerated in a 2 l. flask. The resulting culture was used as inoculum for 8 l. of medium in a 10 l. flask into which passed a glass tube fitted with an aquarium aerator stone. From 9 l. of this culture about 6 g. (wet weight) of organism was obtained after incubation overnight at 30°. All buffer solutions contained 2 g. of KH_2PO_4/l., brought to pH 7·2 with NaOH. Cell-free extracts were prepared from organisms disrupted at −14° without abrasive in the bacterial press

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of Hughes (1951), extracted with buffer and centrifuged at 10,500 *g* for 45 min. to remove insoluble material. Uptake of O_2 and evolution of CO_2 were followed by the conventional manometric procedures (Umbreit, Burris & Stauffer, 1949). Acetoacetic acid was determined by coupling with diazotized *p*-nitroaniline to give a coloured substituted formazan. This reaction is quantitative when carried out according to Walker (1954) and is specific for compounds containing a reactive methylene group; thus, pyruvic acid and many other metabolites do not react. Acetoacetic acid was prepared from freshly-distilled ethyl acetoacetate by the method of Krueger (1952). *m*-Hydroxyphenylacetic acid was prepared according to King & McMillan (1946). Other materials were commercially available. The protein concentrations of cell-free extracts were determined by the method of Sols (1947) with reference to a calibration curve made by using crystalline bovine serum albumin. Concentrations of maleylacetoacetate and fumarylacetoacetate were calculated from measurements of optical densities at 330 mμ, one part of the given solution being brought to pH 1 and the other to pH 13 by addition of HCl and NaOH, respectively. Knox & Edwards (1955) used the known molar extinction coefficients at these two pH values to derive formulae which give the amounts of maleylacetoacetate and fumarylacetoacetate in mixtures of the two. Absorption spectra were read in a Unicam SP500 spectrophotometer using silica cells of 1 cm. light path.

RESULTS

Oxidation of homogentisate by crude cell extracts

Cell-free extracts of the vibrio grown with phenylacetic acid catalysed the rapid oxidation of homogentisic acid (Fig. 1*a*) with an uptake of nearly 1·5 mole O_2/mole homogentisate. Phenylacetic, homoprotocatechuic (3:4-dihydroxyphenylacetic) and mono-hydroxyphenylacetic acids were not oxidized. When uptake of oxygen had ceased the flask contents were cooled in ice, protein precipitated by addition of 0·2 volume of 20 % (w/v) metaphosphoric acid and the precipitate removed by centrifugation and discarded. The contents of the flask containing cell extract but no substrate were treated similarly and served as a control. No reaction was given for homogentisic acid (Neuberger, 1947) but its conversion to a β-oxoacid was shown by a strong positive Rothera reaction (Rothera, 1908) and by the immediate evolution of CO_2 on addition of aniline citrate to solutions in respirometer flasks (Umbreit *et al.* 1949). Similar enzyme preparations from rat liver convert homogentisate to acetoacetate (Knox & Edwards, 1955). When samples of deproteinized reaction mixtures were treated according to the procedure of Walker (1954) the spectrum of the compound formed was very similar to that given by acetoacetic acid similarly treated (Fig. 2). The quantity of acetoacetate agreed with that given by aniline citrate decarboxylation and was equivalent to the homogentisate which disappeared (Table 1).

Oxidation of homogentisate by dialysed cell-free extracts

When crude cell extracts were dialysed for 6 hr. at 5° against phosphate buffer the oxidation of homogentisate was catalysed only when ferrous ions were added and the total oxygen uptake was also decreased but was still greater than 1 mole O_2/mole substrate (Fig. 1*b*). After reaction, solutions were deproteinized with

metaphosphoric acid, neutralized with 2 N-NaOH and equal volumes diluted with distilled water, 3 N-H_2SO_4 and 5 N-NaOH for determination of absorption spectra at pH values of 7, 1, and 13 respectively (Fig. 3). The spectra are similar to those of maleylacetoacetate (Knox & Edwards, 1955) with a maximum at 330 mμ at pH 13, abolished at pH 1. From the published extinction coefficients, about 1·0 μmole

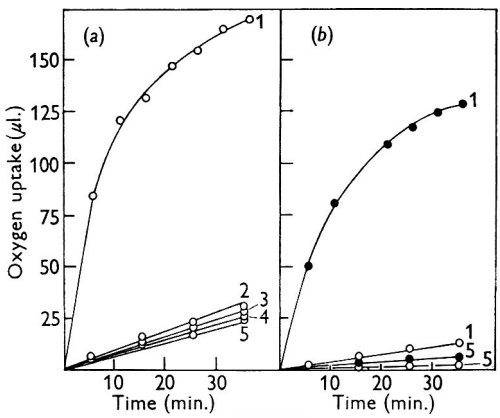


Fig. 1

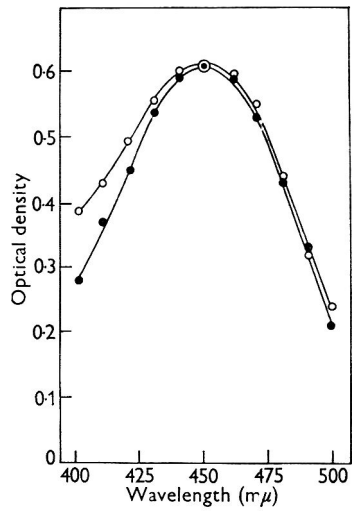


Fig. 2

Fig. 1. Oxidation of homogentisic acid by extracts of vibrio O 1 grown with phenylacetic acid as carbon source. The extract used was either (*a*) untreated or (*b*) dialysed for 6 hr. Warburg flasks at 30° contained extract (equiv. 20 mg. protein) and buffer (pH 7·2) to a total volume of 3 ml. and KOH in centre well. The substrates (5 μmole) added from side bulbs are shown by numbers; 1, homogentisate; 2, homoprotocatechuate; 3, phenylacetate; 4, *o*-, *m*- and *p*-phenylacetate; extract, but no substrate was added in 5. In Fig. 1*b*, mM-$FeSO_4$ was added to mixtures shown by blacked-in circles.

Fig. 2. Absorption spectra of solutions in ethyl acetate after reaction with diazotized *p*-nitroaniline. Acetoacetate was coupled under standard conditions (Walker, 1954) and the reaction product extracted completely into 4 ml. ethyl acetate. Product of enzymic oxidation of homogentisate by untreated vibrio O 1 extract (○—○); 8·5 μg. authentic acetoacetic acid (●—●).

Table 1. *Formation of reaction intermediates in the enzymic oxidation of homogentisate*

Reactions took place in Warburg flasks at 30° containing 5 μmole homogentisate, cell-free extract of vibrio O 1 (20 mg. protein) and buffer, pH 7·2, to a total volume of 3 ml. When O_2-uptake ceased, mixtures were deproteinized by addition of 0·3 ml. 20 % (w/v) metaphosphoric acid. The amounts of acetoacetate (by coupling with diazotized *p*-nitroaniline) and of 4-maleylacetoacetate and 4-fumarylacetoacetate (from extinctions at 330 mμ) were then determined.

Expt.	Enzyme	4-Maleylaceto-acetate (μmole)	4-Fumarylaceto-acetate (μmole)	Acetoacetate (μmole)
1	Untreated	0	0	4·80
2	Dialysed for 6 hr.; mM-$FeSO_4$ added	1·02	0·18	—
3	Dialysed for 12 hr.; mM-$FeSO_4$ added	2·63	0·32	—
4	Dialysed for 12 hr.; mM-$FeSO_4$ + 0·3 mM-GSH added	0	0	4·85

maleylacetoacetate and 0·2 μmole fumarylacetoacetate accumulated; the latter absorbs ultraviolet radiation strongly in acid solution and contributes to the spectrum at pH 1 in Fig. 3. In a similar experiment an extract was dialysed for 12 hr. and the yield of maleylacetoacetate was more than doubled. When reduced glutathione was added, no ultraviolet absorbing compound accumulated and homogentisate was converted almost quantitatively to acetoacetate (Table 1).

The identity of the compound formed from homogentisate by the bacterial extract was confirmed by comparing its reaction with *o*-phenylenediamine to that of maleylacetoacetate obtained by means of the liver preparation. To each 2 ml. of a solution containing about 5 μmole maleylacetoacetate were added 6 ml. of a freshly made solution of 200 mg. *o*-phenylenediamine dissolved in 25 ml. of the following

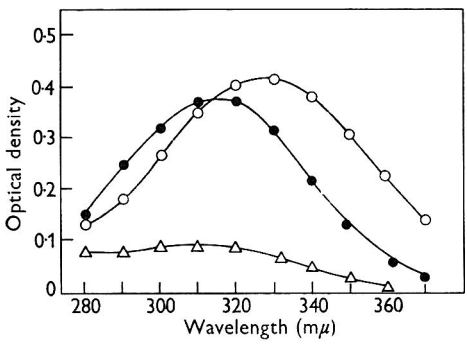


Fig. 3

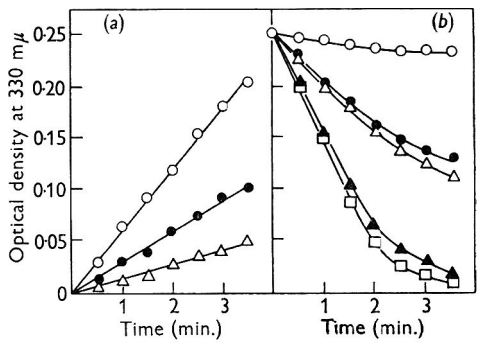


Fig. 4

Fig. 3. Absorption spectra of the product of oxidation of homogentisate by a dialysed vibrio O 1 cell extract. Spectra were read at: pH 7 (●—●), pH 1 (△—△), pH 13 (○—○).

Fig. 4. Formation (*a*), and breakdown (*b*), of maleylacetoacetate catalysed by a partially purified extract (10 mg. protein/ml.) of vibrio O 1. In (*a*), cuvettes contained, in a final volume of 3 ml., 1·0 μmole homogentisate, distilled water and either 0·2 ml. extract, (○—○), 0·1 ml. extract (●—●), or 0·05 ml. extract (△—△). In (*b*), the complete system contained in 3 ml., 0·06 μmole 'liver' maleylacetoacetate, 1·0 μmole GSH, distilled water and either 0·05 ml. extract (△—△) or 0·1 ml. extract (□—□). Similar systems contained 'bacterial' maleylacetoacetate with 0·05 ml. extract (●—●) or 0·1 ml. extract (▲—▲). A control cuvette contained maleylacetoacetate and 0·1 ml. of extract but no GSH (○—○).

phosphate + sulphuric acid reagent: 43·5 g. KH_2PO_4 and 20 ml. 5 N-orthophosphoric acid were dissolved in 500 ml. of 2·4 N-H_2SO_4 and diluted to 1 l. with distilled water. Both solutions of maleylacetoacetate developed a lavender colour which changed slowly to orange (Ravdin & Crandall, 1951).

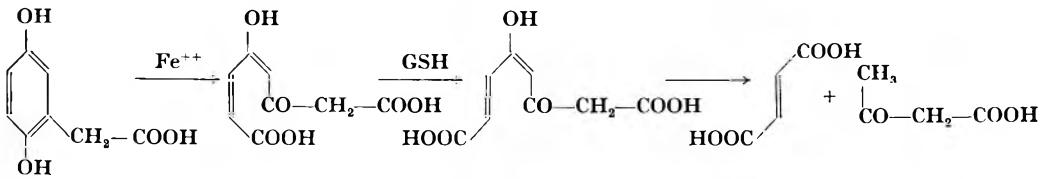
Formation and disappearance of maleylacetoacetate catalysed by partially purified cell extracts

Saturated ammonium sulphate solution, brought to pH 7·3 with ammonia, was added dropwise with stirring to a crude extract of cells until 60 % saturated. The precipitate was centrifuged at 10,000 *g* for 15 min. at 2°, the supernatant fluid discarded and the pellet dissolved in phosphate buffer. The solution was dialysed against changes of buffer at 5° until the dialysis residue no longer gave a reaction for sulphate ions; then 0·4 ml. of 0·033 M-$FeSO_4$ was added to each 10 ml.

of treated cell extract. After incubation for 1 hr. at 5° a slight precipitate was centrifuged down and discarded. In phosphate buffer (pH 7·2) this extract catalysed a linear production with time of maleylacetoacetate; this was followed by measuring the increase in extinction at 330 mμ, from homogentisate at room temperature (Fig. 4*a*). The extract also decomposed both 'bacterial' and 'liver' maleylacetoacetate, but only on addition of reduced glutathione (Fig. 4*b*).

DISCUSSION

The work of Ravdin & Crandall (1951) and Knox & Edwards (1955) established that the following reactions occur in animal liver:



homogentisate → maleylacetoacetate → fumarylacetoacetate → fumarate + acetoacetate

The requirement of homogentisate oxidase for Fe^{++} ions was readily demonstrated by dialysis of extracts of our vibrio O1; Suda & Takeda (1950) made a similar observation for a tyrosine-grown pseudomonad. By contrast, it was necessary to use inhibitors to show participation of Fe^{++} ions in homogentisate oxidation by the enzyme from liver (Knox & Edwards, 1955). These ions may be essential for all known aromatic ring-fission enzymes in bacteria (Dagley *et al.* 1960). Cell-free extracts did not oxidize phenylacetic acid or its monohydroxylated derivatives (Fig. 1); bacterial enzymes which catalyse the introduction of a hydroxyl group into the benzene nucleus are either labile or not readily soluble (Dagley & Patel, 1957). Homoprotocatechuic acid, an isomer of homogentisic acid, is an intermediate in an alternative pathway of oxidation of phenylacetate (Kunita 1955*b*) but it was not oxidized in the presence of extracts of our vibrio O1. Homogentisate was oxidized with the uptake of 1·0–1·5 μmoles O_2/μmole substrate; oxygen may be required both for ring-fission and for oxidation of fumarate which is shown as a product of the above reaction sequence. Although it has not been identified as a product, fumarate is readily oxidized by cell-free extracts of the vibrio, with an uptake of 0·5 μmole O_2/μmole substrate.

Further evidence for the above reaction sequence in phenylacetate-grown vibrio O1 is provided by: (i) the adsorption spectra at various pH values of the product of homogentisate oxidase (Fig. 2); (ii) the fact that this product gives the same colour reaction with *o*-phenylenediamine as authentic maleylacetoacetate; (iii) the ability of partially-purified extracts to decompose the latter only when incubated with reduced glutathione, the known cofactor of maleylacetoacetate isomerase (Fig. 4*b*). Homogentisate was converted almost quantitatively to acetoacetate as predicted by the proposed sequence (Table 1). These reactions were also similar to those studied by Lack (1959), who showed that maleylpyruvate was an intermediate in the oxidation of gentisic acid by bacterial cell extracts.

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An Environmentally-Induced Transition from the Flagellated to the Non-flagellated State in *Salmonella typhimurium*: the Fate of Parental Flagella at Cell Division

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SUMMARY

Bacteria in cultures of *Salmonella typhimurium* LT2 were peritrichously flagellated when grown in nutrient broth at 37°; but most were non-flagellated when grown for 6 mean-generation times or more at 44°. When a culture growing exponentially at 37° was transferred to 44°, growth continued at about the same rate; but the synthesis of new flagella was largely curtailed. The fate of the parental flagella was studied by staining and counting flagella on bacteria from samples taken during growth at 44° of cultures first grown at 37°. After 3 mean-generation-times the average number of flagella/flagellated bacterium had fallen from about 8 to about 2 and the proportion of flagellated bacteria from about 100 % to about 60 %. The distribution of numbers of flagella/bacterium was at all times unimodal, with the mode decreasing from about 8 to 0. In non-growing cultures at 44° there was little or no change in the average number of flagella/bacterium, in the proportion of flagellated bacteria, or in the distribution of numbers of flagella/bacterium. It is inferred that parental flagella are neither rapidly shed at 44° nor retained entirely by one daughter cell at each division but are distributed about equally between the two daughter cells.

INTRODUCTION

Observations on the morphology of bacteria by light and electron microscopy have led some workers to infer that in rod-shaped bacteria the growth of the bacterial cell wall occurs mainly or entirely at one pole. Bisset (1951), Bisset & Pease (1957) and Bisset & Hale (1960) examined, by microscopy, preparations of *Salmonella* spp. and of other rod-shaped organisms. They concluded that during growth and division the portion of the parental cell wall which bore flagella passes in its entirety to one daughter cell, and that the other daughter had a new cell wall and grew new flagella. For ease in discussion of the modes of partition of parental flagella, this view will be called the 'all or none' hypothesis. On the other hand, the results of micromanipulation experiments (Stocker, 1956*a*, *b*; Quadling & Stocker, 1957; Quadling, 1958) on the unilinear transmission of motility and, by inference, of flagella, from parent to daughter cells in *Salmonella* spp. are most readily interpreted by assuming that 'parental' flagella are shared about equally

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between the daughter cells produced at each division. This idea will be referred to as the 'sharing' hypothesis.

We report here an attempt to obtain evidence of the fate of bacterial flagella at cell division, by treating growing cultures of flagellated *Salmonella typhimurium* so that growth continues but no new flagella are produced. If, as Bisset suggested, the original cell wall and all the flagella of a peritrichously flagellated bacterium pass to one of its daughters, a bimodal distribution of numbers of flagella/bacterium would be expected to develop after the cessation of synthesis of flagella, with an increasing proportion of the population (all those with 'new' cell walls) having no flagella, and a decreasing proportion (those with the original cell walls and flagella) having several flagella each. Alternatively, if the flagella of a multiflagellate bacterium be shared between its daughters, a unimodal distribution would be expected, with the modal number of flagella/bacterium decreasing as the original flagella are distributed amongst the increasing bacterial population.

It was found that the synthesis of flagella by growing bacteria could be suppressed by transferring a logarithmic-phase broth culture of a wild-type flagellated *Salmonella typhimurium* strain (LT2) from 37° to 44°. Growth continued at about the same rate as before, but the average number of flagella/bacterium fell from about 8·2 to less than 1, and the proportion of non-flagellated bacteria increased from less than 5 % to 70 %, or more. The changes in distributions of number of flagella/bacterium were also investigated. Though the suppression of synthesis of flagella was less complete and abrupt than had been hoped, the results obtained are compatible with the hypothesis that parental flagella are shared at bacterial division; they cannot, we think, be reconciled with the alternative hypothesis. A preliminary report of this work has already been given (Quadling & Stocker, 1956).

METHODS

Salmonella typhimurium strain LT2 of Zinder & Lederberg (1952) was used. The cultural methods were described by Quadling & Stocker (1957). The growth of cultures was followed by turbidity measurements made with a Hilger 'Spekker' photoelectric absorptiometer or by plate counts of viable bacteria made by the method of Miles & Misra (1938). Cultures were grown in nutrient broth made from a papain digest of lean beef (Asheshov, 1941). Changes in temperature of incubation were effected by diluting cultures into broth at 44°. Cultures were incubated in stationary capped, conical flasks held in a water bath at the appropriate temperature (37°, 44°); they were maintained in exponential growth at populations below 10^8 bacteria/ml. by periodic dilution with prewarmed broth.

Staining and counting of flagella were carried out by a modification of Leifson's (1951) method as described by Quadling (1958). The flagella were counted on each of the first five organisms seen in each of ten or more successive scans of stained smears, starting at arbitrarily chosen points. In some experiments the slides were coded and the counts repeated by one of us who had no knowledge of the times of sampling. This procedure would be expected to decrease observer bias in the selection of organisms for counting. Possible sources of error in observed distributions of numbers of stained flagella were discussed by Quadling (1958). Broth cultures were examined for motility by low-power dark-ground microscopy as described by Stocker

(1956*b*). To determine the percentage of organisms which were motile, suitably diluted samples were examined in a Helber chamber. Separate counts of motile and of non-motile organisms were made and the figures combined to yield a percentage value.

RESULTS

The effect of various treatments on distribution of numbers of flagella

Typical peritrichous flagellation was shown by cultures of strain LT2 grown exponentially in nutrient broth at 37°; the average number flagella/bacterium was about 8 and the proportion of non-flagellated, non-motile, organisms was less than 5 %. Distribution of numbers of flagella/bacterium in such cultures are given in Table 1 (entries 1 and 4) and in Table 2 (times 15 min. and −15 min.). In preliminary experiments it was found that when exponentially growing cultures of strain LT2 were transferred to 44° growth continued at about the same rate as before but the proportion of non-flagellated bacteria increased as growth continued at 44°. To determine whether this increased proportion of non-flagellated bacteria resulted from the suppression of flagellar synthesis or from the rapid shedding of flagella, distributions of numbers of flagella were determined after treatments involving temperature change, with and without growth. It was found that in the absence of growth there was little change in the number flagella/bacterium during incubation at 44° (Table 1). This was shown for a 37°-grown stationary-phase culture held for 4 hr. at 44°, for a formalin-killed 37°-grown log-phase culture held 4 hr. at 44°, and also for a 37°-grown log-phase culture incubated for 4 hr. at 44° with 100 μg. chloramphenicol/ml. (which arrests growth and prevents synthesis of new flagella). We conclude that flagella grown at 37° are not rapidly shed at 44° and that the change from the flagellated to the non-flagellated condition at 44° is associated with growth of the bacteria rather than with adverse effects on the flagella themselves. When cultures grown at 44°, in which less than 1 % of the bacteria were flagellated, were placed at 37° the bacteria rapidly regained flagella. In one such experiment 20 % of the bacteria had demonstrable flagella after 30 min. growth at 37°, and 48 % had flagella after 60 min. Such results are evidence that the loss of flagella at 44° is phenotypic only, and that new flagella are produced on return to a suitable environment.

Effect of growth at 44° on distribution of numbers of flagella

To investigate the effects of continued growth at 44° on the distribution of numbers of parental flagella, the synthesis of new flagella was curtailed by transferring to 44° an unaerated broth culture which was in logarithmic growth at 37°. Such cultures were maintained in logarithmic growth for over 4 hr. by periodic dilution with fresh broth held at 44° in a water bath. Samples for flagella staining were taken at intervals and fixed with formaldehyde. The growth of cultures was followed by turbidity measurements or by plate counts of viable bacteria: the mean doubling time at both temperatures was about 40 min., but varied from experiment to experiment. The distributions of number of flagella/bacterium were determined by counts of flagella present on 50–200 bacteria from each sample in random fields of smears stained by Leifson's (1951) method. Results are given in Tables 2–4 and are illustrated in Figs. 1–3. In general, little change occurred in the mean number of

Table 1. *Effects of various treatments on flagellation of* Salmonella typhimurium *strain* LT2

Culture samples were fixed by addition of formalin to give a final concentration of 0·1 % (w/v) HCHO and stained by Leifson's (1951) method.

	Numbers of bacteria with flagella numbering																			
Treatment	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16 and over	No. bact. examined	Mean no. flagella/ flagellated bacterium	% bacteria flagellated
(1) Log-phase culture grown at 37°	1	0	0	3	1	6	2	4	10	8	6	3	3	0	0	2	1	50	8·4	98
(2) As (1) but then grown 4 hr. at 44°	140	42	10	3	3	1	0	1	0	0	0	0	0	0	0	0	0	200	1·6	30
(3) As (1) but fixed with formalin and held 4 hr. at 44°	0	0	0	0	2	5	4	8	11	5	10	0	2	2	0	0	1	50	9·1	100
(4) Log-phase culture grown at 37°	0	0	1	0	1	8	4	3	12	10	5	2	2	1	0	1	0	50	8·0	100
(5) As (4) but then held 4 hr. at 44° in presence of 100 μg. chloramphenicol/ml.	1	0	1	2	1	5	6	7	11	4	5	1	4	0	0	2	0	50	7·8	98
(6) Stationary phase culture grown at 37°	1	1	2	0	1	1	6	6	9	5	8	2	6	1	0	1	0	50	8·4	98
(7) As (6) but then held additional 4 hr. at 44°	0	0	1	1	4	6	5	9	11	3	6	2	2	0	0	0	0	50	6·9	100

Table 2. *Effect of growth at 44° on distribution of numbers of stained flagella per bacterium in cultures of* Salmonella typhimurium *strain* LT2

Cultures in logarithmic phase at 37° were transferred to 44° at zero time and maintained in logarithmic growth by periodic dilution. Culture samples were fixed at stated times by addition of formalin and stained by Leifson's (1951) method.

	Time from transfer (min.)	Numbers of bacteria with flagella numbering																	
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16 and over	No. of bacteria examined
Expt. 1*	15	—	0	0	2	1	2	4	3	5	3	8	4	7	3	1	2	5	50
	45	—	1	1	2	3	3	1	3	7	7	7	2	4	4	3	0	0	48
	75	—	2	5	5	8	10	8	6	4	1	0	0	0	0	0	0	0	49
	105	—	19	25	25	12	9	6	1	3	0	0	0	0	0	0	0	0	100
	135	—	42	30	13	13	2	0	0	0	0	0	0	0	0	0	0	0	100
	165	—	49	35	11	3	0	2	0	0	0	0	0	0	0	0	0	0	100
	180	—	62	22	10	2	2	1	1	0	0	0	0	0	0	0	0	0	100
	195	—	63	19	10	5	1	0	0	1	1	0	0	0	0	0	0	0	100
	216	—	73	18	8	1	0	0	0	0	0	0	0	0	0	0	0	0	100
	225	—	37	10	2	1	0	0	0	0	0	0	0	0	0	0	0	0	50
	245	—	80	14	2	3	1	0	0	0	0	0	0	0	0	0	0	0	100
	265	—	80	16	3	1	0	0	0	0	0	0	0	0	0	0	0	0	100
	280	—	86	9	3	2	0	0	0	0	0	0	0	0	0	0	0	0	100
Expt. 2	−15	1	1	0	3	1	6	2	4	10	8	6	3	3	0	0	2	1	50
	+30	0	1	3	0	8	4	0	7	5	3	6	3	5	0	2	2	1	50
	60	2	8	5	8	9	8	12	9	5	2	1	0	1	0	0	0	0	70
	120	18	25	19	24	1	8	1	1	2	0	1	0	0	0	0	0	0	100
	180	42	27	16	7	2	3	1	2	0	0	0	0	0	0	0	0	0	100
	240	140	42	10	3	3	1	0	1	0	0	0	0	0	0	0	0	0	200

* In Expt. 1 the data shown are the distribution of no. of flagella/bacterium amongst 50 or 100 flagellated bacteria; the proportion of non-flagellated bacteria (i.e. the omitted 'zero' class of this table) is shown in Table 3, column (*c*).

flagella/bacterium during the first doubling time at 44°, but after 60 min. this number decreased; it continued to decrease until most bacteria had no flagella and the few remaining flagellated bacteria had only one or a small number of flagella each.

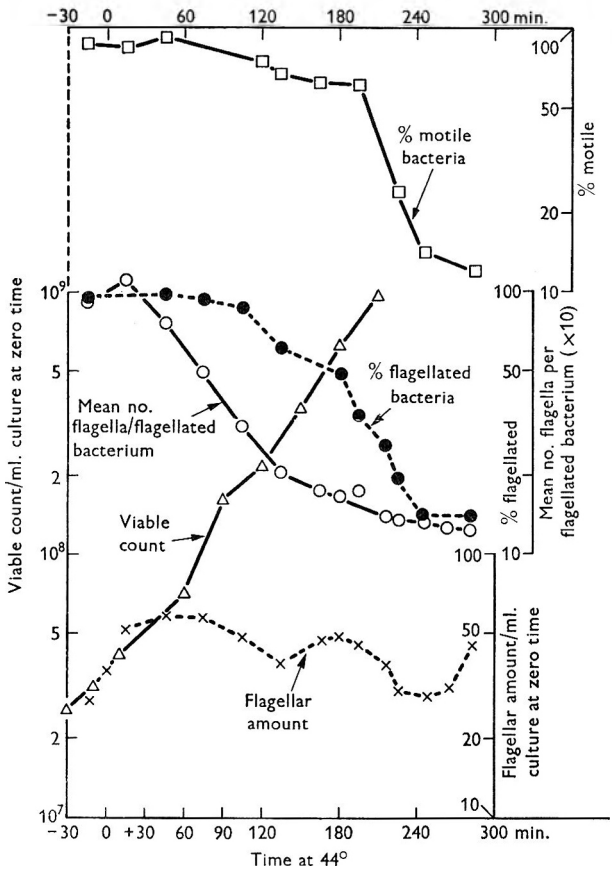

Fig. 1. Effect of growth at 44° on flagellation and motility of a logarithmic phase culture of *Salmonella typhimurium* grown at 37° and transferred to 44° at time zero. Cultures were maintained in logarithmic growth by periodic dilution with pre-warmed broth at 44°. Samples were fixed at stated times by addition of formalin and stained by Leifson's (1951) method. The data are plotted semi-logarithmically and are from Expt. 1, Tables 2 and 3. Viable count as plotted is corrected for dilution during growth.

Data from two experiments are shown in graph form as semi-logarithmic plots against time (Figs. 1, 2). There is an excellent correspondence between estimates of the % motile bacteria and % flagellated bacteria at the times of sampling (Fig. 1). This is evidence for the reliability of the staining technique and for the lack of any significant loss of flagella during preparation and staining, especially at the later times of sampling. For reference, the reciprocal of 'notional' turbidity is plotted in Fig. 2. Notional turbidity was obtained by adjusting observed turbidities to compensate for the periodic dilution of the starting culture which occurred during the experiment. The slope of this line is a measure of the rate at which the culture was diluting its original cell mass by fresh growth. The mean number of flagella/

bacterium decreased from about 45 min. at about the same exponential rate as would be expected if this change were a consequence of dilution by growth. Such a result would be expected if parental flagella were retained only by one daughter bacterium or were shared between both daughters at division. However, the data show that the mean number of flagella *per flagellated bacterium* also decreased steadily towards unity, instead of remaining constant as predicted by the 'all or none'

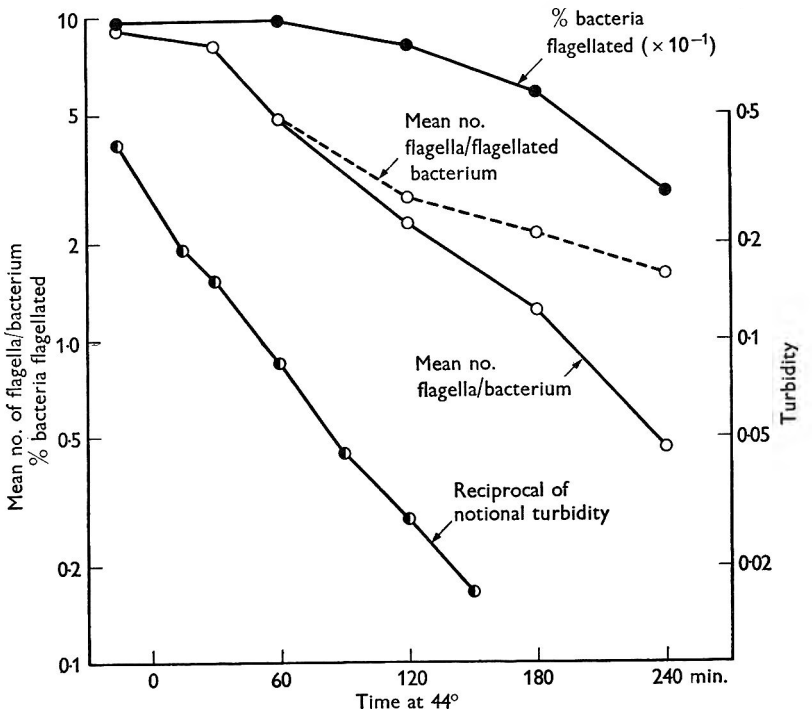

Fig. 2. Effect of growth at 44° on flagellation of a logarithmic phase culture of *Salmonella typhimurium* grown at 37° and transferred to 44° at zero time. Cultures were maintained in logarithmic growth by periodic dilution with pre-warmed broth at 44°. Samples were fixed at stated times by addition of formalin and stained by Leifson's (1951) method. The data are plotted semi-logarithmically and are from Expt. 2, Tables 2 and 4. Notional turbidity is actual turbidity multiplied by a correction factor for dilution during growth.

hypothesis. Such results, taken in conjunction with: (*a*) an absolute decrease in numbers of bacteria with large numbers of flagella (Table 5); (*b*) the demonstrated maintenance of parental flagella at 44° in the absence of growth (Table 1), are difficult to reconcile with the simple 'all or none' hypothesis that parental flagella are retained entirely by one daughter cell at each division. The initial low rate of decline in the proportion of bacteria with flagella also suggests that such 'all or none' partitioning of peritrichously-inserted flagella did not occur. The distributions of number of flagella/bacterium during incubation at 44° are illustrated in histogram form in Fig. 3. These distributions were unimodal and did not become bimodal as predicted by the 'all or none' hypothesis discussed in the Introduction and below.

Residual synthesis of flagella

The experiments mentioned above were designed to distinguish between the hypotheses: (1) 'all or none' partition; (2) 'sharing' of flagella at cell division. It was thought that a decisive test would be to follow the parameter 'mean number flagella/flagellated bacterium' during the transition from almost 100 % of flagellated organisms to the state in which nearly all were non-flagellated. The 'all or none' hypothesis in its simplest form predicts a decrease in this parameter of, at most, a

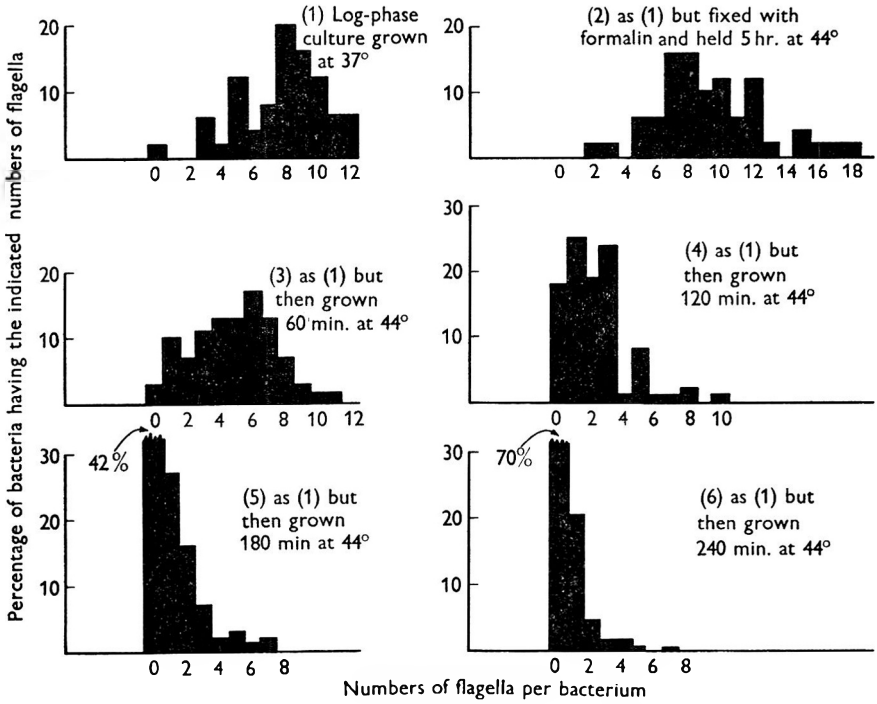

Fig. 3. Effect of incubation at 44°, with and without growth, on the distribution of numbers of flagella per bacterium in *Salmonella typhimurium*. Data of distributions (3) to (6) inclusive are from Expt. 2, Table 2. Cultures in logarithmic growth at 37° were transferred to 44° at zero time and maintained in logarithmic growth by periodic dilution with prewarmed broth at 44°. Samples were fixed at stated times by addition of formalin and stained by Leifson's (1951) method.

half (attributable to completion of bacterial divisions in progress at the time of cessation of flagellar synthesis) and then no further decrease, whilst the 'sharing' hypothesis predicts a steady decline towards unity. Although the changes observed in the mean number flagella/flagellated bacterium clearly favoured the sharing hypothesis, this result was not in itself sufficient to determine the way in which parental flagella were partitioned at cell division, since it was possible to envisage situations in which a slow decline in rate of synthesis of new flagella might lead to a slow decline in the mean number flagella/flagellated bacterium. Accordingly, the extent of the synthesis of new flagella which occurred after the temperature change from 37° to 44° was estimated. The extent of such residual synthesis can be calculated

from the changes in total 'flagellar amount' during experiments. This parameter is estimated by multiplying the mean number flagella/bacterium by the notional turbidity calculated for the culture at the time of sampling or by the calculated notional viable count at the time of sampling (Tables 3, 4). Notional turbidity or

Table 3. *Summary of data* on the effect of growth at 44° on the motility and flagellation of* Salmonella typhimurium *strain* LT2

Cultures in logarithmic growth at 37° were transferred to 44° at zero time and maintained in logarithmic growth by periodic dilution. Entries in columns (*f*), (*g*) and (*h*) are expressed as per ml. culture at zero time, to allow for dilution.

Time from transfer (min.)	Mean no. of flagella/ flagellated bacterium	% bacteria flagellated	% bacteria motile	Mean no. of flagella/ bacterium $\left(=\frac{b \times c}{100}\right)$	Notional viable count ($\times 10^7$)	Smoothed viable count† ($\times 10^7$)	Flagellar amount ($= e \times g$)
(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)
−30	—	—	—	—	2·62	2·6	—
−15	9·1	97 (116/120)	88	8·8	—	3·2	28
−10	—	—	—	—	3·4	3·4	—
0	—	—	—	—	—	4·0	36‡
10	—	—	—	—	4·22	4·6	—
15	10·1	—	85	10·1‡	—	5·0	51‡
45	7·63	98 (118/120)	93	7·5	—	7·9	58
60	—	—	—	—	7·4	9·8	—
75	4·9	93 (130/140)	95	4·6	—	12	55
90	—	—	—	—	16·1	—	—
105	3·04	88 (70/80)	—	2·7	—	18	49
120	—	—	76	—	21·8	24	—
135	2·03	61 (82/134)	67	1·2	—	30	36
150	—	—	—	—	36·3	38	—
165	1·76	—	62	0·97‡	—	48	47‡
180	1·67	49 (90/195)	—	0·82	63·4	60	49
195	1·73	34 (21/62)	51	0·59	—	76	45
210	—	—	—	—	96·6	94	—
216	1·37	25 (32/126)	—	0·34	—	104	35
225	1·34	19 (19/100)	24	0·25	—	118	30
245	1·31	14 (21/150)	14	0·18	—	158	28
265	1·25	10 (16/158)	12	0·13	—	213	28
280	1·21	14 (20/142)	—	0·17	—	265	45

* Expt. 1, Table 2.

† Entries for times −30 min. to 216 min. inclusive are interpolated; the remainder are extrapolated.

‡ In columns *e* and *h* indicates amount calculated from an interpolated value in columns (*b*) and/or (*c*).

( ) In column (*c*) indicates actual figures used in calculation of percentage.

— No observation.

viable count was estimated by taking account of the dilutions which had been made during the experiment and calculating back to obtain the hypothetical value which would have been found had the original culture, undiluted, remained in exponential growth at 44°. Flagellar amount is thus hypothetical and proportional to the total number of flagella/bacterium which would have been present if the whole culture had been maintained in exponential growth. The data in Table 4 (from Exp. 2, Table 2) indicate that total flagellar amount increased slowly throughout 240 min.

of growth at 44°, with the greatest increase occurring within the first 30 min. (about 1 mean generation time) after the temperature change. In contrast, the data in Table 3 (from Exp. 1, Table 2) show an increase in the first 30–60 min. at 44°, followed by a slow decrease in flagellar amount during the next 180 min. of incuba-

Table 4. *Summary of data* on the effects of growth at 44° on flagellation of* Salmonella typhimurium *strain* LT2

Culture in logarithmic growth at 37° transferred to 44° at zero time and maintained in logarithmic growth by periodic dilution.

Time from transfer (min.) (*a*)	No. of bacteria examined (*b*)	Mean no. of flagella/ flagellated bacterium (*c*)	% bacteria flagellated (*d*)	Mean no. of flagella/ bacterium $\left(=\frac{c \times d}{100}\right)$ (*e*)	Turbidity† (*f*)	Flagellar† amount (*e* × *f*) (*g*)
−15	50	8·4	98	8·2	0·25	2·05
0	—	—	—	9·0‡	0·36	3·24
30	50	8·0	100	8·0	0·65	5·20
60	70	4·9	97	4·8	1·18	5·66
120	100	2·7	82	2·2	3·6	7·92
180	100	2·1	58	1·2	11·1	13·3
240	200	1·6	30	0·48	37‡	18·0

* Expt. 2, Table 1.

† Entries in columns (*f*) and (*g*) are expressed as per ml. of culture at −15 min., to allow for subsequent dilution.

‡ Interpolated or extrapolated.

Table 5. *Decrease in absolute numbers of bacteria with higher numbers of flagella during growth of* Salmonella typhimurium *strain* LT2 *at* 44°*

Time after transfer to 44° (min.)	Factor of increase in viable count	No. of organisms in sample with 5 or more flagella		No. of organisms in sample with 8 or more flagella		Sample size (no. of bacteria)
		Present	Expected†	Present	Expected†	
+45	1	41	—	34	—	48
+75	1·57	29	26	5	23	49
+105	2·32	19	35	3	30	100
+135	3·85	2	21	0	18	100

* Data of Expt. 1, Tables 2 and 3.

† Entries are numbers expected on the assumptions that 'all or none' partition of parental flagella occurred at cell division, synthesis of new flagella had ceased, and that parental flagella were not shed during the period of the experiment.

tion at 44°, with some slight increase later. Presumably this decrease was attributable to some shedding of flagella or possibly to lysis of part of the population. It seems that the completeness of the hoped-for cessation of flagella synthesis varied from experiment to experiment. However, in an experiment in which measurable residual synthesis occurred and in experiments in which it did not occur, the mean number flagella/flagellated bacterium decreased steadily, as called for by the 'sharing' hypothesis.

DISCUSSION

It should be noted that the simple hypothesis of 'all or none' partition of parental flagella requires modification if it is to be applied to our material. Bisset (1951) envisaged a situation in which a peritrichously flagellated organism of *Salmonella typhi* reproduced by 'budding'; the parent bacterium retained all the old flagella and the daughter 'bud' grew new flagella. To cover the common case in which nearly 100 % of bacteria are motile (as in strain LT2) one must assume that the new flagella on the daughter are sufficiently long, at the moment of fission, to confer motility and also to be stained and counted. In a culture which is in a steady state with respect to average number flagella/bacterium, the observed mean number flagella/bacterium would result from an averaging of those on bacteria which have just completed fission, of those on bacteria which are just about to divide (and have about twice as many flagella) and of all intermediate classes. On the assumptions that the 'all or none' hypothesis is correct and that synthesis of new flagella ceases abruptly after one mean generation time at 44°, one would expect the mean number flagella/bacterium to decrease to a value between 1 and 0·5 of the original steady-state mean. This decrease would occur as a consequence of divisions initiated before suppression of flagellar synthesis. If a residual synthesis of flagella were to occur in such a system, on the basis of our observations (Table 3) one would expect it to take the form of synthesis of one or a small number of flagella by a minority of the population. Thus the modified 'all or none' hypothesis predicts that at two or three generations after transfer to 44° a bimodal distribution of number flagella/flagellated organism will occur with one mode between 0·5 and 1·0 of the original mean, and on the other, due to residual synthesis, at about unity. No such bimodal distribution was found at appropriate times (i.e. Table 2, Exp. 1, 75 and 135 min.; Exp. 2, 60 and 120 min.).

The conclusion that parental flagella are shared between the daughters is well supported by our quantitative evidence. We have shown that parental flagella are not rapidly shed under the physical conditions of our experiments. We have also shown that during growth at 44° the synthesis of new flagella is curtailed, the mean number flagella/flagellated bacterium decreases steadily towards unity and the absolute numbers of bacteria with higher numbers of flagella decrease. Bisset and co-workers (1951, 1957, 1960) examined rod-shaped and coccoid organisms and concluded that flagella are retained upon 'mother cells' and that 'daughter cells' or 'buds' are devoid of flagella or have them in an early stage of development. This conflict of views remains unresolved; further work may show that either or both view points are oversimplified. Our conclusion is to some extent an oversimplification; clearly one would not expect two flagella inserted close together near one pole of a cell to become separated to different daughters at the succeeding division. We do, however, suggest that flagella located near opposite poles would pass to different daughters and that the greater the proximity of those flagella to opposite poles the greater the probability that they will pass to different daughters at the next division.

Our results could be accounted for under the 'all or none' hypothesis by assuming that the decrease in mean number of flagella/flagellated bacterium is due to a shedding of flagella which occurs during growth but not under other conditions.

Although this possibility cannot be eliminated there is no evidence in its favour. Indeed the stability of the unilinear transmission of motility (Stocker, 1956*a*, *b*; Quadling & Stocker, 1957) provides strong evidence that such shedding is infrequent. It is also conceivable that incubation at 44° may introduce aberrant modes of division. However, studies on the growth of cultures by plate counts, turbidity measurements and by microscopic examination, revealed no abnormality. Our conclusion that flagella are shared out is also supported by indirect evidence obtained during micromanipulation studies on the unilinear transmission of motility from parent to progeny (Stocker, 1956*a*, *b*; Quadling & Stocker, 1957; Quadling, 1958). It was found that in certain situations motile salmonellas transmitted motility to only one or a few descendant bacteria. Evidence was obtained (Quadling, 1958) that such motile descendant bacteria had only one or a small number of flagella each; it was inferred that these flagella were distributed to the daughters at fission until these daughters received one or no flagella each.

Duguid & Wilkinson (1961) examined, by electron microscopy, the distribution of fimbriae amongst dividing enterobacteria growing under cultural conditions in which fimbriation was diminishing. Most dividing organisms were equally fimbriated at each end, were scantly fimbriated at each end, or were completely non-fimbriated. Bacteria which were fimbriated at one end but not at the other were very rare (less than 1 %). Duguid's observations are therefore concordant with our results. Results similar to ours were obtained with salmonellas by Kerridge (1960, 1961) in experiments in which he increased the incubation temperature from 37° to 44°. His work provides evidence for the reproducibility of our results.

Evidence for the sharing of parental cytoplasmic materials between daughters has been obtained by a number of workers. Benzer (1953) showed that the adaptive β-galactosidase of *Escherichia coli* was uniformly distributed, in the absence of inducer, amongst progeny of induced bacteria. Novick & Weiner (1957) working with the same system obtained results which could be interpreted by assuming that permease enzyme molecules were randomly distributed to daughter bacteria. Van Tubergen (1959) and Van Tubergen & Setlow (1961) investigated the distribution of various parental components amongst the progeny during exponential growth of *E. coli*. Specific cell components were labelled by uptake of appropriate tritium-containing nutrient, and the distribution of the labelled parental material amongst the progeny bacteria was determined by microradioautography at intervals after transfer of the bacteria to unlabelled medium. Parental protein (labelled with ^{3}H-proline), ribonucleic acid (labelled with ^{3}H-uridine) and cell-wall material (labelled with ^{3}H-diaminopimelic acid) were distributed randomly amongst the progeny bacteria, whereas parental deoxyribonucleic acid (labelled with ^{3}H-thymidine) was distributed non-randomly in large structures, stable for at least 5 generations. Cell-wall synthesis appeared to take place along the whole length of the cell wall.

The observations of Bergersen (1953) on *Escherichia coli* grown in the presence of chloramphenicol suggest that the mature cell wall is not necessarily a 'dead' structure; he showed that under these conditions additional 'growing points' appeared at the sides of some bacteria. Our observations on the behaviour of Salmonella at the conclusion of the logarithmic phase of growth also bear on this point. The mean size of the organism was observed under the microscope to decrease to about one quarter that of logarithmic-phase bacteria by direct division of the

pre-existing soma. Such observations implied that the walls of the bacterial cells were capable of morphological adjustment and were not 'dead' in the sense of being permanently differentiated.

The relevance of our findings on the fate of parental flagella at cell division to the mode of growth of the cell wall of rod-shaped bacteria is not clear. Assuming that bacterial flagella or their hypothetical basal granules are not able to move independently of the cell wall, our results provide evidence for the eventual dispersion of parental cell walls into discrete fragments, at least as many in number as the mean number of flagella per cell when fully flagellated. Van Tubergen (1959) and Van Tubergen & Setlow (1961) inferred from observations on the distribution amongst the progeny of parental cell-wall material that in *Escherichia coli* the number of 'intact structures' comprising the cell wall of one bacterium was at least 200. There is evidence that some areas of the bacterial cell wall (the sites of future cell division) are more active in synthesis than others (Lederberg, 1957; Mitchell & Moyle, 1957; Murray, Francombe & Mayall, 1959). Our results do not necessarily conflict with such a concept.

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The Preparation and Survival of Almost Bacteria-free Suspensions of *Entodinium caudatum*

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SUMMARY

Suspensions of *Entodinium caudatum* containing less than one viable bacterium per ten protozoa were prepared by aerobic incubation of the protozoa with penicillin, streptomycin, dihydrostreptomycin and neomycin in the presence of autoclaved rumen fluid and rice starch grains. Unfortunately these almost axenic Entodinia could be maintained alive for only 3–4 days. Replacement of the rice starch grains in the medium used for bacterial removal by soluble starch produced almost bacteria-free protozoa which were devoid of food storage materials and died more rapidly than usual. The life of these protozoa was prolonged by adding any of the following: rice starch grains, soluble starch, maltose, glucose and some other sugars.

INTRODUCTION

Sheep rumen Entodiniomorphid (Oligotrich *sensu lato* of Corliss, 1959) protozoa, principally *Entodinium caudatum*, have been maintained dividing every 2 days in the presence of bacteria for over 18 months (Coleman, 1960*a*). There are two reports in the literature of successful attempts to prepare axenic Entodiniomorphid protozoa (Sugden, 1953; Abou Akkada & Howard, 1960), but in each case the demonstration that bacteria were absent depended on the use of a single growth medium. Oxford (1958) treated *Epidinium ecaudatum* with penicillin and neomycin but was unable to obtain the protozoa free from bacteria. The present paper records a successful attempt to obtain almost bacteria-free protozoa, many media being used to test for the absence of bacteria. The protozoa were first incubated with antibiotics to decrease the number of bacteria and then the effects of various incubation conditions on the survival of these protozoa were studied.

METHODS

Source of protozoa. The original source of the protozoa was described previously (Coleman, 1958, 1960*a*) and when used they had been grown on rice starch, grass, autoclaved rumen fluid and chloramphenicol under condition C3 (Coleman, 1960*a*) for over a year. However, whilst most of the experiments described below were being carried out this routine procedure was simplified as follows. The protozoa were grown in 250 ml. centrifuge tubes containing 150 ml. medium and 30 mg. rice starch and about 50 mg. dried grass were added each day. Twice a week two-thirds of the supernatant fluid was sucked off as quickly as possible and replaced by fresh medium (salt solution; Coleman, 1958) to which was added per 100 ml.: 10 ml. auto-

claved rumen fluid (ARF), 5 mg. chloramphenicol, 25 mg. rice starch and about 100 mg. dried grass). After mixing, the culture was divided into two, and one half was made up to the original volume with fresh medium and continued as the stock culture. The other half was combined with a similar half from another culture tube and placed in two 100 ml. centrifuge tubes (to facilitate subsequent manipulations) to form an 'inoculum culture'. These culture tubes were treated each day with 22 mg. rice starch and about 20 mg. dried grass and used on the third day as the source of protozoa. Under these conditions the protozoa formed a white layer at the bottom of the tube and the grass remained as a scum on the surface.

Treatment to remove bacteria from protozoal suspensions. The standard procedure finally adopted is as follows. The medium consisted of 3 ml. salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) in a cotton plugged 5 in. × ½ in. test tube. Immediately after removal from the autoclave and cooling the following additions were made aseptically: 0·2 ml. 1 % (w/v) L-cysteine hydrochloride (neutralized with NaOH and Seitz-filtered just before use), 0·2 ml. 5 % (w/v) $NaHCO_3$, 0·1 ml. 6 % (w/v) rice starch (British Drug Houses Ltd., Poole, heated at 120° for 24 hr.), 0·4 ml. penicillin G (25,000 units/ml.), 0·4 ml. 1 % (w/v) streptomycin sulphate solution, 0·4 ml. 1 % (w/v) dihydrostreptomycin sulphate solution, 0·4 ml. 1 % (w/v) neomycin sulphate solution, and 0·8 ml. protozoa-containing autoclaved rumen fluid (PARF). The antibiotics were dissolved in salt solution B. Where larger quantities of 'bacteria-free' protozoa were required, as in the experiments on the subsequent survival of these protozoa, five times the above quantities were taken in 15 cm. × 2·2 cm. tubes.

The protozoa were taken from the inoculum cultures, after removal of the surface scum and most of the supernatant liquid, and allowed to stand in a suitable test tube for 2 min. until any grass present had sunk to the bottom. The supernatant fluid containing the protozoa was transferred to centrifuge tubes, the residual grass washed once with salt solution B and then the protozoa washed five times in salt solution B which contained 0·3 % (w/v) yeast extract and 0·03 % (w/v) L-cysteine on an angle-head centrifuge at 400 *g* for 1 min. They were finally inoculated at a population density of 30,000–80,000 protozoa/ml. incubation medium, the culture well mixed and incubated aerobically at 39° for 3 days.

Incubation conditions for 'bacteria-free' protozoa. Under standard conditions the medium consisted of 1–4 ml. (adjusted so that the final volume was 7·6 ml.) salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) with 0·9 ml. water and 0·4 ml. 0·05 M-DL-*p*-fluorophenylalanine in a 5 in. × ½ in. cotton-plugged test tube or 7·5 ml. tube (fitted with a ground glass stopper). Immediately after removal from the autoclave and cooling the following additions were made aseptically: 0·2 ml. 1 % (w/v) L-cysteine hydrochloride (neutralized and Seitz filtered), 0·1 ml. 6 % (w/v) rice starch, 0·4 ml. penicillin G (25,000 units/ml.), 0·4 ml. 1 % (w/v) streptomycin sulphate solution, 0·4 ml. 1 % (w/v) neomycin sulphate solution, 1·0 ml. Seitz-filtered rumen fluid and any other additions. The 5 in. × ½ in. test tubes were gassed with 95 % (v/v) N_2 + 5 % (v/v) CO_2 for 1½ min. and then sealed with a sterile rubber bung. Except where stated, all experiments were carried out in these tubes which were inoculated by opening momentarily and then regassing with 95 % (v/v) N_2 + 5 % (v/v) CO_2. The 7·5 ml. glass-stoppered tubes were inoculated before completely filling with sterile salt solution B and sealing with a flamed glass stopper.

The protozoa for inoculation were prepared from the bacterial removal (or first) incubation medium by sucking off 80–90 % of the supernatant fluid (this removed no protozoa) and washing the sedimented protozoa twice on the centrifuge with salt solution B plus 0·03 % (w/v) L-cysteine solution.

Sterile rumen fluid fractions. All rumen fluid was taken from Clun Forest wethers fed on hay or hay and oats. Protozoa-containing autoclaved rumen fluid (PARF) was prepared from fresh rumen contents by straining through two layers of muslin and autoclaving under 95 % (v/v) N_2 + 5 % (v/v) CO_2 in sealed McCartney bottles at 115° for 20 min. Autoclaved rumen fluid (ARF) was prepared similarly to PARF except that the strained material was subsequently centrifuged at 500 *g* for 3 min. before autoclaving. Autoclaved supernatant rumen fluid was prepared similarly to PARF except that the strained material was centrifuged at 12,000 *g* for 45 min. before autoclaving. Autoclaved rumen bacteria fraction was the pellet from the above 12,000 *g* centrifugation, washed once on the centrifuge in salt solution B, and made up to the original volume before autoclaving. Seitz-filtered rumen fluid was prepared as autoclaved supernatant rumen fluid except that sterilization was effected by Seitz-filtration. The number of protozoa was estimated by the method of Coleman (1958). Only those protozoa which showed no signs of distintegration were counted.

All gases were freed from oxygen by the method of Stone & Beeson (1936).

Bacterial culture media. Medium A contained (g./100 ml.): K_2HPO_4, 0·50; KH_2PO_4, 0·39; NaCl, 0·050; $MgSO_4 7H_2O$, 0·007; $CaCl_2 6H_2O$, 0·007; Difco yeast extract, 0·5; Difco Bacto-tryptose, 0·5; glucose, 0·5; sodium lactate, 0·35; 4 ml. PARF (centrifuged to remove solid matter) autoclaved at 115° for 20 min. Medium A was always incubated aerobically in cotton-plugged tubes.

Medium B was Oxoid (Oxo Ltd., London, E.C. 4) thioglycollate (fluid) medium (fluid thioglycollate medium U.S.P.) to which was added 10 % (v/v) PARF (centrifuged to remove solid matter) dispensed in 5 ml. quantities in 5 in. × ½ in. tubes. After inoculation a half-inch layer of sterile liquid paraffin was run on the surface of the medium and the tubes incubated in an anaerobe jar under 95 % (v/v) H_2 + 5 % (v/v) CO_2.

Medium C was based on that of Bryant & Robinson (1961) and contained per 100 ml.: 40 ml. PARF (centrifuged to remove solid matter); 0·5 g. agar (Davis Gelatine Co. Ltd., London E.C. 3); 0·1 ml. 0·1 % resazurin; 3·75 ml. each mineral salt solutions no. 1 and no. 2 (Bryant & Burkey, 1953); 0·025 g. glucose; 0·025 g. cellobiose; 0·05 g. soluble starch; water to 100 ml. This was autoclaved (115° for 20 min.) in 5 ml. quantities in 5 in. × ½ in. tubes and after cooling to 50° 0·25 ml. sterile 8 % (w/v) Na_2CO_3 and 0·25 ml. of a mixture of 0·5 % (w/v) L-cysteine and 0·5 % (w/v) $Na_2S.9H_2O$ (autoclaved together under N_2 after adjustment to pH 11) were added and the medium gassed with CO_2 for 1½ min.

Bacterial viable counts. The standard technique for determining the number of viable bacteria in 'bacteria-free' protozoal suspensions treated under standard conditions was as follows: 0·1 ml. of a well mixed suspension was added to 5 ml. medium B and then 0·05 ml. of this diluted suspension inoculated into media A, B and C followed by two serial tenfold dilutions in media A and B. Each colony in medium C represented 10^3 bacteria/ml. of the original suspension. Where more bacteria were present serial dilutions were made in medium B before inoculation

into media A and C. None of the media maintained the inoculated living protozoa which disintegrated and liberated their ingested bacteria.

All cultures were incubated at 39° for at least 10 days.

RESULTS

Suspensions of *Entodinium caudatum* (10^4–10^5 protozoa/ml.) prepared from growing cultures as described above by washing five times still contained over 10^6 bacteria/ml., excluding those in the gastric sac. Breakage of the protozoa in a Potter homogenizer to liberate these internal bacteria increased the bacterial count 10–100 times on all media. It was therefore necessary to remove most of these internal and external bacteria before it was possible to study the survival and metabolism of the protozoa. A maximum limit of one viable bacterium per ten intact protozoa was arbitrarily chosen; any experiments in which the 'bacteria-free' protozoa contained more bacteria than this were discarded. To demonstrate that the bacteria had been removed it was first essential to grow all the bacteria present; some experiments on the production of media able to grow the bacteria in antibiotic-treated protozoal suspensions are described below.

Media for bacterial viable counts

Preliminary experiments on the development of media A, B and C to give the highest bacterial viable counts were carried out on crude rumen contents and on penicillin-treated protozoa (Coleman, 1960*b*). Subsequent experiments on protozoal cultures treated under standard conditions confirmed that media A, B and C gave higher results than other media tested.

For an aerobic viable count medium A grew 10^8 bacteria/ml. crude rumen fluid and 10^7 bacteria/ml. of growing protozoal cultures. Medium A grew more bacteria from antibiotic-treated protozoal suspensions than did medium B, Oxoid thioglycollate medium (Brewer), Oxoid thioglycollate (fluid) medium, Oxoid reinforced clostridial medium or glucose peptone water incubated aerobically. The addition of 0·05 % (w/v) L-cysteine to medium A and incubation in an anaerobe jar under 95 % (v/v) H_2 + 5 % (v/v) CO_2 decreased bacterial count. Solidification of medium A with 1 % (w/v) Davis agar and incubation aerobically in plates produced no colonies although liquid medium inoculated with the same inoculum grew well. Growth in medium A always began on the surface and sometimes spread throughout the medium.

Media B and C were used routinely for the growth of anaerobes. They grew respectively 10^8–10^9 and 10^9–10^{10} bacteria/ml. crude rumen contents and 10^7–10^8 and 10^8–10^9 bacteria/ml. growing protozoal cultures. Omission of PARF or agar from medium B decreased the bacterial count in penicillin and streptomycin-treated suspensions from, for example, 10^5 to 10^2. The number of colonies produced on medium B solidified with agar was at best 25–50 % of those in medium C and they were almost too small to count under a lens. Medium B grew more bacteria than Oxoid reinforced clostridial medium; neither medium was improved by the addition of 0·1 % (w/v) maltose, cellobiose or 0·5 % (w/v) sodium lactate.

There was always a 24–36 hr. lag before there was visible bacterial growth on any

medium after inoculation from antibiotic-treated suspensions although rumen fluid inoculations grew overnight. All viable count cultures were incubated for 10 days because there was occasionally a 7-day lag before growth began.

Preparation of almost bacteria-free Entodinia

Table 1 shows that to obtain protozoa probably contaminated with less than 10 % of their number of bacteria it was necessary to incubate aerobically in the presence of penicillin + streptomycin + dihydrostreptomycin + neomycin. The addition of dried grass and yeast extract increased the number of surviving protozoa when penicillin (Coleman, 1960*b*) or penicillin + streptomycin were present, but with the four anti-

Table 1. *The effect of various antibiotics and of aerobic incubation conditions on protozoal survival and the removal of bacteria*

Experiment carried out either anaerobically in completely filled and sealed 7·5 ml. tubes or aerobically in cotton plugged 5 in. × $\frac{1}{2}$ in. test tubes containing 7·4 ml. liquid. The inoculum was prepared as described in the text and all tubes were incubated for 3 days. All the tubes contained salts, 0·03 % (w/v) L-cysteine, 0·15 % (w/v) $NaHCO_3$, 0·17 % (w/v) rice starch, 1400 units penicillin/ml. and 12 % (v/v) PARF. The numbers of viable bacteria are those that grew on media A, B and C respectively.

Aerobic or anaerobic	Additional antibiotics*	Protozoa/ml.	Bacteria/ml.
Initial		38,000	10^3, 10^6, 7×10^6
Anaerobic	None	40,000	10^5, $> 10^6$, 10×10^7
Anaerobic	Str	40,000	10^4, 10^4, 10^6
Aerobic	Str	26,000	10^3, 10^3, 20×10^5
Aerobic	Str, Dhs, Neo	12,000	10^3, 10^3, 1×10^3
Anaerobic	Str, Dhs, Neo	21,000	10^4, $< 10^3$, 80×10^3
Aerobic	Neo	17,800	10^3, 10^3, 3×10^3
Aerobic	Str, Neo	16,500	10^3, 10^3, 3×10^3
Aerobic	Dhs, Neo	15,000	10^3, 10^3, 4×10^3
Aerobic	Str, Dhs	27,000	10^3, 10^4, 250×10^3
Aerobic	No penicillin	10,000	$> 10^8$, 10^9, ?

* Str = 570 μg. streptomycin sulphate/ml.; Dhs = 570 μg. dihydrostreptomycin sulphate/ml.; Neo = 570 μg. neomycin sulphate/ml.

biotics together they only increased the number of bacteria. The omission of the antibiotics singly or in pairs showed that penicillin + neomycin were almost as effective as the four antibiotics together (Table 1). Sodium propionate was added routinely to media (in salt solution B) to suppress the growth of moulds (Mrak & Phaff, 1948).

To ensure adequate survival of the protozoa it was essential to add 12 % (v/v) PARF to the incubation medium (Table 2). Other rumen fluid fractions and lower or higher concentrations of PARF were less effective in producing the largest possible number of 'bacteria-free' protozoa.

Figure 1 shows a time course for incubation under aerobic and anaerobic conditions. Although in about half the experiments the same final result was obtained under both conditions, the anaerobic incubation was not used routinely since an extra day was required and frequently the aerobic bacterial count (medium A) was too high.

Since the number of viable bacteria was determined on suspensions of intact protozoa it was possible for the results obtained to be low, since each protozoan that

contained one or more bacteria would give rise to only one colony in the assay. However, breakage of the 'bacteria free' protozoa in a Potter homogenizer before diluting the suspension to determine the number of viable bacteria did not increase the number of bacteria that grew on media A or C. This shows, within the limits of the method, that if a protozoan contained bacteria it contained only one bacterium.

Table 2. *The effect of various rumen fluid fractions on protozoal survival and the removal of bacteria*

Experiment carried out in cotton plugged 5 in. × ½ in. test tubes containing 7·4 ml. medium incubated aerobically for 3 days. All tubes contained the standard incubation medium used for bacterial removal except that the PARF was replaced by various rumen fluid fractions as indicated below. The numbers of viable bacteria are those that grew on media A, B and C respectively. The initial number of protozoa was 40,000/ml.

Rumen fluid fraction	Protozoa/ml.	Bacteria/ml.
None	2,900	10^4, 10^3, 10^3
3 % (v/v) PARF	11,600	10^4, 10^3, $< 10^3$
12 % (v/v) PARF	15,400	10^3, 10^3, $< 10^3$
30 % (v/v) PARF	19,400	10^3, $< 10^3$, 150×10^3
12 % (v/v) Seitz-filtered rumen fluid	9,500	10^3, $< 10^3$, $< 10^3$
12 % (v/v) ARF	8,700	10^3, 10^3, 1×10^3
12 % (v/v) autoclaved supernatant rumen fluid	11,600	10^4, 10^3, $< 10^3$

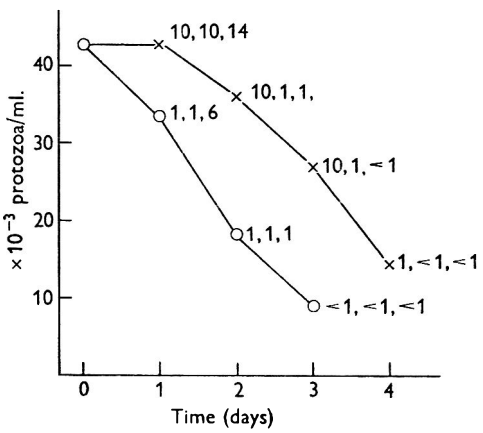

Fig. 1

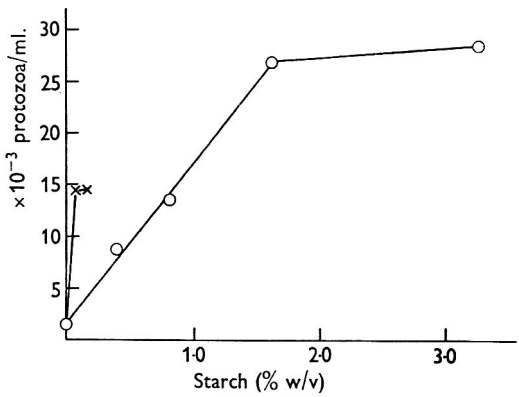

Fig. 2

Fig. 1. Effect of incubation time on the survival of protozoa and the disappearance of bacteria during the incubation used for bacterial removal. O—O, Incubation under aerobic conditions; × — ×, incubation under anaerobic conditions. The figures at each point refer to the number of bacteria (in thousands) that grew on media A, B and C respectively.

Fig. 2. Effect of starch concentration on the survival of protozoa under otherwise standard conditions during the incubation used for bacterial removal. Duration of experiment was 3 days and the initial number of protozoa 60,000/ml. O—O, Soluble starch; × — ×, rice starch grains.

Replacement of the rice starch grains by soluble starch

For these experiments protozoa, the gastric sacs of which contained only a few rice starch grains, were prepared by the addition of only 5 mg. rice starch to the inoculum cultures on the day before that on which the protozoa were required.

Figure 2 shows that in the absence of starch less than 5 % of the protozoa survived after 3 days and that the addition of up to 1·6 % soluble starch linearly increased the number of survivors. Although ten times more soluble starch than rice starch grains was required to obtain the same survival, the maximum number was double that in the presence of rice starch grains. Unfortunately in half the experiments 10^4–10^5 bacteria/ml. survived in the presence of soluble starch. In contrast to the protozoa incubated with 0·04–0·08 % (w/v) rice starch grains or 1·6–3·2 % (w/v) soluble starch, those which survived in the presence of 0–0·8 % (w/v) soluble starch contained no material which stained blue with iodine. These latter protozoa when incubated under the second incubation conditions in the absence of carbohydrate died more quickly than normal protozoa full of rice starch grains and the effect of carbohydrates on their survival could be studied.

The survival of bacteria-free Entodinia

It did not prove possible to maintain the normal 'bacteria-free' protozoa alive for longer than 3–4 days; the numbers steadily declined throughout this period. Nevertheless, various factors influenced the rate at which the organisms died. To obtain maximum survival of the protozoa it was necessary to decrease to a minimum the manipulations of the organisms after removal from the first incubation medium. The best results were obtained by sucking off the supernatant fluid from the first incubation and inoculating the residual protozoa directly into new medium. As the object of the work was to study the effect of various substances on the survival and metabolism of the protozoa, these were usually washed twice before inoculation. Under these conditions damage to the organisms, as measured by the decrease in the number of living protozoa over the first 6 hr., was 20 % as compared with 10 % for unwashed protozoa; subsequently the rate of decrease was the same for both cultures.

The lowest effective concentration of L-cysteine was 0·03 % (w/v). Omission of the L-cysteine decreased the number of survivors after 24 hr. by over 60 % and an increase in concentration to 0·15 % (w/v) had no additional effect.

Omission of the antibiotics singly or all together did not usually result in bacterial growth during the first 24 hr., but the number of protozoa decreased by 5–20 %. DL-*p*-Fluorophenylalanine ($2{\cdot}6 \times 10^{-3}$M) was routinely used in this second incubation medium to suppress the growth of bacteria which subsequently grew specifically on medium C. In the absence of *p*-fluorophenylalanine these bacteria were found in about half the experiments where nutrient materials were added in an attempt to stimulate the protozoa. *p*-Fluorophenylalanine in concentrations up to 0·005M had no effect on the survival of the protozoa whether bacteria grew in its absence or not.

Effect of CO_2 and rumen fluid fractions. Seitz-filtered rumen fluid was more effective than other rumen fluid fractions tested in maintaining 'bacteria-free' protozoa alive (Table 3). The survival in the absence of Seitz-filtered rumen fluid was variable when experiments were carried out in sealed tubes or under 100 % N_2 but more consistent results were obtained with 5 % (v/v) CO_2 in the gas phase. In those experiments in sealed tubes with poor survival in the absence of Seitz-filtered rumen fluid, 5 % (v/v) CO_2 was stimulatory in the presence of 0–14 % (v/v) but not with 42 % (v/v) Seitz-filtered rumen fluid. An increase in the CO_2 to 100 % (plus

the addition of 0·4 % (w/v) Na_2CO_3 to maintain the pH value constant) had no further stimulatory effect (Table 3). The addition of 0·14 % (w/v) $NaHCO_3$ to the sealed tube experiments gave similar results to gassing the medium with 95 % (v/v) N_2 + 5 % (v/v) CO_2.

Table 3. *The effect of carbon dioxide and rumen fluid fractions on protozoal survival during the second incubation*

Experiment carried out either anaerobically in 5 in. × ½ in. test tubes containing 7·4 ml. medium under the appropriate gas or in completely filled and sealed tubes containing 7·5 ml. medium incubated for 24 hr. The standard inoculum and medium was used except where the carbon dioxide tension or rumen fluid fraction was varied. Where 100 % CO_2 was present, 0·4 % (w/v) Na_2CO_3 was added to the medium.

CO_2 added	Rumen fluid fraction	Number of protozoa relative to initial = 100
None*	None	20
None*	14 % (v/v) Seitz-filtered	36
None*	42 % (v/v) Seitz-filtered	50
5 %†	None	42
5 %†	14 % (v/v) Seitz-filtered	65
5 %†	42 % (v/v) Seitz-filtered	49
100 %	None	40
100 %	14 % (v/v) Seitz-filtered	68
5 %†	14 % (v/v) PARF	54
5 %†	14 % (v/v) ARF	52
5 %†	14 % (v/v) Autoclaved supernatant	56
5 %†	14 % (v/v) Autoclaved rumen bacteria	40

* Experiment carried out in completely filled and sealed tubes.
† + 95 % (v/v) N_2.

Although Abou Akkada & Howard (1960) found that salt solutions buffered only with phosphate were harmful to their Entodinia no such effect was noticed in the present studies. Under otherwise standard conditions salt solution B was 10–20 % more effective in the maintenance of the protozoa than was the same solution when the sodium propionate was omitted and the concentration of the other constituents increased to maintain the osmotic pressure, or when propionate was replaced by an equimolar amount of sodium acetate or by 0·1 % (w/v) $NaHCO_3$. Similar results were obtained with growing protozoa (Coleman, 1958, 1960*a*) where sodium acetate could be omitted without harming the protozoa.

Effect of various additives on protozoal survival. None of the following substances at the concentrations indicated prolonged the life of the protozoa incubated under standard conditions: 0·03–0·15 % (w/v) agar (Davis); 0·0005 M-NH_4Cl; 0·3 % (w/v) Bacto-peptone (Difco); 0·3 % (w/v) Bacto-tryptose; a mixture of 0·1–100 μg./ml. each of *iso*butyric acid, valeric acid and *iso*valeric acid (found by Wegner & Foster, 1960, to stimulate certain rumen bacteria); 0·3 % (w/v) Casamino acids (Difco); 0·1 % (w/v) casein; 0·14 % (w/v) cellobiose; 0·001–0·01 % (w/v) cholesterol; 0·14 % (w/v) glucose; a mixture of eleven growth factors (0·1–0·5 μg./ml. each of *p*-aminobenzoic acid, β-alanine, biotin, calcium pantothenate, folic acid, nicotinic acid, nicotinamide, pyridoxin, riboflavin, thiamine, vitamin B_{12}); 0·01 % (w/v) haematin; 5 % (v/v) horse serum; 0·005 M-DL-lactate; 0·3 % (w/v) malt extract;

0·14 % (w/v) maltose; 1 % (v/v) milk (fresh); 0·3 % Tryptone; 0·01–0·1 % (w/v) Tween 80; 7–20 % (v/v) tomato juice; 0·003–0·3 % (w/v) yeast extract (Difco or Oxoid).

Replacement of rice starch grains by other carbohydrates. For these experiments the protozoa were prepared under standard conditions except that on the day before

Table 4. *The effect of various carbohydrates on the survival of 'bacteria-free' Entodinia*

Experiment carried out in sealed 5 in. × ½ in. test tubes under 95 % (v/v) N_2 + 5 % (v/v) CO_2 in the presence of 13 % (v/v) Seitz-filtered rumen fluid. The protozoa differed from those used in other experiments in that they were rendered bacteria-free by incubation in a medium containing soluble starch instead of rice starch and contained no iodine-staining intracellular material. For exact conditions see text.

Carbohydrate	Number of survivors after 24 hr.*	Carbohydrate	Number of survivors after 24 hr.*
0·3–1·0 % Soluble starch	1·00	1·3 % Sorbose	0
0·08 % Rice starch	0·4–0·8	1·3 % Xylose	0·5
1·0 % Amylose	0·8	1·3 % Sorbitol	0·2
1·0 % Amylopectin	0·9	1·6 % Cellobiose	0·3
0·4 % Inulin	0·05	1·3 % Lactose	0·1
0·4 % Dextrin	0·5	1·6 % Maltose	0·7–1·0
1·3 % Arabinose	0·1	1·3 % Melibiose	0·5
1·6 % Fructose	0·2	1·6 % Sucrose	0·6
1·6 % Galactose	–0·6	1·3 % Salicin	0
1·6 % Glucose	0·5–0·9	1·3 % Raffinose	0·1
1·6 % Mannose	0·1		

* The results are quoted as (survivors in presence of carbohydrate − survivors in absence of carbohydrate)/(survivors in presence of soluble starch − survivors in absence of carbohydrate).

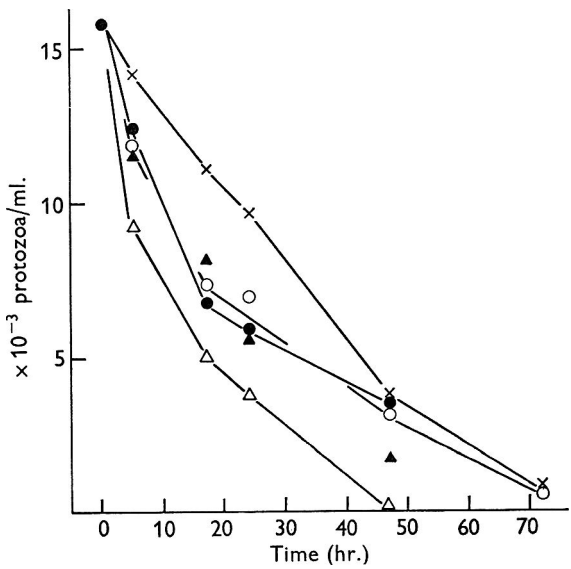

Fig. 3. Effect of various carbohydrates on the survival of starved 'bacteria-free' protozoa. △, no added carbohydrate; ×, 0·8 % (w/v) soluble starch; ●, 0·08 % (w/v) rice starch grains; ○, 1·6 % (w/v) maltose; ▲, 1·6 % (w/v) glucose.

inoculation of the first incubation medium only 5 mg. rice starch was added to each inoculum culture and the rice starch grains in the first incubation were replaced by 0·2 g. soluble starch in each 15 × 2·2 cm. tube. These protozoa died at 2–10 times the usual rate when incubated in the absence of carbohydrate and contained no material which stained blue with iodine. The rate at which the protozoa died was decreased by adding several carbohydrates of which soluble starch was the most effective, followed by maltose and glucose; fructose and the fructose polysaccharide inulin were relatively ineffective (Table 4). There was considerable variation between experiments in the relative effectiveness of these various materials; the figures quoted are averages. Figure 3 shows that the stimulatory effect also varies with time.

DISCUSSION

As far as the author is aware the present work represents the first successful attempt to prepare bacteria-free Entodinia where several media designed to detect rumen bacteria have been used. There is disagreement over the best type of medium for the growth of rumen bacteria. Wilson & Briggs (1955) reported that a rich broth (reinforced clostridial medium) gave the best results of nine media tested, whereas Bryant & Burkey (1953) favoured a poor medium containing a high concentration of autoclaved rumen fluid. Media based on these two types have been tested and the high rumen fluid medium gave the highest results from antibiotic-treated suspensions; King & Smith (1955) obtained similar results with fresh rumen contents. In view of the present findings, it is considered that the two reports of bacteria-free *Entodinium* spp. in the literature are of doubtful validity. Abou Akkada & Howard (1960) treated *Entodinium caudatum* suspensions with chloramphenicol and found no bacteria able to grow in a semi-solid thioglycollate starch medium. This medium is similar, except that glucose was replaced by starch, to the fluid thioglycollate medium used in the present experiments, and in penicillin + streptomycin treated cultures counts of 10^6 bacteria/ml. were obtained on medium C and $0–10^2$/ml. on the fluid thioglycollate medium. It is essential to carry out all tests for viable bacteria on at least one aerobic and one anaerobic medium and that various standard media should be tested to enable the best to be selected.

The absence of any stimulatory effect of ordinary culture medium constituents on 'bacteria-free' protozoa under standard conditions is in contrast to the results reported previously (Coleman, 1960*b*) where penicillin-treated Entodinia were stimulated by the addition of yeast extract and dried grass. These cultures are now known to have contained more bacteria (Table 1) than at first reported and it is likely that the effect of these additives was indirect and was the result of bacterial metabolism.

In previous experiments (Coleman, 1958, 1960*a*) carried out in the presence of bacteria the only source of carbohydrate that produced growth and division was intact rice starch grains. It is likely that only by the use of a solid food material, which was engulfed by the protozoa, was it possible to supply utilizable carbohydrate without extensive bacterial breakdown and acid production before assimilation by the protozoa. In the present experiments where starved 'bacteria-free' protozoa have been produced, an effect of soluble starch and, more important, of glucose and maltose on the survival of the protozoa was found for the first time. The apparent inability of *Entodinium* spp. to utilize soluble carbohydrates has for long

been in contrast to the rapid utilization of sugars, especially glucose, by Holotrich protozoa (Oxford, 1951, 1955).

I wish to thank Mr R. B. Taylor, who fistulated the sheep used in this work; Dr M. P. Bryant for giving me the details of his improved culture medium before publications, and Miss J. M. How and Miss J. G. Pearson for their valuable technical assistance.

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Regulation of Flavin Synthesis by *Escherichia coli*

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SUMMARY

An approach has been made to the problem of how the synthesis of coenzymes is regulated. Two aspects of the problem have been studied, especially as they concern the synthesis of flavins by bacteria:

(1) How are coenzymes prevented from being synthesized as fast as amino acids or nucleic acid bases?

(2) How is coenzyme synthesis adjusted to the often changing physiological needs of bacteria?

Evidence is presented that flavins cannot inhibit the activity of enzymes in the flavin biosynthetic pathway of *Escherichia coli*, but the amount of these enzymes can be made to vary by a factor of at least two. Repression might, therefore, account for the low rate of flavin synthesis. The possibility that repression rather than feedback inhibition also accounts for the low rate of synthesis of other coenzymes is discussed.

Flavin synthesis is not as precisely adjusted to the physiological needs of bacteria as are syntheses of major metabolites for the following reasons:

(1) Flavins are greatly overproduced by bacteria during exponential growth; the ratio of flavins excreted to flavins retained in the cells is between 0·8 and 8 for all strains and cultural conditions tested.

(2) Flavin synthesis is not tightly geared to growth; thus, flavin synthesis goes on uninterrupted for more than an hour when the growth rate of *E. coli* or *Pseudomonas fluorescens* is abruptly reduced from a rapid rate to zero; also growth goes on uninterrupted for over an hour when the flavin supply is abruptly cut off from rapidly growing lactic acid bacteria. Evidently the control mechanism in the flavin pathway is not very sensitive to physiological needs. This conclusion probably applies to other coenzymes as well.

Some incidental findings of interest from other points of view were:

(1) Although internal flavins can get out of *E. coli*, external flavins apparently cannot enter. This could account for the absence of flavinless mutants.

(2) After brief treatment with penicillin, *E. coli* becomes permeable to external flavins while remaining both impermeable to inulin and capable of synthesizing flavins.

(3) Less than 4 % of the intracellular flavins of *E. coli* are free, in a form that can be extracted with *n*-butanol (5 %, v/v), toluene (0·05 %, v/v), cetyl trimethylammonium bromide (0·001 %, w/v) or distilled water. The remaining flavins are bound, in a form that can be extracted with trichloracetic acid (5 %, w/v). The intracellular concentration of free flavins in *E. coli* is estimated to be less than 4×10^{-6} M.

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INTRODUCTION

Coenzymes are synthesized very slowly. For the average coenzyme the rate is under ten molecules per bacterium per second or, about a thousand times less than the rate at which the average amino acid or nucleic-acid base is synthesized by bacteria growing rapidly in minimal media (McIlwain, 1946*a*). One wonders therefore what mechanisms are used to prevent a coenzyme from being synthesized as fast as an amino acid or nucleic-acid base.

Up till now, metabolic control mechanisms have been studied almost entirely in the pathways by which amino acids and nucleic-acid bases are synthesized, as is evident from recent reviews appearing in the *Cold Spring Harbor Symposia on Quantitative Biology*, volume 26 (1962), and elsewhere (Pardee, 1959; Wilson & Pardee, 1962). These studies lead us to suggest four possible mechanisms for explaining the low rate of coenzyme synthesis:

(1) The quantities of some enzymes in the biosynthetic pathway of a coenzyme are very low, as a result of a strong inhibition of their formation exerted by the coenzyme (repression).

(2) The quantity of these enzymes is very low, as a result of a constitutively low rate of enzyme formation, not attributable to inhibition by the coenzyme.

(3) The activity of these enzymes is very low, as a result of strong inhibition of their activity by the coenzyme (feedback inhibition).

(4) The activity of these enzymes is very low, as a result of an inherently low catalytic activity, not attributable to feedback inhibition.

As well as explaining the low rate of coenzyme synthesis, mechanisms 1 and 3 might enable the rate of coenzyme synthesis to be adjusted to *changing* physiological needs.

In this article we present evidence concerning which of the four mechanisms may operate in the biosynthetic pathways of coenzymes, in particular the pathway by which flavins are synthesized in *Escherichia coli* and some other bacteria. This pathway was chosen because of the comparative ease with which flavins can be assayed in the tiny amounts found in small samples of exponentially growing cultures.

METHODS

Organisms. Five different species of bacteria were used, three of which can synthesize flavins, namely *Bacillus subtilis* strain 23, *Pseudomonas fluorescens* strain A312, and the *Escherichia coli* strains B (wild type), C (wild type), 43–5 (leucineless), 1K4 (threonineless), B96 (purineless), P^- (purineless), M45B4 (purineless), ATCC No. 12651 (vitamin-B_6-less), M4834 (*p*-aminobenzoateless), and K12/3000 (thiamineless). The other two species, which cannot synthesize flavins, were *Lactobacillus casei* (ATCC No. 7469) and a nameless *Streptococcus* sp. (ATCC No. 10100).

Media. Stock cultures of *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas fluorescens* were maintained on agar slants containing 'complete' medium (Lederberg, 1950). The two lactic acid bacteria were maintained as stab cultures in the same medium. All stock cultures were stored at 4° between transfers, which were at bimonthly intervals. Experiments were generally carried out with bacteria growing exponentially in liquid culture aerated by swirling in conical flasks either

at 30° (for *P. fluorescens*) or 37° (for the other species). The minimal medium for growth of *E. coli*, *B. subtilis* and *P. fluorescens* was Davis's minimal salt solution (Lederberg, 1950) together with either glycerol, glucose or glycollic acid as the sole carbon source, usually at a concentration of 2 mg./ml. The glycollic acid was sterilized by filtration. For growth of *E. coli* mutants, 15–20 μg./ml. of the required amino acid or purine or 1 μg./ml. of the required vitamin was added to minimal medium. In some experiments *E. coli* was grown on supplemented media: (*a*) the 'rich glycerol' medium, which consisted of glycerol minimal medium together with Difco casamino acids (1 mg./ml.), tryptophan, uracil and adenine (50 μg./ml. of each); or (*b*) the 'rich glucose' medium, which contained peptone, tryptone, yeast extract and beef extract (Difco products, 1 mg./ml. of each) together with glucose (2·5 mg./ml.) $K_2HPO_4.3H_2O$ (3 mg./ml.) and KH_2PO_4 (1 mg./ml.). The lactic acid bacteria were grown in riboflavin assay medium (Snell, 1950) plus riboflavin (0·1 μg./ml.).

Assays. Bacterial growth was determined with a Klett–Summerson colorimeter (green filter) or by measurement of total protein precipitable by cold 5 % (w/v) trichloracetic acid (Lowry, Rosebrough, Farr & Randall, 1951).

Flavins were extracted from bacteria and their concentration determined by the fluorimetric method recommended by Burch (1957) for mammalian cells, but with the following modifications. First, flavins were routinely extracted by exposing bacteria to trichloracetic acid (5 %, w/v) for 15–60 min at 0°. Secondly, reproducible results of satisfactory accuracy and sensitivity could be obtained with cuvettes and test tubes that had been cleaned by heating in beakers of 50 % (v/v) concentrated nitric acid at 100° for 15 min. All other glassware was cleaned with detergent and all reagent solutions were made up in ordinary distilled water.

High concentrations of flavins were determined by optical density at 450 mμ (Peel, 1958), nucleotides by optical density at 260 mμ, inulin by the modified Roe test (Roe, Epstein & Goldstein, 1949) and amino acids by the quantitative ninhydrin method of Troll & Cannan (1953).

Isolation of flavins from culture supernatants. Culture supernatants containing flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin were made approximately 0·1 N with respect to HCl and left at room temperature in darkness for 2 days to hydrolyse FAD to FMN. The FMN and riboflavin were then purified together by chromatography on Florisil (60/100 mesh, for which see Peel, 1958) and after this FMN was hydrolysed quantitatively to riboflavin with acid phosphatase (Worthington Biochemical Corporation). The phosphatase was removed with trichloracetic acid and the riboflavin was precipitated with dithionite, crystallized from 80 % isopropanol and recrystallized several times from dilute acetic acid (Plaut, 1954).

Ultraviolet irradiation. Shallow layers of bacterial suspensions were irradiated at a distance of 25 cm. below a Sylvania 15 W. Germicidal-A ultraviolet lamp.

Radioactive counting. Solutions containing less than 3 mg. ^{14}C-labelled riboflavin were dried on nickel planchets and counted with an end-window gas-flow counter. Each sample was counted long enough to give at least 500 counts above background, which was about 18 counts/min.

RESULTS

Flavin content of bacteria

The flavins of the bacterial cell, like those of cells in general, are in the form of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), although there may be a trace of riboflavin as well (Peel, 1958). This has been verified in the present work with *Escherichia coli* (Table 1). FAD makes up about 60 % of the flavins when *E. coli* is growing on glycerol or glucose and when *Pseudomonas fluorescens* is growing on glucose, but the proportion of FAD rises to about 80 % when *P. fluorescens* is growing on glycollate (Table 1). Peel (1958) found a smaller proportion of FAD, probably because he extracted the flavins with 10 % (w/v) trichloracetic acid at room temperature; under those conditions FAD slowly hydrolyses to FMN.

Table 1. *The flavins of exponentially growing* Escherichia coli *and* Pseudomonas fluorescens

		Type of flavin (%)			
Organism	Growth medium	FAD	FMN	R*	FMN+R
	Intracellular flavins				
E. coli B	Glucose minimal	58	42	0	42
	Glycerol minimal	64	—	—	36†
P. fluorescens A312	Glucose minimal	62	—	—	38†
	Glycollate minimal	81	—	—	19†
	Extracellular flavins				
E. coli B	Glucose minimal	15	52	33	85
	Glycerol minimal	20	—	—	80†
P. fluorescens A312	Glucose minimal	55	—	—	45†

* R means riboflavin.
† FMN and R were not separated.

The flavin content of *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus subtilis*, as determined in the present work, is between 0·21 and 0·33 μmole/g. protein during exponential growth in all media tested and under anaerobic as well as aerobic conditions. These values are similar to Peel's, although he harvested the bacteria in stationary phase and used different strains and culture media from ours. Apparently such variables do not affect the flavin content much.

Although a large proportion of the flavins in yeast and vertebrate cells is bound to protein (Euler & Adler, 1934), in a form that can be extracted with trichloracetic acid (Burch, 1957; Peel, 1958), some flavins are free in solution and some are bound so strongly that they are released only after treatment with proteases (Singer, Kearney & Massey, 1956; Warringa & Giuditta, 1958). However, after *Escherichia coli* was extracted with trichloracetic acid no further flavins were released upon treatment with chymotrypsin or with 6N-HCl for 8 hr. at 105°.

A measure of the size of the soluble flavin fraction has been obtained by treating *Escherichia coli* with cold aqueous solutions of *n*-butanol, toluene or cetyl trimethylammonium bromide (Cetab), agents which break the osmotic barriers of cells apparently without splitting flavins from flavoproteins. Such agents release small

molecules from the intracellular pools of bacteria very effectively and permit added small molecules, including flavins (see below, Table 4), to enter the bacteria freely (Bolton, Britten, Cowie & Roberts, 1955; Mitchell & Moyle, 1956; Newton, 1958). However, only about 5% of the flavins extractable by trichloracetic acid are extracted by these agents (Table 2). Under the same conditions, these agents extract 50% of the soluble nucleotide pool (material absorbing at 260 mμ). (That mild agents do not extract as much material absorbing at 260 mμ as does perchloric acid, is probably because perchloric acid extracts bound coenzymes as well as pool nucleotides; in support of this, pellets extracted with the mild agents retain the creamish beige colour of living *E. coli* whereas the pellets are white after treatment with perchloric acid or trichloracetic acid.)

Table 2. *Extraction of flavins from* Escherichia coli

Escherichia coli B was spun down from samples of a culture growing exponentially in glucose minimal medium and the supernatants were discarded. The walls of the tubes were wiped thoroughly and each pellet of bacteria (8·7 mg. wet weight) suspended in 1 ml. of extracting agent at 0°. After 60 min. the bacteria were spun down at 0° and the supernatants saved for measurement of optical density at 260 mμ and for fluorimetric assay of total flavins. The values presented in the last two columns are the amounts extracted by the various agents, expressed as percentages of the amount extracted by the best extracting agent in the particular experiment.

		Percentage extracted	
Expt.	Extracting agent	Flavin	Material absorbing at 260 mμ
1	Trichloracetic acid, 5% (w/v)	100	—
	Toluene, 0·05% (v/v)	9	—
	n-Butanol, 5% (v/v)	5	—
2	Trichloracetic acid, 5% (w/v)	100	—
	Perchloric acid, 3% (w/v)	73	100
	n-Butanol, 5% (v/v)	4·4	56
3	Perchloric acid, 5% (w/v)	100	100
	Cetab, 0·001% (w/v)	7	46
	Distilled water	5	48

The most accurate figure for the percentage of free flavins in *Escherichia coli* was given by Experiment 2 (Table 2), namely, 4·4%, but even this is an upper limit because the pellets could have contained up to 0·002 mμmole flavin in the interstitial space, i.e. nearly 1% of the total flavin in the pellet. On top of this flavins might be split from flavoproteins to some, as yet unknown, extent, during an hour in contact with these agents. It is therefore not clear how much less than 4% of the intracellular flavins of *E. coli* are in solution. Since *E. coli* contains at least three parts of water to one part of protein (Roberts *et al.* 1955) and about 0·3 μmole flavin/g. protein, there are no more than 0·1 μmole total flavin/ml., and the concentration of free flavins in *E. coli* is less than 4×10^{-6} M.

Flavin synthesis by growing bacteria

Species like *Escherichia coli*, which do not need an external source of flavins for growth, can synthesize flavins from simple compounds in the medium. In fact, flavins are synthesized much faster than they are needed for growth (e.g. Fig. 1).

If samples of the bacteria are spun down or filtered from the medium at intervals during exponential growth, the flavin content is found to stay steady at about 0·27 μmole/g. protein. The rest of the flavin synthesized is easily detected in the medium. In the experiment described in Fig. 1, the extent of flavin overproduction, which is defined here as the ratio of excreted flavin to retained flavin, is 1·5.

Table 3. *Overproduction of flavins by bacteria*

The experimental technique was as described in Fig. 1. The extent of flavin over-production, i.e. the ratio of excreted flavins to retained flavins, was calculated from equation (1). Each value given for *Escherichia coli* B during aerobic growth in minimal media is a mean based on more than ten experiments but other values are derived from one or two experiments.

Species and strain	Growth medium	Extent of flavin over-production	Doubling time (min.)	Potential doubling time supportable by flavin synthesis (min.)
E. coli B	Glucose minimal	1·7	50	18
	Glycerol minimal	1·2	61	28
	Rich glycerol	0·8	35	19
	Glucose minimal (anaerobic)	3·0	77	19
E. coli C	Glucose minimal	2·3	54	16
	Glycerol minimal	1·3	73	32
	Glycollate minimal	8·1	240	28
E. coli K12/3000	Glucose minimal	1·4	77	31
E. coli P⁻	Glycerol minimal	2·0	67	22
E. coli 1K4	Glycerol minimal	1·6	96	35
E. coli 43-5	Glycerol minimal	1·4	52	22
E. coli M45B4	Glycerol minimal	1·2	76	31
E. coli B96	Glycerol minimal	1·6	90	32
B. subtilis 23	Glucose minimal	1·4	77	32
P. fluorescens A312	Glucose minimal	1·5	54	22

Similar results have been obtained with all other bacterial strains tested that are capable of synthesizing flavins. They include seven other strains of *Escherichia coli* and one strain each of *Pseudomonas fluorescens* and *Bacillus subtilis* (Table 3). During exponential growth all strains excreted 0·8 to 8 times as much flavin as they kept inside themselves. Extensive overproduction of flavins is therefore a widespread phenomenon because these three species belong to different bacterial families (Enterobacteriaceae, Pseudomonadaceae and Bacillaceae). Further evidence of the generality of the phenomenon is that it occurs during aerobic growth in all media tested, including minimal media with a variety of carbon sources (glucose, glycerol and glycollate), the rich glycerol medium, and during anaerobic growth in glucose minimal medium (Table 3).

It should be noted that because the flavin content of bacteria stays steady throughout the exponential phase, direct measurements of excreted and retained flavin are not needed to determine the extent of flavin over-production, E; for E can be calculated from the expression

$$E = \frac{\Delta F - \Delta P}{\Delta P}, \qquad (1)$$

where ΔF is the percentage increase in the amount of flavin in the whole culture during a given interval (starting with washed cells at zero time) and ΔP is the percentage increase in the amount of bacterial protein during the same interval. The results presented in Table 3 have mostly been obtained in this way.

Table 3 also shows that, from the growth rate (doubling time) and the extent of

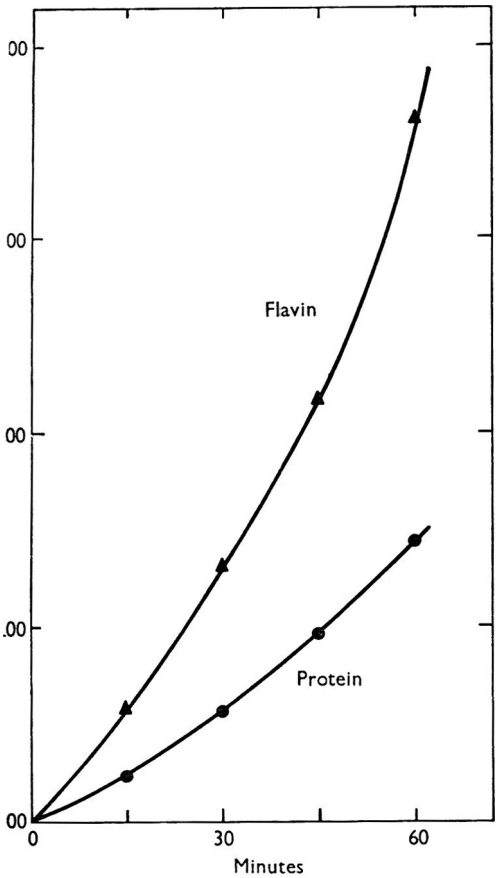

Fig. 1

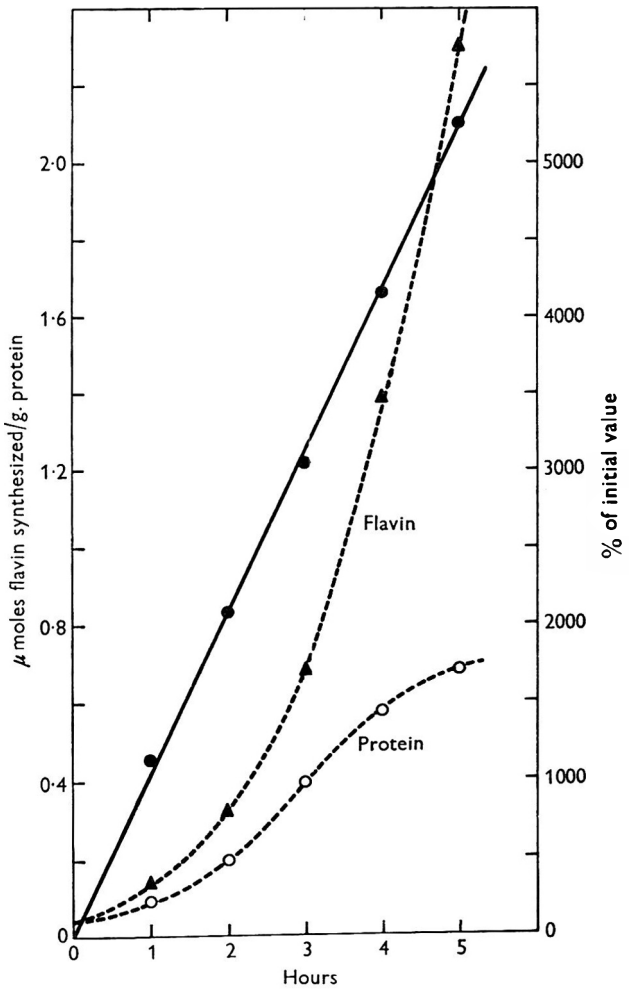

Fig. 2

Fig. 1. Overproduction of flavins by *Escherichia coli* B. Bacteria growing exponentially in glucose minimal medium were centrifuged down and resuspended in fresh warm medium to let exponential growth resume at once. Samples of the fresh culture were taken into cold 5% (w/v) trichloracetic acid at intervals during the succeeding 60 min. for analysis of protein and total flavin.

Fig. 2. The steady rate of flavin synthesis during growth of *Escherichia coli* B. Bacteria growing exponentially in glycerol minimal medium were spun down and suspended in fresh warm medium at 38 μg. protein/ml. to allow exponential growth to resume at once. During the succeeding 5 hr., samples were taken for analysis of protein and total flavin. The rate of flavin synthesis (solid line) has been corrected graphically for protein synthesis (cf. McIlwain, 1946*b*).

flavin overproduction, one can calculate that bacteria would have to grow at nearly their maximum possible rates if they were to retain all the flavins formed.

The rate of flavin synthesis, expressed in more orthodox units, is between 0·3 and 0·9 μmole/g. protein/hr. during exponential growth of all three species. The rate varies with the strain and the medium, and has the same temperature coefficient as the growth rate, at least for *Escherichia coli* B. For a given strain in a given medium at constant temperature, the rate of flavin synthesis is constant throughout exponential phase and beyond, up to the onset of maximum stationary phase, i.e. dF/Pdt = constant, F being flavin, P being protein and t being time. This constancy of rate is not immediately evident in Fig. 1 because of the way in which the data are plotted, but it is evident in Fig. 2, for example, where a correction for protein synthesis has been made. Within 2 hr. after the onset of stationary phase, however, flavin synthesis becomes severely reduced.

Uncoupling of flavin synthesis and growth

Flavin synthesis without growth. The growth rate of a culture in exponential phase can be abruptly reduced to approximately zero by inhibiting the synthesis of macromolecules specifically, through the use of suitable doses of ultraviolet light (Hanawalt & Setlow, 1960; Rushizky, Riley, Prestidge & Pardee, 1960), 5-methyltryptophan (Pardee, Shore & Prestidge, 1956) or chloramphenicol (Gale & Folkes, 1953; Wisseman, Smadel, Hahn & Hopps, 1954), or by withholding the amino acid or nucleic-acid base needed by an auxotrophic mutant for growth. As a virtually immediate result of inhibiting the synthesis of macromolecules, it would be expected that every amino acid and nucleotide would instantly accumulate within the cell and reduce its own synthesis severely by feedback inhibition of the activity of its biosynthetic enzymes. The same might be expected of flavin synthesis because flavins too are, in a sense, constituents of macromolecules (flavoproteins). Indeed it is well known that the synthesis of amino acids and nucleotides does become severely inhibited immediately under such circumstances and this was verified in the present work.

However, flavin synthesis was not inhibited at all when growth of *Escherichia coli* was abruptly inhibited with ultraviolet light (Fig. 3*a*) or 5-methyl-tryptophan (Fig. 3*b*) or by withholding leucine from a leucineless mutant (Fig. 3*c*) or purines from a purineless mutant (Fig. 3*d*). Under these conditions all the flavin synthesized was excreted. Similar results were obtained when threonine was withheld from a threonineless mutant (*Escherichia coli* 1K4), or when purines were withheld from two other purineless mutants (*E. coli* B96 and P^-), or when *Pseudomonas fluorescens* was irradiated heavily with ultraviolet light. But when chloramphenicol was added to exponentially growing *E. coli* B cultures, flavin synthesis was not sustained for long at the rate characteristic of growing bacteria (Fig. 3*b*). Within 15 min. after adding the inhibitor the rate of flavin synthesis began to fall, possibly because of some secondary detrimental effect of chloramphenicol on intermediary metabolism as a whole (cf. Pardee & Prestidge, 1959). What is significant for the problem under consideration is that several independent methods of abruptly reducing the growth rate from a rapid exponential rate to approximately zero do not lead to any adjustment in the rate of flavin synthesis.

Residual growth without vitamins. As is well known, mutants requiring amino

acids stop growing abruptly and completely as soon as the required amino acid is withdrawn from the medium. In the case of purineless and pyrimidineless mutants the situation is only slightly different, in that a slow increase in turbidity, due to protein synthesis, does go on for an hour or so after withdrawing the required base from the medium (Pardee, 1955).

However, when a required vitamin was suddenly withdrawn from the medium of an exponentially growing culture, growth was at first unaffected in any of four cases

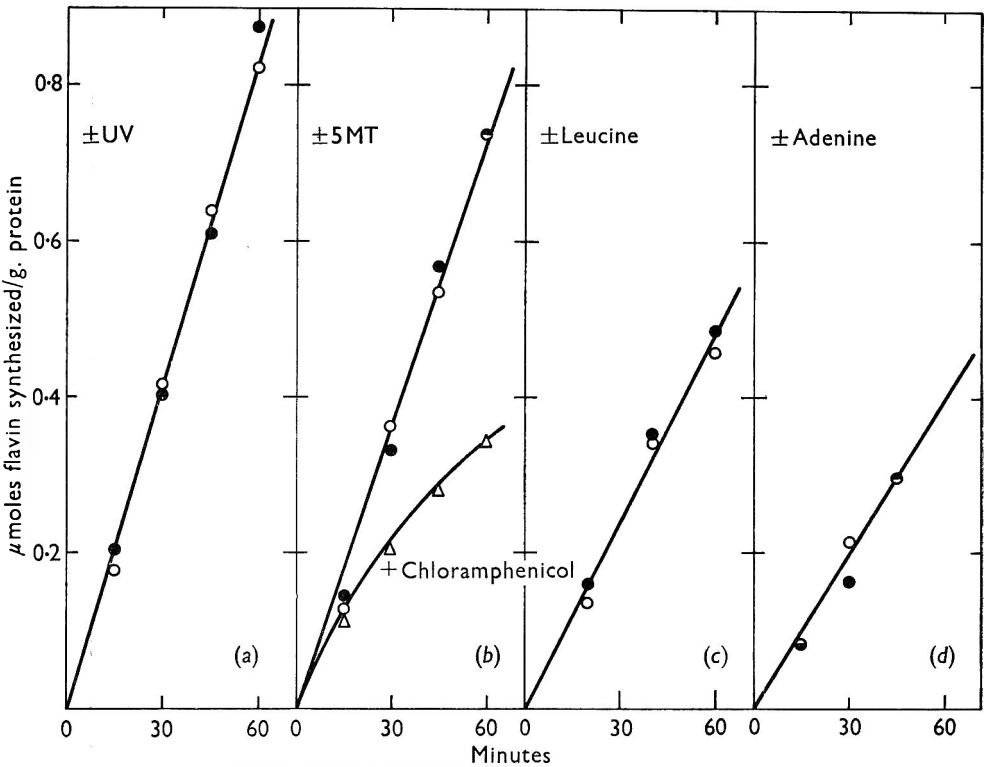

Fig. 3. Synthesis of flavins by *Escherichia coli* before and after abrupt inhibition of rapid growth. *E. coli* B was grown in glucose minimal medium and the mutant strains 43-5 and M45B4 were grown in glycerol minimal medium supplemented with leucine or adenine. When growth became exponential the bacteria were centrifuged down and resuspended in fresh medium. Growth was allowed to resume immediately in one portion of each suspension but inhibited in the other portion either (*a*) by a 60-sec. dose of ultraviolet light (strain B), (*b*) by 100 μg. 5-methyl-DL-tryptophan (5MT) per ml. (strain B) or 50 μg chloramphenicol per ml. (strain B) or (*c*) by omitting leucine (strain 43-5) or (*d*) adenine (strain M45B4). Samples were then taken into cold 5% (w/v) trichloracetic acid at intervals during the first 60 min. for analysis of protein and total flavin. In each case, flavin synthesis by the growing culture (solid points) has been corrected for growth mathematically as described by Brooke, Ushiba & Magasanik (1954) but flavin synthesis by the non-growing culture (empty points) has not been corrected for the trace of protein synthesis that occurred under these conditions.

presented in Fig. 4 or when riboflavin was taken away from the growth medium of *Lactobacillus casei*. For an hour or more, growth went on *exponentially* at the same rate as when the vitamin was present; only later did the growth rate slowly decrease. That growth did finally come to a stop after many hours shows that the

bacteria were not mutants which slowly synthesized the vitamin. Additional evidence against slow flavin synthesis was that the flavin content of the riboflavin-requiring bacteria (*Streptococcus* species and *L. casei*) declined at least in proportion to the amount of growth in the absence of external riboflavin.

That mass increases of several fold can take place in the absence of an external supply of a vitamin has been known for a long time (e.g. Kitay & Snell, 1948). The

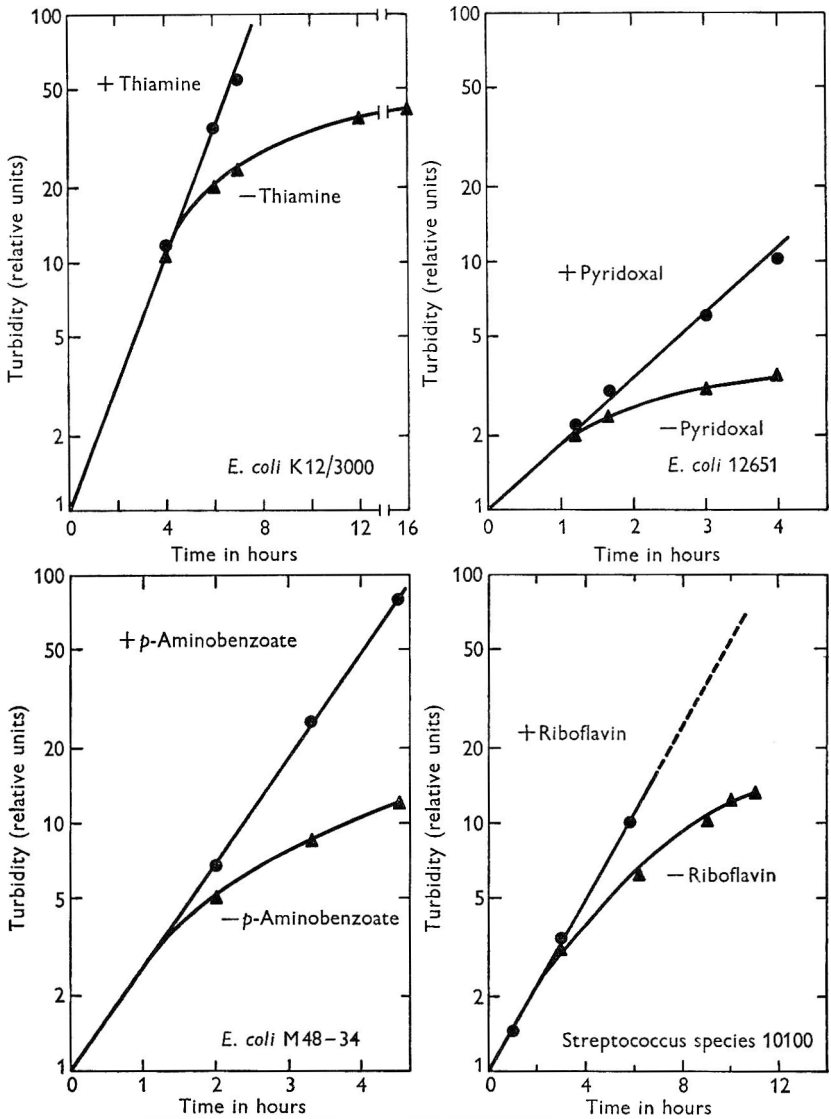

Fig. 4. Residual growth in the absence of an external source of a required vitamin. Four vitamin-requiring strains were grown to exponential phase, centrifuged down from the medium, washed twice by centrifugation in medium lacking the vitamin and finally resuspended in medium with or without the vitamin. The *Streptococcus* sp. was grown in riboflavin assay medium (±0·1 μg. riboflavin/ml.) and the *Escherichia coli* strains were grown in glucose minimal medium (±1 μg. of thiamine, pyridoxal or *p*-aminobenzoate/ml.). Growth was measured turbidometrically.

aim here is to draw attention to the early kinetics of the process, which as shown here, demonstrate that bacteria can function very well if the supply of a vitamin, and hence the supply of the corresponding coenzyme(s), is interrupted even for as long as an hour. This should be equally true if the internal synthesis of a coenzyme were interrupted in bacteria that have no dietary need of vitamins. Indeed, when exponentially growing, wild-type *Escherichia coli* is exposed to folic acid analogues that may inhibit folic acid synthesis, growth goes on at an uninhibited rate for an hour or two before starting to slow down (Webb, 1954). In sum, there is evidence that coenzyme synthesis, including flavin synthesis, can be inhibited for about an hour or more without affecting growth.

Permeability of Escherichia coli *to added flavins*

As a preliminary to testing the effect of added flavins on flavin synthesis, it was necessary to find out whether bacteria are permeable to added flavins.

Pellet method. Substances to which *Escherichia coli* is impermeable can occupy about 30 % of the volume of a centrifugal pellet (the interstitial space), whereas

Table 4. *Permeability of* Escherichia coli *to flavins and inulin*

Escherichia coli B was spun down from a culture growing exponentially in glucose minimal medium and suspended in 40 ml. of minimal salts. Ten ml. of suspension was put in each of four weighed tubes and the bacteria packed into pellets by centrifugation. The tubes were drained, wiped free of all surface liquids, and weighed again to get the weight of each pellet (0·25 g.) and hence its volume (0·25 ml.). The bacteria in each pellet were then suspended thoroughly at room temperature in 0·50 ml. of minimal salts containing inulin (1 mg./ml.) or FMN and riboflavin (each at $2{\cdot}6 \times 10^{-3}$ M), with or without 0·04 ml. *n*-butanol. The bacteria were spun down again after 5 min. and the supernatants analysed for flavins (O.D. $_{450}$) or inulin (Roe test).

	Percentage of pellet volume accessible	
Compound	Untreated bacteria	Butanol-treated bacteria
Riboflavin + FMN	28	89
Inulin	34	30

substances to which *E. coli* is permeable can occupy about 75–80 % of the pellet volume (the interstitial space plus the cell water space). Evidence for this is reviewed by Mitchell & Moyle (1956). Table 4 shows that the percentage of the pellet volume accessible to flavins is about 30 %, the same as for inulin, even when FMN and riboflavin are both present at concentrations that are enormous (5×10^{-3} M) in comparison with the intracellular concentration of free flavins (4×10^{-6} M). Now inulin is a classic example of a substance to which bacteria are impermeable (Mitchell & Moyle, 1956; Pardee, 1957); so there can be no doubt that flavins cannot enter intact cells under these conditions. They can only enter when the osmotic barriers have been broken with an agent like butanol (Table 4). However, one cannot be certain that these conclusions apply to growing bacteria because the bacteria in the permeability tests were in a different physiological state.

Isotopic method. Isotopic experiments with growing bacteria provide some support for the view that *Escherichia coli* is impermeable to added flavins. The specific

activity of the flavins excreted by *E. coli* growing on radioactive glucose was not altered detectably when high concentrations of FMN and riboflavin were added to the medium (Table 5). If the added flavins entered the bacteria they would be expected to displace all the newly synthesized flavins into the medium, thereby raising the specific activity of the excreted flavins by about 50 %. However, the experimental error in measuring the radioactivity of excreted flavins is large (± 15 %) because they account for less than a ten-thousandth of the cell material synthesized from glucose. Hence, a very slow entry of flavins, perhaps accompanied by a small degree of feedback inhibition, would be consistent with the results. Nonetheless, the simplest explanation of the results is that *E. coli* is impermeable to added flavins.

Table 5. *Specific activity of flavins excreted by* Escherichia coli *during growth with or without added flavins*

Escherichia coli B was grown exponentially for 4 hr. from turbidity 9 to 112 in two flasks, each containing 165 ml. of minimal medium with uniformly labelled C^{14}-glucose as the sole carbon source (1 mg./ml., $1{\cdot}5 \times 10^5$ counts/min./mg. carbon); one flask also contained riboflavin (8×10^{-5} M) and FMN (8×10^{-5} M). The bacteria were removed from the media by centrifugation and then non-radioactive riboflavin and FMN were added to bring the total amount in each supernatant to 20 mg. FMN and 15 mg. riboflavin. The flavins in each supernatant were converted to riboflavin which was in turn isolated and crystallized repeatedly as described under Methods.

Number of times crystallized	Specific activity (counts/min./mg. carbon)	
	Culture without added flavins	Culture with added flavins
3	33	35
4	39	35
5	43	40

A permeable preparation of Escherichia coli *capable of synthesizing flavins*

In view of the evidence that *Escherichia coli* is impermeable to external flavins, attempts were made to develop a permeable preparation that retained the ability to synthesize flavins. The best approach seemed to be to find an agent milder than aqueous butanol, one that would weaken the permeability barriers without interfering seriously with metabolism. It was found that penicillin could act in this way. *E. coli* B was treated for a few minutes with penicillin while growing exponentially in a rich glucose medium and then spun down from the medium and suspended at high turbidity in a lactate–salts mixture. When this preparation was shaken at 37° there was no net synthesis of protein but flavins were synthesized steadily for an hour or more (Fig. 5*a*, *b*; solid circles) at about a third of the rate of flavin synthesis by growing *E. coli*.

Besides fulfilling the requirement of synthesizing flavins, the preparation fulfils the permeability requirements. Table 6 shows that bacteria grown for a few minutes in the rich medium with penicillin retain their complete impermeability to inulin, yet have become permeable to both FMN and riboflavin. The permeability to riboflavin would appear to be complete, but the data suggest that FMN may still en-

counter some difficulty in entering. Another test showed that such bacteria are nearly as permeable to *o*-nitrophenyl galactoside as bacteria treated with toluene (cf. Prestidge & Pardee, 1957). Moreover, many small molecules start to leak from *Escherichia coli* after a few minutes of growth in penicillin (Prestidge & Pardee, 1957; Maas, 1959*a*). In conclusion, *E. coli* grown for a few minutes in a rich medium plus penicillin has enhanced permeability towards many small molecules, including flavins; yet the bacteria cannot have lysed because they retained their impermeability to inulin.

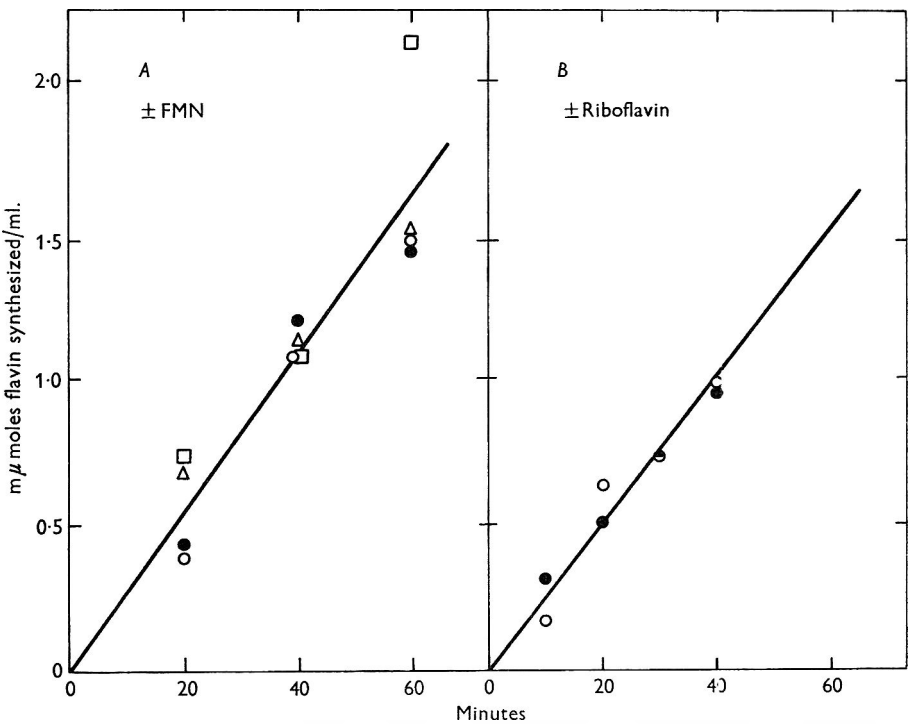

Fig. 5. Effect of added flavins on flavin synthesis by penicillin-treated *Escherichia coli* B. Bacteria were treated with penicillin for 12 min. as described in Table 6, centrifuged down and resuspended at a concentration of about 25 mg. wet weight/ml. in minimal salts plus potassium lactate (10 mg./ml.). Then 2·90 ml. of suspension was put in each of several flasks containing 0·09 ml. minimal salts plus enough flavin to give a final concentration of 0 (●), $2{\cdot}6 \times 10^{-6}$ M (△), $5{\cdot}3 \times 10^{-6}$ M (□) or 8×10^{-6} M (○). The flavin was FMN in Expt. *A* and riboflavin in Expt. *B*. The flasks were shaken at 37° and samples were taken at intervals during the first 60 min. for analysis of total flavin.

To test whether added flavins can inhibit the activity of the flavin-synthesizing enzymes would, therefore, seem possible with this preparation. Such tests have been carried out with FMN (Fig. 5*a*) and riboflavin (Fig. 5*b*). As the figure shows, these flavins at concentrations up to 8×10^{-6} M do not affect the rate of flavin synthesis appreciably. Since the test concentrations are probably at least as high as the normal intracellular concentration of flavins (less than 4×10^{-6} M), and since the intracellular concentration in penicillin-treated bacteria is probably much lower than normal, the results suggest that in intact bacteria flavin synthesis is not normally being held down by feedback inhibition of enzyme activity.

Table 6. *Permeability of penicillin-treated* Escherichia coli *to flavins and inulin*

Escherichia coli B was grown to exponential phase in a rich glucose medium. Then penicillin was added to a portion of the culture to give a concentration of 100 μg./ml. After 10–12 min. of further growth the bacteria were spun down and suspended in minimal salts. The bacteria in aliquots of the suspensions were then packed into pellets by centrifugation in weighed tubes and the percentage of the pellet volume accessible to inulin, FMN and riboflavin was measured as described in Table 4.

		Percentage of pellet volume accessible	
Expt.	Compound	Untreated bacteria	Penicillin-treated bacteria
1	Riboflavin	35	73
	FMN	37	61
2	Riboflavin	—	81
	FMN	—	57
	Inulin	—	27

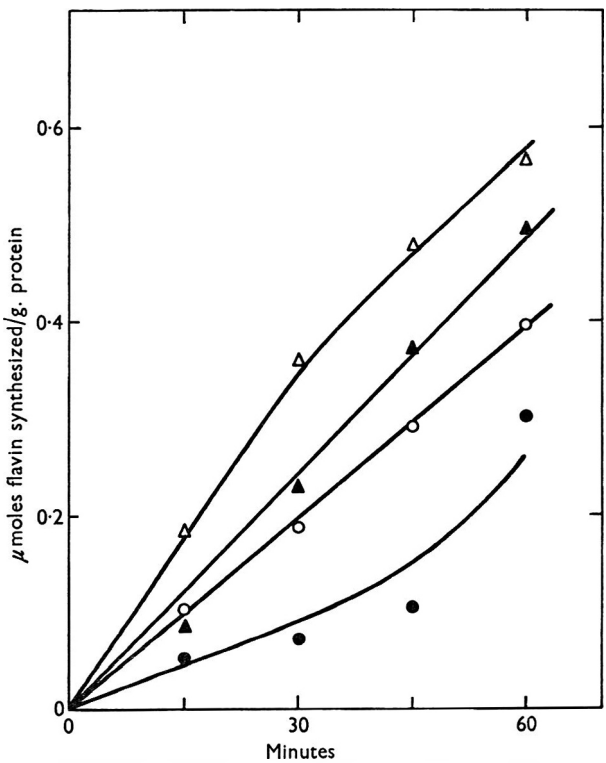

Fig. 6. Flavin synthesis after growth in rich and poor media. *Escherichia coli* B was grown to exponential phase in the 'rich glycerol' medium (triangles) and in glycerol minimal medium (circles), centrifuged down and resuspended in fresh media of both kinds. During the succeeding 60 min. of growth samples were taken from each culture for analysis of protein and total flavin. Solid symbols refer to flavin synthesis in the rich medium and empty symbols to flavin synthesis in the minimal medium.

Effect of rich and poor media on flavin synthesis by Escherichia coli

Several attempts have been made to find out whether the flavin-synthesizing enzymes can vary in amount according to the cultural conditions. Success has been achieved so far in only one type of experiment (Fig. 6). *Escherichia coli* B was grown to exponential phase in minimal medium and in a rich medium lacking flavins and then the bacteria from each culture were spun down and resuspended in media of both types. During the succeeding 30 min. in minimal medium the bacteria from the rich medium synthesized flavin twice as fast as bacteria from the minimal medium. During the same time in rich medium somewhat lower rates of flavin synthesis were observed, but again bacteria from the rich medium synthesized flavins twice as fast as bacteria from minimal medium. Afer 30 min. the rates of flavin synthesis began to adapt to the new media. When the experiment was repeated by suspending bacteria, grown in the minimal or the rich medium, in minimal medium containing chloramphenicol to prevent enzyme synthesis, the twofold difference in rate of flavin synthesis was maintained for at least 60 min. It is likely that this difference in rates of flavin synthesis reflects a difference in the amount of flavin-synthesizing enzymes in bacteria grown on rich and minimal media.

The presence of high concentrations of both riboflavin and FMN (8×10^{-5} M each) during many generations of growth in the rich and minimal media had no effect on the rate at which *Escherichia coli* B synthesized flavins in minimal medium containing chloramphenicol. This is consistent with the evidence that *E. coli* is impermeable to added flavins.

DISCUSSION

Mechanisms underlying the low rate of flavin synthesis

Feedback inhibition. Flavins evidently do not inhibit the activity of flavin-synthesizing enzymes. One line of evidence is provided by the experiments with *Escherichia coli* made permeable to flavins by penicillin. A second line of evidence comes from the experiments with specific inhibitors of the synthesis of macromolecules. The internal concentration of free flavins must rise markedly in the presence of such inhibitors because flavins are not being drained off into flavoproteins, yet the observation was that flavin synthesis was not inhibited at all. It is therefore unlikely that feedback inhibition is responsible for the low rate of flavin synthesis in comparison with amino acid synthesis.

Ideally, other experiments could be done to test whether flavins inhibit the activity of flavin-synthesizing enzymes, even though none of the early enzymic steps of flavin synthesis are known, for example, isotopic competition experiments (Roberts *et al.* 1955) and experiments on the excretion of biosynthetic intermediates by mutants with a genetic block in the pathway. Unfortunately, because *Escherichia coli*, like *Ashbya gossypii* (Maley & Plaut, 1959), is impermeable to added flavins it has been impossible to carry out isotopic competition experiments or to isolate flavinless mutants, despite deliberate attempts by ourselves and others (e.g. Davis, 1950) to do so.

Further evidence against feedback inhibition can be drawn, nevertheless, from work that has been done on the terminal steps in flavin synthesis. Studies with the purified enzyme that catalyses the conversion of ribityl lumazine to riboflavin

suggest that its specific activity (uninhibited) in intact cells is about 1 μmole riboflavin formed/g. protein/hr. in *Escherichia coli* and several other bacteria and fungi (Plaut, 1960 and unpublished). And the last two enzymes in the pathway, flavokinase and FAD synthetase, have specific activities of probably less than 1 μmole substrate converted/g. protein/hr. in intact brewer's yeast (Schrecker & Kornberg, 1950; Kearney & Englard, 1951) and *Lactobacillus arabinosus* (Snoswell, 1957). Consequently, the enzymes are not in appreciable excess over the amount needed to support the observed rate of flavin synthesis by rapidly growing bacteria (0·3–0·9 μmole/g. protein/hr. for *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas fluorescens*). Yet a huge excess of enzyme would be expected if feedback inhibition alone accounted for the low rate of flavin synthesis.

Feedback inhibition may also be unimportant in the biosynthetic pathways of other coenzymes. Thus, one enzyme in the pathway of pyridine nucleotide synthesis, nicotinic-acid-mononucleotide pyrophosphorylase, has a specific activity of about 1 μmole nicotinic acid mononucleotide formed/g. protein/hr. in *Escherichia coli*, an organism that synthesizes pyridine nucleotides at the rate of about 1 μmole/g. protein/hr. (Imsande, 1961). And at least two enzymes in the pathway of folic acid synthesis have similarly low activities in *Lactobacillus arabinosus* (Shiota, 1959) and yeast (Weiss & Srinivasan, 1959).

Repression. The formation of enzymes in the flavin biosynthetic pathway is evidently influenced by nutritional conditions. Resting-cell suspensions of *Escherichia coli* synthesize flavins more slowly if the bacteria have been grown previously in minimal medium instead of a rich medium (cf. Fig. 6). Now in minimal medium growth is slower and a smaller fraction of the flavin synthesized during growth is incorporated into flavo proteins than in a rich medium. Because of this the intracellular concentration of free flavins during growth might be higher in minimal medium than in a rich medium. Consequently, repression by flavins might account for the apparently low level of flavin-synthesizing enzymes in bacteria grown in minimal medium.

Also, the large increase in the final yield of riboflavin synthesized by *Ashbya gossypii* brought about by surface active agents might be due to de-repression of the flavin-synthesizing enzymes (Smith, Smith & Papadoupoulou, 1961).

Much better evidence is available for some other coenzymes, especially pyridine nucleotides which can inhibit their own synthesis in *Escherichia coli* by inhibiting the synthesis of nicotinic-acid-mononucleotide pyrophosphorylase (Imsande & Pardee, 1962). The level of pyridoxal phosphokinase in vitamin-B_6-requiring lactic-acid bacteria can vary according to the concentration of vitamin B_6 in the growth medium (MacCormick, Gregory & Snell, 1961). Enzymes of folic acid synthesis may also be repressible because when wild-type bacteria grow in the presence of sulphonamides, or when *p*-aminobenzoateless mutants grow on limiting amounts of *p*-aminobenzoate, they become deficient in folic acid and at the same time markedly improve their ability to synthesize folic acid from *p*-aminobenzoate in resting-cell suspensions (Nimmo-Smith, Lascelles & Woods, 1948; Lascelles & Woods, 1952). If these latter findings were to be substantiated they would become of historical interest because they antedated by several years the first reports of repression in any biosynthetic pathways.

Other mechanisms. There is no evidence concerning whether mechanisms other

than repression, for example, mechanisms 2 and 4, have a role in determining the low rate of coenzyme synthesis. Mechanism 2 (constitutively low rate of enzyme formation) would seem more likely than mechanism 4 (enzymes with low turnover number) to have such a role because it would be uneconomical for bacteria to synthesize huge amounts of enzymes with a very low turnover number.

The adjustment of flavin synthesis to physiological needs

Granting that the rate of coenzyme synthesis is about 1000 times lower than the rate at which the average amino acid or nucleic-acid base is synthesized, we can ask how precisely this low rate is adjusted to the physiological needs of bacteria. The precision of adjustment is rather poor by comparison with that in the synthesis of amino acids and nucleic-acid bases, according to several criteria.

Overproduction. One such criterion is excretion or overproduction. The excretion of a biosynthetic end product signifies that the control mechanisms in the pathway are too weak to prevent the compound from being overproduced (Moyed, 1960; Ennis & Gorini, 1961). Moreover, the extent of overproduction, i.e. the ratio of end product excreted to end product retained, should be a measure of the weakness of these control mechanisms. According to this criterion, the control mechanisms in large-scale biosynthetic pathways are strong, because, as is well known, their end products are not found in easily detectable amounts outside the cells of bacteria growing exponentially in minimal media. Thus, sensitive methods have revealed that the extent of overproduction is about 0·01 or less for many amino acids synthesized by *Escherichia coli*, *Pseudomonas aeruginosa* and a *Vibrio* sp. (Roberts *et al.* 1955; Dagley & Johnson, 1956), less than 0·01 for the porphyrins synthesized by the photosynthetic bacterium, *Rhodopseudomonas spheroides* (Lascelles, 1961) and about 0·05 or less for the nucleic-acid bases synthesized by *E. coli* (Wilson & Pardee, unpublished results).

By comparison, however, flavins are greatly overproduced, as the present work has shown; the ratio of excreted to retained flavin ranges between 0·8 and 8 during exponential growth under different conditions. Several other vitamins and coenzymes are greatly overproduced during exponential growth, for example, biotin by *Proteus vulgaris* (Thompson, 1942), pantothenic acid (McIlwain, 1946*b*; Maas, 1959*b*), vitamin B_6 (Nurmikko & Laaksonen, 1961) and pyridine nucleotides (Imsande, 1961; Imsande & Pardee, 1962) by *Escherichia coli*, and inositol by a strain of cultured mammalian cells (Eagle, Agranoff & Snell, 1960; Eagle, personal communication). Considering how few attempts have been made to detect overproduction of vitamins or coenzymes by exponentially growing cultures, it would seem quite possible that all such compounds are overproduced to an extent that is large in comparison with the extent to which amino acids and nucleic-acid bases are overproduced.

In support of this, vitamins are, as a general rule, found largely in the filtrates of stationary cultures of bacteria that can synthesize them (Thompson, 1942; Knight, 1945; Van Lanen, 1948). As Thompson pointed out, this may often result from overproduction and excretion of vitamins during growth, as well as from autolysis. Vitamins are present in relatively large amounts in the filtrates of stationary cultures of fungi too, especially the flavins synthesized by ascomycetes such as *Eremothecium ashbyii* (Goodwin & Pendlington, 1954), *Ashbya gossypii* (Maley & Plaut, 1959;

Smith *et al.* 1961), and *Candida* sp. (Enari, 1958; Goodwin & McEvoy, 1959). These findings probably also apply to coenzymes because the analytical techniques used have generally not distinguished coenzymes from vitamins.

Uncoupling of growth and coenzyme synthesis. Further evidence that coenzyme synthesis is not precisely adjusted to physiological needs is given by experiments which showed that growth and coenzyme synthesis are not tightly coupled to each other. On the one hand, there is residual growth in the absence of a required vitamin, and on the other hand, there is the evidence that when growth is inhibited flavin synthesis goes on uninterrupted. Some data in the literature suggest that, like flavins but unlike amino acids and nucleic-acid bases, other vitamins and coenzymes are synthesized at a rate which cannot be adjusted quickly to a change in growth rate. Thus although there are no reports fully comparable to the present one concerning what happens to coenzyme synthesis when growth is abruptly halted, it is clear that non-growing bacteria can often synthesize vitamins in the absence of special supplements (McIlwain, 1946*b*; Nimmo-Smith *et al.* 1948; Lascelles & Woods, 1952; Morris, 1959). Indeed, McIlwain found that when *Escherichia coli* or *Pseudomonas aeruginosa* were grown in minimal medium, then harvested in stationary phase and suspended in fresh medium lacking the nitrogen source (NH_4^+), they would synthesize pantothenic acid at about the same rate as exponentially growing bacteria.

Disadvantages of precise adjustment. Bacteria would probably not benefit from stricter controls over the synthesis of flavins and other coenzymes. In the first place, the waste of energy and materials entailed in overproducing flavins and other coenzymes is small. The absolute rate at which the average amino acid or nucleic-acid base is excreted is approximately as great or greater. In this sense, therefore, the controls in coenzyme pathways are as strict as those in large-scale pathways.

To avoid even this small absolute rate of excretion, control mechanisms would have to be stricter; that is, the concentration of end product required to inhibit its own synthesis (by whatever means) would have to be much lower. Perhaps lower concentrations would be too low for the end products to act as substrates for the synthesis of macromolecules. The flavin case illustrates this possibility well because, as shown in the present work, the internal concentration of free flavins in *Escherichia coli* is already less than 4×10^{-6} M.

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Characterization of a Propionic Acid Producing Actinomycete, *Actinomyces propionicus*, Sp.nov.

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SUMMARY

An anaerobic actinomycete isolated from the lachrymal duct of a case of human lachrymal canaliculitis is described. Although the organism was described previously as *Actinomyces israelii*, more recent observations show it differs significantly from members of this genus in metabolism, physiology and cell-wall composition. The organism is similar to the *Actinomyces* in its production of true branching mycelial elements *in vivo* and *in vitro*, catalase negativity, pathogenicity for experimental animals, and amino acid cell-wall composition. It is similar to the propionic acid bacteria in its fermentation of glucose to propionate, acetate, and CO_2, its morphological variation, formation of a dull orange colour, and the presence of diaminopimelic acid in its cell wall. Other characteristics such as the formation of DL-lactic acid, absence of a CO_2 requirement either anaerobically or aerobically, inability to ferment glycerol or lactate, and sugar and amino sugar composition of the cell wall also serve to differentiate this organism from other strains of *Actinomyces* and from *Propionibacterium*. In view of these observations the organism is reclassified and named *A. propionicus*, sp.nov. The possible phylogenetic relation of *A. propionicus* to certain other branching filamentous organisms is discussed.

INTRODUCTION

From studies of human canaliculitis, Pine & Hardin (1959) and Pine, Hardin, Roberts & Turner (1960) have described two of the etiological agents as *Actinomyces israelii*. Both organisms were found to possess morphological and physiological characters typical of the species. However, one of the organisms, strain 699, fermented glucose to form propionic acid in addition to the acetic, formic, lactic, and succinic acids described for other strains (Pine & Howell, 1956). Because of this difference, the morphological, physiological and biochemical characteristics of strain 699 have been studied further. Although strain 699 possessed many of the characters of recognized species of *Actinomyces* (*Bergey's Manual*, 1957; Howell, Murphy, Paul & Stephan, 1959; Pine, Howell & Watson, 1960; Waksman, 1961) the more recent findings showed it had major differences in metabolism, physiology, and cell-wall composition. In many respects it was closely related to members of the genus *Propionibacterium*. After consideration of these findings we were unable to assign the organism to a previously described species of either of these genera. As a result, we have concluded that the organism warrants inclusion in the genus *Actinomyces* and propose the name *Actinomyces propionicus*, sp.nov.

METHODS

Actinomyces propionicus (*A. israelii*, strain 699) was isolated as described previously by Pine & Hardin (1959). Sources of other species of *Actinomyces* were described by Pine, Howell & Watson (1960). *Propionibacterium arabinosum*, 4965, and *P. pentosaceum*, 4875, were obtained from the American Type Culture Collection. The Casitone medium of Pine & Watson (1959) with no added lipoic acid was used throughout this study. The procedures for the preparation of inocula and growth were those described by Howell & Pine (1956).

Methods of growth studies

Amounts of growth were determined by preparing a 1/10 or 1/20 dilution of homogenized cultures with distilled water and reading the absorbancy at 660 mμ on a Spectronic-20 colorimeter against a corresponding dilution of uninoculated medium. One growth unit = optical density unit divided by the dilution, and was equivalent to 0·3 mg. dry wt. organism. Forty-eight hr. cultures of *Actinomyces bovis* (P1S) and *A. naeslundii* (279) and 72 hr. cultures of *A. propionicus* and *A. israelii* (895) were diluted with sterile distilled water to an optical density = 0·5 on a Spectronic-20 colorimeter. Test tubes containing 5 ml. Casitone medium were inoculated with one drop of this suspension. Anaerobic cultures were incubated under a pyrogallol + Na_2CO_3 seal. Aerobic cultures were either unsealed or sealed with KH_2PO_4(M) + Na_2CO_3 (10 %, w/v) as indicated and incubated on a rotary shaker. All incubations were conducted at 37°.

The methods used to determine fermentative and physiological characters were as previously described (Pine & Howell, 1956). The size of inoculum required for aerobic growth was determined according to the procedure of Howell *et al.* (1959).

For pathogenicity studies, organisms from 72 hr. cultures of *Actinomyces* spp. and from 48 hr. cultures of *Propionibacterium* were harvested by centrifugation and washed twice with sterile distilled water. Packed organisms (0·5 ml.) were brought to a volume of 5 ml. with physiological saline (NaCl, 0·85 %, w/v). White female mice ranging from 5 to 6 weeks of age were inoculated intraperitoneally with 0·2 ml. or 0·5 ml. of this suspension, depending upon the experiment. After 18 days animals were sacrificed and examined for internal lesions. When lesions were found these were recorded, isolation plates were streaked with pus, and Gram stains of the pus made. Isolations were made on brain heat infusion agar (Difco) plates which were incubated 1–3 days under an atmosphere of 5 % (v/v) CO_2–95 % (v/v) N_2.

For product analysis fermentations were carried out in duplicate; one of each duplicate contained uniformly labelled ^{14}C-glucose of known specific activity. All fermentation vessels were inoculated with 2 mg. equivalent of dry wt. organism and incubated for 3 days at 37°. For anaerobic studies, the organisms were grown in fermentation tubes (Pine & Howell, 1956) containing 50 ml. of medium. Tubes were sealed with 1 ml. each of Na_2CO_3 + pyrogallol or NaOH + pyrogallol. For aerobic studies organisms were grown on a rotary shaker in a 160 ml. Erlenmeyer flask containing 50 ml. medium and stoppered with a vaccine stopper. At the end of the

incubation period 1 ml. of 50 % (w/v) CO_2-free NaOH was injected through the vaccine stopper to make the liquor strongly alkaline.

Carbon dioxide was determined manometrically by the method of Peters & Van Slyke (1932). Volatile acids were quantitatively estimated by the method of Friedemann (1938) as modified by Rabinowitz & Barker (1956). After formate oxidation, propionate and acetate concentrations were calculated by solving simultaneous equations based on Duclaux values. Glucose was determined by the method of Folin & Malmros as given by Umbreit, Burris & Stauffer (1957).

Succinate and lactate were quantitatively estimated from fermentations conducted with radioactive glucose. They were collected by 48 hr. ether extraction and separated and identified by means of a Dowex-1 column with a formic acid gradient according to a method modified from that of Busch, Hurlbert & Potter (1952). This method effects repeatable separation of lactic and succinic acids with no overlapping of peaks. The formic acid eluant was removed by evaporation on a steam bath, and the non-volatile acids were quantitatively determined by titration with 0·1 N-NaOH to the phenol red end-point.

Glucose incorporation into cellular material was calculated from organisms grown on ^{14}C-labelled glucose which were collected by centrifugation, washed 5 times with distilled water, pipetted on to planchets and dried under infrared heat. Samples were counted at infinite thinness. Radioactivity was measured with a continuous flow counter equipped with a 'micromil' window (Nuclear-Chicago).

The lactic acid used for determination of optical rotation was obtained from a 6 l. flask of fermentation liquor. The flask contained a modified Casitone medium in which acid-hydrolysed casein (Nutritional Biochemical Co., Cleveland, Ohio) was substituted for Casitone and the minor-element solution omitted. Lactate was collected by 48 hr. ether extraction and crystallized as the zinc salt (Pederson, Peterson & Fred, 1926). Optical activity was determined with a solution of zinc lactate in a Model BO polarimeter (O.C. Rudolph and Sons Inc., Caldwell, N.J.).

Cell walls were isolated and acid-hydrolysed according to the method of Cummins & Harris (1956). In most cases amino acids and amino sugars were identified solely by R_f values on two-dimensional paper chromatograms developed as described by these investigators. Diaminopimelic acid and lysine were more definitely identified by co-chromatography with known compounds. Sugars as well as amino sugars were identified by R_f values from unidirectional paper chromatograms developed in butanol + pyridine + water (6 + 4 + 3 by vol) and butanol + acetic acid + water (5 + 1 + 2 by vol). Sugar and amino sugar spots were located on duplicate sheets and were characterized by their comparative reactions using a modified silver nitrate dip (Dr R. W. Wheat, unpublished results) and a ninhydrin spray (0·25 %, w/v, ninhydrin in 95 %, v/v, ethanol in water).

A culture of *Actinomyces propionicus* 699 has been deposited in the American Type Culture Collection, 2112M Street, N.W., Washington, D.C., under the number ATCC 14157.

RESULTS

As a result of preliminary fermentation studies, it became evident that *Actinomyces propionicus* fermented glucose in a manner typical of many species of *Propionibacterium*. Moreover, certain morphological and physiological characteristics

common to the propionibacteria became apparent. Although a brief description of the morphological and physiological characters of *A. propionicus* was presented previously (Pine & Hardin, 1959), it subsequently became necessary to extend these studies and to compare the organism directly to known species of *Actinomyces* and *Propionibacterium*.

Cellular morphology

As shown in Pl. 1, fig. 1, *Actinomyces propionicus* formed branched mycelia typical of other members of the genus (Howell *et al.* 1959; Pine, Howell & Watson, 1960). However, when the organism was grown in 1 l. volumetric flasks under Na_2CO_3 + pyrogallol seals to prepare organisms for enzymic studies, a marked change in morphology occurred. For reasons which are not known, *A. propionicus* changed under these conditions from a short branching rod in the initial stages of growth to a long thread-like element which tangled to form a cotton-like mass (Pl. 1, fig. 2). As growth progressed occasional bulbous ends were observed on some cells. In sugar fermentation tests the sugar fermented greatly influenced the morphology. After 10–14 days of incubation in glucose fermentation tubes, forms identical with those of *Propionibacterium pentosaceum* were observed (Pl. 1, fig. 3). Here the individual cells in a hyphal element swelled with apparent spheroplast formation. With raffinose as a substrate virtually all cells rounded and swelled to form solid masses of spherical cells (Pl. 1, fig. 4). Although the number of spherical cells or hyphal-like threads which were formed varied from one substrate to another, it apparently was not directly related to the amount of growth or acid produced on any given substrate. While further studies were necessary to determine what factors influenced the morphology of the organism, the morphological similarity of *A. propionicus* to certain of the propionic acid bacteria as originally described by Van Niel (1938) and by Hitchner (1932, 1934) was quite obvious. However, it may be pointed out that spherules such as those shown in Pl. 1, fig. 4, were much larger than any observed with propionibacteria. In addition, they were rigid structures resistant to the osmotic effects of distilled water or 20 % (w/v) sucrose.

Colony morphology

Of the taxonomic criteria used in the identification of species of *Actinomyces*, the morphology of the micro-colony is of paramount importance (Wright, 1905; Emmons, 1935; Howell *et al.* 1959; Pine, Howell & Watson, 1960). Consequently, the colony morphology of *Actinomyces propionicus* was compared to that of *Propionibacterium pentosaceum* and *P. arabinosum* (Pl. 2, figs. 6, 7, 8, 9). Under low power the mycelial nature of *A. propionicus* was readily apparent, while the colony of *P. arabinosum* was round with an unbroken periphery. Under high power, the colony of *A. propionicus* was definitely mycelial with long hyphal elements which branched repeatedly (Pl. 2, fig. 8). Under the same conditions colonies of *P. arabinosum* and *P. pentosaceum* appeared to be mycelial although the hyphal elements were short and branching was not apparent (Pl. 2, fig. 9). Moreover, when a coverslip was placed over these colonies, they immediately disintegrated to form a free-flowing mass of individual cells having no semblance of colonial structure. On the other hand, *A. propionicus* tenaciously maintained its intact mycelial structure

under these conditions. These characteristics indicated that while *A. propionicus* produced definite mycelial colonies, cells of the propionic acid bacteria were very loosely connected and did not form a true mycelium.

Relative pathogenicity

To the authors' knowledge there have been no experimental pathogenicity studies conducted with species of *Propionibacterium*. Consequently, comparative pathogenicity studies were made with species of *Propionibacterium* and *Actinomyces*. The results of animal inoculations with *Actinomyces propionicus* strongly indicated that it was no less pathogenic than any of the other species of *Actinomyces* tested (Table 1). The results given in Table 1 suggested, and this was supported by several additional experiments, that *A. propionicus* was slightly more pathogenic than the other strains of *Actinomyces* tested. On the other hand, failure to observe typical mycelial elements or any Gram-positive organisms in the lesions of animals inoculated with species of *Propionibacterium*, and the failure to isolate these organisms, showed that the propionibacteria were non-pathogenic. The fact that spontaneous infections in animals or man due to propionibacteria have not been reported (Topley & Wilson, 1957) support these observations. From the appearance of *A. propionicus* in animal lesions (Pl. 1, fig. 5) there was no doubt that it was similar morphologically to other species of *Actinomyces* in clinical material although it has often shown a greater propensity for being thread-like (Pine & Hardin, 1959).

Sugar fermentations

The results of the utilization of various substrates for growth and acid production by *Actinomyces propionicus* and related organisms are presented in Table 2. The organism was found to follow the same general pattern of other *Actinomyces* in these respects. While there were other major differences between the anaerobic actinomycetes and members of the genus *Propionibacterium*, the complete inability of *Actinomyces* species to ferment and utilize glycerol for growth and acid production was the most pronounced (Howell *et al.* 1959; Pine, Howell & Watson, 1960). All species of *Propionibacterium* ferment glycerol and lactate with the formation of acid (*Bergey's Manual*, 1957); *A. propionicus* could not use these substrates for growth in many repeated experiments. In its fermentation of sugars, *A. propionicus* was similar to *A. naeslundii* for it fermented raffinose but failed to ferment xylose (Howell *et al.* 1959). The ability to ferment mannitol was not shown by strains of *A. naeslundii* whereas mannitol always supported good growth of *A. propionicus* although acid production was variable.

Physiological characteristics

The compared physiological characteristics of the *Actinomyces* and *Propionibacterium* spp. are recorded in Table 3. Colonies of *A. propionicus* had a dull orange colour when grown on the surface of aerobic or anaerobic tubes, whereas all other species of *Actinomyces* remained white. *A. propionicus* also produced large amounts of H_2S; it was similar to the facultative anaerobe, *A. naeslundii*, in that both reduced nitrate to nitrite. *A. propionicus* was consistently catalase negative. On the other hand, the propionic acid bacteria were catalase positive as previously described (*Bergey's Manual*, 1957; Van Niel, 1928).

Table 1. *Comparative pathogenicity of species of* Actinomyces *and* Propionibacterium

Organism	Size of inoculum packed cells ml./mouse	Mice incubated (days)	Mice inoculated (no.)	Mice having abscesses (no.)	Description of infections*	Gram+ filaments no. of mice	Positive isolation no. of mice†
Actinomyces propionicus 699	0·05	18	5	5	All mice showed multiple lesions on stomach, kidney, intestinal tract, and/or liver. One mouse had 2 cm. encapsulated abscess on lower peritoneal wall with numerous lesions on dorsal part of liver	5	n.d.
	0·05	33	3	3	One mouse with massive intestinal abscess adherent to spleen, multiple lesions throughout. Second mouse with two large abscesses, third with single small abscess on lower intestine	3	n.d.
A. naeslundii 279	0·05	18	6	3	Two mice each with encapsulated abscess diameter greater than 2 cm., one on intestine, one on liver. Multiple small abscesses in addition. Third mouse with large intestinal abscess and small lesions on liver and mesentery	3	n.d.
A. bovis P1S	0·05	32	6	1	Small lesions adherent to intestinal wall	1	n.d.
A. propionicus 699	0·02	25	4	4	Small abscesses at point of inoculation and on liver	4	4
	0·02	32	4	4	As above but two mice each with single encapsulated lesion 0·5–0·7 cm. in diameter. One in liver, one on the peritoneal wall	4	4
A. naeslundii 279	0·02	25	5	2	One with two small lesions on peritoneal wall, one with single lesion at point of inoculation	2	n.d.
	0·02	32	5	5	Small lesions on peritoneal cavity or at point of inoculation	5	5
A. israelii 895	0·02	25	4	3	One mouse with large encapsulated abscess attached to liver and stomach. Second with multiple lesions throughout abdominal cavity. Third with three small lesions on liver and one on kidney	4	n.d.
	0·02	32	3	0	No infections	0	n.d.
Propionibacterium arabinosum ATCC 4965	0·02	21	7	4	One mouse with multiple lesions on liver, three mice with single small lesion at point of inoculation	0	0
Propionibacterium pentosaceum ATCC 4875	0·02	21	6	3	One mouse with multiple lesions on liver, two mice with single lesion at inoculation site	0	0

* Pus from the largest and most developed lesions was taken from infected mice and mounted in 10 % (w/v) KOH for microscopic examination. Although in some cases mycelial clumps were observed, typical sulphur granules were not seen in any of the preparations.

† n.d. = Quantity not determined.

Table 2. *Comparison of the growth and fermentative ability of* Actinomyces propionicus *with other* Actinomyces *and* Propionibacterium *spp.*

+ = Acid and no gas (bromcresol purple indicator). Relative growth expressed in units ranging from a minimum = 1 to a maximum = 5. Duplicate tubes containing 5 ml. were inoculated with 1 drop of an homogenized 48–72 hr. culture and incubated under pyrogallol + Na_2CO_3 seal for 7–10 days at 37°. Basal medium was the Casitone medium with 1·0 % (w/v) substrate. Strain numbers are indicated below the name of each species.

	Organism											
	A. propionicus (699)		*A. israelii* (895)		*A. bovis* (P1S)		*A. naeslundii* (279)		*P. arabinosum* (ATCC 4965)		*P. pentosaceum* (ATCC 4875)	
Substrate	A.*	G†	A.	G.	A.	G.	A.	G.	A.	G.	A.	G.
Glucose	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5
Fructose	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5
Mannose	+, +	5, 5	n.d.‡	n.d.	n.d.	n.d.	n.d.	n.d.	+, +	5, 5	+, +	4, 4
Galactose	+, +	3, 3	±, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	4, 4	+, +	5, 5
Lactose	+, +	5, 5	+, +	3, 3	+, +	5, 5	+, +	5, 5	+, +	4, 4	+, +	5, 5
Maltose	+, ±	5, 5	±, +	5, 5	+, +	5, 5	±, +	5, 5	+, +	4, 4	+, +	4, 4
Mannitol	+, −	5, 5	±, ±	4, 4	−, −	1, 1	−, −	5, 5	+, +	4, 4	+, +	4, 4
Sucrose	±, ±	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	4, 4	+, +	4, 4
Xylose	−, −	1, 1	+, +	5, 5	−, −	1, 1	−, −	3, 3	−, −	2, 2	+, +	4, 4
Raffinose	±, ±	5, 5	+, +	5, 5	−, −	1, 1	+, +	5, 5	−, −	1, 1	+, +	4. 4
Arabinose	+, +	5, 5	−, −	1, 1	−, −	1, 1	−, −	1, 1	−, −	1, 1	+, +	4, 4
Ribose	±, ±	3, 3	+, +	5, 5	−, −	1, 1	−, −	1, 1	+, +	4, 4	+, +	4, 4
Rhamnose	−, −	3, 3	−, −	3, 3	±, ±	5, 5	−, −	1, 1	+, ±	3, 3	+, +	3, 3
Salicin	−, −	2, 2	+, +	5, 5	±, ±	1, 1	±, ±	5, 5	±, ±	3, 3	±, ±	3, 3
Starch	±, ±	5, 5	±, ±	5, 5	+, +	5, 5	±, ±	5, 5	−, −	2, 2	±, ±	4, 4
Glycogen	−, −	1, 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−, −	1, 1	−, −	2, 2
Lactic acid	−, −	1, 1	−, −	1, 1	−, −	1, 1	−, −	1, 1	+, +	5, 5	+, +	5, 5
Glycerol	−, −	1, 1	−, −	1, 1	−, −	1, 1	−, −	1, 1	+, +	5, 5	+, +	5, 5
Cellulose	−, −	1, 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* A = Acid production. † G. = Growth. ‡ n.d. = quantity not determined.

Carbon dioxide requirement for anaerobic and aerobic growth

All species of *Actinomyces* have been shown to require CO_2 for maximum anaerobic growth, while *A. naeslundii*, a facultative anaerobe, manifests a similar requirement aerobically (Pine & Howell, 1956; Howell *et al.* 1959). Consequently, the CO_2 requirement for *A. propionicus* was investigated, both anaerobically and aerobically. As seen in Table 4, this organism was similar to *A. naeslundii* in that it grew well aerobically although anaerobic growth was induced by a smaller inoculum than that required for aerobic growth. In contrast to *A. naeslundii* (Howell *et al.* 1959) CO_2 had no stimulating effect on aerobic growth of *A. propionicus*. A comparison of the growth curves of *A. naeslundii* grown aerobically in the presence and absence of added CO_2 with those of *A. propionicus* showed conclusively that *A. propionicus* did not have the CO_2 requirement for aerobic growth so consistently demonstrated for *A. naeslundii* (Fig. 1). Similarly, fermentations carried out under a NaOH + pyrogallol seal revealed that *A. propionicus* did not require CO_2 for anaerobic growth (Table 5).

Table 3. *Physiological characteristics of* Actinomyces propionicus *and* Actinomyces *and* Propionibacterium *spp.*

Physiological tests were carried out as described previously (Pine & Howell, 1956). With the exception of litmus milk, basal medium was the Casitone medium with 1·0 % (w/v) glucose. Conditions of growth as given in legend to Table 2.

Character	*A. propionicus* (699)	*A. israelii* (895)	*A. bovis* (P1S)	*A. naeslundii* (279)	*P. arabinosum* (4965)	*P. pentosaceum* (4875)
Catalase	−*	−	−	−	+	+
Colour	Dull orange	White	White	White	Orange-yellow	Cream
Gelatin liquefaction	−	−	−	−	−	−
Starch hydrolysis	−	−	+	−	−	−
Indole production	+	+	+	+	−	−
Nitrate reduced to nitrite	+	−	−	+	+	−
Acetoin production	−	−	−	−	−	−
H_2S formation	+	−	−	−	±	−

* Catalase was negative whether the organism was grown aerobically or anaerobically in the presence or absence of added haemin.

Table 4. *Effect of inoculum size on anaerobic and aerobic growth of* Actinomyces propionicus

A 48 hr. culture of *A. propionicus* was homogenized and diluted with sterile distilled water to an optical density = 0·5. This suspension was serially diluted with water as indicated. Duplicate tubes containing 5 ml. of Casitone medium with 0·5 % (w/v) glucose were inoculated with 1 drop of each dilution and incubated for 84 hr. at 37°. Aerobic cultures were grown on a rotary shaker. Relative growth expressed for duplicate tubes in units ranging from a minimum = 1 to a maximum = 5; − signifies no growth.

Growth conditions	Dilution used for inoculum: 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
	Relative growth								
Anaerobic:									
Na_2CO_3 + pyrogallol seal	5, 5	5, 5	4, 4	4, 4	3, 3	2, 1	2, 1	2, 1	2, 1
Aerobic:									
$Na_2CO_3 + KH_2PO_4$ seal	5, 5	5, 5	3, 2	5, 2	1, −	−, −	−, −	−, −	−, −
Aerobic: no seal	5, 5	5, 5	2, 2	1, 1	−, −	−, −	−, −	−, −	−, −

Comparative growth of species of Actinomyces

Figure 2 shows the comparative growth curves of species of *Actinomyces*. Anaerobically the rate of growth of *A. propionicus* was not usually as great as that of *A. naeslundii* and was usually less than that of *A. bovis* or a granular strain of *A. israelii*. However, as shown in Fig. 2 the yields of *A. propionicus* grown anaerobically were comparable to those of *A. naeslundii* and much greater than those of *A. israelii* and *A. bovis*. Generally, species of *Actinomyces* utilize only 25–30 μmole glucose/ml. medium (Pine & Howell, 1956). This has been shown to be due to sensitivity of these organisms to the acid formed; greater glucose utilization with

increased yields of growth occurred in an adequately buffered medium (Buchanan & Pine, unpublished results). However, *A. propionicus* utilized only 16 μmole glucose/ml. in Casitone medium, yet the growth yields as judged by optical density measurements were greater than those observed for the other species (Fig. 2). This ability of *A. propionicus* to form more organisms from less glucose would appear to be related to a more efficient pathway of glucose dissimilation (Bauchop & Elsden, 1960). Usually growth yields of *A. naeslundii* doubled in the presence of oxygen (Pine & Howell, 1956; Buchanan & Pine, unpublished results). However, without changing the medium, growth of *A. propionicus* in air did not increase significantly (Figs. 1, 2), although all the glucose (27 μmole/ml.) was utilized (Table 5). The reason for this is unknown at present; it may be due to the limitation of growth factors in the medium.

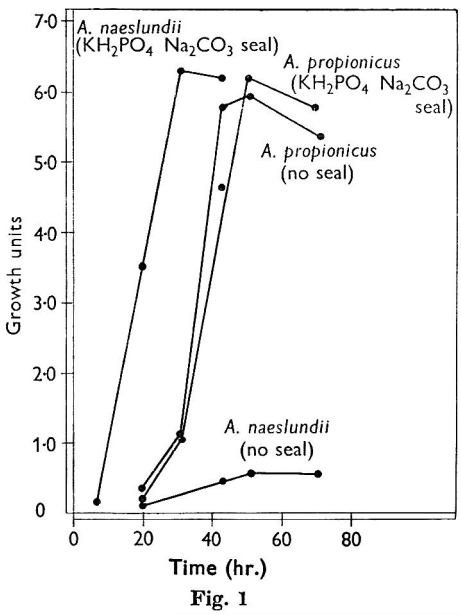

Fig. 1

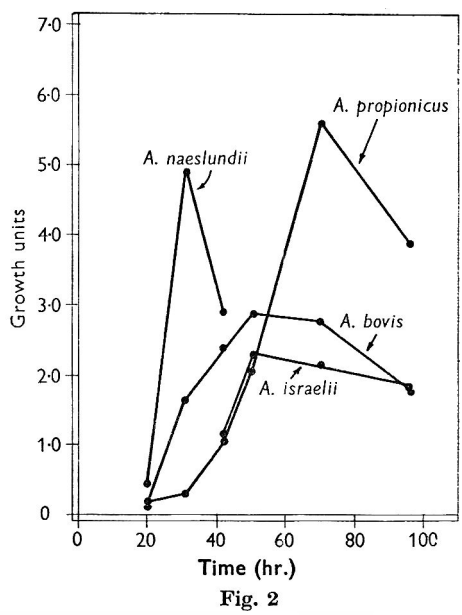

Fig. 2

Fig. 1. Comparative aerobic growth of *Actinomyces propionicus* and *A. naeslundii* with and without added CO_2.

Fig. 2. Comparative anaerobic growth of species of *Actinomyces*.

Fermentation balances

The results of the glucose fermentations of *Actinomyces propionicus* and related organisms are given in Table 5. Anaerobically, in the presence or absence of added CO_2, *A. propionicus* produced principally CO_2, acetic and propionic acids, with a ratio of products that was typical of many strains of *Propionibacterium*. However, the reported utilization of glucose by *Propionibacterium* spp. was much greater than that observed for *A. propionicus* (Wood & Werkman, 1936). From 70 to 80 % of the total carbon utilized could be accounted for by these products. While their concentrations were quite small, lactate and succinate were definitely produced under these conditions, although the amounts of lactate produced varied. After consideration of the data of Wood & Werkman (1936) and Wood, Stone & Werkman

(1937) it was concluded that there was no significant qualitative or quantitative difference between glucose fermentations of *A. propionicus* and species of *Propionibacterium* other than the total amount of glucose fermented.

Aerobically in the absence of added CO_2, *Actinomyces propionicus* fermented glucose to yield stoichiometric amounts of acetic acid and CO_2. While there was a small percentage of carbon which remained unaccounted for, this was not present in the ether-extractable non-volatile fraction. Absence of radioactivity in any remaining fraction other than whole cells indicated that no other products were formed in significant amounts.

Table 5. *Glucose fermentation balances for* Actinomyces propionicus *and related organisms*

Organism	Growth conditions	Glucose fermented (μ mole/ml.)	Expressed in μmole/100 μmole glucose fermented: CO_2 fixed	CO_2 produced	Acetate	Propionate	Formate	Lactate	Succinate
A. propionicus	Anaerobic: Na_2CO_3+ pyrogallol seal	15·9	—	53·5	55·3	114·5	4·4	2·5	3·1
		16·4	—	54·9	53·7	111·0	0·0	2·4*	3·0*
	Anaerobic: NaOH+ pyrogallol seal	14·4	—	n.d.	71·5	118·7	0·0	2·8	4·9
		13·9	—	n.d.	48·9	133·8	9·4	2·9*	5·0*
	Aerobic: no seal	27·0	—	175·9	144·8	0·0	0·0	0·5	0·5
		27·5	—	169·4	157·4	0·0	0·0	0·5*	0·5*
A. israelii, 295‡	Anaerobic: Na_2CO_3+ pyrogallol seal	26·6	32·7	—	23·7	0·0	29·3	139·5	32·3
Propionibacterium arabinosum (34 W_1)§	Anaerobic: under N_2-atmosphere	145·3	—	63·6	17·8	139·1	—	—	13·2
P. pentosaceum (49 W_3)§	Anaerobic: under N_2-atmosphere	155·9	—	52·3	16·6	127·8	—	—	21·1
P. shermanii (52 W_8)§	Anaerobic: under N_2-atmosphere	163·9	—	47·0	45·9	106·1	—	1·5	15·0

Organism	Growth conditions	Glucose fermented (μ mole/ml.)	Expressed in μmole/100 μmole glucose fermented: Non-reducing material	Cellular glucose incorporation	Ratio: CO_2/acetate	Ratio: propionate/acetate	Carbon recovery (%)	Redox index
A. propionicus	Anaerobic: Na_2CO_3+ pyrogallol seal	15·9	—	15·7	0·97	2·07	104·4	1·00
		16·4	—	15·2*	1·02	2·07	101·0	1·02
	Anaerobic: NaOH+ pyrogallol seal	14·4	—	14·6	—	1·66	102·4†	—
		13·9	—	15·1*	—	2·74	104·7†	—
	Aerobic: no seal	27·0	—	15·6	1·21	—	93·2	—
		27·5	—	15·3*	1·08	—	96·0	—
A. israelii, 295‡	Anaerobic: Na_2CO_3+ pyrogallol seal	26·6	—	—	—	—	98·7	1·06
Propionibacterium arabinosum (34 W_1)§	Anaerobic: under N_2-atmosphere	145·3	2·8	—	3·59	7·82	97·6	1·01
P. pentosaceum (49 W_3)§	Anaerobic: under N_2-atmosphere	155·9	2·8	—	3·15	7·70	95·0	0·98
P. shermanii (52 W_8)§	Anaerobic: under N_2-atmosphere	163·9	4·1	—	1·02	2·33	91·2	1·02

* Assumed values from preceding radioactive glucose fermentation. † Co_2 not considered in fermentation balance. ‡ Data of Pine & Howell (1956). § Data of Wood & Werkman (1936). n.d. = quantity not determined.

Optical rotation of lactic acid

Although the amount of lactic acid produced by *Actinomyces propionicus* varied, it was generally quite small in large fermentations. From 6 l. of fermentation liquor about 6 g. zinc lactate were obtained, of specific rotation +0·10. Because of relations to be discussed subsequently, the lactic acid formed by *Bacterionema*

Table 6. *Cell-wall composition of* Actinomyces propionicus *and related organisms*

	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Fucose	Ribose	Glucosamine	Galactosamine	Muramic acid	Aspartic acid	Alanine	Glutamic acid	Glycine	Lysine	Diaminopimelic
Actinomyces propionicus	–	–	+++	+++	–	–	tr.	+	+	?tr.	++	+++	+++	–	tr.	++
*A. israelii**	–	–	++	–	–	–	NR	+	–	+	.	+++	+++	.	+++	.
*A. bovis**																
NCTC 9430	–	++	tr.	–	tr.	++	NR	+	–	+	+++	+++	+++	.	+++	.
NCTC 9431	–	++	+	–	–	–	NR	+	–	+	+++	+++	+++	.	+++	.
*Propionibacterium pentosaceum** NCIB 8070	–	–	++	++	tr.	–	NR	+	+	+	.	+++	+++	+++	.	+++
*P. jensenii** NCIB 8069	–	+	++	+++	tr.	–	NR	+	tr.	+	.	+++	+++	+++	.	+++
Bacterionema matruchotii†‡ (*Leptotrichia dentium*)	+	–	–	+	–	–	NR	tr.	–	NR	–	+	+	–	–	+
Nocardia salivae§	–	–	+	–	–	NR	NR	.	tr.	.	tr.	++	++	tr.	++	–

tr. = Trace; NR = not reported; . = amino acids not considered as major constituents.

* Data of Cummins & Harris (1956, 1958) for representative strains. † Data of Davis & Baird-Parker (1959). ‡ Synonymy of names given by Gilmour, Howell & Bibby (1961). § Data of Davis & Freer (1960).

matruchotii (*Leptotrichia dentium*); Baird-Parker & Davis, 1958 (Gilmour, Howell & Bibby, 1961) was converted to the zinc salt and optically assayed as described for *A. propionicus*. A specific rotation of −0·63 was found. Since the zinc salt of the pure optically active isomer has a specific rotation of + or −8·25 (Pederson, Peterson & Fred, 1926) and because of the low solubility of the zinc lactates of the two organisms tested, it was concluded that both *B. matruchotii* and *A. propionicus* formed DL-lactic acid.

Cell-wall analysis

Cummins & Harris (1958, 1959), Davis & Baird-Parker (1959) and Davis & Freer (1960) have shown cell-wall composition to be a general taxonomical character relevant to the classification of Actinomycetales. Consequently, cell walls of *Actinomyces propionicus* were isolated and analysed. The results of this analysis and those of several related organisms as reported by other workers are given in Table 6. Alanine, glutamic, aspartic, and diaminopimelic acids were the major amino acid components of the *A. propionicus* cell-wall hydrolysate, while there was a small but definite amount of lysine. There appeared to be a trace of muramic acid as suggested by the colour formed with ninhydrin and its position on the paper, but this was not further identified. Galactose and glucose and glucosamine and galactosamine represented the major sugar and amino sugar constituents, respectively. No attempt was made to determine the stereoisomeric form of the diaminopimelic acid.

DISCUSSION

From the results of experiments comparing pathogenicity of *Propionibacterium* spp. and *Actinomyces* spp., it is obvious that *Actinomyces propionicus* is readily classified in the genus *Actinomyces*, although pathogenicity is not usually a taxonomic characteristic used to separate genera. Stanier & Van Niel (1941) and Howell *et al.* (1959) emphasized morphology as the primary diagnostic character pertaining to the classification of members of the Actinomycetales. Primarily on the basis of its colony morphology and its morphological appearance in animals, *A. propionicus* was initially classified as a strain of *A. israelii* (Pine & Hardin, 1959). In the original spontaneous infection and in inoculated animals the long branching mycelial threads were typical of strains of *A. bovis*, *A. israelii* and *A. naeslundii*.

On the basis of cellular morphology *in vitro*, there is little to distinguish *Actinomyces propionicus* from *Propionibacterium arabinosum* or *P. pentosaceum*, although during the logarithmic growth phase there is a much greater tendency for *A. propionicus* to form true branching rods and hyphal elements. Subsequent to the stationary phase of growth or dependent upon cultural conditions and media, the propionibacteria form spherical cells which may be normal or greatly enlarged in diameter. In this respect the cells of *A. propionicus* may be identical with those of *P. arabinosum* (Skerman, 1959), although the cells of *A. propionicus* continue to enlarge under certain conditions of substrate and age to form huge spheres not unlike the cystites of *Arthrobacter* species (Chaplin, 1957; Skerman, 1959). Although there have been no direct experiments to determine the nature of these morphological changes, the impression gained from general observation strongly suggests that these coccoidal or swollen forms are a result of abnormal cell-wall formation and do not

constitute a normal phase of the organism's growth. Similar types of cells have been observed in cultures of *A. bovis* (Pine, Howell & Watson, 1960) although these lacked the rigidity of structure observed in cells of *A. propionicus*.

Perhaps one of the strongest diagnostic characteristics identifying the anaerobic actinomycetes infecting man is the formation within a few days of small delicate mycelial colonies on the agar surface. In this regard, *Actinomyces propionicus* formed a colony typical of that formed by strains of *A. israelii*, whereas such a colonial structure was completely absent in the colonies of *Propionibacterium arabinosum* and *P. pentosaceum*. However, similar to the colonies of *P. arabinosum* (*Bergey's Manual*, 1957), the colonies of *A. propionicus* grown aerobically or anaerobically on the surface of agar media gradually assumed a dull orange colour within several weeks while all other strains of *Actinomyces* spp. remained white or cream.

In its physiological and fermentative aspects *Actinomyces propionicus* does not show distinctive characters of its own but combines several of the identifying characteristics of the genera *Actinomyces* and *Propionibacterium*. Like all members of the genus *Actinomyces* (Pine & Howell, 1956; Howell *et al.* 1959; Pine, Howell & Watson, 1960) *A. propionicus* does not ferment lactic acid or glycerol, whereas these substrates are fermented by all *Propionibacterium* spp. (*Bergey's Manual*, 1957). Like most strains of *A. naeslundii* (Howell *et al.* 1959), *A. propionicus* ferments raffinose but does not ferment xylose or mannitol, although growth of *A. propionicus* on mannitol is invariably good and substantial acid formation is occasionally observed. Like all strains of *A. naeslundii* tested, *A. propionicus* reduces nitrates to nitrites.

Species of *Actinomyces* are generally recognized as being catalase negative (Suter, 1956; Hazen & Little, 1958; Pine & Howell, 1956; King & Meyer, 1957; Howell *et al.* 1959; Pine, Howell & Watson, 1960). Similarly *Actinomyces propionicus* was found to be catalase negative. Since catalase may be an inducible enzyme (Clayton, 1960) or be dependent upon the presence of essential factors (Biberstein & Gills, 1961) many attempts were made to demonstrate the formation of catalase by *A. propionicus* grown anaerobically or aerobically in the presence and absence of added haemin; these attempts failed and catalase formation was not demonstrated. On the other hand, catalase activity of *Propionibacterium arabinosum* and *P. pentosaceum* has been described as being extremely limited (*Bergey's manual*, 1957). Although this was true of *P. arabinosum* when it was received, the initial catalase activity of *P. pentosaceum* was high. However, with further culturing on the Casitone medium, the catalase activity of *P. arabinosum* also became strongly positive.

All strains of *Actinomyces* studied previously required substrate amounts of CO_2 to ferment glucose, forming formic, acetic, lactic and succinic acids (Pine & Howell, 1956; Pine, Howell & Watson, 1960). Unlike the *Actinomyces*, but similar to the glucose fermentations of species of *Propionibacterium*, *Actinomyces propionicus* fermented glucose with the formation of CO_2, acetic and propionic acids, and small amounts of formic, lactic and succinic acids. Thus unlike the *Actinomyces*, CO_2 was not required for anaerobic growth but was a major product of fermentation. Like the fermentations of *Propionibactcrium* (Wood *et al.* 1937) significant amounts of lactic acid were not consistently formed, whereas this is a major product of all *Actinomyces* fermentations. Like *A. naeslundii* (Buchanan & Pine, unpublished

results) and *P. arabinosum* (Ichikawa, 1957), *A. propionicus* was facultative and fermented glucose in the presence of oxygen to form stoichiometric amounts of CO_2 and acetic acid. However, unlike *A. naeslundii*, *A. propionicus* did not require CO_2 to grow and ferment glucose in the presence of oxygen.

Table 7. *A suggested phylogenetic progression in the order Actinomycetales*

Organism	Characters
Lactobacillus bifidus	Branching cells, no true mycelium. Anaerobic, catalase negative. Ferments glucose with formation of large amounts of lactic (L+) and acetic acid, traces of formic and succinic also formed. Lysine in cell wall. Parasitic (Norris, Flanders, Tomarelli & György, 1950; Frank & Skinner, 1954; Pine & Howell, 1956; Cummins, Glendenning & Harris, 1957).
Actinomyces israelii	Branching cells, true mycelium formed. Anaerobic, catalase negative. Ferments glucose and CO_2 to form acetic, formic, lactic (L+), and succinic acids. Lysine in cell wall. Parasitic and pathogenic. (Pine & Howell, 1956; Cummins & Harris, 1958).
A. naeslundii	Branching cells, true mycelium formed. Facultative, catalase negative. Ferments glucose and CO_2 to form lactic (L+), formic, acetic and succinic acids. Aerobically, glucose is oxidized in the presence of CO_2 to form CO_2 and acetic acid. Lysine in cell wall. Parasitic and pathogenic (Pine & Howell, 1956; Cummins & Harris, 1958; Howell *et al.* 1959; Buchanan & Pine, unpublished results).
A. propionicus	Branching cells, true mycelium formed. Facultative, catalase negative. Ferments glucose to form CO_2, acetic and propionic acids with small amounts of lactic (DL) and succinic acids. Aerobically, glucose is oxidized to form CO_2 and acetic acid. Diaminopimelic acid in cell wall. Parasitic and pathogenic.
X (*Propionibacterium*) species	Branching cells, true mycelium not formed. Facultative, catalase positive. Ferments glucose to form CO_2, acetic, succinic and propionic acids. Lactic acid fermented. Aerobically, glucose is oxidized to form CO_2 and acetic acid. Diaminopimelic acid in cell wall. Enzymes oxidizing citric acid cycle intermediates present. Saprophytic (Hitchner, 1934; Wood & Werkman, 1936; Delwiche & Carson, 1953; Ichikawa, 1957; Cummins & Harris, 1958).
Y (*Corynebacterium*) species	Branching cells, true mycelium not formed. Facultative to aerobic, catalase positive, cytochrome transport system present. Ferments glucose in decreased oxygen supply to form CO_2, acetic, formic, lactic, succinic, and propionic acids. With adequate oxygen, these products oxidized to CO_2 and H_2O. Diaminopimelic* acid in cell wall. Parasitic, pathogenic, saprophytic (Tasman & Brandwijk, 1940; Pappenheimer & Hendee, 1947; Cummins & Harris, 1956).
Bacterionema matruchotii (*Leptotrichia dentium*)	Branching cells, true mycelium formed. Facultative to strict aerobes, catalase positive. Ferments glucose in decreased oxygen supply to form CO_2, formic, acetic, propionic, lactic (DL) and succinic acids. With adequate oxygen, glucose and lactic acid oxidized presumably to CO_2 and water. Diaminopimelic acid present in cell wall. Parasitic. (Davis & Baird-Parker, 1959; Gilmour, 1961; Pine & Howell, 1961).
Nocardia and *Streptomyces* species	Branching cells, true mycelium formed with production of chains of endospores. Strict aerobes, glucose and various carbohydrates oxidized to CO_2 and H_2O. Catalase positive. Diaminopimelic acid in cell wall. Saprophytic and pathogenic (Gordon & Mihm, 1958; Gilmour, Butterworth, Noble & Wang, 1955; Butterworth, Gilmour & Wang, 1955; Cummins & Harris, 1958; Moore & Chapman, 1959).

* Of ten species of *Corynebacterium* studied by Cummins & Harris (1956), two (*C. pyrogenes* and *C. hemolyticum*) had lysine instead of diaminopimelic acid.

Hungate (1946) described a propionic acid fermentation by the strict anaerobe *Micromonospora propionici* (*Actinobacterium propionici*, Prévot, 1957) isolated from the gut of a wood termite. Although its anaerobic fermentations were identical with those of *Actinomyces propionicus* and its morphology similar, it would appear that *M. propionici* is a different organism from *A. propionicus* on the basis of habitat, anaerobic requirements and use of cellulose as a substrate.

The form of lactic acid formed by a species has been used for taxonomic differentiation particularly among the members of the family Lactobacteriaceae. There has been no report on the kind of lactic acid formed by strains of *Propionibacterium*. However of the *Actinomyces* spp. studied, *A. naeslundii*, *A. israelii*, and *A. bovis* form L(+)-lactic acid. On the other hand, *A. propionicus* was found to produce DL-lactic acid, as did *Bacterionema matruchotii*, which is a genus considered phylogenetically intermediate between *Actinomyces* and *Nocardia* (Gilmour *et al.* 1961; Gilmour 1961; Gilmour & Beck, 1961; Howell & Pine, 1961). This observation serves to relate these two species (Table 7) as does their common ability to form propionic acid (Howell & Pine, 1961) and similar cell-wall constituents (Davis & Baird-Parker, 1959).

Cummins & Harris (1956, 1958) and Davis & Baird-Parker (1959) have emphasized the taxonomic value of cell-wall composition to determine species and generic relationships among members of the actinomycetes. In this regard, cell walls of *Actinomyces propionicus* were found to contain glucose, galactose, small amounts of glucosamine and galactosamine, and aspartic and diaminopimelic acids. This composition is similar to that of species of *Propionibacterium* except the latter had glycine and no aspartic acid (Cummins & Harris, 1956, 1958). The cell-wall composition of *A. propionicus* was essentially the same as that of *Bacterionema matruchotii* (*Leptotrichia dentium*) except the walls of the latter contained no galactose or aspartic acid. *A. propionicus* differed from all other members of the genus *Actinomyces* in the sugars found in the cell wall and in the fact that all other *Actinomyces* species had lysine instead of diaminopimelic acid (Cummins & Harris, 1958, 1959). Therefore, on the basis of cell-wall composition *A. propionicus* is distinct from all related organisms.

It is apparent that the interest in *Actinomyces propionicus* lies in its phylogenetic relationship to other actinomycetes and to propionibacteria. Starting with the simple branching anaerobic *Lactobacillus bifidus* var. *pennsylvanicus* (Sundman, Björksten & Gyllenberg, 1959), one may make a morphological and physiological progression to the more complex sporulating actinomycetes having an aerobic metabolism (Table 7). Table 7 shows the relationships of members of the genus *Actinomyces* to some other organisms which have been classified in the Actinomycetales, or which have been recognized by various investigators as being related to members of this order. So far there have been described no species forming true mycelium which might fill the gaps X and Y in Table 7, and which might have the progressively more complicated metabolic characteristics found in the genera *Propionibacterium* and *Corynebacterium*. Although the relationships of members of these genera to members in the genus *Actinomyces* have long been recognized on a morphological and physiological basis (*Bergey's Manual*, 1934, 1939; Stanier & Van Niel, 1941), these taxonomic schemes have been complicated by the assignment of a more complex cellular structure to *Actinomyces*, because of the formation

of a true mycelium. Consequently on a morphological basis, *Propionibacterium* and *Corynebacterium* have been considered to precede the more complex *Actinomyces* (Stanier & Van Niel, 1941; Prévot, 1957).

However, in the light of present knowledge about the physiological basis of morphology and its relationship to cell-wall composition (Duguid & Wilkinson, 1961; Glick, Sall, Zilliken & Mudd, 1960), the formation of branching cells with true hyphal elements may be considered the result of a decrease, rather than an increase, in enzymic complexity. Formation of buds or branches due to defective cell-wall synthesis and the formation of branched hyphae and even mycelium due to delayed cross-wall formation might represent an impaired operation of cell-wall synthesizing enzymes. This functional loss need not necessarily benefit the organism nor be conducive to its survival. On the other hand, the formation of chains of catenulae (oidia) observed in strains of *Nocardia* or *Streptomyces* (Gordon & Mihm, 1958; Moore & Chapman, 1959) most certainly indicates a more complex morphological development pertinent to the life cycle and increased survival of the organism. From these considerations it is not unreasonable to substitute *Propionibacterium* for X in Table 7 as a metabolically more complex genus following *Actinomyces* (*A. propionicus*), and *Corynebacterium* for Y in the postulated phylogenetic progression from *Lactobacillus* to *Streptomyces* shown in Table 7. Further clarification of the taxonomic relationships of these genera with more recently described genera and species such as *Bacterionema matruchotii* and *Nocardia salivae* will require additional information about the intermediary metabolism of these organisms and members of the genus *Actinomyces*.

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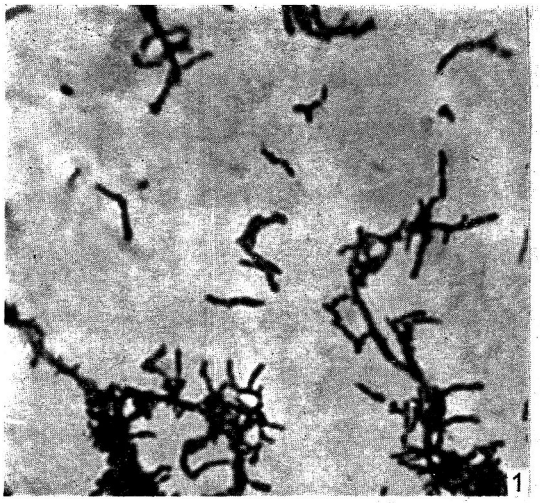
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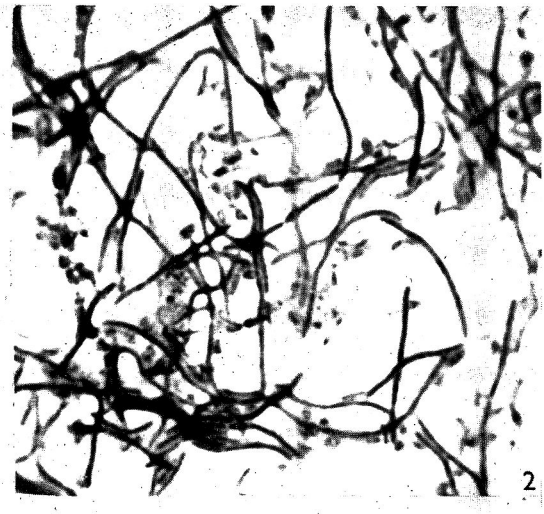
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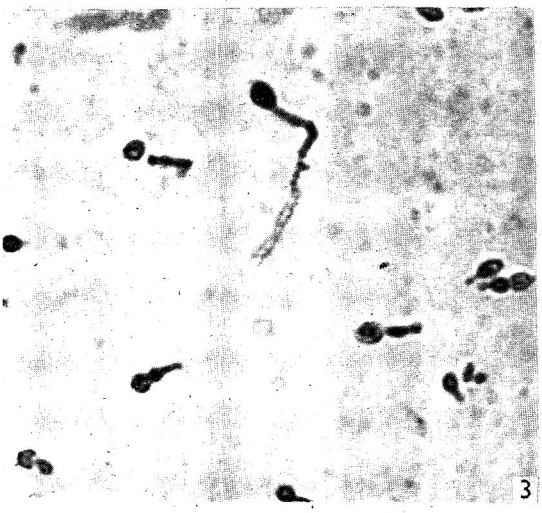
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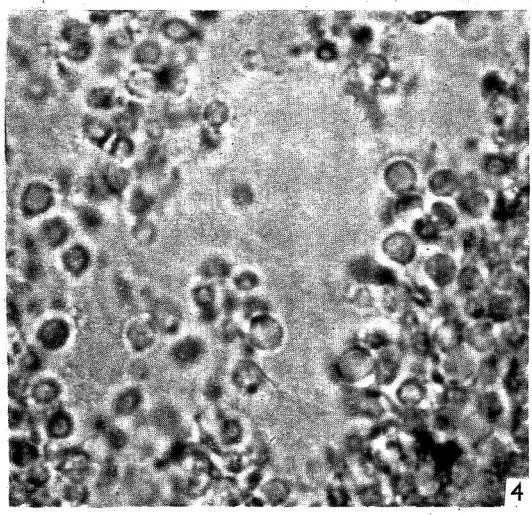
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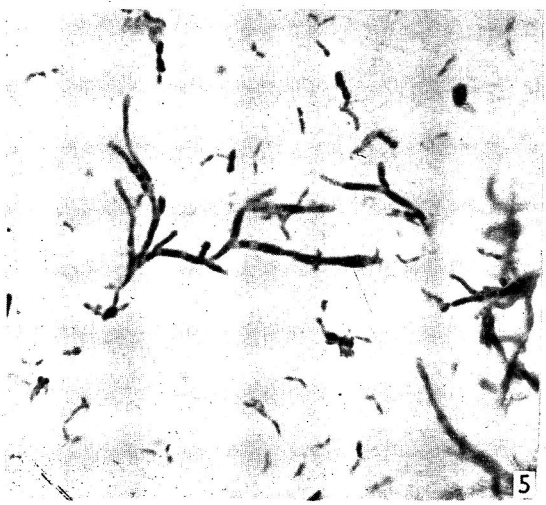
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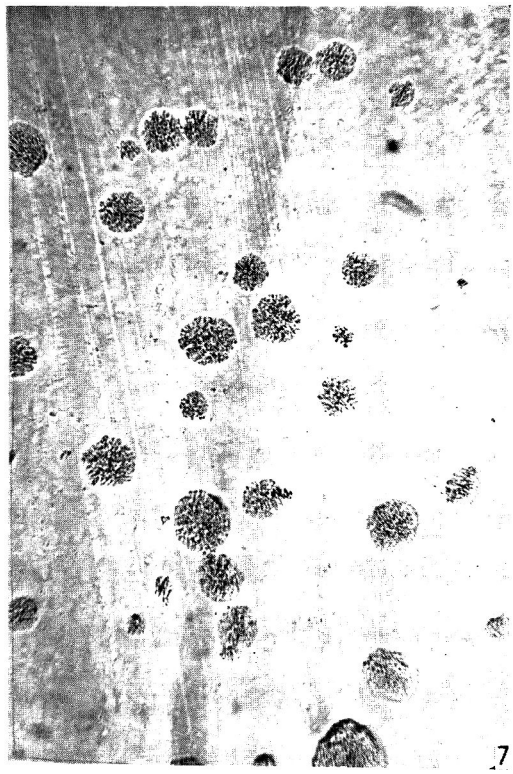
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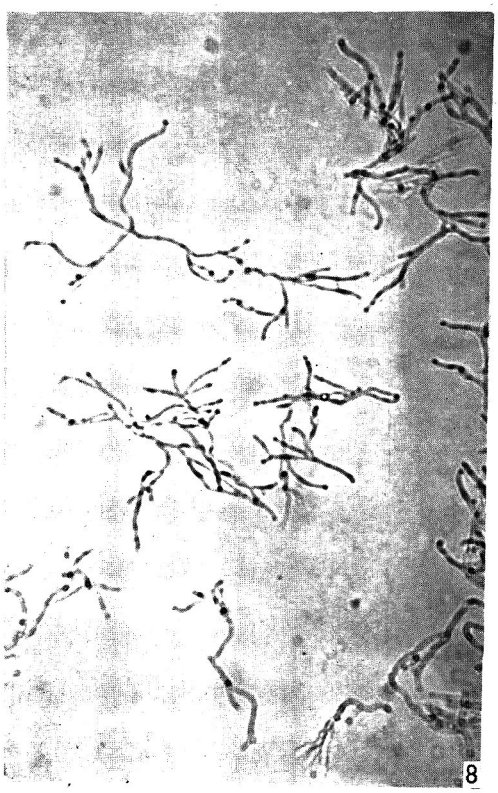
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EXPLANATION OF PLATES

Plate 1

Cellular morphology of *Actinomyces propionicus*

Fig. 1. Typical branching cells grown anaerobically for 72 hr. on 5 ml. of 1 % (w/v) glucose + Casitone medium. Gram stain, × 970.

Fig. 2. Long filamentous forms grown anaerobically for 72 hr. in 1 l. fermentation flasks of 0·5 % (w/v) glucose + Casitone medium. Gram stain, × 970.

Fig. 3. Typical organisms grown anaerobically for 10 days in 5 ml. of 1 % (w/v) glucose + Casitone medium showing terminal formation of spheroplasts. Methylene blue wet mount, × 970.

Fig. 4. Cells grown for 10 days on 5 ml. of 1 % (w/v) raffinose + Casitone medium showing formation of greatly enlarged spheroplasts. Methylene blue wet mount, × 970.

Fig. 5. Pus from lesion in mouse inoculated with *A. propionicus*. Mouse was killed after 33 days. Gram strain, × 970.

Plate 2

Comparison of colony morphology of *Actinomyces propionicus* and *Propionibacterium arabinosum*

Fig. 6. Colonies of *A. propionicus* grown 24 hr. on 1 % (w/v) glucose + Casitone agar. × 100.

Fig. 7. Colonies of *P. arabinosum* grown 24 hr. on 1 % (w/v) glucose + Casitone agar. × 100.

Fig. 8. Colonies of *A. propionicus* grown 24 hr. on 1 % (w/v) glucose + Casitone agar. × 430.

Fig. 9. Colonies of *P. arabinosum* grown 24 hr. on 1 % (w/v) glucose + Casitone agar. × 430.

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With 1 *plate*
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Morphological Changes and Resistance induced in *Saccharomyces pastorianus* by the antibiotic cycloheximide

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(*Received* 29 *August* 1961)

SUMMARY

The growth of *Saccharomyces pastorianus* was 50% inhibited by 0·013 p.p.m. cycloheximide, and completely inhibited by 0·100 p.p.m. in liquid medium after 24 hr. at 28°. At subinhibitory concentrations of cycloheximide abnormally large cells and cell aggregates were formed, probably as a result of interference by cycloheximide with the synthesis of structural cell-wall materials. When *S. pastorianus* was grown in media with successively higher concentrations of cycloheximide partially resistant organisms were developed. These did not form aggregates and giant cells in the presence of cycloheximide, but on back inoculation to cycloheximide-free media they were 'slow growers' and formed small colonies on nutrient agar. After a few transfers on cyclo heximide-free media the resistant organisms reverted to the normal 'fast growing' type. The resistance to cycloheximide was not the result of any acquired ability to decompose or inactivate the compound.

INTRODUCTION

The growth inhibitory effect and the action mechanism of cycloheximide (actidione) on *Saccharomyces pastorianus* was investigated by Whiffen (1948), Coursen & Sisler (1960) and others. However, no descriptions are given in these papers of the morphology of organisms grown at subinhibitory concentrations of the compound. Since it was found in this laboratory that the action of cycloheximide on the metabolism of *S. pastorianus* was reflected in anomalies in the growth and reproduction of sensitive organisms it was thought useful to give a description of this secondary effect of cycloheximide. Some observations on induced resistance of *S. pastorianus* towards cycloheximide will also be described.

METHODS

The organism used was *Saccharomyces pastorianus* Hansen, strain ATCC 2366. Stock cultures were maintained on a solidified yeast extract medium (pH 6·0): glucose, 10 g.; yeast extract (Difco), 2·5 g.; KH_2PO_4, 1 g.; Bacto-agar (Difco) 20 g.; distilled water to 1000 ml. This medium, and a liquid medium of the same pH value and composition, but without the agar, was used for all the experiments. The media were sterilized by autoclaving at 115° for 15 min. and the cultures incubated at 28°. Unless otherwise stated all cultures were static.

Stock solutions of cycloheximide (β-(2-(3,5-dimethyl-2-oxo-cyclohexyl)-2-hydroxyethyl)-glutarimide; synonym: actidione) were prepared by dissolving 0·25 g. pure cycloheximide (obtained from The Upjohn Company, Kalamazoo, U.S.A.) in 5 ml. ethanol and diluting this to 100 ml. with M/15 KH_2PO_4. The 2500 p.p.m. solution was sterilized by filtration and stored at 4°. Fresh solutions were made about every 4 weeks. Dilutions were always made in sterile M/15 KH_2PO_4 and not more than 0·1 ml. was added to 5 ml. liquid medium or to 25 ml. of solid medium to give the final concentration.

A bio-assay of cycloheximide was made by placing 13 mm. filter paper disks (pads) moistened with solutions of known cycloheximide concentration on the surface of 15 ml. agar medium containing about 10^4 *Saccharomyces pastorianus* organisms. These organisms (from a 24 hr. culture) had been suspended in a larger batch of melted agar cooled to 45–50° before they were pipetted out into sterile Petri dishes. After 24 hr. of incubation the inhibition zones were measured. Within the concentration range of 0·5–10 p.p.m. cycloheximide the diameters of the inhibition zones were proportional to log concentration. The cycloheximide contents of unknown samples were determined by directly reading their inhibition zones after proper incubation.

Turbidimetric readings were made at wavelength 550 mμ in a Beckman model B spectrophotometer. The numbers of organisms/ml. corresponding to optical densities between 0·075 and 0·500 were checked by counting in a Buerker blood-cell counting chamber.

RESULTS

Growth inhibition in liquid media

With an inoculum giving 10^3 viable organisms/ml. medium at zero time the growth of *Saccharomyces pastorianus* in presence of increasing concentrations of cycloheximide was determined turbidimetrically after incubation for 24 hr. (Fig. 1). The effective dose for 50 % inhibition (ED 50) was 0·013 p.p.m. No growth occurred at 0·1 p.p.m. cycloheximide.

The sediment of organisms, formed in cultures containing 0·025 p.p.m. and higher concentrations of cycloheximide differed from normal sediments in being flocculent rather than compact and without an entire edge. This different appearance of the sediment pointed towards some anomaly in the growth of the organism in media containing cycloheximide. Microscopical examination of the flocculent sediment revealed that most of the organisms occurred in aggregates containing from 5 to 30 organisms sticking together. Although difficult to make out with certainty it seemed that the aggregates had formed by repeated budding without complete separation of the daughter cells in the same way as in the formation of a pseudomycelium. The aggregated cells showed considerable differences in size, some (the oldest?) having a diameter three or more times the diameter of normal cells (Pl. 1, fig. 2–6).

The internal structure of these cells sometimes appeared more granulated than normal, grains apparently being present both in vacuoles and in the cytoplasm. In several of the giant cells the vacuole made up nearly the whole cell volume, the protoplasm being restricted to a fairly thin layer between the vacuole and the cell wall. In some cells the cell wall seemed to have been damaged in some way, giving

the cell a shrivelled surface. Sometimes no cell wall could be seen at all, the cell appearing naked (Pl. 1, fig. 3). In media containing cycloheximide cells of normal size and appearance were also seen mixed up with the cell aggregates and giant cells, especially on prolonged incubation (2–3 days) These 'normal' cells probably were cells resistant to cycloheximide as evidenced in experiments described later.

Aggregation and cell morphology at subinhibitory and inhibitory concentrations of cycloheximide

A sample (0·05 ml.) of a 24 hr. old culture of *Saccharomyces pastorianus* was used as inoculum for liquid media containing no cycloheximide, or 0·06 p.p.m. or 1 p.p.m. cycloheximide. These media were incubated for 24 hr. and examined by the microscope. As expected, cells grown in the presence of 0·06 p.p.m. cycloheximide showed the usual aggregation and swellings previously described. No growth occurred in the 1 p.p.m. media but it was observed that the inoculum cells were affected. As compared with the number of aggregates found with subinhibitory concentrations of cycloheximide few and smaller aggregates had formed, but most of the intact cells were considerably enlarged. In many cells the cell wall was ruptured, usually at one point only (the budding scar?) and more or less of the cell contents had been emptied into the surroundings (Pl. 1, figs. 5, 6). Several shrivelled cell envelopes were seen and groups of grains and diffuse material showed that some of the cells had been more or less emptied.

Although 1 p.p.m. cycloheximide inhibits the growth of *Saccharomyces pastorianus* metabolic processes still proceed at a high rate when nutrients are available (Coursen & Sisler, 1960). As it was of interest to know whether cycloheximide affected the cell size and morphology, irrespective of metabolic activity, the above experiment was repeated with starved cells suspended in a nutrient-free medium. Portions of 24 hr. cultures were centrifuged and the sediment washed several times with M/15 phosphate (pH 4·5). After resuspension the organisms were starved at 28° for 24 hr. before cycloheximide, 1 and 10 p.p.m., was added. At intervals the sediments of organisms were examined under the microscope. During a 30 hr. period no swellings or other abnormal changes were seen. It was therefore concluded that cycloheximide had no destructive effect on preformed cell walls.

Strength of the cell wall of cycloheximide-grown cells

The purpose of the following experiment was to find out whether the cell wall of cycloheximide-grown organisms had been weakened during contact with the compound. If this were true it might be expected that the cells (or at least some cells) would burst because of increased turgor when washed and transferred to distilled water. Samples (15 ml.) of liquid medium without and with addition of 0·05 p.p.m. cycloheximide were inoculated with a fairly heavy suspension (5×10^7 organisms) prepared from a 24 hr. culture of *Saccharomyces pastorianus* on agar medium. After 24 hr. the actual cell numbers were determined in a blood-cell counting chamber. Samples (10 ml.) of the cultures were simultaneously centrifuged at 3000 rev./min. for 15 min., the supernatant fluid carefully removed and the sediment washed and centrifuged twice with 10 ml. distilled water. After final resuspension in 10 ml. distilled water the cell numbers were again counted in the blood-cell counting chamber. In several experiments of this type no significant

decrease in cell number was ever found. Negative results were also obtained with 1 p.p.m. cycloheximide. It was also seen that the volume of cycloheximide-grown cells did not increase further after replacement of the nutrient medium by water, as might have been the case if cycloheximide caused a softening of the otherwise rigid cell wall.

Effect of cycloheximide on growth on solid medium

A sample (0·05 ml.) of a suspension of *Saccharomyces pastorianus* containing about 150 organisms was spread on the surface of 25 ml. agar plates containing different amounts of cycloheximide. The plates were incubated at 28° and the colonies counted and measured daily during 14 days. A lag in the onset of growth resulted

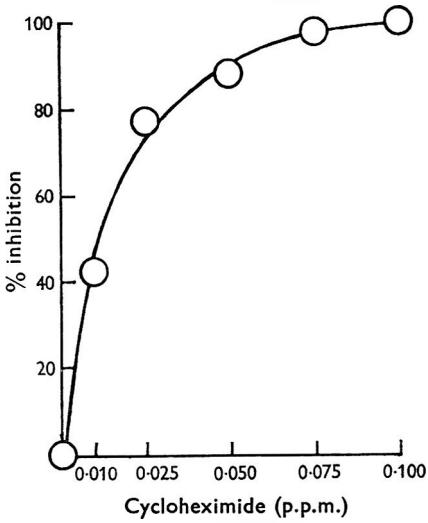

Fig. 1

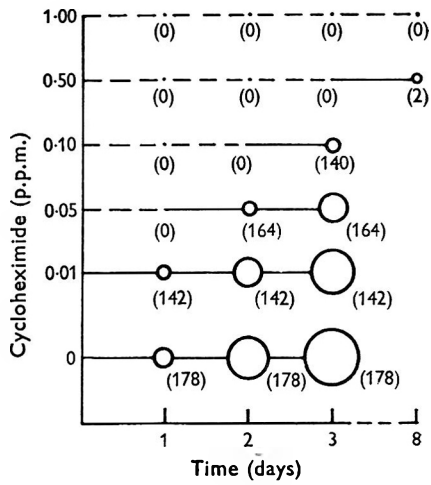

Fig. 2

Fig. 1. Growth of *Saccharomyces pastorianus* in liquid yeast extract medium with varying amounts of cycloheximide. 24 hr., 28°.

Fig. 2. Growth of *Saccharomyces pastorianus* on yeast extract agar with varying amounts of cycloheximide. The circles show the sizes of the colonies at different times and different concentrations of cycloheximide. Figures in brackets are the total number of colonies on the agar plates.

when cycloheximide was incorporated in the media, but the final colony numbers were not decreased within the concentration range 0·01–0·1 p.p.m. With 0·5 p.p.m. cycloheximide not more than 2 % of the inoculum organisms gave rise to colonies which were visible only after an appreciable time (8–10 days). When examined microscopically, cells from these colonies were seen to be considerably enlarged, but aggregates were not formed. With 1 p.p.m. cycloheximide in the media no colonies were formed within 4 weeks. The relative growth of *S. pastorianus* on solid media is illustrated diagrammatically in Fig. 2.

Isolation and growth of cycloheximide-resistant organisms

Saccharomyces pastorianus was allowed to grow for 72 hr. in liquid medium without and with addition of 0·05 p.p.m. cycloheximide. At the end of the incubation period the cultures were diluted in sterile saline to about 10^3 organisms/ml. and

0·05 ml. spread on agar medium without any cycloheximide. After 48 hr. of incubation the plates were examined. It was seen that colonies of two different sizes had formed on the plates which had been inoculated with material from the medium containing cycloheximide. The larger colonies corresponded in size and appearance to those formed on plates inoculated with organisms from cycloheximide-free media. The colonies of the second type had the same white colour and regular smooth appearance as the larger colonies, but were definitely smaller in size (Pl. 1, fig. 7).

The three kinds of colonies were picked and streaked on agar medium. After incubation for 24 hr. cell suspensions were prepared from the different growths and 0·05 ml. samples pipetted into 5 ml. lots of liquid medium containing different amounts of cycloheximide. Growth in these media was determined turbidimetrically after 24 and 48 hr. of incubation at 28° (Table 1). The cells of the smaller colonies showed slower growth also in the liquid cycloheximide-free medium. In none of the media was there any difference in growth between normal organisms and the organisms of the larger colonies. But in the media containing 0·05 p.p.m. and higher concentrations of cycloheximide the growth of the small-colony type organisms was less inhibited than that of the other two.

Table 1. Saccharomyces pastorianus: *growth in liquid yeast extract medium with different amounts of cycloheximide*

		Cycloheximide (p.p.m.)							
	Initial no. organisms/ml. ($\times 10^4$)	0	0·05	0·10	0·50	0	0·05	0·10	0·50
		Organisms $\times 10^6$/ml.							
Inoculum		24 hr.				48 hr.			
Not cycloheximide-grown organisms	3·5	80	10	0	0	70	58	1·6	0
Cycloheximide-grown organisms									
Small colony	3·3	52	25	3	0	63	59	61	0·7
Large colony	3·0	76	7	0	0	63	57	1·1	0

This experiment showed that in liquid medium containing 0·05 p.p.m. cycloheximide organisms are developed which readily became resistant to cycloheximide-concentrations 2–10 times higher than the original concentration. These resistant organisms gave rise to small colonies on cycloheximide-free media. In the same cultures viable non-resistant organisms, resembling normal ones in growth rate and response to cycloheximide, were also present. Whereas normal organisms and non-resistant cycloheximide-grown organisms when exposed to 0·05 or 0·10 p.p.m. cycloheximide displayed the usual picture of swollen cells and aggregates in abundance, the resistant organisms were of normal size and appearance and did not form aggregates in the presence of 0·10 p.p.m. cycloheximide.

The persistence of cycloheximide in cultures of resistant organisms

Although it seemed not very probable that cycloheximide-resistant organisms had been enzymatically adapted to decompose or inactivate cycloheximide this possibility could not be entirely ruled out. Therefore, an experiment was carried out in which the cycloheximide concentration was determined before and after growth of resistant organisms. The quantitative determination of cycloheximide was made by the bio-assay described in Methods. However, as the range most reliable for assay is between 2 and 5 p.p.m. cycloheximide it was necessary to increase further the resistance of the cycloheximide-resistant organisms obtained in earlier experiments. This was done by inoculation into liquid medium containing 1 p.p.m. cycloheximide from the 0·5 p.p.m. culture. When good growth (about 10^6 organisms/ml.) had occurred in the higher concentration, inoculation was made into 2 p.p.m. cycloheximide and so on, until the organism would grow in 5 p.p.m. cycloheximide. The time needed to obtain good growth was longer the higher the concentration. Thus, to go from 4 to 5 p.p.m. 6 days were required, although the time needed to obtain good growth in subcultures of the same concentrations of cycloheximide decreases with the number of subcultures.

Saccharomyces pastorianus which had in this way been trained to grow in the presence of 5 p.p.m. cycloheximide was now inoculated into liquid medium (25 ml. in conical flasks) containing 3 p.p.m. cycloheximide. Experiments were made with static cultures incubated at 28° and with cultures at 25° on a reciprocal shaker. After growth for 7 days in 3 p.p.m. cycloheximide the cultures were centrifuged and the cycloheximide content of the supernatant culture fluid tested against uninoculated medium initially containing the same amount of cycloheximide. Controls were taken from cycloheximide-free media, some uninoculated, and some inoculated with cycloheximide-resistant and non-resistant organisms. No removal of cycloheximide from the cultures of cycloheximide-resistant organisms was found in any of these experiments. This negative result is evidence that acquired resistance to cycloheximide does not imply enzymic adaptation or any other kind of inactivation of cycloheximide by *S. pastorianus*.

DISCUSSION

Reasons for the formation of giant cells in *Saccharomyces pastorianus* in the presence of cycloheximide might include: (1) cycloheximide has dissolved or softened the preformed rigid cell walls so that these will no longer resist the turgor pressure of the protoplast; (2) by altering the cellular metabolism or the permeability of the cell membranes cycloheximide has caused an elevation of the osmotic value of the cells, which again has resulted in increased turgor pressure followed by a reversible swelling; (3) cycloheximide has affected the synthesis of structural cell wall materials, resulting in irreversible enlargement of the 'cell wall'.

The results of the present work give little support to the first two of these hypotheses. Cycloheximide caused morphological changes only in growing organisms and in organisms which had not been deprived of reserve nutrients, but not in starved organisms suspended in a nutrient-free medium. The same results were obtained with the filamentous fungus *Fomes annosus* in which cycloheximide has

a similar effect on the cell wall (Gundersen, 1961). If cycloheximide had a dissolving effect on preformed cell walls it might be expected that this effect would also be obtained with resting starved cells.

If the second explanation of the giant cell formation were correct it might be expected that these cells would split or expand further on being transferred from a medium of relatively high osmotic value (the culture fluid) to a medium of zero osmotic value (distilled water); this was not the case. The second hypothesis also presupposes a considerable resilience of the cell wall. It may be calculated that the surface of the largest cells increased 10-fold as a result of the cycloheximide treatment. Such a degree of resilience could hardly be expected in the cell walls of a yeast.

The last hypothesis, an effect of cycloheximide on the synthesis of cell wall materials, has not been proved nor disproved in this work. However, it is supported by the observation that when cycloheximide-grown organisms are plasmolysed in 0·5 M sucrose only the protoplast contracts, whereas the cell wall preserves its original size.

The formation of cell aggregates in cycloheximide-grown *Saccharomyces pastorianus* appears to be a result of the cycloheximide effect on the cell wall. In the phase-contrast microscope it could be seen that the cytoplasmic strands connecting neighbouring cells in these aggregates usually were much thicker than normal. Also the budding scars seemed to be enlarged. These changes suggest that the severance of cells was rendered difficult. However, the circumstances relating to cell division in cycloheximide-grown *S. pastorianus* probably are more complicated than this. Kerridge (1957, 1958) showed that in *S. mandshuricus* and in *S. carlsbergensis* cycloheximide inhibited the synthesis of protein and deoxyribonucleic acid at minimum growth-inhibitory concentrations. If this effect is also valid for *S. pastorianus* cycloheximide must have a profound influence on the whole process of cell division, including the severance of the cells.

Organisms resistant to cycloheximide always develop in media containing cycloheximide. This has been observed by Whiffen (1948) in *Saccharomyces pastorianus* and by Monreal (1961) in *S. cerevisiae* var. *ellipsoideus*. Resistance to cycloheximide was also obtained in *Fomes annosus* (Gundersen, 1962) and in *Sclerotinia fructicola* and *S. laxa* (Grover & Moore, 1961). In these organisms the resistance was obtained slowly by transfer of cell material (or mycelium) from medium of lower cycloheximide concentration to medium of slightly higher concentration. No absolute tolerance towards cycloheximide was obtained in any of these cases. In *S. pastorianus* even prolonged growth and serial subcultures in 5 p.p.m. cycloheximide did not produce organisms with a growth rate as high as that of non-resistant organisms. With *Fomes annosus* the upper limit of tolerance was found at about 10 p.p.m. cycloheximide. This sluggishness in the formation of resistant organisms, and the absence of formation of a cycloheximide-splitting enzyme, makes it seem probable that many factors are involved. Monreal (1961) used the term 'modification' for resistant organisms formation in *S. cerevisiae*. But it remains to be explained what is implied in this term.

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EXPLANATION OF PLATE 1

Effect of cycloheximide on *Saccharomyces pastorianus*. Figs. 1–6 are phase contrast micrographs.

Fig. 1. Normal organisms, 72 hr. 28°.

Fig. 2. Cell aggregate with enlarged cells formed during growth in the presence of 0·05 p.p.m. cycloheximide, 72 hr. 28°.

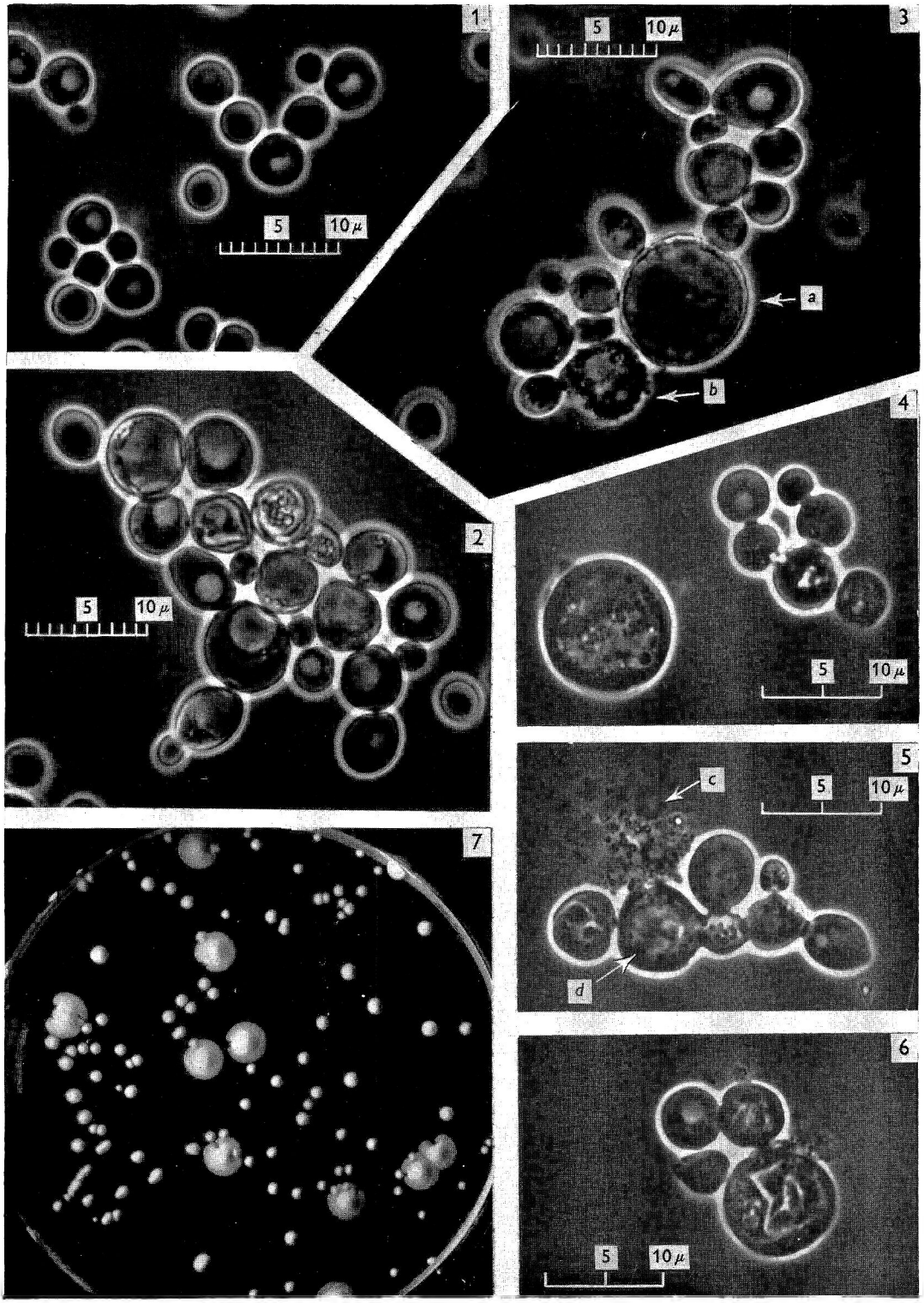
Fig. 3. Cell aggregate formed in 0·05 p.p.m. cycloheximide with giant cell containing one large vacuole (*a*), and lysed cell (*b*), 72 hr. 28°.

Fig. 4. Cell aggregate and single giant cell formed in 1 p.p.m. cycloheximide, 48 hr. 28°.

Fig. 5. Cell aggregate formed in 1 p.p.m. cycloheximide. One cell has lysed (*c*), or the mass of cytoplasm comes from neighbouring cell (*d*), 48 hr. 28°.

Fig. 6. Shrivelled and punctured cells with extruding cytoplasm from 1 p.p.m. cycloheximide, 48 hr. 28°.

Fig. 7. Colonies on yeast extract agar developed from cells previously grown in liquid medium containing 0·05 p.p.m. cycloheximide. Small colonies: cells resistant to cycloheximide; large colonies: cells not resistant to cycloheximide. 7 days 28°.



K. GUNDERSEN AND T. WADSTEIN

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Adansonian classification of mycobacteria

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SUMMARY

The physiological properties of 229 strains of mycobacteria (photochromogens, scotochromogens, non-photochromogens and rapid growers) and others already classified were analysed. According to their metabolic capacities and using the method proposed by Sneath, three branches were established.

Branch I is formed by micro-organisms with a high metabolic capacity and rapid rate of growth. It includes *Mycobacterium smegmatis*, *M. phlei*, *M. peregrinum* sp.nov. and many unnamed strains with great disparity in their characteristics, that were placed in a separate branch labelled 'irregular'.

Branch II includes micro-organisms that utilize only a limited number of carbohydrates. Belonging to this branch are *Mycobacterium fortuitum*, *M. marinum*, *M. piscium* and *M. thamnopheos*. Three new species are described, two of them rapid growers, *M. acapulcensis* sp.nov. and *M. runyonii* sp.nov. and a scotochromogen, *M. flavescens* sp.nov.

Branch III, formed by *Mycobacterium kansasii* (photochromogen), *M. avium* (non-photochromogen), *M. marianum* (scotochromogen) and *M. gordonae* sp.nov. (scotochromogen). All are slow growing micro-organisms.

Neotype strains of old species are proposed and holotypes of the new species are designated. A key for the identification of these species and a taxonomic tree of mycobacteria are described.

INTRODUCTION

The relationships existing between the different species of mycobacteria have not been adequately established. It is difficult to decide how to distinguish between two related species. This problem arises from the deficiency of many of the earlier descriptions and in other instances, from the difficulty in interpreting the data. Utilizing the Adansonian method, it has been possible to classify in a logical manner the groups belonging to the rapidly growing mycobacteria (Bojalil & Cerbón, 1961; Cerbón & Bojalil, 1961). The advantages of using the method proposed by Sneath (1957) for the analysis of the different characters of bacteria have been shown in several previous reports (Sneath & Cowan, 1958; Hill, 1959).

The present study aims to analyse the physiological properties of the mycobacteria in order to define the existence and value of previously described species, to determine if the preliminary groups described by Runyon (1955) are homogeneously formed and to establish their place among the Mycobacteriaceae.

METHODS

In all, 229 strains regarded as Mycobacterium (acid-fast bacilli) were studied. Some of these strains were received with a species name, but they were considered as unclassified to avoid any bias in the interpretation of the results.

They were first tentatively grouped according to their rate of growth and pigment production in the presence and absence of light.

The following divisions were made: 115 strains were rapid growers (group IV of Runyon). Of these 69 were non-pigmented and 46 were pigmented. 114 strains showed a slow rate of growth: 44 were scotochromogens (group II of Runyon), 45 were non-photochromogens (group III of Runyon) and 25 were photochromogens (group I of Runyon).

They were maintained in Löwenstein–Jensen medium (Wheeler, 1951), checked for viability and purity by microscopic examination and subculture in different media. Single cell cultures of the strains were used to test their physiological properties in triplicate series.

The biochemical methods used were, in general, those described in a previous report (Bojalil & Cerbón, 1961). The exceptions were as follows: strains that developed better at temperatures of 28° were tested at this temperature; the urea solution described by Singer & Cysner (1952) was used in the differentiation of photochromogens and non-photochromogens. The characters used were as follows.

Acid production from carbohydrates: glucose, mannose, fructose, galactose, L-arabinose, xylose, rhamnose, lactose, sucrose, trehalose, melibiose, raffinose, mannitol, sorbitol, *m*-inositol, erythritol and dulcitol.

Utilization of organic acids as carbon sources: benzoate, citrate, tartrate, succinate, pyruvate and propionate.

Temperatures of growth: 28°, 37°, 45° and 52°.

Survival to 60° for 4 hr.

Rate of growth: all the strains showing visible growth in 48–72 hr. in Löwenstein–Jensen medium were considered as rapid growers; the presence of visible growth after one or more weeks was considered slow growth.

Pigment production: those strains producing definitely more pigment when grown in the light were considered photochromogens; those uninfluenced by light as scotochromogens and those without pigment as non-photochromogens.

The methods used for the Adansonian classification and the system for quantitative notation were those described by Sneath (1957).

RESULTS

The results obtained from the different tests were tabulated: a strain × strain ($i \times i$) table, was prepared from the similarity indexes obtained, which were expressed in percentages. In sorting the characters of the 229 strains, various distinct patterns were at once discernible. These are shown diagrammatically in Fig. 1, in which each group is represented by only five entities and the intermediate strains are eliminated in order to simplify the interpretation. Subsequently, a taxonomic tree was constructed (Fig. 2), the branches of which may be considered as species. Several species previously characterized remained as groups, or were placed in one of the groups, when subjected to Adansonian analysis.

The Figs. 1 and 2 show the existence of three major branches of strains which are related at S levels of *c.* 60 %. One, formed by micro-organisms of high metabolic capacity (branch I), includes two well-defined species, *Mycobacterium smegmatis* and *M. phlei*; it also includes a large group labelled 'irregular branch' in which each strain could represent a species. The strains numbered 6020 and 128–1 are of particular interest because they are non-pigmented, vigorously fermentative mycobacteria; they have been named *M. peregrinum* sp.nov. All the strains belonging to this branch are rapid growers.

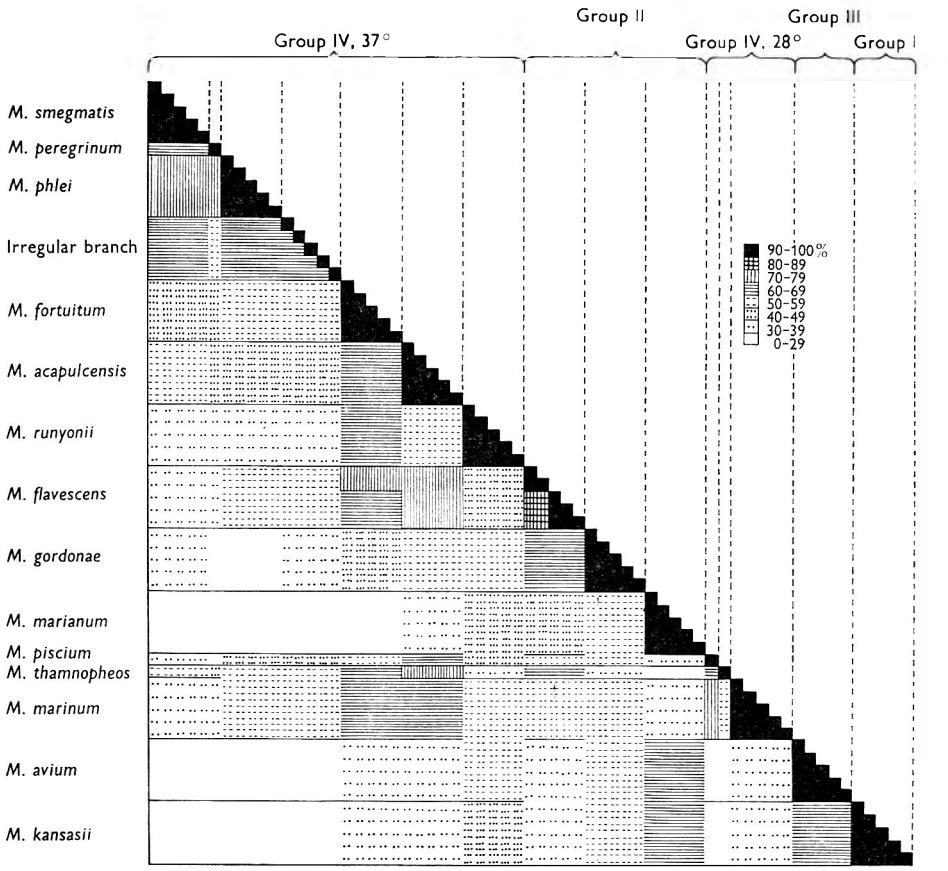

Fig. 1. Diagrammatic representation of the full % S value table.

A second and extensive branch II is constituted principally of rapid growers. Belonging to this branch are *Mycobacterium fortuitum*, *M. piscium*, *M. marinum* and *M. thamnopheos* among the species previously described.

The strains received as *Mycobacterium balnei* are grouped together with *M. marinum* because they show a high index of similarity. Other groups included in branch II are the following: under the name of *M. acapulcensis* sp.nov. and *M. runyonii* sp.nov. the previously described groups 2 and 3 (Cerbón & Bojalil, 1961) of the rapidly growing mycobacteria, and a small group of scotochromogens named *M. flavescens* sp.nov. which has a metabolism similar to *M. fortuitum*.

Table 1

Laboratory number, name and source.

Strains identified as *Mycobacterium smegmatis* (Trevisan) Lehmann and Neumann

W-113. *M. smegmatis*, ATCC 101 Rutgers University, S. A. Waksman
439, 11, 912, 153, 429. *Mycobacterium* sp., from different pathological products, Unidad de Patología Hosp. Gral. (UPHG), México
St-169. *M. phlei*, Tuberkulose Forschungsinstitut Börstel, R. Bönicke
W-106. *M. ranae*, National Trudeau Bank, W. Steenken jr.
70. *M. lacticola*, Escuela Nacional de Agricultura, Chapingo, México
110. *M. ranae*, ATCC 110, National Trudeau Bank, W. Steenken jr.
607, 599. *Mycobacterium* sp., Rutgers University, S. A. Waksman
114. *M. friedmanii*, ATCC 114
ATCC 65. *Mycobacterium* sp., leprae-65, ATCC
362. *M. butyricum*, ATCC 362
281. *M. stercoris*, ATCC 281

Strains identified as *Mycobacterium peregrinum* sp.nov.

6020, 128-1. *Mycobacterium* sp., from a child bronchial aspiration, and from nasal exudate of cattle, UPHG

Strains identified as *Mycobacterium phlei*, Lehmann and Neumann

NTB, W. *M. phlei*, National Trudeau Bank, W. Steenken jr.
W-1. *M. phlei*, Rutgers University, S. A. Waksman
1787, 637. *Mycobacterium* sp., lung biopsy, UPHG

Strains identified as *Mycobacterium piscium* (Dubard) Bergey *et al.*

9819. *M. piscium*, ATCC 9819

Strains identified as *Mycobacterium marinum* Aronson

7010. *M. marinum*, NTCC 7010. Neon tetra 1
927. *M. marinum*, ATCC 927
10011, 10010. *M. balnei*, NTCC 10011, 10010

Strains identified as *Mycobacterium fortuitum*, Cruz

11. *M. fortuitum*, Hektoen Institut for Med. Research (HIMR) Cruz original isolate. ATCC 6841, S. McMillen
6, 6*a*. *M. fortuitum*, Medical Research Council Unit, Oxford 6-Wells and Weiss 6*a*-Wells strains NCTC 8573, HIMR, S. McMillen
5, 7. *M. fortuitum*, 5 leg biopsy Wise; 7 Sputum Schumpert
10. *M. fortuitum*, *M. minettii* Penso, HIMR, S. McMillen
14, 15, 16, 17. *M. fortuitum*, 14, 17, bovine mastitis; 15 soil; 16 NCTC 8697, HIMR, S. McMillen
20, 38, 39, 41. *M. fortuitum*, 38, halibut strain NCTC 2291; 20, 39, 41 from sputum. HIMR, S. McMillen
156. *M. fortuitum*, Communicable Disease Center, Atlanta, Georgia, G. Kubica
HI-5836. *Mycobacterium* sp., UPHG. Bronchial aspiration
66038. *Mycobacterium* sp., AFIP, Mayo Clinic, A. G. Karlson
274. *Mycobacterium* sp., Communicable Disease Center Atlanta, G. Kubica
CAR, CC. *Mycobacterium* sp., Western Fish Disease Laboratory, Seattle, Washington., Carson Strains, (CAR), and Chambers Creek strains (CC), J. Ross
SN-413. *Mycobacterium* sp., Tuberkulose Forschungsinstitut, Börstel. R. Bönicke
1216. *Mycobacterium* X, University of Florida, E. Suter
8, 131, 231, 232, 405, HI-501, HI-503, 400, RD-112, AC-164, AC-242, AC-328, AC-621, AC-624, AC-625, AC-674, AC-681, AC-713, AC-833, AC-1523, 1919. *Mycobacterium* sp., from different pathological products

Table 1 (*cont.*)

Strains identified as *Mycobacterium thamnopheos* Aronson

4445. *M. thamnopheos*, ATCC 4445

Strains identified as *Mycobacterium flavescens* sp.nov.

D-50, D-25, D-15, 2159, 1078, 787. *Mycobacterium* sp., from guinea-pigs with tuberculosis arrested by chemotherapy (D-50, D-25 and D-15) and from human biopsies, UPHG

Strains identified as *Mycobacterium acapulcensis* sp.nov.

343, 103, 102, 465, 358. *Mycobacterium* sp., from sputum. Acapulco Campaign against tuberculosis, UPHG

Strains identified as *Mycobacterium runyonii* sp.nov.

380, 518, 481. *Mycobacterium* sp., Veterans Administration Hosp. Salt Lake City, E. H. Runyon
29. *Mycobacterium* sp., Hepatic abscess, Cuba, A. Curbelo
AC-561. *Mycobacterium* sp., Acapulco Campaign against tuberculosis

Strains identified as *Mycobacterium gordonae* sp.nov.

P-15, 251. *Mycobacterium* sp., Veterans Administration Hosp., E. H. Runyon
RNGO, LLE/RAS, 17. *Mycobacterium* sp., Colombia, G. Muñoz Rivas
R-41, PTB, D-13, CN-16, 168, CN-21, SIM, 591, SMG, 166, CN-30. *Mycobacterium* sp., from different pathological materials, UPHG

Strains identified as *Mycobacterium marianum* Penso

1313. *M. marianum*, Inst. Pasteur of Lyon, M. Suzanne
2465, 4333. *M. scrofulaceum*, University of Montreal, E. H. Prissick and A. M. Masson
227, 27, HI-5887, AC-134. *Mycobacterium* sp., from different clinical material, UPHG
128, 23, 55, 24, 80, 16. *Mycobacterium* sp., from cattle with symptoms of respiratory disease, UPHG
8138. *M. marinum* (Battaglini strain), ATCC 8138

Strains identified as *Mycobacterium avium* Chester

4109, 4110, 4133, Kirchberg. *M. avium*, Mayo Clinic, A. G. Karlson. W. H. Feldman
Sheard. *M. avium*, National Trudeau Bank, W. Steenken jr.
1, 3, 7. *Mycobacterium* sp., from tuberculous chickens, UPHG
P-17, P-20, P-23, P-25. *Mycobacterium* sp., from tuberculous chickens, UPHG, G. Kubica
77. *Nocardia intracellularis*. Communicable Disease Center, Atlanta, L. Ajello.
P-2, P-3, 171, 223, A-225, 248, P-7, 277, 470, 477, 487, 509, 513, 514, 520. 568, 584, 585, 586, 587. *Mycobacterium* sp., Veterans Administration Hosp., E. H. Runyon, non-photocromogens
SN-405. *Mycobacterium* sp., Tuberkulose Forschungsinstitut, Börstel, R. Bönicke
AC-2, AC-32, AC-123, AC-271, AC-289. *Mycobacterium* sp., UPHG, from sputa of people with respiratory disease
SN-418. *Mycobacterium suis*, Tuberkulose Forschungsinstitut, Börstel, R. Bönicke

Strains identified as *Mycobacterium kansasii* Hauduroy

P-16, P-18, P-24. *Mycobacterium* sp., Communicable Disease Center, Atlanta, G. Kubica
P-22, P-1, CAL-265, 266 Ohio, P-16, P-8, 488, 410, 79, 212, 22, 259, 184, 365. *Mycobacterium* sp., Veterans Administration Hospital, Runyon
SN-501, SN-504, SN-506, SN-510, SN-511. *Mycobacterium* sp., Tuberkulose Forschungsinstitut, Börstel, R. Bönicke
685-4, C-931, C-457. *Mycobacterium* sp., strains RW, VM, and VS from Tuberculosis Research Institute of Prague, Czechoslovakia, M. Kubin

The third branch (III) includes micro-organisms that show a slow rate of growth and a very low capacity to utilize carbohydrates; belonging to this branch are *Mycobacterium kansasii* (photochromogens), *M. avium* (non-photochromogens), *M. marianum* (scotochromogens) and *M. gordonae* sp.nov. (scotochromogens).

A list of the strains studied is shown in Table 1. They are grouped under the specific epithet that, according to our results, corresponds to each of them. It was possible to classify 187 strains out of a total of 229 studied; while the remaining 42 strains belonged both to the irregular branch (16 strains) and to intermediates between categories (26 strains).

DISCUSSION

The use of the Adansonian analysis of the physiological properties of mycobacteria permits their separation into twelve different categories. The differences between them depend on the combination of various characters.

Some variations can exist in each category between strains or groups of strains, with the consequent formation of subgroups as has been mentioned previously (Bojalil & Cerbón, 1961). For instance, in the category *Mycobacterium fortuitum* there are two subgroups, one capable and the other incapable of producing acid from mannitol. However, the high similarity index shown by these two sub-groups makes it impracticable to separate them, and recognition of the subgroups would unnecesarily increase the number of taxa.

The presence of strains intermediate between categories suggests the existence of other strains that could form a continuous metabolic spectrum, that would make difficult the separation of related categories. Thus, some groups now considered as species might constitute, in the future, subcategories or varieties. If, however, the categories are formed only with micro-organisms showing a high similarity index, a useful taxonomic schema can be obtained. In practice, it is not difficult to distinguish several categories that differ only in one or two absolute characters. For instance, *Mycobacterium flavescens* sp.nov. shows a metabolism similar to that of *M. fortuitum*, differing only in that the former is pigmented and grows slowly and that the latter is non-pigmented and grows rapidly. Therefore, it is impossible to place a scotochromogen into a group of rapidly growing non-pigmented mycobacteria. Furthermore, a better delimitation of some of these groups could be obtained with the use of other additional tests, such as the presence of amidases (Bönicke, 1960).

An Adansonian classification based on metabolism, clearly shows the relationships between species. Micro-organisms that exhibit a greater metabolic capacity correspond to rapidly growing mycobacteria, while those with a lesser metabolic capacity generally show a slow rate of growth. This fact permits the formation of three metabolic branches: each of them includes species that form natural groups and can be accepted as logically classified. Other natural groups were also observed in each of these three branches and were constituted by unnamed strains. The new categories so formed were named because their characteristics do not correspond to any previously known species.

The designation of neo-types of co-type for known species, of holotypes for those newly described and the general properties of the different categories of the mycobacteria, are noted in Table 2. Branch I is constituted by rapidly growing micro-organisms, and includes *Mycobacterium smegmatis*, and *M. phlei* that have been

Table 2. *General properties of mycobacteria*

	Branch 1				Branch 2							Branch 3			
Species and type strain...	*M. smegmatis*, NeT. W-113.	*M. peregrinum*, H.T. 6020.	*M. phlei*, NeT. NTB.	Irregular branch.	*M. fortuitum*, C.T. ATCC 6841.	*M. runyonii*, H.T. 380.	*M. acapulcensis*, H.T. 103.	*M. flavescens*, H.T. D-25.	*M. piscium*,	*M. marinum*, C.T. ATCC 927.	*M. thamnopheos*, C.T. ATCC 4445.	*M. gordonae* H.T. P-15.	*M. marinum*, NeT. 1313.	*M. avium*, N.T. 4109.	*M. kansasii* N.T. P-22.
Pigment in Löwenstein	Pale orange	Pale straw	Yellow	Yellow orange	Pale straw	Pale straw	Brilliant yellow	Brilliant orange	Yellow	Yellow	Slight yellowish	Brilliant orange	Reddish orange	Pale straw	Yellow
Property:															
Light															
Influenced	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+
Rapid grower	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−
Slow grower	−	−	−	−	−	−	−	+	−	−	−	+	+	+	+
Acid from:															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Fructose	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−
Galactose	+	+	+	−	−	−	−	−	−	−	+	−	−	−	−
L-arabinose	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
Xylose	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Lactose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Maltose	−	+	−	±	−	−	−	−	−	−	−	−	−	−	−
Sucrose	−	+	−	±	−	−	−	−	−	−	+	−	−	−	−
Trehalose	+	+	+	+	+	−	+	∓	+	∓	+	−	−	−	−
Melibiose	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Raffinose	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannitol	+	+	+	+	±	−	+	+	−	−	+	−	−	−	−
Sorbitol	+	+	+	+	−	−	+	±	−	−	+	−	−	−	−
m-Inositol	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Erythritol	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Dulcitol	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Utilization of:															
Benzoate	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Citrate	+	−	+	±	+	−	−	+	−	−	+	−	−	−	−
Succinate	+	+	+	+	+	+	+	+	−	±	+	−	−	−	−
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propionate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth temperatures:															
28°	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±
37°	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
45°	+	−	+	±	−	−	−	−	−	−	−	−	−	±	−
52°	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Resistance to 60° 4 hr.	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−

NeT., proposed neotype; H.T., holotype; C.T., cotype; +, positive; − negative; (±) the majority gave positive results; (∓) the majority gave negative results.

adequately characterized (Gordon & Smith, 1953; Penso *et al.* 1951), and a group of other strains that show the greatest metabolic capacity of all the strains studied, being therefore distinct from the other species. For this group, the name of *M. peregrinum* sp.nov. is proposed (Latin adjective, strange, foreign) because they are the only non-pigmented strains in branch I. The properties of one of these last strains has been previously reported, as strain number 40 (6020) (Bojalil & Cerbón, 1961, p. 341).

Besides the species mentioned, there are a number of unnamed micro-organisms (irregular branch) that also show a high metabolic capacity and a rapid rate of growth, and are, therefore, included in this branch. The disparity between the characters of members of this group does not allow their inclusion in any of the aforementioned species, nor do they form new groups.

Branch I can easily be differentiated from the other branches by an absolute distinctive character: the utilization of pentoses (xylose, L-arabinose and rhamnose).

Branch II includes: *Mycobacterium fortuitum*, *M. acapulcensis* sp.nov. and *M. runyonii* sp.nov. The last two corresponding to groups 2 and 3 previously described (Cerbón & Bojalil, 1961). These groups retain their individuality in the present comparative study and are therefore named acapulcensis, from Acapulco, a town on the Pacific coast of Mexico, where they were isolated, and runyonii, after R. E. Runyon who established the preliminary subdivision of the atypical mycobacteria.

Other rapidly growing strains belonging to this branch are *Mycobacterium marinum*, and *M. thamnopheos*, but they have an optimal growth temperature of 28° to 32°. After several transplants, *M. marinum* can grow well at 37°. Also included in branch II are a small group of scotochromogen strains, named *M. flavescens* sp.nov. (L. part. adj. becoming yellow). Although slow growers, they show a greater index of similarity with the species of this branch than to those of branch III, which are formed exclusively by slow-growing micro-organisms.

Strains received as *Mycobacterium balnei* (Linell & Norden, 1954) showed similar properties to *M. marinum* (Aronson, 1926) as has been already pointed out (McMillen & Kushner, 1959; Bojalil, 1959). Therefore, *M. balnei* was considered as synonymous with *M. marinum*. The physiological properties described in Table 2 for this species are not entirely in accord with those reported by others who showed that they utilize L-arabinose (Gordon, 1937), and that they do not use mannose and fructose (Ross, 1960). The strains here studied did not utilize L-arabinose and they did utilize mannose and fructose.

The strain received as *Mycobacterium marinum* ATCC 8138 (Battaglini strain) is shown not to belong to this species, because it differs in various characters from the other strains. According to its characteristics it belongs to the species *M. marianum* of branch III.

Mycobacterium piscium (Dubard, 1897; Bergey *et al.* 1923) and *M. thamnopheos*, (Aronson, 1929) apparently are two valid species. However, it is necessary to study a large number of strains to define better their properties, since the results obtained differ from other observations (Gordon, 1937; Ross, 1960).

Some species of branch II utilize trehalose, while others do not use it. Apparently a relationship exists between the utilization of this substrate and the general metabolic capacity, since the micro-organisms that utilize it show a greater ability to use other substrates.

Branch III is formed by *Mycobacterium gordonae* sp.nov. (scotochromogen), *M. marianum* (scotochromogen), *M. avium* (non-photochromogen) and *M. kansasii* (photochromogen). All show a low metabolic capacity and grow slowly. This branch could include *M. ulcerans*, *M. tuberculosis* and *M. bovis* since they do not utilize trehalose or most of the other substrates used in this work.

Mycobacterium gordonae sp.nov. (after Miss R. E. Gordon of Rutgers University who has done so much in establishing the classification of mycobacteria) is the species that shows the highest metabolic capacity; it utilizes mannose and fructose, which are not used by other members of this branch. All species utilize glucose, although some strains of *M. avium*, *M. kansasii* and *M. marianum* do not utilize this substrate. The differentiation between the last three species is difficult when it is based only on their utilization of carbohydrates. However, they can be distinguished: *M. marianum* is a scotochromogen, *M. avium* does not produce pigment, at least in young cultures, and *M. kansasii* produces pigment only in the presence of light. The differences between these categories can be made more evident by the use of other tests such as the capacity to metabolize amides. *M. kansasii* decomposes urea but not pyrazinamide, while *M. avium* does not decompose urea but liberates ammonia from pyrazinamide (Bönicke, 1960). However, deamidation tests are not always able by themselves to delimitate the different categories of mycobacteria. An analysis of the results obtained by the use of these tests will be published later.

Non-photochromogen mycobacteria were included under the specific name of *Mycobacterium avium*, because of the great similarity between them (Bojalil & Cerbón, 1960). This similarity is based mainly on negative data but other findings seem to confirm it. Bönicke (1960) states that these strains have similar capacities by which they metabolize amides. Palmer, Edwards, Hopwood & Edwards (1959) and Smith *et al.* (1961) report that the response of many individuals to different tuberculins from Battey or avian strains is practically the same and Smith *et al.* (1960) have shown the presence of the same special type of lipid. Other similarities have also been found (Feldman, Davis, Moses & Adberg, 1943; Engbaek, Friis & Søeborg-Ohlsen, 1957; Takeya, Zinnake, Yamura & Toda, 1960).

There is some tendency to separate *Mycobacterium avium* from the Battey type micro-organisms. One of the most discussed facts has been the degree of virulence for chicken, which is high for *M. avium* but non-photochromogenic strains show little pathogenicity for this animal. This is not an absolute criterion, since non-virulent strains of *M. avium* exist (Winn & Petroff, 1933; Branch, 1933; Will, Froman, Krasnow & Bogen, 1957; Doer, Smith & Altman, 1959). Other slight differences have been reported in arylsulphatase activity (Kubica & Beam, 1961), catalase activity (Bojalil & Cerbón, 1960) and sensitins (Magnusson, Engbaek & Bentzon, 1961). These differences are of degree only, since there are representative strains of both *M. avium* and the Battey type with identical enzymic activity. It is believed, therefore, that in the absence of sufficient proof that would permit the separation of these micro-organisms, they still must be considered as a single group.

A key for a practical identification of the different categories was designed (Fig. 3); this is based on the most constant characters of each group. However, if a strain is aberrant in some of its properties, it must be classified considering the characters listed in Table 2.

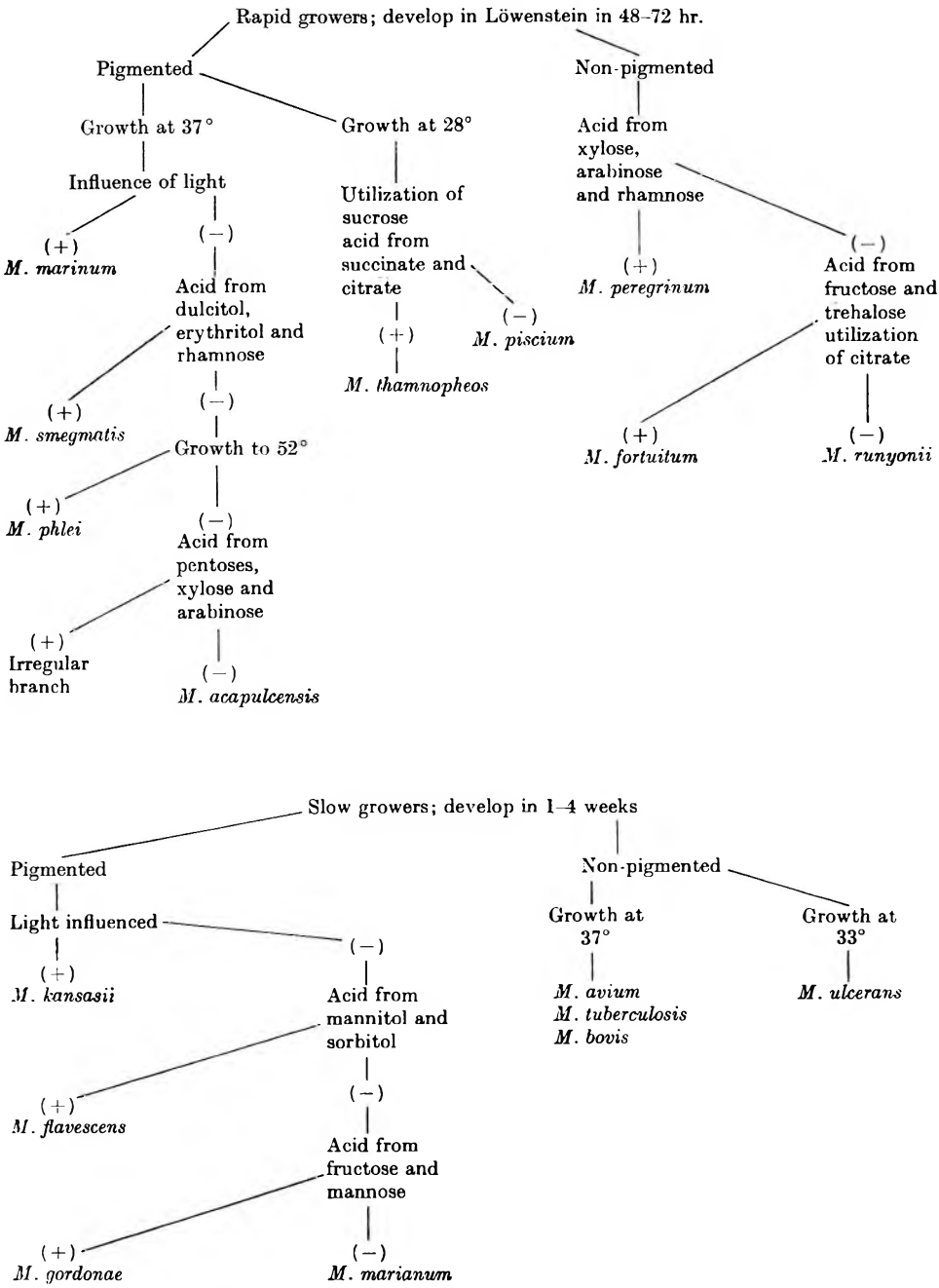

Fig. 2. Simplified scheme for the identification of mycobacteria.

DESCRIPTION OF NEW SPECIES

Only the most outstanding and distinctive characters will be mentioned, since all the properties studied are noted in Table 2.

Branch I. *Mycobacterium peregrinum* sp.nov. (L. adj. peregrinus, strange, foreign).

Rapid growing, acid-fast bacilli, with visible growth in 24–48 hr. in most ordinary culture media.

Löwenstein–Jensen: abundant, confluent, smooth, buff or pale straw-coloured growth.

Temperature relations: optimum for growth, 37°; grows at 28° but not at 45°; does not survive 4 hr. at 60°.

Glycerol agar slants; good growth.

Acid from glucose, mannose, fructose, galactose, L-arabinose, xylose, rhamnose, maltose, sucrose, trehalose, melibiose, raffinose, mannitol, sorbitol, *m*-inositol. No acid from lactose, erythritol or dulcitol.

Utilizes succinate, pyruvate and propionate but not benzoate or citrate.

Proposed holotype: strain 6020 (Unidad de Patología, Hospital General, México).

Distinctive characters. This species may be distinguished from closely related species such as *Mycobacterium smegmatis* by its ability to produce acid from maltose, sucrose, melibiose and raffinose. It is non-pigmented and does not utilize benzoate or citrate and does not produce acid from erythritol or dulcitol.

Source: one strain was isolated from bronchial aspiration of a child with respiratory symptoms and another from nasal exudate of a cow.

Branch II. *Mycobacterium acapulcensis* sp.nov. (From Acapulco, a town on the Pacific coast of México.)

Rapid growing acid-fast bacilli, with visible growth in 24–48 hr. in most ordinary laboratory media.

Löwenstein–Jensen: abundant, wrinkled, waxy, intense yellow colonies.

Glycerol agar slants: spreading growth, dry with slightly discoloured yellow pigment.

Temperature relations: grows well from 28° to 37°; optimal temperature 37°; does not grow at 45° and does not survive 4 hr. at 60°.

Acid from glucose, mannose, fructose, trehalose, mannitol, and sorbitol, but not from galactose, L-arabinose, xylose, rhamnose, lactose, maltose, sucrose, melibiose, raffinose, *m*-inositol, erythritol and dulcitol.

Utilizes succinate, pyruvate and propionate but not benzoate or citrate.

Proposed holotype: strain AC-103 (Unidad de Patología, Hospital General, México).

Distinctive characters: these strains are distinguished from *Mycobacterium thamnopheos*, a closely related species in that they do not produce acid from galactose or sucrose, or utilize citrate, and they grow at 37°.

Source: isolated from sputum of people with pulmonary diseases, usually tuberculosis.

Mycobacterium runyonii sp.nov. (After E. H. Runyon, The Veterans Administration Hospital, Salt Lake City.)

Rapid growing acid-fast bacilli, that grow in most ordinary culture media in 24–48 hr.

Löwenstein–Jensen: good growth, smooth, confluent, soft consistency, non-pigmented.

Glycerol-agar-slants: good growth.

Temperature relations: optimal for growth 37°; grows at 28° but not at 45°; does not survive 4 hr. at 60°

Acid from: glucose and mannose only.

Utilizes succinate, pyruvate and propionate, but not benzoate or citrate.

Proposed holotype: strain number 380 (E. H. Runyon, Vet. Admin. Hosp., Salt Lake City).

Distinctive characters: non-pigmented, rapidly growing micro-organisms with cultural properties similar to those of *Mycobacterium fortuitum*, from which they differ in that they are unable to produce acid from fructose, trehalose, mannitol and do not utilize citrate.

Source: isolated from sputum (in absence of tubercle bacilli) from people with pulmonary lesions.

Mycobacterium flavescens sp.nov. (L. v., flavesco, to become golden yellow).

Slowly growing acid-fast bacilli, that does not grow well in ordinary culture media.

Löwenstein–Jensen: the development is detected after one or more weeks; colonies are of soft consistency, butyrous, adherent, difficult to remove and of intense orange colour (scotochromogen).

Glycerol agar slants: scarce development, orange pigment at 37°, does not grow at 28°.

Temperature relations: optimal growth at 37°, scanty growth at 28° in Löwenstein–Jensen; does not grow at 45° or survive 4 hr. at 60°.

Acid from: glucose, mannose and fructose; some strains produce acid from trehalose, sorbitol and mannitol.

Utilizes citrate, succinate, pyruvate and propionate but not benzoate.

Proposed holotype: strain number D-25 (Unidad de Patología, Hospital General, México).

Distinctive characters: these strains are closely related to *Mycobacterium fortuitum* from which they can be distinguished by their intense pigment production, their slow rate of growth and their inability to grow well in glycerol agar both at 28° and 37°.

Source: isolated from guinea-pigs with tuberculosis arrested by chemotherapy and from autopsy material.

Branch III. *Mycobacterium gordonae* sp.nov. (After Miss Ruth E. Gordon of Rutgers University.)

Slowly growing acid-fast bacilli that do not grow in ordinary culture media. Development is detected only after 9–12 days.

Löwenstein–Jensen: abundant, soft consistency, orange coloured, adherent growth (scotochromogen).

Glycerol agar slants: scarce growth at 37°, orange coloured and generally no growth at 28°.

Temperature relations: optimal growth 37°, scanty at 28° in Löwenstein; does not grow at 45 or survive 4 hr. at 60°.

Acid from: glucose mannose and fructose only.

Utilizes pyruvate and propionate but not benzoate, citrate or succinate.

Proposed holotype: strain number P-15 (E. H. Runyon, Vet. Admin. Hosp., Salt Lake City).

Distinctive characters: their capacity to produce acid from mannose and fructose makes possible their differentiation from its most related scotochromogen species, *Mycobacterium marianum*.

Source: isolated from human pathological material.

The authors wish to thank all research workers who kindly provided strains for study. To Dr P. H. A. Sneath (National Institute for Medical Research, London, for correction of the typescript and valuable suggestions, and Dr H. Brandt (Unidad de Patología, Hospital General, México) for help in the preparation of the manuscript.

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Physiological and Biochemical Studies on Streptomycin Dependence in *Escherichia coli*

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SUMMARY

The exponential growth rate of a streptomycin-dependent strain of *Escherichia coli* was proportional to streptomycin concentration until a critical concentration was reached, above which it was independent of streptomycin concentration. The value of the critical concentration changed with a change either in the carbon source, or in the temperature of cultivation. Below the critical concentration, the macromolecular composition of the cells was affected by the external streptomycin concentration: as this decreased, the ribonucleic acid (RNA) content of the organisms increased, and the protein content decreased. When external streptomycin was removed, streptomycin-dependent organisms continued to grow for many hours. Growth was at first exponential, the extent and duration of this phase being functions of the concentration of streptomycin to which the organisms had previously been exposed. This phase was followed by a much longer period of arithmetic growth, unaccompanied by cell division, during which the organisms elongated progressively. Growth in the absence of streptomycin caused changes in the macromolecular composition of the organisms which were similar in nature to those produced by growth with a subcritical concentration of streptomycin, but much more pronounced. The greatly increased total RNA content of these organisms was not accompanied by grossly detectable qualitative changes in the RNA content of the organisms. In the absence of streptomycin, the synthesis of some enzymes was either arrested or decreased in rate; the synthesis of others was unaffected. This leads to an imbalance in the enzymic constitution. These differential effects on enzyme synthesis appeared to be random. Growth in absence of streptomycin did not seem to affect deoxyribonucleic acid (DNA) synthesis or function, as shown by the ability of a lysogenic streptomycin-dependent strain to produce infective phage under such conditions. The re-introduction of streptomycin to a culture growing arithmetically as a consequence of streptomycin depletion caused a resumption of DNA synthesis at the normal exponential rate. The rate of protein synthesis also soon increased, but attained its normal exponential rate more slowly. RNA synthesis was wholly arrested until the RNA content of the organisms had fallen to a normal value, and then resumed at the normal exponential rate. Grown in the presence of a greater than critical concentration of streptomycin, the streptomycin-dependent organism bound irreversibly about 250,000 molecules of streptomycin, half of which could be extracted with hot water, and the remainder with hot perchloric acid. A new hypothesis concerning the location and nature of the genetically determined intracellular lesion which results in streptomycin dependence is developed on the basis of these facts.

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INTRODUCTION

Not long after the discovery of streptomycin, Miller & Bonhoff (1947) reported the isolation from a streptomycin-sensitive meningococcus of a mutant strain which had an absolute requirement for streptomycin as a growth factor. Similar streptomycin-dependent strains have subsequently been isolated from many other naturally streptomycin-sensitive bacterial species; there are thus good reasons to believe that mutation to an absolute dependence on this substance is a widespread genetic potentiality of bacteria. Dependence on streptomycin is one of three alternative genetically determined responses of a bacterium to the presence of streptomycin in its environment; the two other possible responses being sensitivity and resistance. The category of resistance includes several different states, which are distinguishable from one another both genetically and phenotypically (Watanabe & Watanabe, 1959*a*, *b*; Hashimoto, 1960; Matney, Goldschmidt & Bausum, 1960); but the available data suggest that dependence is monogenically determined, and hence a phenotypically uniform state. In *Escherichia coli*, genetic analysis has shown that sensitivity, dependence and single-step resistance to a high concentration of streptomycin ('indifference') are determined by multiple alleles at a single locus, known as the Sm locus (Newcombe & Nyholm, 1950; Lennox, 1955; Hashimoto 1960). This genetic fact implies that the three phenotypic states in question are all determined, in the last analysis, by alternative structural modifications of a single chemical substance within the cell, and that each of these modifications has a series of specific functional consequences which cause the cell to respond in a specific way to streptomycin.

Attempts to elucidate the mode of action of streptomycin have led to many different studies of its physiological and biochemical effects on bacteria, and a considerable number of divergent hypotheses have been put forward on the basis of the resultant findings. Most of these studies have been done with strains of the sensitive and resistant phenotypes. However, the genetic facts which have been outlined above suggest that analysis of the relatively neglected, bizarre and clinically unimportant phenomenon of dependence has an equal intrinsic probability of furnishing clues to the mechanism of streptomycin action in the bacterial cell. Such reasoning resulted in the investigation of the dependent phenotype of *Escherichia coli* which will be described in the present paper.

The principal facts established by earlier work about the physiological and biochemical properties of streptomycin-dependent bacteria may be summarized as follows. (1) The growth rate of a dependent strain is a direct function of the concentration of streptomycin in the medium; the amount required to permit a maximal growth rate is very substantial, generally of the order of several hundred μg./ml. (Paine & Finland, 1948; Schaeffer, 1950). (2) When streptomycin is removed from the medium, cell division soon ceases; the amount of residual cell division is a function of the concentration of streptomycin in which the cells were previously grown (Demerec, Wallace, Witkin & Bertani, 1949; Bertani, 1951). After cell division ceases, further arithmetic growth occurs; the organisms increase in length, and nuclear divisions continue (Delaport, 1949; Demerec *et al.* 1949; Schaeffer, 1950; Simon, 1955). (3) In the absence of streptomycin, the synthesis of some enzymes ceases. Generally, the oxidative capacity of a dependent culture does not

increase in the absence of streptomycin (Schaeffer, 1949*a*, *b*, 1950, 1952), and the synthesis of active cytochrome oxidase is blocked under these conditions (Engelberg & Artman, 1961). The same studies also showed, however, that the synthesis of certain enzymes associated with fermentative activity was not influenced by streptomycin deprivation. (4) The growth of streptomycin-dependent organisms does not diminish appreciably the streptomycin content of the medium (Rubin & Steinglass 1951). Attempts to show uptake of streptomycin by streptomycin-dependent organisms during growth have so far failed (Szybalski & Mashima, 1959); but it should be noted that the sensitivity of these measurements was not sufficiently great to exclude the intracellular accumulation of very small amounts.

METHODS

Organisms. The strains used in all experiments were derived from *Escherichia coli* strain K-12. Strain W1709 was obtained from Dr J. Lederberg; it requires threonine, leucine, thiamine and streptomycin for growth, and is unable to grow at the expense of lactose or maltose. The failure to use lactose is a consequence of the inability of this strain to form the permease necessary for entry of lactose into the cell. Strain W1709 is also lysogenic for bacteriophage λ. Strain CS-1 is a β-galactosidase constitutive recombinant selected from a mating between W1709 (F^-) and W3300 (a Hayes-type Hfr which is streptomycin-sensitive, constitutive for the production of β-galactosidase and requires thiamine). Strain W3300 was obtained from Dr A. J. Clark, Dept. of Bacteriology, University of California, Berkeley, California, U.S.A. The recombinant was selected from the mating mixture by plating on minimal agar supplemented with streptomycin + thiamine and containing lactose as sole carbon source. Strain CS-2 is a spontaneous mutant of W1709 which has regained the ability to grow with lactose as sole carbon source, and produces β-galactosidase inducibly. It was isolated by spreading about 10^6 organisms from a glucose-grown culture of strain W1709 on plates of a defined medium containing lactose as sole carbon source, and supplemented with threonine + leucine + thiamine + streptomycin. On this medium, CS-2 forms large colonies, whereas the parental strain W1709 does not grow appreciably.

Media. The defined medium used for all growth experiments had a mineral base of the following composition: NH_4Cl, 0·1% (w/v); $MgSO_4.7H_2O$, 0·025% (w/v); $KH_2PO_4 + Na_2HPO_4$ buffer (pH 7·0), 0·05 M. After sterilization, this base was supplemented with separately sterilized solutions of required growth factors, carbon source and streptomycin. The growth factors were used at the following final concentrations: DL-threonine, 100 μg./ml.; DL-leucine, 100 μg./ml.; thiamine hydrochloride, 0·5 μg./ml. The carbon source (glucose, sodium succinate or glycerol) was used at a final concentration of 0·4% (w/v). The concentrations of streptomycin used are specified for each experiment. The complex medium, used for estimating number of viable organisms, had the following composition: Difco yeast extract, 0·5% (w/v); Difco peptone, 0·3% (w/v); $KH_2PO_4 + Na_2HPO_4$ buffer (pH 7·0), 0·02 M; agar, 2·0% (w/v).

Methods of cultivation and measurement of growth. Cultures were grown in shallow layers in Erlenmeyer flasks incubated on a rotary shaker, and growth was estimated turbidometrically with a Klett-Summerson colorimeter. Klett readings and bacterial

numbers were proportional up to a Klett reading of 50; when the measured optical density exceeded this value, a correction curve was used to determine the true population density. A Klett reading of 50 corresponded to a viable count of $3{\cdot}7 \times 10^8$ bacteria/ml. (equiv. to 0·12 mg. dry wt. cell material/ml.) in exponentially growing cultures. Unless otherwise stated, all growth experiments were done at 30°.

When a growth experiment was done in the absence of streptomycin, or when the streptomycin concentration was changed during the course of an experiment, the bacteria were carefully washed to remove absorbed streptomycin by two successive centrifugations in M/20 phosphate buffer (pH 7·0). These operations were carried out as rapidly as possible in sterile screw-capped tubes at room temperature. The efficiency of this procedure in the removal of ^{14}C-streptomycin is shown in Table 1. Bacteria treated in this way immediately resumed exponential growth when placed in fresh pre-warmed medium of the same composition as that from which they had been removed.

Table 1. *Removal of adsorbed streptomycin by washing*

Escherichia coli strain CS-1 was suspended at 5×10^8 bacteria/ml. in the medium shown in the first column containing the indicated concentration of ^{14}C-streptomycin. The bacteria were washed by successive centrifugations in the same volume of phosphate buffer.

Medium	Streptomycin added (μg./ml.)	Supernatant fluid	First wash	Second wash	Total recovery (%)
		Streptomycin recovered after sedimentation of cells (μg./ml.)			
0·01 M-phosphate buffer (pH 7·0)	630	618	14·2	0·6	100
Defined	350	347	4·8	0·1	100
Defined	20	16·4	3·6	1·0	105

Analytical methods. The protein content of the bacteria was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). For the determination of nucleic acids, bacteria were fractionated with perchloric acid by the procedure of Burton (1956), after which ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined in the hot acid-soluble fraction by the procedures of Mejbaum (Schneider, 1957) and Burton (1956), respectively.

Preparation of cell-free extracts and measurement of enzymic activity. Bacteria which were to be used for the preparation of enzymically active extracts were harvested by centrifugation in the cold and were washed twice with cold phosphate buffer (M/100; pH 7·5). The extracts were prepared by suspending the bacteria in ten times their own weight of the same buffer and treating them in the French Pressure Cell (American Instrument Company, Silver Springs, Maryland, U.S.A.) at a pressure of 20,000 lb./sq. in. Unbroken bacteria and the large cellular debris were removed by centrifugation for 20 min. at 7000 *g* in the cold. A portion of the crude extract thus prepared was then subjected to further centrifugation at 100,000 *g* for 90 min. The supernatant fluid from this centrifugation (soluble fraction) was carefully decanted; the sediment (particulate fraction) was washed with the same buffer by centrifugation. In certain instances enzyme activity was determined in bacteria ruptured with toluene. In such cases, 0·1 ml. toluene was added directly to a sample of the culture and this sample was then shaken for

10 min. at 30°. Enzymic activities were measured directly on portions of this toluenized suspension.

Enzymic assays were carried out according to standard procedures. Manometric measurements were done in the Warburg apparatus at 30°. Spectrophotometric assays were done in the Beckman model DU spectrophotometer fitted with a circulating water bath which maintained the cuvette compartment at 30°. The enzymes measured, the fraction of cell-extract routinely used for assay, the measurement of activity for each enzyme, and the reference to the method used are listed in Table 2. In all cases enzymic activity was determined by the measurement of the initial rate of reaction under conditions in which enzyme concentration was the rate-limiting factor.

Table 2. *Techniques employed for the analysis of enzymic activities*

Enzyme	Cell fraction assayed	Measurement of activity	Reference
Threonine deaminase	Toluenized cells	Keto-acid formation	Pardee & Prestidge, 1955
Glutamic dehydrogenase	Soluble	TPN reduction	Strecker, 1955
5-Dehydroshikimic reductase	Soluble	TPN reduction	Yaniv & Gilvarg, 1955
Tryptophan synthetase	Crude extract	Indole disappearance	Yanofsky, 1955
Dihydroorotic dehydrogenase	Particulate or crude extract	Orotic acid production	Yates & Pardee, 1957
Isocitric dehydrogenase	Soluble	TPN reduction	Barban & Ajl, 1952
DPNH oxidase	Particulate or crude extract	DPNH oxidation	Slater, 1950
Succinic dehydrogenase	Particulate	DIP* reduction	Slater & Bonner, 1952; Price & Thimann, 1954
Glucose-6-phosphate dehydrogenase	Soluble	TPN reduction	DeMoss, 1955
β-Galactosidase	Crude extract or toluenized cells	ONPG† hydrolysis	Lederberg, 1950

* Dichlorophenolindophenol; † *o*-Nitrophenyl-β-D-galactoside.

Preparations of fractions for analysis of intracellular RNA distribution. Bacteria were harvested and fractionated by a procedure adapted from that used by Bolton, Britten, Cowie & Roberts (1958) and Bolton *et al.* (1959). The bacteria were harvested, washed twice with TSM buffer (2-amino-2-hydroxymethylpropane-1:3-diol, 0·01 M; succinic acid, 0·004 M; magnesium acetate, 0·01 M; pH 7·6), and resuspended in the same buffer at a concentration equivalent to about 20 mg. dry wt./ml. Crude extracts were prepared in the French Pressure Cell as described, and this crude extract then subjected to differential centrifugations which yielded three fractions: the *membrane fraction* consisting of material sedimented in 15 min. at 100,000 *g*; the *ribosome fraction* consisting of the material sedimented in 120 min. but not in 15 min. at 100,000 *g*; the *soluble fraction* consisting of the material not sedimented in 120 min. at 100,000 *g*. Ribosomes were washed by resuspension in TSM buffer and recentrifugation for 120 min. at 100,000 *g*.

Ultracentrifugal analyses were performed on crude extracts in the Spinco model E analytical Ultracentrifuge.

Chemicals. ^{14}C-streptomycin ($CaCl_2$ salt) uniformly labelled with a specific activity of 0·079 μc/mg. of streptomycin base was a gift from Dr B. Woodruff (Merck, Sharp & Dohme, Inc., Rahway, New Jersey, U.S.A.). Isopropyl-β-D-thiogalactoside (IPTG) was a gift from Dr A. B. Pardee.

RESULTS

Binding of streptomycin during growth by streptomycin-dependent organisms

A culture of *Escherichia coli* strain W1709 was allowed to grow for ten to twelve generations in the defined medium containing 560 μg. ^{14}C-streptomycin/ml. The bacteria were then harvested, washed three times by centrifugation with M/20 phosphate buffer, and then collected in quantities equivalent to 1·0–1·5 mg. dry wt. on Millipore filters. They were further washed on the filters with the same buffer containing 50 μg. non-radioactive streptomycin/ml. until the washings no longer possessed detectable radioactivity. These samples were then dried and their radioactivity determined. This experiment showed that the amount of bound streptomycin corresponded to about 250,000 molecules/bacterium. Extraction of the bacteria with hot water (95°, 10 min.) removed 50% of the bound streptomycin. The remainder could be extracted by hot perchloric acid (0·5N, 70°, 15 min.) but not by cold perchloric acid (0·5N, 0°, 15 min.).

Effect of streptomycin concentration on growth rate

The strains of *Escherichia coli* used in this work grew exponentially in the defined medium provided that the concentration of streptomycin was greater than 10 μg./ml. When glycerol or succinate was the carbon source, the growth rate was a direct function of the streptomycin concentration up to about 100 μg./ml., at which value streptomycin was no longer the rate-limiting nutrient. When glucose was the carbon source, streptomycin remained rate-limiting up to about 250 μg./ml. At rate-limiting concentrations of streptomycin, the growth rate was independent of the nature of the carbon source. These effects of streptomycin concentration are illustrated in Fig. 1. The growth rate in this figure is expressed as k, the exponential growth constant, calculated by means of the equation: $k = \ln 2/G$, where G is the generation time in hours. For the purposes of this paper, we shall define as 'normal bacteria' of a streptomycin-dependent strain, bacteria which are growing exponentially in a medium containing a concentration of streptomycin that is equal to or greater than the rate-limiting concentration for growth. We shall define as a 'critical concentration' of streptomycin that concentration which is just sufficient to support growth at maximal rate in any given medium.

Effect of streptomycin concentration on cellular composition

Cultures of *Escherichia coli* strain W1709 were grown in the defined medium with glycerol as carbon-source, and with streptomycin concentrations which ranged from 10 to 1000 μg./ml., thus including both sub- and supra-critical concentrations. While in the course of exponential growth, the bacteria from each culture were harvested and analysed to determine their content of RNA, DNA and protein. At subcritical concentrations of streptomycin, the RNA content of the

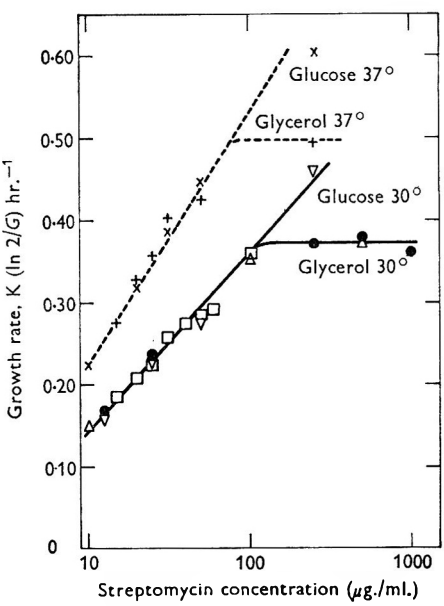

Fig. 1

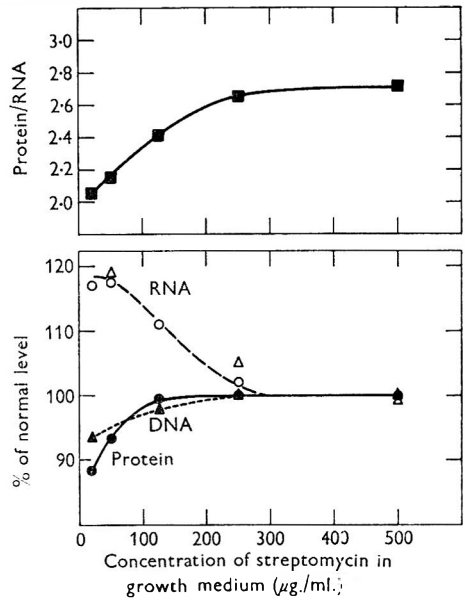

Fig. 2

Fig. 1. The effect of streptomycin concentration on the growth rate of *Escherichia coli* strain CS-2 in defined medium with different carbon sources. (×) glucose, 37°; (+) glycerol, 37°; (□, ▽) glucose, 30°; (△, ●) glycerol, 30°.

Fig. 2. The effect of streptomycin concentration on the macromolecular composition of *Escherichia coli* strain CS-1. The concentration of each component is expressed as percentage of the normal dry wt. of cells grown in media containing 500–1000 μg. streptomycin/ml.

bacteria was higher and the protein content lower than in bacteria grown with supra-critical streptomycin concentrations. The DNA content was only slightly affected by changes in streptomycin concentration, decreasing slightly at very low concentrations. These variations in cellular composition as a function of streptomycin concentration are shown in Fig. 2.

The phenomenon of deprived growth

When bacteria were harvested during the exponential phase of growth, washed to remove adsorbed streptomycin and then re-inoculated into a streptomycin-free medium of otherwise identical composition, a characteristic sequence of events took place. The cell mass increased exponentially at the maximal rate for a short period, the duration of which was directly proportional to the streptomycin concentration of the medium from which the bacteria had been taken. This was followed by a period of growth at a progressively declining rate which lasted for 1–2 hr. Finally, there was a period of arithmetic growth which lasted for 16–20 hr., during which the cell mass increased about sixfold. The bacteria then entered a stationary phase. The extent of growth, i.e. the ratio between the final and the initial cell mass, was directly proportional to the streptomycin concentration in which the bacteria were grown, and was the same for all cultures grown at a given streptomycin concentration. This growth in the absence of streptomycin will be termed 'deprived

growth'; and bacteria in the period of arithmetic deprived growth will be termed 'deprived bacteria'. The effects of streptomycin concentration on subsequent deprived growth are shown in Fig. 3. In the graphical presentation of the data from experiments on deprived growth (as in Fig. 3), it is convenient to express the increase either in cell mass, or in chemical constituents of the cell, as a relative increase, rather than as an absolute one. This device permits a direct comparison of the data from different experiments by eliminating the otherwise confusing graphical effects caused by differences in inoculum size.

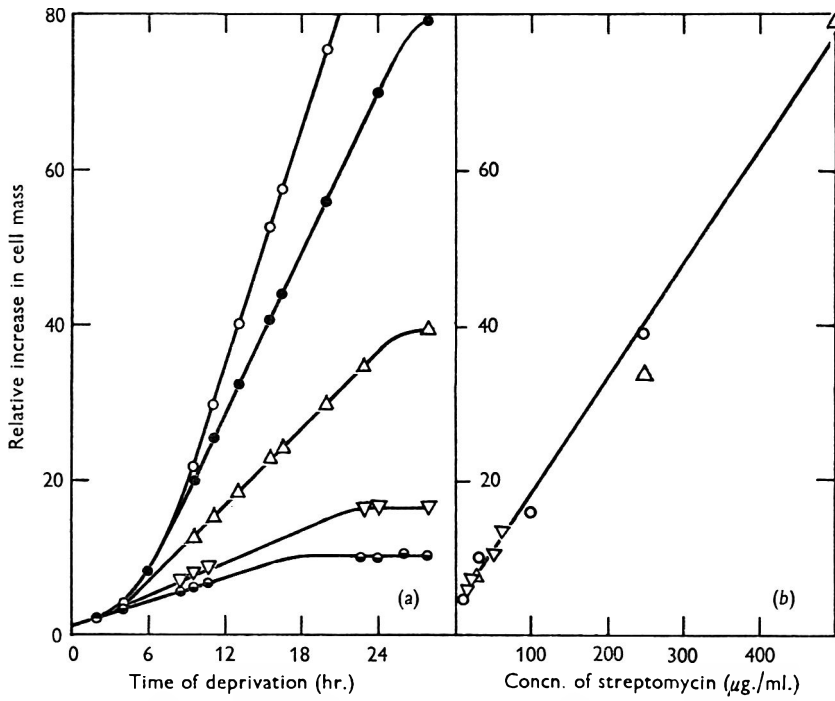

Fig. 3. The effect of streptomycin concentration on deprived growth of *Escherichia coli* strain CS-1. (*a*) Deprived-growth curves of cultures pre-grown in glycerol defined medium containing streptomycin at concentrations of 1000 μg./ml. (○); 500 μg./ml. (●); 250 μg./ml. (△); 100 μg./ml. (▽); 30 μg./ml. (◒). (*b*) Relation between extent of deprived growth and streptomycin concentration. Glycerol defined medium.

Viable counts performed during the period of deprived growth revealed an initial short period of multiplication, roughly coincident with the period of exponential growth. Thereafter, the number of colony-forming units in the culture remained constant throughout the period of arithmetic growth and only began to decrease several hours after the onset of the stationary phase. The relation between growth and viable count is shown in Fig. 4. As deprived growth proceeded all of the bacteria became converted into filaments which eventually attained a length about ten times the normal length. Each of these filaments contained several nuclear bodies, generally 6 or 8 by the time the stationary phase was reached.

Analysis of cellular composition during the initial period of deprived growth revealed drastic changes in relative concentrations of different macromolecular constituents. These changes were qualitatively similar to those which resulted from

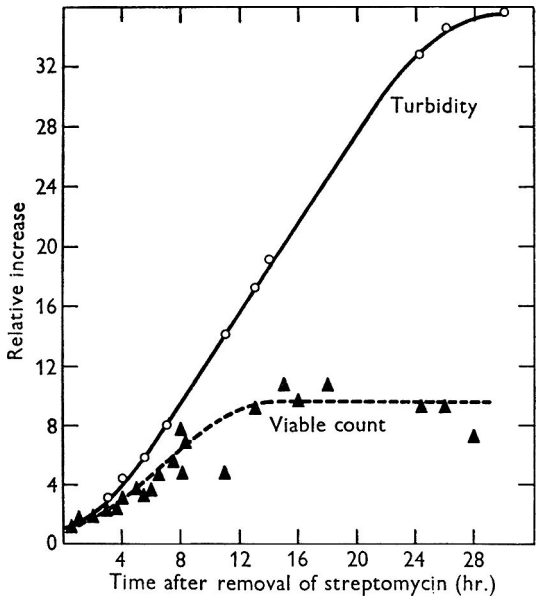

Fig. 4

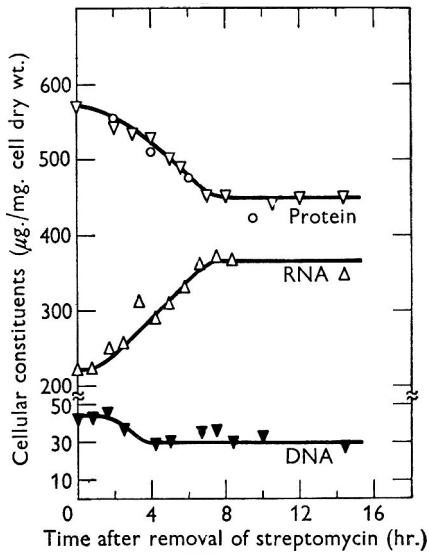

Fig. 5

Fig. 4. Increase in colony-forming units during deprived growth of *Escherichia coli* strain CS-1. The culture was grown in glycerol defined medium containing 250 μg. streptomycin/ml.

Fig. 5. Change in concentration of cellular constituents during deprived growth of *Escherichia coli* strain CS-1. The culture was grown in glycerol defined medium containing 250 μg. streptomycin/ml.

growth in media with rate-limiting streptomycin concentrations, but ultimately attained a much greater magnitude. They ceased shortly after arithmetic growth began, and thereafter the macromolecular composition of the bacteria remained constant (Fig. 5). The RNA/protein ratio in streptomycin-deprived bacteria reached a value more than twice that of normal cells. Although this ratio is directly related to the streptomycin concentration in bacteria growing exponentially in subcritical concentrations of streptomycin, it never exceeded a value 1·3 times that of normal bacteria at the lowest concentrations of streptomycin which would support exponential growth (see Fig. 3).

Spectrophotometric analysis of the medium during deprived growth revealed no significant excretion of material which absorbed in the ultraviolet region.

Effects of streptomycin deprivation on the nature and distribution of RNA within the cell

A comparison of extracts prepared from normal and deprived bacteria showed that the abnormally high concentration of RNA in deprived bacteria was not accompanied by any gross changes in the intracellular distribution of RNA. In both kinds of bacteria, 12–13% of the total RNA was in the soluble fraction, the remainder being associated with particulate (i.e. ribosomal) material. Analysis of washed ribosomal particles from normal and deprived bacteria showed no differences

in gross chemical composition (Table 3). The values found in both instances agree with values previously published for the ribosomes of *Escherichia coli* (Bolton *et al.* 1958; Tissières, Watson, Schlessinger & Hollingworth, 1959). Ultracentrifugal analyses of crude extracts prepared from normal and deprived bacteria showed no qualitative differences in the distribution of macromolecular components. Both extracts gave the same pattern of ribosomal peaks (Fig. 6). Exactly the same peaks, with the same characteristic sedimentation constants, have been found in similarly prepared extracts of a wild type (streptomycin-sensitive) strain of *E. coli* (Bolton *et al.* 1958). The relative sizes of the peaks attributable to ribosomes in the extract from deprived bacteria are approximately the same as in the extract of normal bacteria, suggesting that streptomycin deprivation had no effect on the quantitative distribution of material among the ribosomal fractions of different molecular weight. It can be seen (Fig. 6) that in the extract of deprived bacteria the total amount of ribosomal material (peaks 1–5) was much larger relative to the total amount of other proteins (peak 6) than in the extract of normal bacteria. This is a necessary consequence of the high RNA content of deprived bacteria and of the unchanged gross intracellular distribution of the RNA.

Table 3. *Analysis of washed ribosomes from normal and deprived bacteria of* Escherichia coli *strain CS*-1

Time of deprivation (hr.)	Composition of washed ribosomes		
	protein (mg./ml.)	RNA (mg./ml.)	ratio RNA/protein
0	3·34	5·54	62/38
9	2·66	3·75	59/41
20	2·50	3·45	58/42

Effects of streptomycin deprivation on enzymic constitution

As just described, a much larger fraction of cellular protein was bound into ribosomes in deprived bacteria than in normal bacteria. Since ribosomal protein is, at least in large part, not enzymically functional, this change of protein distribution necessarily implies that the total enzymic activity of the deprived bacteria must be considerably lower on a weight basis than that of a normal bacterium. The question may therefore be asked, whether the synthesis of all cellular enzymes has been affected to an equal extent, or whether the effects of streptomycin deprivation on enzyme synthesis are to some extent selective. To examine this question, measurements were made of the relative increase in the activities of a representative group of enzymes during deprived growth. By comparison with the relative increase in the total protein of the bacteria, it was then possible to determine the extent to which the synthesis of each enzyme studied had been affected by streptomycin deprivation. Typical data for a few of the enzymes assayed are presented graphically in Fig. 7. It is evident that the effect of streptomycin deprivation on enzyme synthesis was highly selective: for certain enzymes the rate of relative increase in activity closely paralleled the rate of increase of total protein, while for others it was decreased to a greater or lesser extent. The selective inhibition of the synthesis of certain enzymes became evident only during the transition to arithmetic growth; during the brief initial period of rapid exponential growth, the activities of all the

enzymes assayed increased at the same rate as did total protein. The values in Table 4 show the extent to which the synthesis of each enzyme studied was affected. In Table 4 the enzymes studied have been classified in three categories: enzymes with a biosynthetic function; enzymes with a function in respiratory metabolism; enzymes without any clearly attributable function under the growth conditions used. With respect to the third class, it should be noted that glycerol was used as carbon source, and that the experiment was conducted with *Escherichia coli* strain CS-1, in which the synthesis of β-galactosidase is constitutive.

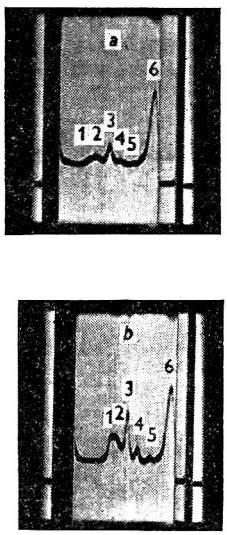

Fig. 6

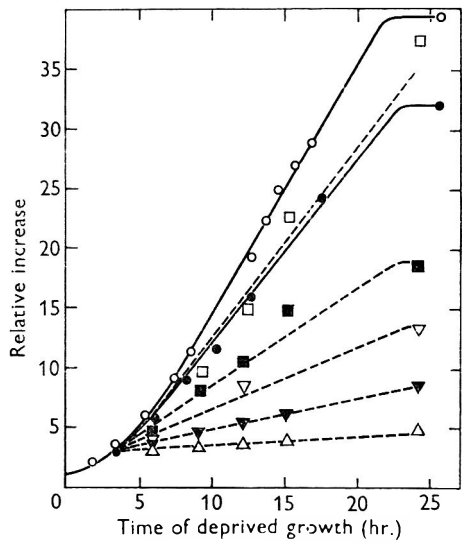

Fig. 7

Fig. 6. Sedimentation diagrams of crude extracts of normal bacteria of *Escherichia coli* strain CS-1 (*a*) and of bacteria of the same strain after 16 hr. of deprived growth (*b*). The peaks are numbered to correspond to the nominal sedimentation constants previously described for *E. coli*: 1, 100S; 2, 85S; 3, 70S; 4, 50S; 5, 30S; 6, 4S. The photographs were taken 8 min. after reaching a final speed of 49,330 rev./min. Temperature 21°.

Fig. 7. Increase in the total activity of a selected group of enzymes during deprived growth of *Escherichia coli* strain CS-1. (△) β-galactosidase; (▼) tryptophan synthetase; (▽) succinic dehydrogenase; (■) dihydroorotic dehydrogenase; (□) 5-dehydroshikimic reductase. Solid lines indicate increase in turbidity (○) and in total protein (●) in parallel cultures. Cultures were grown in glycerol defined medium containing 250 μg. streptomycin/ml.

Although the number of enzymes studied is admittedly limited, perusal of Table 4 suggests that there was no correlation between the physiological function of an enzyme and the degree to which its synthesis was affected by streptomycin deprivation. The severe inhibition of the synthesis of the biosynthetic enzyme tryptophan synthetase is in itself sufficient to explain the arithmetic nature of deprived growth. Schaeffer's (1949*a*, 1950) observations that the Q_{O_2} decreased during deprived growth of streptomycin-dependent bacteria was confirmed for our streptomycin-dependent strains of *Escherichia coli*. This observation is in accord with the substantial inhibition of specific respiratory enzymes shown in Table 4.

Table 4. *The inhibition of enzyme synthesis by deprived growth of* Escherichia coli *strain CS-1*

Figures represent degree of inhibition in the rate of enzyme synthesis relative to the rate of total protein synthesis. Cultures were grown in defined medium with glycerol as the carbon source.

Enzymes with a biosynthetic function		Enzymes functional in respiratory metabolism		Enzymes with no immediately apparent function	
Enzyme	Inhibition (%)	Enzyme	Inhibition (%)	Enzyme	Inhibition (%)
Threonine deaminase	0	Isocitric dehydrogenase	40	Glucose-6-phosphate dehydrogenase	5
Dehydroshikimic reductase	0	DPNH oxidase	40	β-Galactosidase	95
Glutamic dehydrogenase	50	Succinic dehydrogenase	60		
Tryptophan synthetase	80				
Dihydroorotic dehydrogenase	40				

Effect of streptomycin deprivation on the induced synthesis of β-galactosidase

The capacity of the inducible strain CS-2 of *Escherichia coli* to synthesize β-galactosidase during deprived growth was studied by introducing the inducers isopropyl-β-D-thiogalactoside (IPTG) and thiomethyl-β-D-galactoside (TMG) into cultures at various times after the onset of deprivation. As seen in Fig. 8, the inducibility of the organism declined rapidly during deprivation. Inducibility with IPTG was better maintained during deprivation than was inducibility with TMG. It should be noted that the entry of TMG into the cell, and consequently its ability to act as an inducer, is dependent on the activity of a specific inducible permease. IPTG, on the other hand, can enter the cell in the absence of the permease. Consequently β-galactosidase activities measured after induction with IPTG afford a direct measure of the inducible synthesis of β-galactosidase alone, whereas activities measured after induction with TMG reflect the synthesis not only of β-galactosidase, but also of the specific permease. In confirmation of the earlier reports of Polglase (Polglase, Peretz & Roote, 1956) it is evident that streptomycin deprivation severely affected the capacity of a streptomycin-dependent strain of *Escherichia coli* to synthesize inducibly β-galactosidase. In the light of his findings, Polglase assumed that streptomycin deprivation specifically affects the capacity of streptomycin-dependent organisms to respond to induction. This interpretation now appears to be questionable, since the constitutive synthesis of β-galactosidase was likewise very severely affected by deprivation, as shown in Table 4.

Effect of streptomycin deprivation on lysogenic induction

The production of phage by a lysogenic organism requires both the expression of genetic information, and the synthesis of considerable amounts of biologically active DNA. The maintenance of DNA function during streptomycin deprivation can therefore be to some extent assessed by examining the ability of the bacteria

to support phage development. Accordingly the capacity of deprived bacteria of *Escherichia coli* strain W1709 to produce phage λ following ultraviolet (u.v.) irradiation was investigated. Even after 9 hr. of deprivation, the bacteria responded to induction by lysis, with the release of infectious particles. When u.v. induction was applied at various times during the course of streptomycin deprivation, a progressive lengthening of the latent period and a decline in burst size were observed. These effects are probably best explained as resulting from a general decline in the capacity for cellular syntheses, rather than from any specific effect of deprivation on the process of phage formation.

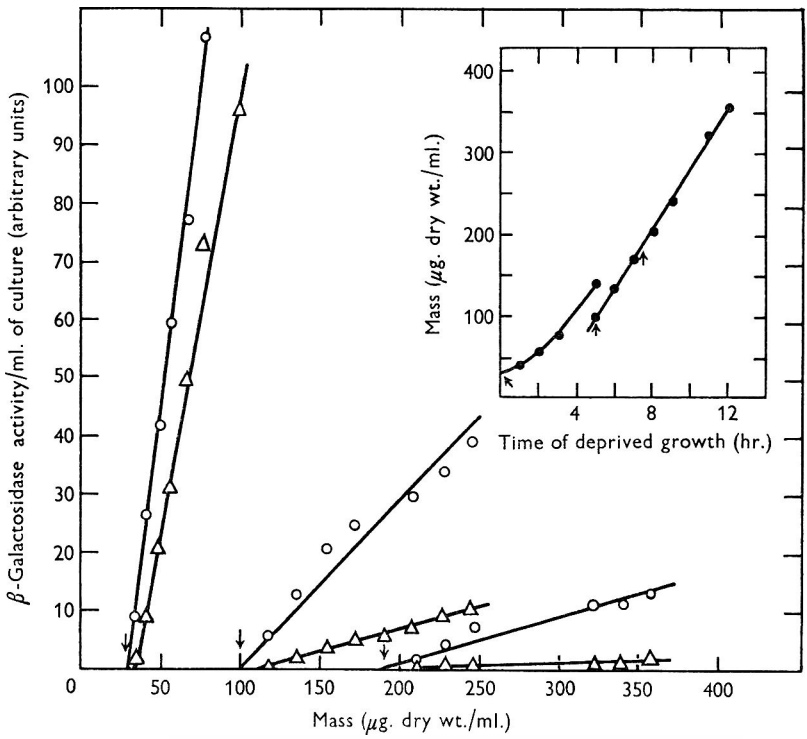

Fig. 8. Induction of β-galactosidase during deprived growth of *Escherichia coli* strain CS-2. Total activity/ml. culture after addition of IPTG (○) or TMG (△) each at 5×10^{-4} M is plotted as a function of cell mass. Inset shows growth of parallel cultures. Time of addition of inducers indicated by arrows.

Recovery

When streptomycin was added to a deprived culture, there was a lag before the rate of increase in turbidity returned to the normal exponential value. The length of this lag was directly proportional to the length of time during which the bacteria had been deprived; it was as little as a few minutes when streptomycin was added early in the arithmetic phase, or it was as long as 6–8 hr. when streptomycin was added toward the end of the arithmetic phase. The cellular changes which accompanied recovery are illustrated in Figs. 9 and 10 for a culture of *Escherichia coli* strain W1709 which was given streptomycin late in the course of arithmetic deprived growth. After the addition of streptomycin, the number of colony-forming units

remained constant for about 2 hr. It then increased rapidly until the relationship between turbidity and viable count characteristic of a normal exponentially growing population had been attained (Fig. 9). As shown by direct microscopic examination, the rapid rise in viable count was preceded by the transformation of the filaments into chains of bacteria, and was accompanied by the fragmentation of those chains. Once an exponential growth rate had been re-established, only very rare filaments were detected in the culture by microscopical examination.

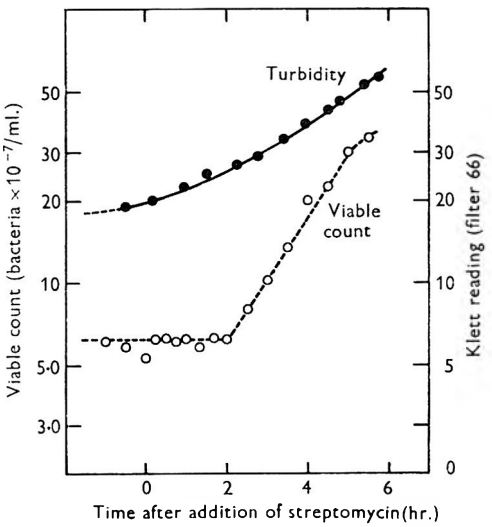

Fig. 9

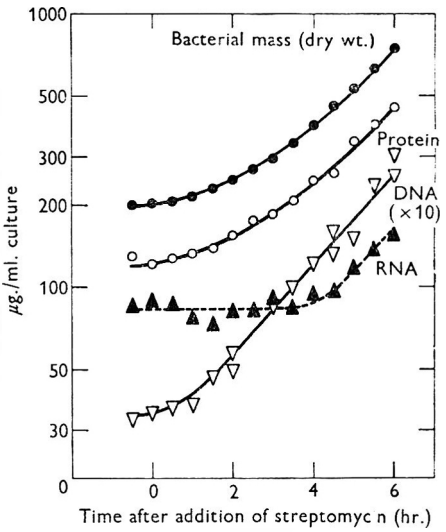

Fig. 10

Fig. 9. Increase in colony-forming units in a culture of *Escherichia coli* strain CS-1 during recovery after 16 hr. of deprived growth. Streptomycin was added at time 0 at 250 μg./ml.

Fig. 10. Increase in RNA, DNA, cell mass and protein in a culture of *Escherichia coli* strain CS-1 during recovery after 16 hr. of deprived growth. Streptomycin was added at time 0 at 250 μg./ml.

The restoration of normal macromolecular composition in the bacteria during recovery involved a distinctive pattern of synthesis (Fig. 10). Following the addition of streptomycin, the rate of synthesis of DNA soon increased to its normal exponential value. The rate of protein synthesis also began to increase soon after the addition of streptomycin, but approached more slowly its normal exponential value. Net synthesis of RNA did not take place until the RNA content of the bacteria had decreased to its normal value as a result of preferential synthesis of the other components of the cell. As soon as this value was reached, RNA synthesis resumed at the normal exponential rate.

DISCUSSION

Previous studies on streptomycin-dependent bacteria have led to two suggestions about the manner in which streptomycin maintains the normal functioning of a dependent organism. Schaeffer (1952) suggested that streptomycin is required either for the synthesis or for the function of the particulate centres of respiration in the

cell. Polglase *et al.* (1956) proposed that streptomycin is specifically required for the synthesis of inducible enzymes. The results which we report here make possible a somewhat broader assessment of the way in which streptomycin regulates the enzymic constitution of streptomycin-dependent organisms. When streptomycin becomes limiting an enzymic imbalance, which affects all classes of enzymes in the cell, becomes established. This imbalance is a consequence of the fact that the synthesis of some specific enzymes is either partly or wholly suppressed by a lack of streptomycin, whereas the synthesis of others continues at a normal differential rate. The selective effects of streptomycin deprivation on enzyme synthesis seem to be random, when examined in terms of enzymic function. There is no reason to believe that the inducibility of constitutivity of an enzyme has any bearing on the way its synthesis will be affected by deprivation.

We also found that streptomycin deprivation had a major effect on the RNA metabolism of the cell. It caused a marked enhancement of the differential rate of synthesis of RNA, which resulted in the overproduction of soluble and ribosomal RNA. As judged by relatively gross criteria, deprivation did not, however, affect the intracellular state of RNA, which cannot be so far distinguished from that in a streptomycin-dependent organism grown with a critical concentration of streptomycin, or in a sensitive organism grown without streptomycin.

The remaining effects which accompanied streptomycin deprivation can be interpreted as secondary consequences of the enzymic imbalance characteristic of the deprived organism. The differential rate of DNA synthesis began to decline markedly only when streptomycin had become severely limiting. Even under these circumstances, nuclear division continued, and the DNA that was synthesized was still functional (as shown by the ability of the deprived lysogenic cell to produce active phage upon induction). There was no cellular lysis or leakage of cellular constituents during deprivation, which suggests that the formation of a normal cell wall and cell membrane continued. Although the specific factors which lead to the cessation of cell division are not known, this consequence of deprivation can also be plausibly regarded as a secondary one. Cell division is readily inhibited by adverse environmental conditions, and many inhibitory agents which differ from one another in their primary mode of action on the organism (e.g. u.v. irradiation, penicillin, mitomycin C) share the common property of blocking cell division.

We are accordingly left with only two biochemical effects which can be considered to stem specifically and more or less directly from the withdrawal of streptomycin: the partial or complete suppression of the synthesis of many enzymes; the high differential rate of synthesis of all forms of RNA. Taken in conjunction, these two effects suggest that in the streptomycin-dependent organism streptomycin plays a role at some point in the interrelated processes of protein and RNA synthesis.

To make a more specific inference about the location of streptomycin action, we must consider the implications of the quantitative effects of streptomycin on the growth of streptomycin-dependent organisms. The first of these is the extremely regular correlation of the exponential growth rate with streptomycin concentration over a wide range of concentrations, and its abrupt change to insensitivity at the critical concentration. The second is the quantitative relation of deprived growth to the concentration of streptomycin to which the streptomycin-dependent organisms were previously exposed. The existence of this relationship with respect to the

extent of residual cell division was first shown by Demerec *et al.* (1949). We have found a similar relationship with respect to the duration and the extent of residual exponential growth. The interpretation of all these facts has become easier with the discovery that the streptomycin-dependent cell can bind streptomycin irreversibly, and that growth with a supra-critical concentration of streptomycin results in the binding of this compound in two forms distinguishable by the means required to extract them from the cells.

It is difficult to avoid the conclusion that the streptomycin-dependent organism contains a number of specific sites, all of which must be combined with streptomycin for the maintenance of normal cellular function. The critical concentration of streptomycin can then be interpreted as that external concentration which just suffices to keep all the specific sites within a growing population combined with streptomycin. At subcritical concentrations, the external streptomycin concentration is too low to saturate all the internal specific sites, and the bacteria suffer a functional impairment which is grossly expressed as exponential growth at a submaximal rate. At supra-critical concentrations, the bacteria contain more streptomycin than the amount required to saturate the specific sites, and consequently possess an internal pool, the size of which is directly related to the external streptomycin concentration. Our data on streptomycin binding by streptomycin-dependent organisms grown with a supra-critical concentration of streptomycin fit very well with this assumption. The fraction of bound streptomycin which can be extracted from the organisms with hot water might be considered to represent the streptomycin of the internal pool, and the fraction extractable only with hot perchloric acid to represent the streptomycin bound to specific sites. It should be noted, however, that even the former fraction is not able to diffuse out of the cell, since it cannot be exchanged with external streptomycin. The internal pool is therefore probably not a 'free' one, but is non-specifically bound by other internal constituents of the cell.

The quantitative aspects of deprived growth by streptomycin-dependent organisms previously grown with a supra-critical concentration of streptomycin can be explained on the basis of the assumptions made in the last paragraph. Under conditions of deprivation, the non-diffusible internal pool of streptomycin can be drawn upon to saturate newly formed specific sites. Normal growth and function can thus be maintained for a limited period, the duration of which is proportional to the size of the internal pool, which is in turn a function of the external concentration of streptomycin to which the organism had been previously exposed.

Is it possible to make any inferences concerning the nature of the postulated specific sites from the available data? In view of the magnitude of streptomycin binding by the streptomycin-dependent organism, we suggest that the number of specific sites is large: i.e. of the order of 10^4 to 10^5. Each streptomycin-dependent bacterium contains about 120,000 molecules of firmly bound streptomycin, not extractable by hot water. Even if we assume that several of these molecules are attached to each site, the number of sites must therefore be relatively great. A second inference about the sites can be drawn from our observations about the critical concentration of streptomycin. Under any given set of environmental conditions, this critical concentration was constant, but its magnitude changes with the nature of the carbon source. The critical concentration was considerably higher

for glucose, the carbon source which supports the highest growth rate of *Escherichia coli*, than for succinate and glycerol which support lower (and similar) growth rates. Since we interpret the critical streptomycin concentration as that concentration which is just sufficient to saturate all the specific sites in the organism, it follows that the number of specific sites in each organism is not absolutely fixed, but can fluctuate within certain limits in response to a change in the nature of the carbon source.

Both these inferred properties of the specific combining sites can be explained by the hypothesis that they are the ribosomes. We accordingly propose that the miscellaneous biochemical and physiological peculiarities of a streptomycin-dependent organism ultimately reflect a genetic impairment of ribosomal structure, the functional consequences of which can be specifically overcome by combination of the ribosomes with streptomycin. The postulated impairment of structure must obviously be a minor one (perhaps the change of a single amino acid in the ribosomal protein) since the ribosomes of streptomycin-dependent organisms showed no gross abnormalities of composition or sedimentation behaviour.

Under conditions of streptomycin deprivation, impaired ribosomal function causes an imbalance in the pattern of enzyme synthesis, coupled with a decline in the rate of gross protein synthesis. This, in turn, provokes an over-production of RNA through the derangement of the feed-back control mechanism which normally keeps protein and RNA synthesis in metabolic balance. We shall not further develop the strictly biochemical aspects of this hypothesis here, since they are treated at length in another paper, which is devoted to a general theoretical interpretation of streptomycin sensitivity, resistance and dependence in bacteria (Spotts & Stanier, 1961).

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Relationship of Sodium Chloride Tolerance and Serological Group of Staphylococcal Phages

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SUMMARY

Twenty-two staphylococcal bacteriophages representing serological groups A, B, F and L were examined for activity after incubation of free phage in 10 % (w/v) NaCl Trypticase soy broth for 24 hr. at 30°. The mean decrease in plaque-forming units for ten phages of serological group A was 13 %; in no instance was inactivation of any group A phage greater than 33 %. All but one of nine group B phages were 88 % or more inactive after incubation in the 10 % NaCl broth; the single exception was phage 83 with slightly more than 40 % inactivation in both 0·5 % or 10 % NaCl broth. Serological group F phage 42D resembled phages of group A, whereas phage 77 of group F and group L phage 187 were intermediate between group A and B phages with respect to NaCl stability.

INTRODUCTION

The effect of the ionic environment on bacteriophages has been the subject of many investigations. Relatively few of the studies reported, however, have been concerned with the stability of free phages in the presence of high concentrations of salt. Bronfenbrenner (1925) observed that concentrations of NaCl greater than 1 % inactivated 99 % of phage, although the particular phage tested and time required for inactivation were not specified in his report. Adams (1949) examined the inactivating effects of temperature on *coli* phages, particularly T5, in concentrations of NaCl ranging from 0·01 to 2 N. He found that inactivation of phage T5 was almost complete after 1 hr. at 37° in solutions containing less than 0·2 N-NaCl, whereas at concentrations of 0·4 N and 0·8 N there was no significant decrease in plaque count. Increase in sodium ion concentration resulted in increased stability of phages to the inactivating effects of temperature (about 50° to 70°); phage T5 was equally stable in 2 N-NaCl solution and in nutrient broth. In studies on the effect of electrolytes on the adsorption of staphylococcal phages, Rountree (1951) noted that, in the free state, some phages were partially inactivated by 1 % sodium citrate. Experiments to determine the quantity and kind of electrolytes giving maximum adsorption included 20 min. incubation of free phages, as well as phage + bacteria mixtures, in aqueous solutions which contained a high percentage of NaCl. Some staphylococcal phages were unstable in such simple salt solutions although adsorption occurred in certain instances.

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The present work was begun when it was observed that staphylococcal phage 81 lysed its propagating staphylococcus on agar medium containing concentrations of NaCl up to 10 %, whereas some other staphylococcal phages were inactive at NaCl concentrations greater than 3 %.

METHODS

Phages and staphylococci. Phages were selected on the basis of preliminary observations and as examples of each of the staphylococcal typing groups. Arranged according to serological group the phages used were as followss

Serological group	Phages
A	3A, 3B, 3C, 7, 42B, 42E, 47, 47C, 75, 81
B	29, 52, 52A, 79, 80, 55, 71, 44A, 83
F	42D, 77
L	187

Staphylococci used were the phage propagating strains originally obtained, together with the phages, through the courtesy of Dr J. E. Blair.

Preparation of phage suspensions. Corresponding dilutions of each phage, designed to contain from 100 to 300 plaque-forming units/0·1 ml., were made in 10 ml. amounts of (1) commercially prepared (Baltimore Biological Laboratories; BBL) Trypticase soy broth of 0·5 % (w/v) NaCl and (2) the same broth to which sufficient NaCl was added to make a final concentration of 10 % (w/v). At 10 % NaCl concentration the pH value of the broth was decreased from 7·2 to 6·8. No adjustment of pH value was made since, in previous experiments, variation of pH value within this range had no effect on the lytic activity of ten representative phages tested in media containing 0·5, 3, 7·5 and 10 % (w/v) NaCl.

Plaque counts. Within 5–10 min. after dilution samples (0·3 ml.) of each phage broth were withdrawn and dispensed in 0·1 ml. amounts to each of three 13 × 100 mm. tubes which contained 0·1 ml. of a 6-hr., 37°, Trypticase soy broth culture of the appropriate staphylococcus. Immediately thereafter 3 ml. melted cooled soft nutrient agar (Trypticase soy broth + 0·6 %, w/v, agar) was added to each tube; the contents were mixed and layered over the surface of regular Trypticase soy agar (BBL) in a standard Petri plate. When the soft agar layer was firm, the plates were inverted and incubated at 30°. All plaque counts except those of phages 71 and 79 were made after 24 hr. incubation. Plaques of phages 71 and 79 were counted after 18 hr. because further incubation resulted in overgrowth by the staphylococci. After withdrawal of the initial (0) sample, each phage-broth was incubated at 30°. At the end of 24 hr. incubation, plaque counts of both the 0·5 % and 10 % NaCl broth suspensions were made according to the method used for determining the initial counts. In a series of similar experiments plaque counts were made at intervals throughout the 24 hr. period.

RESULTS

The results of plaque counts before and after 24 hr. incubation at 30° in 0·5 % and 10 % NaCl broth indicated that phages of serological group A were more stable in the presence of 10 % NaCl than were the group B phages. Of the ten group A

phages studied only phage 47 C showed as much as 33 % decrease in plaque-forming units, whereas the others of this group retained 72 % or more of their activity after exposure to the higher concentration of NaCl (Fig. 1). The mean survival of the ten group A phages, after 24 hr. in 10 % NaCl broth, was 87 %. Conversely, eight of the nine group B phages tested showed 88 % or more inactivation under the same conditions (Fig. 2). The one exception, phage 83, though less sensitive to

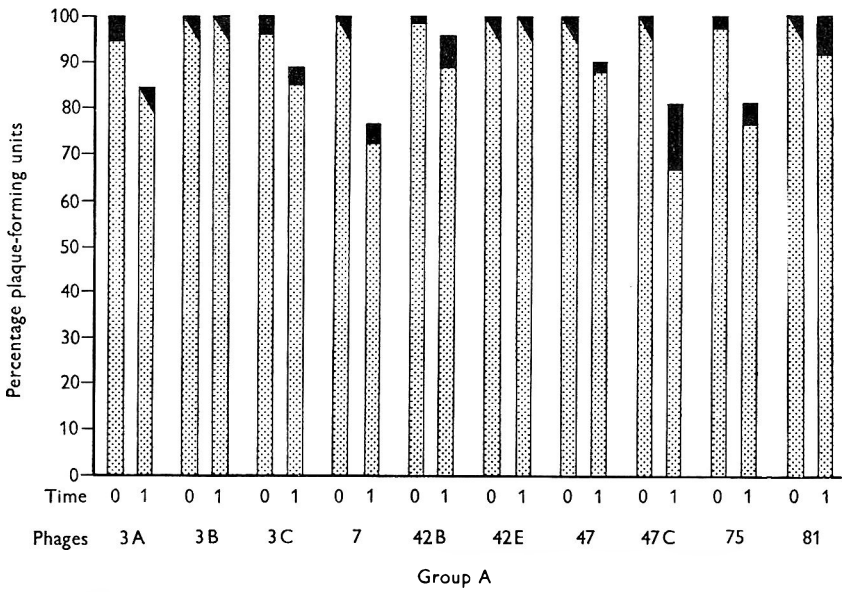

Fig. 1. Activity of serological group A staphylococcal phages after incubation of free phage in 0·5 % NaCl or 10 % NaCl in Trypticase soy broth at 30°. Percentages based on plaque counts of initial phage suspension (0) and after 24 hr. (1). ▬, 0·5 % NaCl broth; ▨, 10 % NaCl broth.

NaCl than the rest of the group B phages, was inactivated to about the same degree in 0·5 % NaCl broth as in 10 % NaCl broth, 41 and 44 % decrease, respectively. (Results with phage 83 do not appear in Fig. 2 because its serological group became known to us, through personal communication from Miss Miriam Carr, after completion of the figure.) The NaCl sensitivity of the group B phages was also shown by a decrease in the number of plaque-forming units within 5–10 min. after initial contact of most of these phages with 10 % NaCl broth. Such an immediate effect was not produced by exposure of group A phages to the same 10 % NaCl broth. Results with group F phages (42 D and 77) and group L phage 187 indicated that they were more NaCl tolerant than the group B phages, but only phage 42 D resembled a typical group A phage in this respect (Fig. 2).

Comparison of the mean percentage decrease in plaque counts after holding the group A and group B phages in 0·5 % NaCl broth and in 10 % NaCl broth for 24 hr. is presented in Table 1; the extremes in variation of the stability of individual phages within each group are also shown. It will be noted that survival of active group A phages in 0·5 % NaCl broth and in 10 % NaCl broth are remarkably similar. Most of the group B phages, as well as those of group A, appeared to be

quite stable in 0·5 % NaCl broth, although plaque count decrease in the case of phage 55 after 24 hr. incubation in 0·5 % NaCl broth was 59 %, as compared to 88 % in 10 % NaCl broth. The instability of phage 55 in ordinary Trypticase soy broth was exceeded only by that of group L phage 187, which proved to be 67 % inactive after 24 hr. in 0·5 % NaCl broth and 72 % inactive after the same time in 10 % NaCl broth.

In other experiments, plaque counts of samples taken at intervals throughout the incubation period revealed that, when the activity of a phage was decreased after

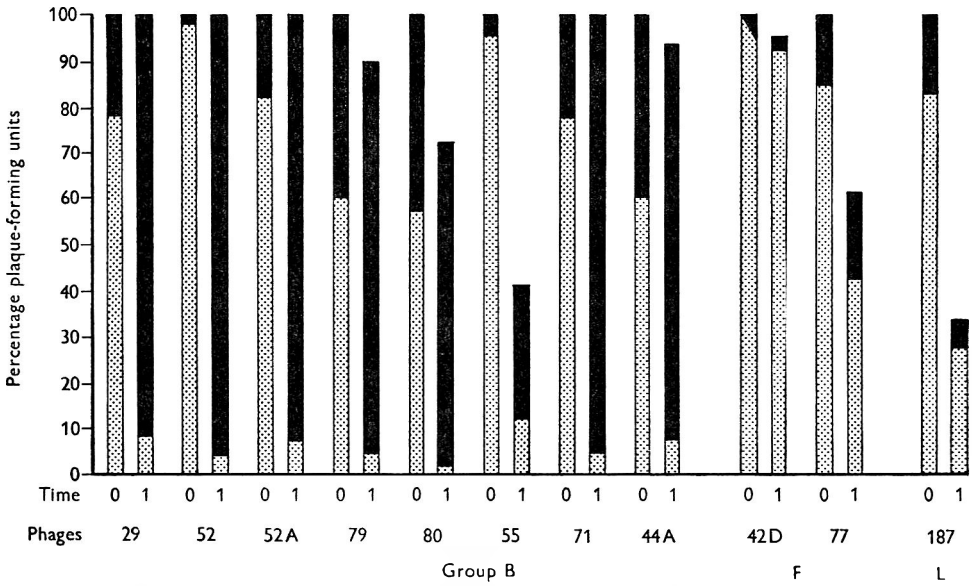

Fig. 2. Activity of serological group B, F and L staphylococcal phages after incubation of free phage in 0·5 % NaCl or 10 % NaCl in Trypticase soy broth at 30°. Percentages based on plaque counts of initial phage suspension (0) and after 24 hr. (1). ▬, 0·5 NaCl broth; ▒, 10 % NaCl broth.

Table 1. *Percentage inactivation of staphylococcal phages after 24 hr. incubation in 0·5 or 10 % NaCl Trypticase soy broth at 30°*

Serological group	% NaCl in broth	Group mean	Most stable phage	Least stable phage
		After 24 hr., 30°		
		% decrease in plaque count		
A (10 phages)	0·5	11	0	24
	10	13	0	33
B (9 phages)	0·5	17	0	59
	10	88	44	99
F (2 phages)	0·5	—	4	39
	10	—	8	58
L (1 phage)	0·5	—	67*	—
	10	—	72*	—

* Only one phage (187) studied.

24 hr. incubation in the presence of either 0·5 % or 10 % NaCl, a continuous decline in the quantity of active phage occurred throughout this period. Some typical results of plaque counts made during the first half of the period are given in Table 2. In the case of phage 29, for example, diminution in number of plaque-forming units after incubation in 10 % NaCl broth for 4 hr. was 24 %, after 12 hr. it was 70 % and over 90 % inactivated after 24 hr.

Table 2. *Plaque counts of typical staphylococcal serological group A phages* (42B *and* 81) *and group B phages* (29 *and* 70) *after initial dilution and incubation at* 30° *in* 0·5 % *or* 10 % NaCl *Trypticase soy broth*

	Staphylococcal phages							
	Phage 42B		Phage 81		Phage 29		Phage 80	
Time (hr.)	0·5 % NaCl broth	10 % NaCl broth	0·5 % NaCl broth	10 % NaCl broth	0·5 % NaCl broth	10 % NaCl broth	0·5 % NaCl broth	10 % NaCl broth
	Number of plaque-forming units							
0	250	247	137	137	188	178	203	179
4	247	260	128	141	171	135	142	43
10–12*	217	223	120	112	168	53	103	16

* All 12 hr. counts except those of phage 81 which were made after 10 hr.

DISCUSSION

In addition to distinctive antigenicity, serological groups of staphylococcal phages are characterized by other properties including host range, plaque size, ability to form lysogenic systems and stability (Rippon, 1956). Rountree (1949) pointed out the relative instability of serological group B phages, particularly their marked sensitivity to heat and storage at 4°, as compared with that of the group A and F phages. In the first serological investigation of staphylococcal phages Burnet & Lush (1935) observed that these phages could be divided into two categories, citrate-sensitive and citrate-insensitive. Rountree (1951) reported that most staphylococcal phages, when in the free state, were inactivated to some degree by 1 % (w/v) Na citrate and those most completely inactivated belonged to serological group B. In the same study, which was concerned chiefly with the effect of electrolytes on adsorption, evidence was presented that each phage had a characteristic calcium requirement. In all but two of the phages examined Na citrate inhibited phage adsorption and this effect was attributed to the action of the citrate radical which, it was suggested, bound the calcium on the surface of the phage and thus blocked the adsorption site. The absolute calcium requirement for propagation of serological group B phages was reported by Blair & Williams (1961); phage 47C (group A) and phage 187 (group L) were the only other staphylococcal phages in this category.

In the present work the staphylococcal phages which were least stable when incubated without staphylococci in 10 % NaCl Trypticase soy broth were also those of serological group B. The marked diminution in activity of these phages might be due to the effect of the NaCl on free phage before contact with the staphylococci

or to the failure of adsorption because of the NaCl introduced with phage to the phage + bacteria mixture. If inhibition of adsorption as proposed by the latter explanation were solely responsible, equal diminutions in plaque counts should occur in the initial (0) sample and the 24 hr. sample which were both taken from 10 % NaCl broth. Mixing of 0·1 ml. of this broth with an equal amount of the broth culture of the propagating strain of staphylococcus, followed by immediate addition of 3 ml. soft agar, quickly diluted the concentration of NaCl to about 0·8 %. The quantity of NaCl was further decreased on plating the soft agar. Rountree (1951) found that with the citrate-sensitive phages adsorption in NaCl was variable and, when it occurred, recovery of active phage from the bacteria infected in NaCl was less than from bacteria infected in $CaCl_2$. These conclusions were based on experiments in which phages and bacteria were held 20 min. in aqueous solutions of NaCl at lower concentrations than those in the phage + bacteria mixtures tested here. In our experience the most unstable phages of group B lyse susceptible staphylococci when 1 % NaCl Trypticase soy agar is used in the routine typing procedure.

If inactivation of free phage occurs in the NaCl broth before mixing with the staphylococci, one would expect a continuing decrease in the number of plaque-forming units with increase in time. This was observed to occur. In the case of the group B phages there was an immediate decrease, followed by progressive decrease in plaque count until, at the end of 24 hr., 12 % or less of the original activity remained. Group A phages showed little, if any decrease in activity on initial contact with 10 % NaCl broth; when the activity was decreased after 24 hr. plaque counts made at 4–8 hr. intervals became successively lower. The inactivation of staphylococcal phages observed here appears to be an effect of NaCl on free phage.

The NaCl instability of serological group B staphylococcal phages observed in this work parallels their requirement for calcium. Two phages other than those of serological group B, which have an absolute calcium requirement are 47C (group A) and 187 (group L) (Blair & Williams, 1961). Phage 47C proved to be the most NaCl sensitive of the group A phages tested, but it was much more NaCl stable than any group B phage. The salt tolerance of phage 187 and of the exceptional group B phage 83 was intermediate between that of the average group A and group B phages, but they were almost as unstable in 0·5 % NaCl broth as in 10 % NaCl broth. In general, inactivation in the higher concentration of NaCl was independent of stability in regular broth. Most phages retained their activity in 0·5 % NaCl broth, although there were individual differences within each group. No phage was more stable in 10 % than in 0·5 % NaCl broth. NaCl tolerance appears to be a characteristic of certain staphylococcal phages, particularly those of serological group A, and this property may be useful in presumptive identification of serological group or in other classification schemes.

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Saccharomyces hienipiensis, a New Melibiose-fermenting Yeast, Unable to Assimilate Raffinose

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(*Received 5 September* 1961)

SUMMARY

A new *Saccharomyces* species has been isolated from 'alpechin'. It is distinguished from other species of the genus by its ability to ferment and assimilate glucose, maltose and melibiose. Galactose, sucrose, lactose and raffinose are not assimilated. The melibiase found in *S. hienipiensis* can hydrolyse raffinose.

INTRODUCTION

A new melibiose-fermenting yeast has been isolated from 'alpechin' (aqueous solution separated during the manufacture of olive oil), which apparently cannot be identified with previously described species. I propose to name the new yeast by the latin name (Hienipa) of Alcalá de Guadaira, the village in the south of Spain (Sevilla) from which the first strain was obtained.

METHODS AND RESULTS

Origin of the strain. Two strains were studied, both isolated from 'alpechin' received in December 1959 from Alcalá de Guadaira, Sevilla, Spain. The isolations were effected by streaking the alpechin directly on potato glucose agar (Difco) acidified to pH 3·5 with sterile tartaric acid (10 %, w/v solution) after sterilization.

Methods. The characters were examined by the methods of Lodder & Kreger-van Rij (1952), with some exceptions as regards the procedure for studying sugar assimilation. For these latter tests the method of Wickerham (1951) was followed, as well as for the vitamin deficiency tests, growth at moderate osmotic pressure, etc.

Description of Saccharomyces hienipiensis

Growth on malt extract. After 3 days at 25° cells are oval to long oval 2·6–6·5 × 3·9–14·5 μ, occurring singly. A sediment is formed (Fig. 1).

On malt agar the streak after 1 month is filiform, cream coloured, soft, smooth and shining. Margin smooth.

Slide culture. No pseudomycelium is formed.

Sporulation. In MA 29 sporulation medium (Santa Maria, 1959; MA 29 sporulation medium = Kleyn medium (Kleyn, 1954) + 0·1 %, w/v DL-serine, British Drug Houses Ltd.), spores are formed moderately after 7 days of incubation at 25°; 1–4 spores per ascus (Fig. 2). The spores are round or oval and have a smooth surface. No cases of conjugation were observed before spore formation, but only partheno-

genetic production of asci. Yet, isogamous or heterogamous conjugation of cells, without spores, were sometimes observed. The asci do not rupture at maturity.

Fermentation. Glucose +; maltose +; melibiose +; galactose −; sucrose −; lactose −; raffinose −.

Fermentative power: After 19 days of anaerobic incubation at 25°, yeast water with 13·6 % (w/v) glucose is fermented and the ethanol produced is 8·9 % (w/v).

Assimilation. Glucose, maltose, trehalose, melibiose and pyruvic acid (latent and weak) are assimilated. Compounds not assimilated: galactose, L-sorbose, sucrose, cellobiose, lactose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine hydrochloride, ethanol, glycerol, i-erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, alpha-methyl-D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, ethyl acetoacetate, i-inositol.

Assimilation of KNO_3. Negative.
Growth in a vitamin-free medium. None.
Growth in the osmotic pressure medium. None
Growth at 37°. Positive.

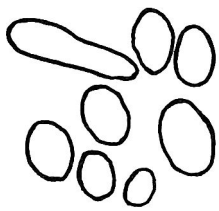

Fig. 1

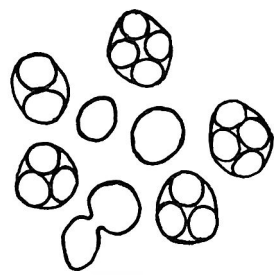

Fig. 2

Fig. 1. *Saccharomyces hienipiensis* sp.nov. After 3 days in malt extract medium at 25°. ×2000.

Fig. 2. *Saccharomyces hienipiensis* sp. nov. Ascospores on MA 29 sporulation medium after 7 days at 25°. ×2000.

The strains studied must undoubtedly be included in the genus *Saccharomyces*, given their general characteristics (method of vegetative reproduction, non-formation of pellicle, non-assimilation of KNO_3) and their capacity for fermentation. Up to the present, no species is known in this genus whose biochemical characteristics for fermentation and assimilation correspond to those described here; therefore it would seem that these strains ought to be considered as a new species.

Until Van Uden & Assis-Lopes (1957) described *Saccharomyces italicus* var. *melibiosi*, no yeast was known with the ability to ferment raffinose but not sucrose. Later Santa Maria (1958) described a new species, *S. oleaginosus*, with a similar biochemical spectrum. A comparative study of these two yeasts awaits publication. The species here described is a yeast capable of fermenting melibiose but which neither ferments nor assimilates sucrose or raffinose.

To test the action of the melibiase of this yeast on raffinose, the residual sugars in the assimilation tubes were identified chromatographically, by the following paper

chromatographic technique. The descending method was used, with development for 48 hr. with solvent *n*-butanol + acetic acid + water (4 + 1 + 5 by vol.) on Schleicher 2040 A paper. The papers after drying were sprayed with aniline hydrogen phthalate for aldohexoses and with urea solution for hexuloses, and heated at 105° for 10 min.

The normal test-tubes for assimilation of melibiose and raffinose were examined chromatographically after 21 days of incubation. The sugar present in the melibiose tube was galactose; and in the raffinose tube was raffinose. Because of this result, two assimilation tubes with melibiose and raffinose, respectively, were each inoculated with growth from the whole of a streak of 48-hr. growth on a malt agar slope. The sugar tubes were incubated for 11 days; then by chromatography it was found that the residual sugars were: in the melibiose tube, galactose; and in the raffinose tube, raffinose, sucrose and galactose. Finally, sterile glucose 0·1 % (w/v) was added to the raffinose assimilation tubes which were inoculated in the usual way and incubated for 21 days at 25°. The residual sugars in this case were also raffinose, sucrose and galactose.

It seems, therefore, that the melibiase of *Saccharomyces hienipiensis* is a constitutive enzyme capable of hydrolysing the raffinose to sucrose and galactose. The negative result obtained in the normal raffinose tubes was undoubtedly due to the fact that few organisms inoculated were incapable of development, as they had no assimilable carbon source and their ability to hydrolyse raffinose was not sufficient to be shown by chromatography. When the amount of cellular material was increased, either by direct addition or by stimulating its development in presence of raffinose, a clear hydrolysis was produced.

We are of the opinion, therefore, that *Saccharomyces hienipiensis* is not only a new species, but may also elucidate points in the genetics of the fermentation of melibiose (Roberts, Ganesan & Haupt, 1959).

One of the two strains of *Saccharomyces hienipiensis* has been chosen as the type of the species and is maintained under reference no. Le Ac 132, in the culture collection of the Sección de Bioquímica, Instituto Nacional de Investigaciones Agronómicas, Madrid. A subculture has been deposited in the Yeast Collection of the Centraal Bureau voor Schimmelcultures in Delft.

Latin diagnosis

Saccharomyces hienipiensis, sp.nov.

In musto maltato cellulae ovoideae aut long ovoideae 2·6–6·5 × 3·9–14·5 μ, singulae. Sedimentum formantur.

In agaro maltato cultura (post unum mensem, 17°) mollis, glabra, nitida, flavalbida. Margine glabro.

Pseudomycelium nullum.

Asci formantur ex transformatione cellularum vegetativarum diploidearum; ascosporae rotundae aut ovoideae; 1–4 in asco.

Glucosum, maltosum et melibiosum fermentatur at non galactosum, saccharosum, lactosum et raffinosum.

In medio minerali glucosum, maltosum, trehalosum, melibiosum et acidum pyruvicum (exiguum) assimilantur, at non galactosum, sorbosum, saccharosum, cellobiosum, lactosum, raffinosum, melezitosum, inulinum, amylum solubilis,

xylosum, arabinosum, ribosum, rhamnosum, alcohole aethylicum, erythritolum, adonitolum, dulcitolum, mannitolum, sorbitolum, alpha-methyl-glucosidum, salicinum, acidum lacticum, acidum succinicum, acidum citricum, acetoacetate aethylicum et inositolum.

Nitras kalicus non assimilatur.

Necessariae ad crescentiam sunt vitamina externae.

Isolate ex amurca in Alcalá de Guadaira (Sevilla, España).

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Catabolism of Nitrogenous Compounds by Pseudomonas

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SUMMARY

The rate of O_2 utilization by both pigmented and non-pigmented strains of *Pseudomonas aeruginosa* was greater with 2% (w/v) Casitone as substrate than with 0·1 M-glucose as substrate. *P. aeruginosa* readily catabolized Casitone, amino acids and other nitrogenous compounds with the production of an alkaline reaction even in the presence of 0·5% (w/v) glucose. Other species of Pseudomonas studied varied in their utilization of these compounds, suggesting a different and possibly satisfactory approach to the identification and classification of species within the genus *Pseudomonas*.

INTRODUCTION

The ability of *Pseudomonas aeruginosa* to produce acid from carbohydrates, in particular from glucose, in amounts sufficient to lower the pH value of the commonly used carbohydrate media is generally variable and to a large extent depends upon the nitrogenous compounds present in the medium. Simon (1956) stated that the production of blue-green pigment and failure to ferment any of the usual coliform test sugars, except occasionally glucose, are generally regarded as characteristic of '*P. pyocyanea*'. She found that the fermentation of sugars by Pseudomonas cultures was masked in the peptone medium by the production of free ammonium and a consequently high pH value. The recent studies of Warren, Ells & Campbell (1960) indicated that *P. aeruginosa* grown in a glucose ammonium phosphate medium accumulated nitrogenous materials which were oxidized during endogenous respiration. Rhodes (1959) found that after incubation for 7 days in peptone water the pH values of the media of all Pseudomonas strains studied were between pH 8·3 and pH 8·7. Sherris, Shoesmith, Parker & Breckon (1959) showed that nearly all of the Pseudomonas strains they tested were able to break down arginine more rapidly than could most other Gram-negative bacilli and suggested that this characteristic might prove to be a useful diagnostic feature. It is evident, therefore, that many of the Pseudomonas species produce an alkaline reaction when grown in the presence of peptides or amino acids. The present study was undertaken to determine the utilization of nitrogenous compounds by several species of Pseudomonas and in particular *P. aeruginosa*.

METHODS

The strains of *Pseudomonas aeruginosa* studied were obtained from our stock culture collection and from the diagnostic laboratory. All cultures were identified by the methods described by Gaby & Free (1953, 1958) and included pigmented and non-pigmented varieties. The strains, selected at random, were grown on nutrient agar or in nutrient broth at 37°. Species other than *P. aeruginosa* obtained

from the American Type Culture Collection were *P. saccharophilia* no. 9114, *P. oleovorans* no. 8062, *P. ovalis* no. 950 (grown at 25° and 37°), *P. fragi* no. 4973, *P. fluorescens* no. 11251, and *P. mucidolens* no. 4685 (grown at 25°).

Suspensions of organisms for manometric studies were obtained by growing the bacilli on agar slopes or in shaken flask broth cultures for 18 hr. at 25° or 37°. The organisms were washed thoroughly with saline or phosphate buffer, resuspended in 0·1 M-phosphate buffer, and diluted to give an optical density of 1·35 at 515 mμ on a Colman Junior Spectrophotometer Model 6A. This dilution is equivalent to $6{\cdot}87 \times 10^9$ viable organisms, 12·5 mg. wet weight or 2·2 mg. dry weight of organisms per ml. Oxygen consumption was measured by conventional Warburg techniques.

To determine alkalinity produced by the Pseudomonas species from peptides and amino acids, the various strains were inoculated into a liquid medium containing (%, w/v) Casitone (Difco) 1·0, glucose 0·5, amino acid 0·5, adjusted to pH 7·2 and containing 0·02% phenol red indicator. The amino acids studied were DL-alanine, DL-serine, and DL-methionine (see Table 1). Asparagine, arginine, leucinamide and glycinamide were also included because of their different amino groupings. The cultures were incubated at either 25° or 37°, depending upon their optimum temperature requirement, and colour changes of the pH indicator were noted for periods up to 72 hr.

RESULTS

The results shown in Fig. 1 illustrate the stimulation in the rate of O_2 consumption of a representative pigmented strain of *Pseudomonas aeruginosa* by increasing concentrations of Casitone from 0·1 to 2·0% (w/v). Three pigmented and three non-pigmented strains tested under these conditions gave essentially the same results. On the other hand, the rate of O_2 utilization of four brown (brownish-purple) pigmented strains was only one-half to three-quarters of that shown in Fig. 1. The results in Fig. 2 show that with both pigmented and non-pigmented strains the rate of O_2 utilization was greater with 2% (w/v) Casitone as substrate than with 0·1 M glucose. All the strains produced a slight amount of acid in a glucose + peptone basal medium on primary isolation. It is evident from these results that the six *P. aeruginosa* strains tested not only oxidized Casitone at a faster but also more uniform rate than glucose. The results at least partially explain the frequently reported observations that *P. aeruginosa* strains may or may not produce acid in a glucose medium, particularly when the medium contains peptone.

Since Casitone contains amino acids and peptides, experiments were carried out to determine the rate of O_2 utilization of *Pseudomonas aeruginosa* with various single amino acids as substrate. The results in Table 1 show that the rate of O_2 utilization by *P. aeruginosa* varied considerably among the 18 different amino acids tested, ranging from 18·5 μl. O_2 uptake/90 min./mg./dry wt. bacteria with DL-methionine to 74·1 μl. O_2 uptake with DL-serine. These results are in agreement with those of Silberman & Gaby (1961) which showed a correlation between the rate of O_2 consumption by *P. aeruginosa* of various amino acids as substrates and the uptake of the corresponding ^{14}C labelled amino acid by the lipid complex of *P. aeruginosa*.

The results in Table 2 list the reactions of the cultures in the media containing the various nitrogenous compounds. All strains of *Pseudomonas aeruginosa* gave an alkaline reaction within 24 hr. in the media containing asparagine, arginine, leucinamide and glycinamide with the exception of four strains which required

48 hr. to produce an alkaline reaction in the media containing glycinamide and leucinamide. Four of the *P. aeruginosa* strains produced either an acid reaction or no indicator colour change in the media containing the four amino acids. The reactions of the other Pseudomonas species in the media differed considerably, not only from *P. aeruginosa*, but also indicated distinct differences between individual species. When peptone was substituted for Casitone in the test medium the results were similar with the exception that alkaline production was less rapid. The other Gram-negative bacilli which normally ferment glucose with the production of much stronger acids continued to do so in the presence of the nitrogenous compounds tested.

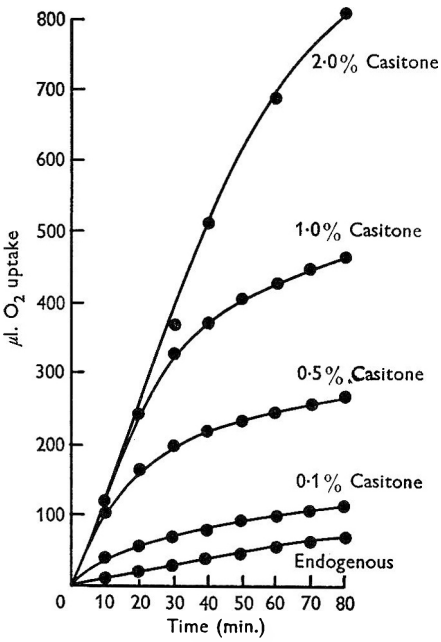

Fig. 1

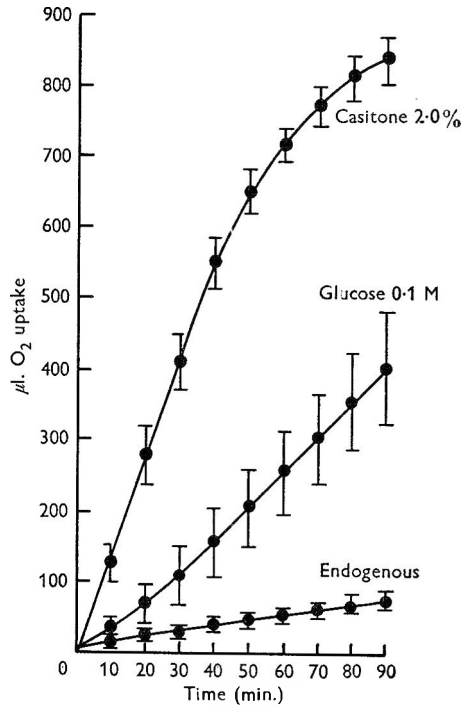

Fig. 2

Fig. 1. The effect of increasing concentrations of Casitone on rate of O_2 utilization by *P. aeruginosa*. See legend Table 1, p. 382 for conditions.

Fig. 2. Comparison of the rate of O_2 utilization by 6 strains of *P. aeruginosa* with 2% Casitone and 0·1 M glucose as substrates. Vertical lines represent the extent of individual strain variation. See legend Table 1, p. 382 for conditions.

DISCUSSION

The results emphasize the ability of *Pseudomonas aeruginosa* to catabolize nitrogenous compounds. It is of interest to note that there was an excellent correlation found between pigmentation, ability of these strains to utilize nitrogenous compounds, and mouse pathogenicity. The green pigmented and non-pigmented strains of *P. aeruginosa* were found to be weakly pathogenic for mice, whereas 0·5 ml. of an 18–24 hr. undiluted broth culture of the brown pigmented strains failed to kill when injected intraperitoneally into mice (Gaby & Logan, 1961).

Table 1. *Effect of amino acids on O_2 uptake by washed suspension of* Pseudomonas aeruginosa

The Warburg vessels contained, 2·0 ml. suspension in phosphate buffer (pH 7·0); 1·15 mg. amino acid in 0·3 ml. phosphate buffer in the side arm; 0·2 ml. 20 % KOH in the centre well; atmosphere, air; bath temperature 37°.

DL-Amino acid substrate	O_2 uptake (μl./90 min./mg. bacterial dry wt.)		Ratio: substrate/ endogenous
	Endogenous	Substrate	
Methionine	15·7	18·5	1·18
Hydroxyproline	20·0	24·2	1·21
Histidine	20·0	27·4	1·37
Cystine*	20·8	28·9	1·39
Tyrosine	15·7	22·6	1·44
Leucine	21·8	32·5	1·49
Tryptophane	18·3	29·3	1·60
Lysine	21·0	37·6	1·79
Glycine	16·4	30·8	1·84
Isoleucine	20·8	40·5	1·94
Phenylalanine	21·2	45·0	2·03
Valine	17·8	36·5	2·05
Glutamic acid	19·1	39·8	2·08
Cysteine	18·3	37·6	2·11
Threonine	15·7	33·5	2·13
Asparagine	21·2	45·6	2·15
Alanine	21·2	70·3	3·31
Serine	26·4	74·1	3·63

* Added in a slurry.

The limited number of Pseudomonas species studied, other than *Pseudomonas aeruginosa*, indicated differences in their ability to catabolize nitrogenous compounds. These reactions were consistent on repeated testing and indicate the possibility of using this type of procedure as an aid in differentiating the numerous species with the Pseudomonas genus. There is general agreement among those working in this field that the species comprising the genus are ill-defined, due in part to the emphasis which has been placed on the ability of these bacilli to attack glucose and other sugars either oxidatively or fermentatively. The inadequacy of these commonly used procedures emphasizes the obvious need for the development of new techniques and methods.

Recent publications by Colwell & Liston (1961) and Rhodes (1961) using the electron computer technique to study the taxonomic relationship among the Pseudomonas, stress the difficulty of species identification within the genus. Most of the cultures examined by Colwell & Liston (1961) fell into four groups. They were unable, however, to identify most of their freshly isolated strains with named strains listed in *Bergey's Manual* (Breed, Murray & Smith, 1957). Rhodes (1961) also found that the strains which she examined fell into groups, but concluded that none of the groups merit species rank but should perhaps be regarded merely as variants of *Pseudomonas fluorescens.*

All of the Pseudomonas strains tested in this laboratory have given a positive cytochrome oxidase test (Gaby & Hadley, 1957) while Colwell & Liston (1961) found that all of the strains within their four groups were positive by both the

Table 2. *Change of reaction produced by Pseudomonas and other species on incubation for 72 hr. in media containing Casitone + glucose + added nitrogenous compounds*

Organisms	Asparagine	Arginine	Leucinamide	Glycinamide	Alanine
P. aeruginosa 21 strains	alk	alk	alk	alk	1-n 2-a 18-alk
P. saccharophilia	alk	alk	alk	alk	n
P. oleovorans	n	n	n	n	alk
P. ovalis	alk	alk	a	a	a
P. fragi	alk	alk	a	a	a
P. fluorescens	alk	alk	alk	a	a
P. mucidolens	a	a	n	a	a
E. coli	a	a	a	a	a
Salmonella*	a	a	a	a	a
Shigella*	a	a	a	a	a
Proteus*	a	a	a	a	a

Organisms	Serine	Lysine	Methionine	Glucose (peptone-base)	Xylose (peptone-base)
P. aeruginosa 21 strains	2-n 2-a 17-alk	1-n 1-a 19-alk	4-n 1-a 16-alk	7-a 14-n	2-a 19-n
P. saccharophilia	n	n	n	a	a
P. oleovorans	n	n	n	n	n
P. ovalis	alk	a	n	a	a
P. fragi	a	a	a	a	a
P. fluorescens	a	a	a	a	n
P. mucidolens	a	a	a	a	n
E. coli	a	a	a	a	a
Salmonella*	a	a	a	a	a
Shigella*	a	a	a	a	a
Proteus*	a	a	a	a	a

alk = alkaline; a = acid; n = neutral; no change in indicator.

* Several strains and species tested.

oxidase test (Kovacs, 1956) and cytochrome oxidase test (Gaby & Hadley, 1957). These results would seem to indicate that it should not be too difficult to identify a Gram-negative bacillus as a Pseudomonas. Proper emphasis, however, has not been placed on the catabolism of nitrogenous compounds by this group of micro-organisms.

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