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Studies of the Nutritional Requirements of *Poteriochromonas stipitata*

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SUMMARY

The nutritional requirements of the phagotrophic phytoflagellate *Poteriochromonas stipitata* are defined. Minerals, glucose, ammonium-nitrogen and the vitamins thiamine, biotin and vitamin B_{12} are essential for the growth of this chrysomonad. The addition of L-isoleucine, L-leucine, L-valine and L-phenylalanine increases the yield of organism at least fivefold. Further augmentation with adenine, L-glutamic acid, L-histidine and L-arginine provides optimal nutrition for the organism.

INTRODUCTION

This paper reports a study of the nutritional requirements of *Poteriochromonas stipitata* American Type Culture Collection (ATCC) no. 11531, a freshwater phytoflagellate closely related to *Ochromonas malhamensis* (Pringsheim, 1952).

METHODS

Poteriochromonas stipitata ATCC no. 11531 was maintained in a medium consisting of (g./l.): powdered milk, 2·0; trypticase (Baltimore Biological Laboratories, BBL, Baltimore, Md., U.S.A.), 0·5; sucrose, 1·0; adjusted to pH 5·0. Ten ml. volumes of medium were dispensed in 25 ml. Erlenmeyer flasks fitted with cottonwool plugs and sterilized by autoclaving at 121° for 15 min. Thioglycollate broth (BBL), prepared and autoclaved according to the instructions of the manufacturer, could also be used for maintenance. The defined medium described by Hutner, Provasoli & Filfus (1953) for use with freshwater chrysomonads served as a medium for comparison (Table 1). It was dispensed in 10 ml. quantities into micro-Fernbach flasks, which were then closed with inverted glass tumblers and autoclaved for 15 min. at 121°. The basal medium A for evaluating possible nutrients was composed as shown in Table 1. The basal media were always prepared without the trace metals and vitamin solutions and autoclaved for 15 min. at 121°. The medium was adjusted to pH 5 or 7. The 100 × concentrated trace metal and vitamin solutions (Table 1) were sterilized by filtration through ultrafine sintered glass, and stored in sterile glass-stoppered brown bottles. The trace metal and vitamin solutions were added in 10 ml/l. amounts after the rest of the medium had been autoclaved.

Nutritional experiments were done in 25 ml. micro-Fernbach flasks, covered with inverted rimless tumblers. The total volume of medium in these flasks never exceeded 10 ml. The experimental vessels were incubated in stainless steel transfer hood

provided with a glass window and illuminated by two 50 W. daylight fluorescent bulbs. Temperature in this hood ranged from 22–25°. Cells for inoculation were washed thrice with sterile M/30 phosphate buffer (pH 5·6), the original volume restored and each experimental vessel inoculated with 0·05 ml. Aseptic precautions were observed throughout. For studies in the dark a 25° incubator was used. Since illumination exerted no appreciable effect on yields, all results represent experiments conducted in the light unless stated otherwise.

Table 1. *Constituents of various media used (g./l.)*

	Hutner *et al.* (1953)	Basal medium					
		A	B	C	D	E	F
Ethylenediamine-tetra-acetic acid	0·64	0·5	0·5	0·5	0·5	—	0·5
$CaCO_3$	0·15	0·15	0·15	0·15	0·15	—	0·15
$MgSO_4.7H_2O$	1·6	0·6	0·6	0·6	0·6	0·6	0·6
KH_2PO_4	0·3	0·3	0·3	0·3	0·3	0·3	0·3
NH_4HCO_3	0·4	0·3	0·3	—	0·3	—	—
Na_2CO_3	0·84	—	—	—	—	—	—
Glucose	—	—	10·0	10·0	10·0	20·0	10·0
Sucrose	12·0	—	—	—	—	—	—
Casein hydrolysate	—	—	2·0	—	—	—	—
L-Isoleucine	—	—	—	—	0·5	0·5	—
L-Leucine	—	—	—	—	0·5	0·5	—
L-Valine	—	—	—	—	0·5	0·5	—
L-Phenylalanine	—	—	—	—	0·5	0·5	—
L-Glutamic acid	3·0	—	—	—	—	0·6	—
L-Histidine	0·5	—	—	—	—	0·1	—
L-Arginine	0·4	—	—	—	—	0·1	—
L-Methionine	0·3	—	—	—	—	—	—
Adenine sulphate	—	—	—	—	—	0·5	—
Ammonium citrate	1·2	—	—	—	—		—
NH_4Cl	—	—	—	—	—	1·0	—
$ZnSO_4.H_2O$*	0·875	0·175	0·175	0·175	0·175		0·175
$MnSO_4.H_2O$*	0·0923	0·1846	0·1846	0·1846	0·1846		0·1846
$CuSO_4.H_2O$	0·0195	0·039	0·039	0·039	0·039		0·039
$FeSO_4(NH_4)_2SO_4.6H_2O$*	0·00702	0·01404	0·01404	0·01404	0·01404	—	0·01404
$CoSO_4 7H_2O$*	0·00141	0·00282	0·00282	0·00282	0·00282	—	0·00282
$(NH_4)_6Mo_7O_{24}.{}_4H_2O$*	0·00324	0·00648	0·00648	0·00648	0·00648	—	0·00648
Thiamine HCl†	0·002	0·004	0·004	0·004	0·004	—	0·002
Biotin†	0·000005	0·000001	0·00001	0·00001	0·00001	—	0·00002
Vitamin B_{12}†	0·0000005	0·000001	0·000001	0·000001	0·000001	—	0·0000005

* Trace-metals prepared as concentrate, sterilized by filtration and added as 10·0 ml./l. after autoclaving.

† Vitamins prepared separately but exactly as trace metal solution and added after autoclaving. pH 5 or pH 7.

All materials which were known to be stable when autoclaved at 121° for 15 min. were added before heat sterilization. Heat-labile materials were usually dissolved in the basal medium A and passed through sterile ultrafine sintered-glass filters. All

experiments were done in quadruplicate and repeated five times. Modifications of media were made as required.

Tests for sterility were performed on all materials and solutions and the inocula were examined for contaminants; the media used for these purposes were Eugon (BBL) and anaerobic (BBL) agars; solid media were preferred since the chrysomonad itself is inhibited by agar.

To ensure inocula of uniform physiological activity and age, 2 ml. of maintenance culture were transferred to 2 l. Fernbach flasks containing 600 ml. basal medium B (Table 1). A sterile Tephlon-coated iron bar was then introduced and the mixture gently agitated with a magnetic stirrer. Measurement of growth of *Poteriochromonas stipitata* was determined after 14 days incubation by the estimation of total cellular-nitrogen according to the method described by Umbreit, Burris & Stauffer (1957) with albumin (Armour) and ammonium sulphate as standards. Organisms were prepared for analysis by transferring 10 ml. of culture to 15 ml. conical tubes and centrifuging at 1150 *g* at 5° for 10 min. The packed organisms were washed thrice with M/30 phosphate buffer (pH 5·6).

Chlorophyll was determined according to methods described by Wolken & Palade (1953) and Strain (1949). Since the acetone extracts displayed greater differences of extinction, this solvent was used. For quantitative results, nitrogen determinations were made on samples of the acetone extracts.

RESULTS

Carbon sources

Table 2 lists the few compounds which satisfied the carbon requirements of *Poteriochromonas stipitata*. The following materials (at 0·056 M) did not fulfil this requirement: lactose, ribose, xylose, rhamnose, calcium, gluconate, potassium pyruvate, sodium lactate, sodium citrate, α-ketoglutarate, sodium succinate,

Table 2. *Total cell-nitrogen of* Poteriochromonas stipitata *produced while growing on various single carbon sources*

Basal medium A was used and the compounds added to 0·056 M unless stated otherwise. Experiments were made at pH 5·0 in the light. The numbers represent the mean values of five experiments each performed in quadruplicate. Basal medium A without an organic carbon source did not support the growth of the organism.

Carbon source	Total cell-N (μg./ml. culture)	Carbon source	Total cell-N (μg./ml. culture)
Glucose	41·83	Starch (5 %, w/v)	95·29
Fructose	33·81	Glycogen (5 %, w/v)	75·86
Galactose	41·83	Raffinose	51·31
Mannose	11·27	Glycerol	35·39
Sucrose	43·75	Sodium acetate	14·19
Maltose	44·71	Ethanol	15·96

sodium fumarate, sodium propionate, sodium butyrate, glycollic acid, sodium glutamate, L-asparagine, L-glutamine, cholesterol, sitosterol, ribonucleic acid, deoxyribonucleic acid, acetone, methyl formate, ethyl formate, *n*-propyl formate, *n*-butyl formate, ethylene glycol, acetaldehyde, tricarboxylic acid and hexose monophosphate shunt intermediates, simple esters, fatty acids, steroids, and nucleic acids.

When judged on the basis of μg total cell-N per carbon atom of substrate, acetate and ethanol were as effective as the hexoses, while glycerol was better. Thus the total cell-N per carbon atom of glycerol was almost $12\,\mu g.$, while glucose yielded only $7\,\mu g.$ on the same basis. The ineffective carbon sources were re-examined for their action on the growth of *P. stipitata* in the presence of glucose. None had any effect except citrate which inhibited growth, probably because it bound trace metals; the joint addition of iron and magnesium abolished the inhibition.

The optimal concentration of glucose (Fig. 1) was determined for several media. Regardless of the nutrient environment and presence or absence of illumination the optimal glucose concentration was about 20 g./l., i.e. 0·112 M. In the basal medium B a slight but definite increase in total cell-N occurred with illumination.

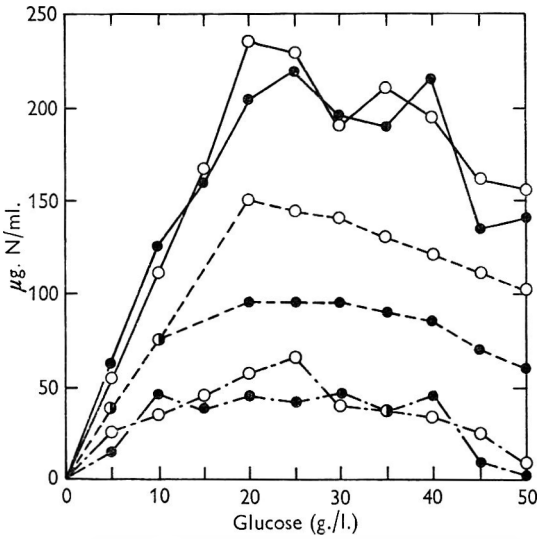

Fig. 1. Response of *Poteriochromonas stipitata* to glucose while growing in various media at pH 5·0. ○——○, Medium E + trace metals + vitamins in light; ●——●, medium E + trace metals + vitamins in dark; ○---○, Hutner's defined medium (Hutner *et al.* 1953) in light; ●---●, Hutner's defined medium in dark; ○-·-○, basal medium B in light; ●-·-●, basal medium B in dark.

The ability of *Poteriochromonas stipitata* to utilize acetate as a sole carbon source suggested a possible connexion between this organism and the 'acetate flagellates' (Hutner & Provasoli, 1951). Figure 2 shows that 0·1 M acetate was definitely inhibitory for *P. stipitata*. Since the 'acetate flagellates' flourish at this concentration of the compound, *P. stipitata* cannot be considered a member of this group. Ethyl formate was not used as a carbon source, although this ester is in equilibrium with ethanol and formic acid when in solution and the organism can utilize ethanol as a carbon source. Ethyl formate did not inhibit the growth of *P. stipitata* when added to media containing glucose, but the ester as well as formic acid prevented proliferation of *P. stipitata* when ethanol or acetate were substituted for the hexose.

Nitrogen sources

Basal medium C was inadequate for growth. Several nitrogenous compounds were then examined for their ability to serve as nitrogen sources, either alone, with casein hydrolysate (BBL), or with casein hydrolysate (BBL) + ammonium bicarbonate. The latter is the major nitrogen source advocated by Hutner *et al.* (1953). The findings are presented in Table 3. Nitrate or nitrite in the concentrations shown inhibited growth. However, sodium nitrate 1·0 g./l. resulted in good crops. Nitrite did not support growth at any concentration tested. The capacity of the chrysomonad to utilize urea as sole nitrogen source at pH 5 and 7, and to the same degree or better than on the casein hydrolysate (BBL) and casein hydrolysate (BBL) + ammonium bicarbonate controls, was surprising; no extracellular urease was demonstrable in the culture fluid. When such cell-free supernatant fluid was added to urea in a neutral buffer, liberation of ammonia did not occur even after prolonged incubation.

Table 3. *The growth of* Poteriochromonas stipitata *with various nitrogen sources*

The basal medium C at pH 5·0 was used. The flasks were illuminated during the experiment. Casein hydrolysate (BBL) 2 g./l. and NH_4HCO_3 0·3 g./l. were added to basal medium C. The numbers represent the mean values of five experiments each performed in quadruplicate.

Nitrogen source (g./l.)		Media: Basal C alone	Basal C + casein hydrolysate	Basal C + hydrolysate + NH_4HCO_3
		Total cell-N (μg./ml. culture)		
No addition	0	1·96	18·27	26·66
$NaNO_3$	2·3	1·03	13·02	19·95
$NaNO_2$	1·86	1·89	1·79	1·83
Urea	0·8	30·73	61·04	38·50
$NH_2OH.HCl$	1·88	1·87	1·87	1·87
Human serum	10	11·09	22·54	46·20
Gelatin	2·5	38·08	31·41	37·45
Trypticase (BBL)	2·5	30·31	39·38	73·41
Phytone (BBL)	2·5	23·36	33·08	42·00
Polypeptone (BBL)	2·5	40·46	54·43	60·69
Thiotone (BBL)	2·5	33·08	40·86	61·25

Amino acid requirements. Table 3 also shows that the stimulatory effect of casein hydrolysate (BBL) 2 g./l. was considerable, regardless of the nature of the other nitrogen sources tested, except in the cases of nitrite and hydroxylamine, which were inhibitory. Therefore, various amino acids were tested for stimulatory activity. The following L-amino acids were added instead of casein hydrolysate to basal medium C in amounts which corresponded to their concentration in 2 g./l. casein hydrolysate (g./l.): glutamic acid, 0·432; proline, 0·232; glycine, 0·038; serine, 0·125; threonine, 0·105; tyrosine, 0·127; isoleucine, 0·122; leucine, 0·189; valine, 0·139; aspartic acid 0·153; histidine, 0·061; arginine, 0·078; methionine, 0·058; cystine, 0·007; alanine, 0·067; phenylalanine, 0·099; tryptophan, 0·026. Leucine, isoleucine, valine, and phenylalanine additions resulted in yields of organism comparable with those obtained with casein hydrolysate. Therefore, these amino acids were added in

increasing concentrations singly and in mixtures to basal medium C. Results showed that 0·5 g./l. was the most favourable concentration for these amino acids. Total cell-N values greater than for casein hydrolysate were obtained almost uniformly with these amino acids by themselves or in various mixtures as shown in Table 4.

Table 4. *The influence of casein hydrolysate and amino acids on total cell-nitrogen yields of* Poteriochromonas stipitata

Casein hydrolysate (BBL) content was 2 g./l. while the amino acids were added in 0·5 g./l. amounts each for the same volume. The basal medium C was used at pH 5·0, and the flasks were illuminated.

Amino acid(s)	Total cell-N (% as compared with casein hydrolysis)	Amino acid(s)	Total cell-N (% as compared with casein hydrolysis)
Casein hydrolysate	100·0	Leucine, isoleucine	128·1
Phenylalanine, valine, leucine, isoleucine	181·7	Phenylalanine, leucine, isoleucine	161·2
Phenylalanine	106·3	Phenylalanine, valine, isoleucine	191·2
Valine	111·3		
Leucine	105·6	Phenylalanine, valine, leucine	86·5
Isoleucine	107·5		
Valine, leucine	128·1	Valine, leucine, isoleucine	139·5
Valine, isoleucine	122·3		

Table 5. *The optimal concentration of certain amino acids for adequate and optimal nutrition of* Poteriochromas stipitata

The basal medium for testing these amino acids was identical with that used in Table 4; the experiments were conducted at pH 7·0 as well as pH 5·0, with illumination. Casein hydrolysate served as the control. The values obtained with these combined concentrations of the amino acids exceeded the control yields by at least 100 %.

Amino acids	g./l.	Requirement* for Adequate nutrition	Requirement* for Optimal nutrition
L-Isoleucine	0·5	+	+
L-Leucine	0·5	+	+
L-Valine	0·5	+	+
L-Phenylalanine	0·5	+	+
L-Glutamic acid	0·6	.	+
L-Histidine	0·1	.	+
L-Arginine	0·1	.	+

* See Table 11 for definition of nutritional states.

It is quite difficult to consider these findings without comparing them with results obtained with Hutner's defined medium (Hutner *et al.* 1953, Table 1). At pH 5, comparable yields of organism were obtained; yet arginine, glutamic acid, histidine and methionine were present in Hutner's defined medium without the amino acids found to be stimulatory in the present work. When the above amino acids of Hutner's defined medium were added to basal medium D (Table 1) or to the basal medium B, four times the total cell-N obtained with Hutner's defined medium was

obtained, and at least twice the total cell-N with the basal medium B. To establish the optimal concentration of stimulatory amino acids, tolerance tests with each amino acid singly or in mixtures were performed. The results of these determinations indicated that optimal growth of *Poteriochromonas stipitata* was obtained with the concentrations shown in Table 5. These concentrations were equally effective at pH 5 and 7. The remaining amino acids found in casein exerted no stimulatory effect. Indifferent amino acids could, however, substitute as nitrogen sources in the absence of the stimulatory amino acids. Under these conditions yields of organism amounted to about one-third to one-half those obtained with casein hydrolysate (2 g./l.). Methionine by itself, however, was found to decrease the total yields of organism considerably and in concentrations as low as 0·1 g./l. to abolish chlorophyll production. On the other hand, glutamic acid increased the chlorophyll content (acetone-extractable fraction absorbing at 650 mμ), the production of which was enhanced by this addition regardless of the conditions of illumination. This effect could not be reproduced with succinic acid, glycine or δ-amino levulinic acid.

Table 6. *The effect on the growth of* Poteriochromonas stipitata of *precursors, derivatives and antagonists of purines and pyrimidines*

The medium used for these experiments was basal medium A to which glucose and casein hydrolysate were added in the usual concentrations, at pH 5·0 and with illumination.

Substance	Concentration (g./l.)	Total cell-N (% as compared with no addition = 100)	Substance	Concentration (g./l.)	Total cell-N (% as compared with no addition = 100)
No addition	0	100·0	Ureidosuccinic acid	0·39	111·4
Sodium ribonucleate	20	180·9	4-amino imidazole-5-carboxamide. HCl	2·17	67·2
Sodium deoxyribonucleate	0·5	264·9			
Adenine	0·7	193·2	6-Chloropurine	0·2	71·5
Hypoxanthine	0·5	111·1	6-Mercaptopurine	0·18	69·4
Guanine	0·6	97·4	Barbital	0·22	180·0
Xanthine	0·2	160·0	Kinetin	0·15	183·3
Uric acid	0·19	66·4	Furfurylacetate	0·196	80·9
Allantoin	0·375	105·3	Furfurylamine	0·122	149·2
Thymine	0·135	125·1	Furan	0·095	100·9
Uracil	0·15	110·9	2-Furamide	0·155	56·1
Cytosine	2·0	129·6	Furoic acid	0·157	36·2
Orotic acid	3·88	109·8	Furfuryl alcohol	0·137	90·4
Cytidylic acid	1·39	68·4	2-Furaldehyde	0·135	73·7
Adenylic acid	0·31	267·0	Hydrofuramide	0·159	0·0
Thymidine	0·293	103·7	Furacin	0·005	83·1
Uridine	0·5	79·8	Furadantin	0·005	70·3
Adenine sulphate	2·11	268·9	Furoxone	0·005	90·2

Purines, pyrimidines and related compounds. The effects of derivatives of purines and pyrimidines and related compounds were examined; the results of these experiments are presented in Table 6. It is clear that adenine-containing compounds stimulated *Poteriochromonas stipitata* growth. The increase of total cell-N given by sodium ribonucleate and sodium deoxyribonucleate is explainable on the basis of their content of adenine. The increased yields obtained with hypoxanthine, uric acid,

barbital and kinetin may be related to their structural similarity to adenine. Since kinetin was stimulatory, one wondered whether the effect might not also be a reflexion of the growth-promoting activity of this compound, similar to that observed in green plants. To this end, furan derivatives, which affect growth in plants, were tested (see Table 6). Of these, only furfurylamine exhibited stimulatory effects. The adenine moiety of kinetin, therefore, was probably responsible for the stimulation of *P. stipitata* observed.

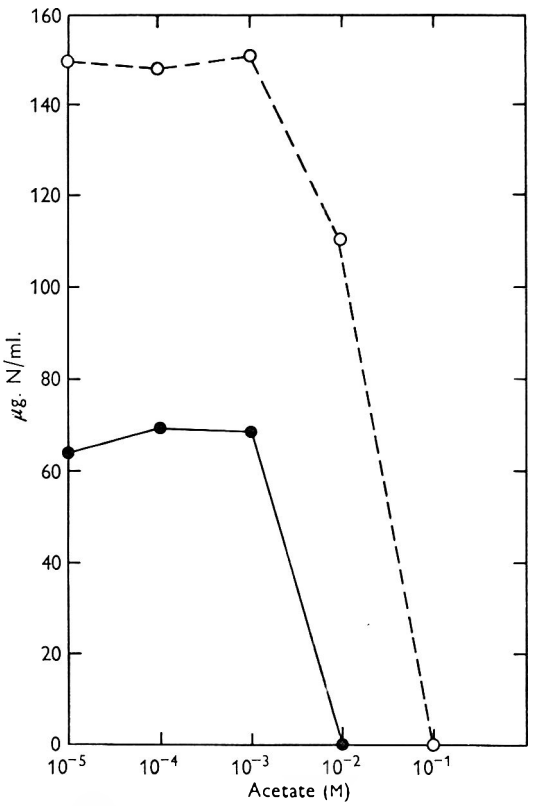

Fig. 2. Reaction of *Poteriochromonas stipitata* to increasing amounts of acetate at pH 5·0.

Vitamin requirements

The present work confirmed the vitamin B_{12}, thiamine and biotin requirements established by Hutner *et al.* (1953). No response was obtained with any of the other B vitamins, ascorbic acid or fat soluble vitamins. As can be seen in Fig. 3 the amount of the nutrient designated by Hutner *et al.* (1953) (0·5 × concentrations, Table 1) for freshwater chrysomonads was not in every case the optimum amount for *Poteriochromonas stipitata.* It had been noted earlier in the work that doubling the amount of the vitamin mixture increased the yields of organism. The 1 × concentration in Fig. 3 reflects this doubling; the figure shows, however, that vitamin B_{12} was most effective at a concentration originally suggested by Hutner (Hutner *et al.* 1953). Response to thiamine was similar to vitamin B_{12}. However, biotin was indeed

most stimulatory at the highest concentration tested. Table 7, in which the omission of these materials is presented, revealed the absolute need for the 3 vitamins by the flagellate.

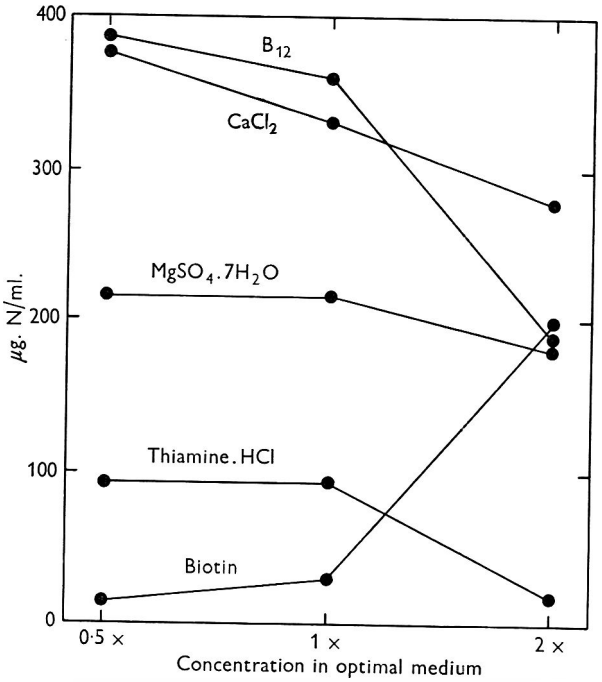

Fig. 3. The effect of varying vitamin and cation concentrations on cell-N yields of *Poteriochromonas stipitata*. The medium is the basal medium E at pH 5·0. The 1 × concentrations were as follows: vitamin B_{12}, 10 μg./l.; thiamine HCl, 4 mg./l.; biotin, 40 μg./l.; $CaCl_2$, 0·8 g./l.; $MgSO_4.7H_2O$, 0·6 g./l., while 0·5 × concentration represents their concentration in Hutner's medium (1951, see Table 1). Trace metals were added in their usual concentration.

Table 7. *The effect of omission of individual metals and vitamins on cell-N yields of* Poteriochromonas stipitata

Basal medium E at pH 5·0 was used with illumination. All cations and vitamins were then added with the exception of the single substance under scrutiny. The results were the average of ten determinations with an error of less than ±1·5 %

Substance omitted	Usual concentration (g./l.)	Total cell-N when material was omitted (% of positive control)
$MgSO_4.7H_2O$	0·6	70·3
$CaCl_2$	0·24	70·3
$ZnSO_4.7H_2O$	0·18	110·2
$MnSO_4.H_2O$	0·18	110·2
$CuSO_4.5H_2O$	0·059	107·1
$FeSO_4(NH_4)_2SO_4.6H_2O$	0·042	21·2
$CoSO_4.7H_2O$	0·038	77·1
$(NH_4)_4Mo_7O_{24}.4H_2O$	0·0007	83·9
Thiamine	0·004	12·7
Biotin	0·00003	16·1
Cobalamine (B_{12})	0·000015	27·9
None present (control)	.	11·8
All present (control)	.	100·0

Mineral requirements

Figure 3 also shows that calcium and magnesium elicited an optimal response at the concentrations advocated by Hutner *et al.* (1953). Table 7 shows the effect of the omission of cations from basal medium E (Table 1), which contained the same non-metabolizable chelating agent as basal medium A. Ethylenediaminetetra-acetate has admittedly low chelating efficiency at acid pH values with the alkaline earth metals, but it can still remove contaminating traces from those metals present. It became obvious that the most critical metal was iron. A lesser dependency on magnesium, calcium, cobalt and perhaps molybdenum was also evident. Zinc, manganese and copper may have exerted some toxic influence since their absence

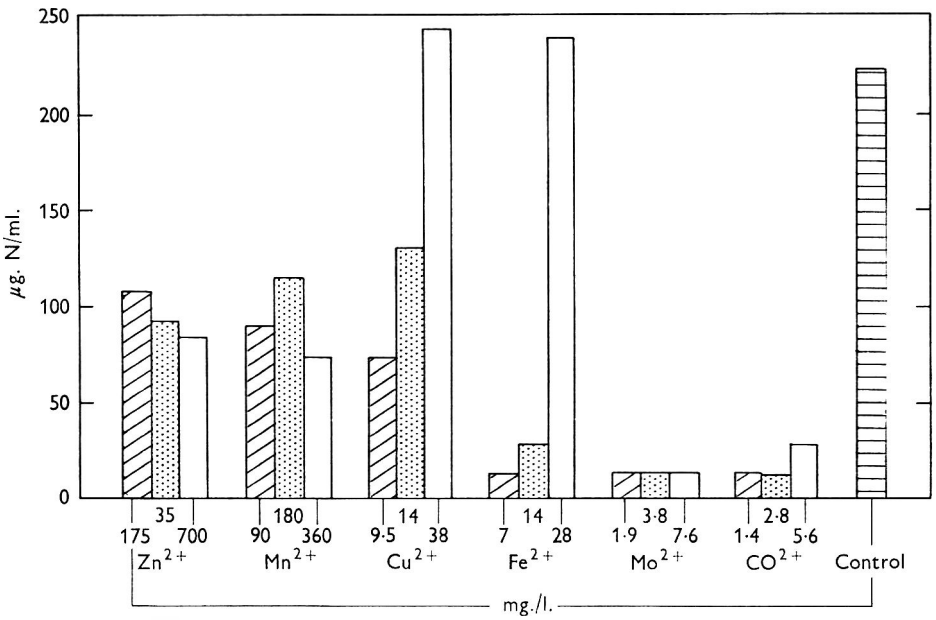

Fig. 4. The growth response of *Poteriochromonas stipitata* to varying concentrations of trace metals at pH 5·0 with illumination. The medium used corresponds to the one described in Table 7 to which were added thiamine. HCl, 2 mg./l.; biotin, 0·02 mg./l., vitamin B_{12}, 0·005 mg./l.; $CaCl_2$, 0·8 g./l.; $MgSO_4.7H_2O$, 0·6 g./l. For the control all trace metals were added in the amounts represented by the centre bar. The results were the average of ten determinations, with an error of less than $\pm 1{\cdot}5\,\%$.

resulted in slightly enhanced yields of organism. Addition of increasing amounts of trace metals confirmed these findings (Fig. 4). Zinc gave the best results in its lowest concentrations; manganese in the medium range; the concentration of cobalt mattered little. Molybdenum and copper, and especially iron, gave the best results in the highest concentrations tested. The findings with copper were surprising in view of the effect its absence exerted; repeated experiments gave similar results consistently. When optimal concentrations of trace metals were used a 50 % increase in total cell-N over the control represented in Fig. 4 was obtained.

Hutner's defined medium (Hutner *et al.* 1953) contained 0·3 g. KH_2PO_4/l. (Table 1). Figure 5 showed that yields of organism obtained with this amount were about 50 %

of those with 1 g. phosphate/l.; this finding was independent of the cation used with the phosphate.

The medium proposed by Hutner *et al.* 1953 (Table 1) only supported the organism when the initial pH was about 5. Using the media devised in the present work the organism grew well at pH 5 and produced a final pH value of $3{\cdot}3 \pm 0{\cdot}3$, regardless of substrate or nature of the carbon source. Duplicate samples were examined at 2-day intervals for 30 days. While the maximum yields of organism were obtained at different times from each medium, the final pH value was reached within 2 days and was identical for all.

It was found early in this work that *Poteriochromonas stipitata* could be maintained in thioglycollate broth (BBL) at pH 7. All attempts to grow the organism in Hutner's defined medium (Hutner *et al.* 1953;) at this pH value failed, while the basal medium B supported the chrysomonad at pH 7. The *a priori* hypothesis that the decreased oxidation-reduction potential found in thioglycollate broth enabled *P. stipitata* to grow near neutrality could not be proved by using compounds such as L-cystine, mercaptoethanol, glutathione, sodium sulphite, thioglycollate, L-cysteine and cystamine, either singly or in mixtures.

Table 8. *Tolerance of* Poteriochromonas stipitata *to ammonium salts at acid and neutral pH in basal medium F*

Compound	Concentration of compound ($\times 10^{-3}$ M)	pH value initial	pH value final	μg. total cell-N/ml.	Difference pH 7·0/pH 5·0 (%)
Ammonium citrate	4·7	5·0	3·6	49·0	.
		7·0	3·8	37·3	76
Ammonium carbonate	5·7	5·0	2·6	65·3	.
		7·0	3·3	41·2	63
Ammonium chloride	11·4	5·0	2·7	74·7	.
		7·0	3·0	74·3	100
Ammonium sulphate	5·7	5·0	2·7	66·9	.
		7·0	3·0	59·5	89

However, the chrysomonad medium of Hutner *et al.* 1953 includes several amino acids, ammonium citrate and ammonium carbonate which are omitted from the defined media devised during the present work. This difference suggested that ammonia might exert a deleterious effect at pH 7. Several ammonium salts (Table 1) were tested in the basal medium F at pH 5 and pH 7, resulting in findings summarized in Table 8 which illustrates the role played by ammonia in permitting protozoan growth at neutral pH. The volatility of ammonia at pH 7 was considerably depressed by the presence of the more acidic anions which would not only maintain ammonia in an ionized state but also make it available as a nitrogen source. When ammonium chloride, as sole nitrogen source, was added in increasing amounts to the basal medium F at pH 7 (Fig. 6), no inhibition was demonstrable. Replacement of the ammonium ion by other forms of nitrogen in Hutner's defined medium (Hutner *et al.* 1953) resulted in increases in yield of total cell-N at pH 7, even in the presence of the same concentration of citrate ion. Thus, while ammonium citrate in its usual concentration (Table 1) resulted in 20 μg. total cell-N/ml., urea 0·34 g./l. and sodium citrate 1·67 g./l. increased yields to 31 μg./ml. while 51·7 μg. of

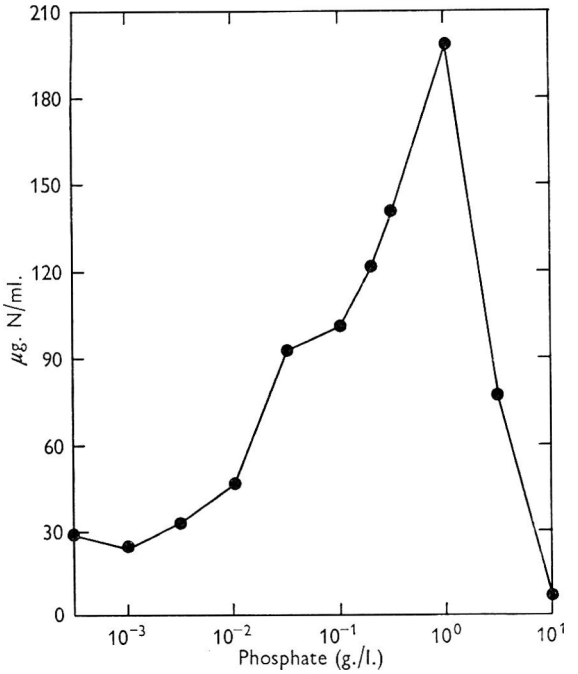

Fig. 5. The phosphate response of *Poteriochromonas stipitata* in basal medium B at pH 5·0 with illumination.

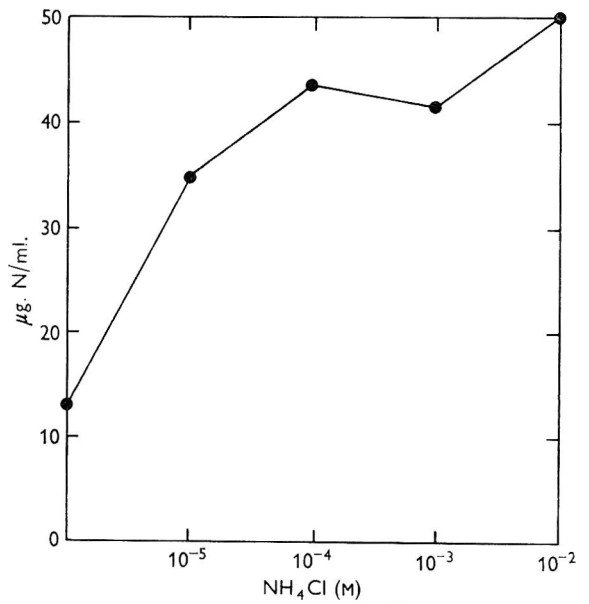

Fig. 6. The effect of ammonium chloride as only source of nitrogen in basal medium F at pH 7·0.

total cell-N/ml. were formed with sodium nitrate 0·97 g./l. and sodium citrate 1·67 g./l.

The ammonium salts of weaker acids present in Hutner's defined medium (Hutner *et al.* 1953) offered only a partial explanation for the inability of the chrysomonad to grow at pH 7. Comparison of that basal medium with basal medium D evolved in the present work showed the presence of additional organic nitrogen in

Table 9. *The effect of organic nitrogen on the growth of* Poteriochromonas stipitata *in the presence and absence of ammonium citrate*

Experimental basal medium D was used; the amino acids and citrate were present in the concentrations found in Hutner's defined medium (Hutner *et al.* 1953).

	pH values			
	5		7	
	Ammonium present	Citrate absent	Ammonium present	Citrate absent
	Yield of organism (*μ*g. total cell-N/ml. medium)			
L-Arginine	161·8	152·46	15·4	23·4
L-Glutamic acid	159·5	81·6	92·5	90·0
DL-Histidine	161·0	127·4	12·4	96·3
DL-Methionine	143·9	107·7	4·7	52·5
Control	77·0	75·0	65·7	65·3

Table 10. *The effect of amino nitrogen on* Poteriochromonas stipitata *at* pH 7·0 *in basal medium D*

	*μ*g total cell-N/ml., medium		
Compound	Alone	Ammonium citrate, 1·2 g./l.	NH_4Cl, 0·57 g./l.
Glutamic acid*	38·1	8·4	52·5
L-Arginine HCl	87·2	7·8	59·3
DL-Histidine	45·1	9·4	40·8
DL-Methionine	36·5	7·7	53·0
DL-Ornithine HCl	108·1	5·6	45·1
DL Citrulline	63·8	7·3	30·0
L-Lysine HCl	80·1	6·6	33·5
Glycine	51·4	6·4	35·4
L-Cysteine HCl	74·6	15·2	22·5
L-Glutamine	43·7	3·6	26·5
L-Asparagine	53·6	7·4	27·2
Control	51·9	47·9	54·8

* Monosodium salt used.

the form of L-arginine HCl 0·4 g./l., L-glutamic acid 3·0 g./l., DL-histidine 1·0 g./l., and DL-methionine 0·6 g./l. The effect of these compounds on total cellular N/ml. in the basal medium D at pH 5 and pH 7 was tested in the presence and absence of ammonium citrate (Table 9). It is quite evident that although at pH 5 the nitrogen sources were all utilizable and omission decreased the amount of total cell-N/ml., at pH 7 amino acids such as arginine, histidine and methionine were especially detrimental to protozoan proliferation in the presence of ammonium citrate. Additional

evidence that amino acid nitrogen enhanced ammonia toxicity was sought experimentally by increasing the concentration of the amino acids added in the basal medium D to 1·0 g./l. Other amino acids, including the dibasic ones, and the amides of dicarboxylic amino acids were included. The action of these amino acids was evaluated in the presence of ammonium chloride and compared with results obtained with ammonium citrate at pH 7. Table 10 shows that the increase in amino acid concentration resulted in good yields of organism in the absence of ammonium salts. The presence of ammonium citrate led to almost complete cessation of growth; decreased yields of organism were observed with equimolar ammonium chloride. The deleterious effect of ammonia was even shown by substituting the monosodium salts of dicarboxylic acids for the acid, as shown with monosodium glutamate in Table 10. This suggests that any substance capable of shifting the pH toward neutrality favours the conversion of ammonium ion to ammonia which results in toxic effects.

DISCUSSION

The nutritional requirements of *Poteriochromonas stipitata* can be considered in three categories (Table 11). The basal or most essential nutrients could not be replaced

Table 11. *The nutritional requirements of* Poteriochromonas stipitata

Compound	g./l.	Compound	g./l.
Essential nutrients (to obtain 10–20 μg./ml. total cell-N)		Adequate nutrition (to obtain at least 100 μg./ml. total cell-N)§	
Glucose*	10·0	L-Isoleucine	0·5
NH_4Cl†	1·0	L-Valine	0·5
Ethylenediaminetetra-acetic acid‡	0·64	L-Leucine	0·5
		L-Phenylalanine	0·5
KH_2PO_4	0·03		
$CaCl_2$	0·08	Optimal nutrition (to obtain in excess of 200 μg./ml. total cell-N)‖	
$MgSO_4.7H_2O$	0·06		
Thiamine HCl	2·0 mg.	Glucose	10·0
Biotin	0·02 mg.	KH_2PO_4	0·7
Vitamin B_{12}	0·005 mg.	Adenine sulphate	0·5
$ZnSO_4.H_2O$	175·0 mg.	L-Glutamic acid	0·6
$MnSO_4.H_2O$	184·6 mg.	L-Histidine	0·1
$CuSO_4.5H_2O$	39·0 mg.	L-Arginine	0·1
$FeSO_4(NH_4)_2SO_4.6H_2O$	28·1 mg.		
$CoSO_4.7H_2O$	1·9 mg.		
$(NH_4)_6Mo_7O_{24}.4H_2O$	1·4 mg.		

pH 7·0 or 5·0; incubated in the presence or absence of light.

* Other suitable carbon sources may be substituted.

† Urea, nitrate or most casein amino acids may be substituted in equimolar amounts.

‡ This compound, though not metabolized, required listing since it is essential for maintaining minerals in a utilizable state.

§ Added to the essential nutrients.

‖ Added to all previously listed nutrients.

or diminished in concentration to any great degree; the yields of organisms on this basal medium were equivalent to 10–20 μg. total-N/ml. medium. The addition of a few amino acids in moderate amounts led to a disproportionate increase (5–30-fold) of protozoan growth relative to the amounts of nitrogen and carbon supplied; other amino acids supplied in similar amounts failed to achieve the 5–30-fold increase in yield. The response of the organism was modified still further by adding more carbon and phosphate as well as additional amino acids and adenine. These latter supple-

ments resulted in at least a further doubling of total cell-N. The presence or absence of light and maintenance of acid or neutral pH values did not appear to influence the yields of *P. stipitata* to any degree.

It seems doubtful whether *Poteriochromonas stipitata* encounters these optimal concentrations and kinds of nutrient in nature. Indeed, it is found experimentally that the less-than-optimal nutrition produces protozoa less sensitive to toxic materials introduced into the environment (Isenberg, Berkman & Sundehim, 1962); nevertheless the upper and lower limits of nutritional response should be established.

The ability to utilize exogenous material is a first approximation of the physiology and biochemistry of an organism (Knight, 1936). The limited number of compounds which serve *Poteriochromonas stipitata* as carbon and energy sources reveal the biochemical limitations of this organism. No β-glycosidases could be demonstrated. However, the organism was able to utilize all α-glycosides supplied. When high concentrations of starch (200 g./l.) were used in an attempt to cultivate the phytoflagellate on a solid medium, liquefaction of the starch gel was rapid, suggesting the production of an amylase. Similarly, proteinases were produced, as indicated by rapid liquefaction of gelatin media. Further evidence for the elaboration of extracellular proteinases and peptidases was given by the variety of proteins and peptides which could supply the nitrogen requirements of the organism. The production of extracellular enzymes apparently supplements the phagotrophic capacities of this chrysomonad. The organism's ability to utilize glucose and galactose with equal ease is interesting, especially since none of the pentoses are utilized. The selectivity of the cell with respect to tri-carboxylic acid cycle compounds and intermediates of hexose oxidation, and fatty acid metabolism is noteworthy in view of the ease with which glycerol, acetate and ethanol were metabolized. Glycerol utilization especially warrants attention. *P. stipitata* and *Ochromonas malhamensis* have been considered 'sugar' flagellates (Pringsheim, 1952). Yet the better yields obtained with glycerol suggest that this compound may be a preferred carbon source. Since, in addition, gradual loss of chlorophyll function is an outstanding characteristic of these organisms (Pringsheim, 1952; Wolken & Schwartz, 1953), these 'most primitive animals' (Hutner & Provasoli, 1951), may reflect another step in the evolution of heterotrophic chemo-organotrophy (Lwoff, 1951) from autotrophic or mesotrophic autotrophy in protozoa. While acetate was not tolerated in concentrations resembling those of 'acetate flagellates' (Hutner & Provasoli, 1951), the concentrations of glucose found optimal were more suggestive of fungal physiology. Phase-contrast microscopy of *P. stipitata* cells grown on such a high carbohydrate diet revealed large storage bodies which were lacking in cells grown in thioglycollate broth. In manometric studies, not reported here, cells with such inclusions showed prolonged enhanced endogenous respiration.

The present work indicates that ammonia and urea or nitrate can supply the nitrogen requirements of *Poteriochromonas stipitata*. The rate of growth is influenced markedly by the presence of certain amino acids; the most important of these, leucine, isoleucine, valine, phenylalanine, are all ketogenic. Their importance as nutrients seems more likely to be a reflexion of the branched or aromatic nature of their carbon skeletons. The other amino acids and adenine which promoted optimal growth probably reflect the greater ease with which organisms grow in the presence of non-essential amino acids (Oginsky & Umbreit, 1959).

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Comparative Carbohydrate Catabolism in Arthrobacter

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SUMMARY

The catabolic pathways for the utilization of glucose and gluconate in one representation of each of five species of Arthrobacter were studied by the respirometric method (Wang *et al.* 1958). The results indicate that these Arthrobacter organisms can be classified into two groups on the basis of their catabolic behaviour. The first group (*Arthrobacter ureafaciens*, *A. globiformis*) relies primarily on the operation of the Embden–Meyerhof–Parnas pathway and, to some extent, the hexose monophosphate pathway for the assimilation of glucose. In the second group (*A. simplex*, *A. pascens*, *A. atrocyaneus*) glucose is catabolized primarily by way of the intermediary formation of gluconate; the Entner–Doudoroff and the hexose monophosphate pathways appear to be the major routes for the assimilation of glucose and gluconate.

INTRODUCTION

Conn (1928) first called attention to the occurrence in soil of a bacterium which exhibited a very striking pleomorphism in the sense of changing from a distinct rod in young cultures to a coccoid form in older cultures, after passing through an elementary life cycle. Although morphologically resembling Corynebacterium, Conn named the soil bacterium in question *Bacterium globiforme*. Subsequently, Conn & Dimmick (1947) proposed the name Arthrobacter for the inclusion of the pleomorphic soil micro-organisms which, in contrast with the animal diphtheroids, liquefy gelatin, do not produce acid from fermentable sugars, are urease negative and grow well on simple defined media with inorganic nitrogen sources. The new genus has been given recognition in the current edition of *Bergey's Manual* (1957). Although many microbiologists felt that the soil globiform group ought not to be included in the genus *Corynebacterium*, the present taxonomic status of the genus *Arthrobacter* is still a subject of much controversy. Lochhead & Burton (1957) and Lochhead (1958), while investigating the dominant soil microflora, isolated and characterized several bacterial species, some of which require amino acids, others being dependent to different degrees upon growth factors. A few of these species were assigned to the genus *Arthrobacter* on the ground of morphological resemblance, in spite of pronounced differences in nutritional requirements. There are nine Arthrobacter species listed in *Bergey's Manual* (1957) which have been brought together mainly on account of their peculiar cell morphology, uneven staining and incidental motility; species characterization according to their physiological behaviours has not been very rewarding. Taylor (1938), summarizing his extensive

work on the utilization of sugars by strains of *B. globiforme*, stated that the findings were so inconsistent and changeable that it was practically impossible to classify these organisms on the basis of acid production from sugars. In fact, one finds in the literature many detailed descriptions of the unusual pleomorphism and life cycles exhibited by this interesting soil organism (Conn & Dimmick, 1947; Lochhead & Burton, 1953; Sacks, 1954; Chaplin, 1957; Lockhead, 1958; Sundman, 1958), whereas studies of its physiology are notably scanty. Moreover, the genus *Arthrobacter* has gradually expanded beyond the commonly held definition of the type-species, *A. globiformis*, an organism which possesses remarkable synthetic abilities and grows well in simple media containing inorganic nitrogen and no growth factors. Thus, the organism, *A. terregens* (Lochhead & Burton, 1953), requires amino nitrogen, biotin, thiamin, pantothenic acid and the 'terregens factor' for proliferation, but is nevertheless included in the genus *Arthrobacter*.

The Arthrobacter organisms constitute an important fraction of the soil indigenous microflora, yet little is known with regard to the catabolic mechanisms functioning in these organisms. With *Arthrobacter ureafaciens* (*Corynebacterium creatinovorans*), Ghiretti & Barron (1954) reported that hexoses were catabolized by a pathway involving pentose and sedoheptulose as key intermediates. The occurrence of the Embden–Meyerhof–Parnas (EMP) pathway in this organism was ruled out on the grounds that glucose cannot be utilized by this organism under anaerobic conditions, although several enzymes in the EMP sequence were reported to be present. In so far as the catabolism of breakdown products of glucose is concerned, these authors suggested that acetate is oxidized *via* the 'dicarboxylic acid cycle'. However, Fukin & Vandermark (1952) reported evidence supporting the operation of the tricarboxylic acid cycle (TCA) in this organism. Morris (1960) reported that in *A. globiformis* glucose is catabolized mainly (65 % of the substrate glucose) by the EMP pathway and to a limited extent (35 %) by the hexose monophosphate (HMP) pathway. Pyruvate, a key degradation product of glucose, was reported to be oxidized mainly via the TCA cycle mechanism. In the present work, the catabolic mechanisms for the assimilation of glucose and gluconate in single strains of each of five species of Arthrobacter have been examined comparatively by the radiospirometric method (Wang *et al.* 1958). The findings provide information for a better understanding of biochemical behaviour and phylogenetic relationships with regard to these organisms.

METHODS

Organisms. One strain of each of five species of the genus *Arthrobacter* were used in this study. They were: *Arthrobacter globiformis*, ATCC No. 8010; *A. ureafaciens*, ATCC no. 7562; *A. simplex*, ATCC no. 6946; *A. pascens* n. sp.; *A. atrocyaneus* sp. The first three were obtained from the American Type Culture Collection (ATCC). *A. pascens* and *A. atrocyaneus* (Kuhn & Starr, 1960) were obtained through the courtesy of the Microbiology Research Institute, Department of Agriculture, Ottawa, Canada, and the Department of Bacteriology, University of California at Davis, respectively. The stock cultures were maintained on nutrient agar slopes (with 0·05 % yeast extract) and kept in the refrigerator (at about 6°) until ready to use.

Media. The media used for growing the organisms are given in Table 1. Glucose and some of the other constituents were sterilized separately by autoclaving for 10 min. at 120°, and mixed asceptically when cool.

Growth of organisms. All organisms were grown in about 100 ml. of liquid media in 250 ml. Erlenmeyer flasks and incubated on a rotary shaker at 28°. Three serial transfers from the stock culture, at suitable time intervals, were made before harvesting the organisms. Organisms in the logarithmic stage of growth were used for the radio-respirometric experiments.

Table 1. *Growth conditions for the Arthrobacter strains used*

Organism		
Arthrobacter ureafaciens	Medium used by Morris, slightly modified. $(NH_4)_6Mo_7O_{24}.4H_2O$ and $Co(NO_2)_2.6H_2O$ were substituted for $Na_2MoO_4.2H_2O$ and $CoSO_4$, respectively. K gluconate was used as sole source of carbon. pH 7·0	Morris (1960), Clark (1955)
A. globiformis	As above but with glucose used as sole carbon source	Morris (1960)
A. simplex	Morris medium with phosphate concentration reduced to 1/8 and addition of yeast extract (0·04 %) and a trace of vitamin B_{12}. Glucose as sole source of carbon	Morris (1960)
A. pascens and *A. atrocyaneus*	Medium used of Loehhead & Burton slightly modified. Dibasic potassium phosphate (0·650 g./l.) and monobasic potassium phosphate (0·350 g./l.) were used; yeast extract, 0·01 %; pH 6·8	Lochhead & Burton (1957)

Harvesting and preparation of organisms. The organisms were removed from the growth medium by centrifugation and immediately resuspended in a carbohydrate-free medium, otherwise identical to the respective growth media. The final concentrations of organisms, as determined with a Klett–Summerson colorimeter, ranged from the equivalent of 0·5 to 1·5 mg. dry wt./ml. medium.

^{14}C*Labelled compounds.* [1-^{14}C]glucose, [2-^{14}C]glucose, [3-^{14}C]glucose and [6-^{14}C]-glucose were obtained from the National Bureau of Standards through the kind co-operation of Dr H. S. Isbell. [3,4-^{14}C]Glucose was prepared in this laboratory from liver glycogen of rats metabolizing $^{14}CO_2$ according to the method of Wood, Lifson & Lorber (1945). [1-^{14}C]Gluconate was purchased from the Nuclear Chicago Corporation. [2-^{14}C]Gluconate, [3-^{14}C]gluconate, [3,4-^{14}C]gluconate and [6-^{14}C]-gluconate were prepared from the corresponding labelled glucose samples by the method of Moore & Link (1940).

Radio-respirometry. The radio-respirometric experiments were carried out according to the procedures described by Wang *et al.* (1958) with 10 ml. of a given suspension of organisms for each of the experiments. Respiratory CO_2 produced by the organisms was trapped with 10 ml. of 0·25 N-ethanolic hyamine hydroxide (trade name for *p*-diisobutylcresoxyethoxyethyl, Rohm and Haas Co. Philadelphia, Pa., U.S.A.) which was replaced at hourly intervals. The radio-respirometric experiment was terminated when the assay of the radioactivity of $^{14}CO_2$ indicated that the labelled substrate had been exhausted from the medium. At that time, the suspension of organisms in the incubation flask was chilled with ice for about 15 min. and the organisms separated from the incubation medium by centrifugation. The clear medium and the washed organisms were then processed for the measurement of radioactivity.

Assay of radioactivity. The radioactivity in the respiratory CO_2, in the form of ethanolic hyamine carbonate, was assayed by means of the liquid scintillation counting technique in the manner described by Wang & Ikeda (1961). Countings were carried out over a sufficient period of time so that the counting data would carry a standard deviation no greater than 2%. The radioactivity in organisms and incubation media was also counted by the use of the liquid scintillation counter. The counting samples in the nature of thioxotropic gel, were usually prepared with 1 ml. of either the aqueous suspension of organisms or the incubation medium with 14 ml. of the gel preparation made according to the procedures of White & Helf (1956). The radioactivity of the labelled substrate was determined by procedures for sample preparation and counting identical with those used for $^{14}CO_2$ samples or organism and medium samples, respectively. The counting efficiencies of the liquid scintillation counting with respect to the various forms of counting samples were determined by the use of internal ^{14}C standards. The details of the liquid scintillation counting procedures are published elsewhere (Wang, 1962).

RESULTS

The complete substrate inventory of the radio-respirometric experiments on the utilization of glucose or gluconate for the five Arthrobacter species are given in Table 2. The data presented are those observed at the end of each experiment. The time-course plots of radio-respirometric data are given in Figs. 1–4 as typical examples. An examination of the data leads one to conclude that the five Arthrobacter strains examined in the present work can be divided into two major groups. In the first group, which consists of *Arthrobacter globiformis* (Figs. 1, 2) and *A. ureafaciens*, one finds that the radio-respirometric patterns for the utilization of glucose are basically different from the patterns for the assimilation of gluconate, whereas in the second group, which consists of *A. simplex*, *A. pascens* and *A. atrocyaneus*, there exists a remarkable resemblance when the radio-respirometric patterns for

Table 2. *Substrate inventory of the radio-respirometric experiments on the utilization of glucose or gluconate by 5 Arthrobacter species.*

Organism		Substrate		Substrate inventory,* (%)			
Species	Conc. of organism mg. dry wt./ 10 ml.)	Labelling	mg./ 10 ml.	Resp. CO_2	Organ-isms	Medium	Total
Arthrobacter ureafaciens	8	[1-^{14}C]Glucose	5	34	57	4	95
		[2-^{14}C]Glucose	5	28	57	8	93
		[3-^{14}C]Glucose	5	49	38	9	96
		[3,4-^{14}C]Glucose	5	57	34	5	96
		[4-^{14}C]Glucose†	—	65	—	—	—
		[6-^{14}C]Glucose	5	29	60	5	94
		[1-^{14}C]Gluconate	2·5	79	2	20	101
		[2-^{14}C]Gluconate	2·5	25	65	8	98
		[3-^{14}C]Gluconate	2·5	28	55	2	85
		[3,4-^{14}C]Gluconate	2·5	44	47	3	94
		[4-^{14}C]Gluconate†	—	59	—	—	—
		[6-^{14}C]Gluconate	2·5	23	68	6	97

Table 2 (*cont.*)

Organism		Substrate		Substrate inventory,* (%)			
Species	Conc. of organism mg. dry wt./ 10 ml.)	Labelling	mg./ 10 ml.	Resp. CO_2	Organ-isms	Medium	Total
A. globiformis	8	[1-^{14}C]Glucose	8	48	45	3	96
		[2-^{14}C]Glucose	8	28	69	2	99
		[3-^{14}C]Glucose	8	32	65	5	102
		[3,4-^{14}C]Glucose	8	43	47	4	94
		[4-^{14}C]Glucose†	—	54	—	—	—
		[6-^{14}C]Glucose	8	21	69	4	94
		[1-^{14}C]Gluconate	10	82	4	13	99
		[2-^{14}C]Gluconate	10	37	51	5	93
		[3-^{14}C]Gluconate	10	25	64	9	98
		[3,4-^{14}C]Gluconate	10	39	56	4	99
		[4-^{14}C]Gluconate†	—	54	—	—	—
		[6-^{14}C]Gluconate	10	18	69	6	93
A. simplex	15	[1-^{14}C]Glucose	2·5	77	22	3	102
		[2-^{14}C]Glucose	2·5	35	59	3	97
		[3-^{14}C]Glucose	2·5	24	70	3	97
		[3,4-^{14}C]Glucose	2·5	32	58	5	95
		[4-^{14}C]Glucose	—	40	—	—	—
		[6-^{14}C]Glucose	2·5	28	65	3	96
		[1-^{14}C]Gluconate	2·5	74	12	16	102
		[2-^{14}C]Gluconate	2·5	39	54	3	95
		[3-^{14}C]Gluconate	2·5	24	72	4	100
		[3,4-^{14}C]Gluconate	2·5	33	57	5	95
		[4-^{14}C]Gluconate†	—	43	—	—	—
		[6-^{14}C]Gluconate	2·5	22	72	3	97
A. pascens	12	[1-^{14}C]Glucose	10	80	17	7	104
		[2-^{14}C]Glucose	10	51	35	9	95
		[3-^{14}C]Glucose	10	53	35	9	97
		[3,4-^{14}C]Glucose	10	65	21	12	98
		[4-^{14}C]Glucose†	—	77	—	—	—
		[6-^{14}C]Glucose	10	32	52	8	92
		[1-^{14}C]Gluconate	5	82	5	6	93
		[2-^{14}C]Gluconate	5	50	37	10	97
		[3-^{14}C]Gluconate	5	43	47	8	98
		[3,4-^{14}C]Gluconate	5	55	30	10	95
		[4-^{14}C]Gluconate†	—	66	—	—	—
		[6-^{14}C]Gluconate	5	31	50	8	89
A. atrocyaneus	12	[1-^{14}C]Glucose	5	88	12	4	104
		[2-^{14}C]Glucose	5	57	33	5	95
		[3-^{14}C]Glucose	5	51	42	5	98
		[3,4-^{14}C]Glucose	5	58	36	3	97
		[4-^{14}C]Glucose†	—	65	—	—	—
		[6-^{14}C]Glucose	5	22	64	8	94
		[1-^{14}C]Gluconate	5	80	3	10	93
		[2-^{14}C]Gluconate	5	63	28	4	95
		[3-^{14}C]Gluconate	5	50	43	5	98
		[3,4-^{14}C]Gluconate	5	53	34	8	95
		[4-^{14}C]Gluconate†	—	56	—	—	—
		[6-^{14}C]Gluconate	5	27	62	8	97

* Substrate inventory data are those collected at the end of each radio-respirometric experiment.

† Data were calculated from experimental findings from the 3-^{14}C glucose and the 3,4-^{14}C glucose experiments.

glucose utilization (Fig. 3, Table 2) and those for the utilization of gluconate (Fig. 4, Table 2) are compared.

It appears that in the Arthrobacters of the first group, glucose is not catabolized by way of gluconate or phosphorylated gluconate, whereas, in the second group, gluconate is the predominant, if not exclusive, intermediate for glucose catabolism. In as much as there exists a drastic difference in the catabolic behaviours of these two groups of Arthrobacter, detailed analysis of the radio-respirometric patterns, with respect to the identification of catabolic pathways functioning in these organisms, will be presented separately under the two stated different groups. To facilitate the analysis of the radio-respirometric data collected in the present work previous findings about the extent of CO_2 formation from each of the carbon atoms

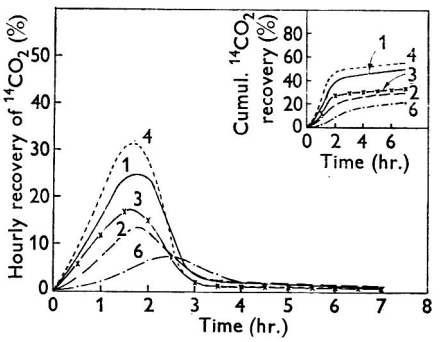

Fig. 1

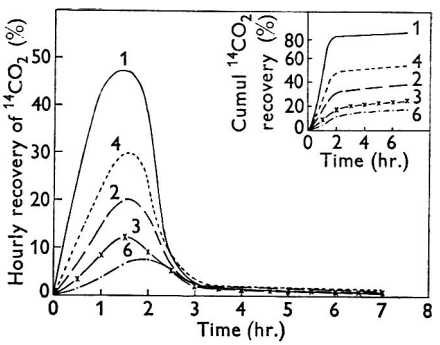

Fig. 2

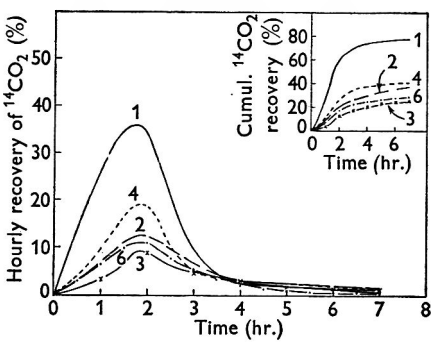

Fig. 3

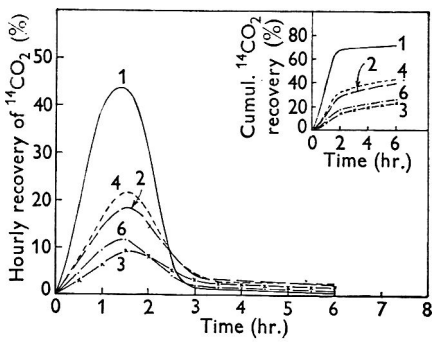

Fig. 4

Fig. 1. Radio-respirometric pattern for the utilization of glucose by *Arthrobacter globiformis*. [1-^{14}C]Glucose,———; [2-^{14}C]Glucose, - - - - -; [3-^{14}C]Glucose, - × - × - × -; [4-^{14}C] Glucose, ---------; [6-^{14}C]Glucose,·····.

Fig. 2. Radio-respirometric pattern for the utilization of gluconate by *Arthrobacter globiformis*. [1-^{14}C]Gluconate,———; [2-^{14}C]Gluconate, - - - - -; [3-^{14}C]Gluconate, - × - × - × -; [4-^{14}C]Gluconate, ---------; [6-^{14}C]Gluconate, ·····.

Fig. 3. Radio-respirometric pattern for the utilization of glucose by *Arthrobacter simplex*. [1-^{14}C]Glucose, ———; [2-^{14}C]Glucose, - - - - -; [3-^{14}C]Glucose, - × - × - × -; [4-^{14}C]Glucose, ---------; [6-^{14}C]Glucose, ·····.

Fig. 4. Radio-respirometric pattern for the utilization of gluconate by *Arthrobacter simplex*. [1-^{14}C]Gluconate,———;[2-^{14}C]Gluconate,- - - - -;[3-^{14}C]Gluconate,- × - × - × -; [4-^{14}C]Gluconate, ---------; [6-^{14}C]Gluconate, ·····.

of glucose by various pathways operative in micro-organisms are summarized in Fig. 5, which serves as a reference for recognizing the major glucose pathways in Arthrobacter.

Arthrobacter species of group 1

Arthrobacter ureafaciens. With glucose as substrate, the extensive recovery of C-4 and C-3 of glucose in CO_2 (Fig. 1, Table 2) points to the operation of the EMP pathway in conjunction with the oxidative decarboxylation of pyruvate as the principal route for glucose assimilation. The product of this sequence, acetate, is presumably catabolized by the TCA cycle. Preliminary work with *A. ureafaciens* indicated that acetate was readily utilized, although it is not a carbon source which supports proliferation. The fact that C-1 of glucose is converted to CO_2 to an extent

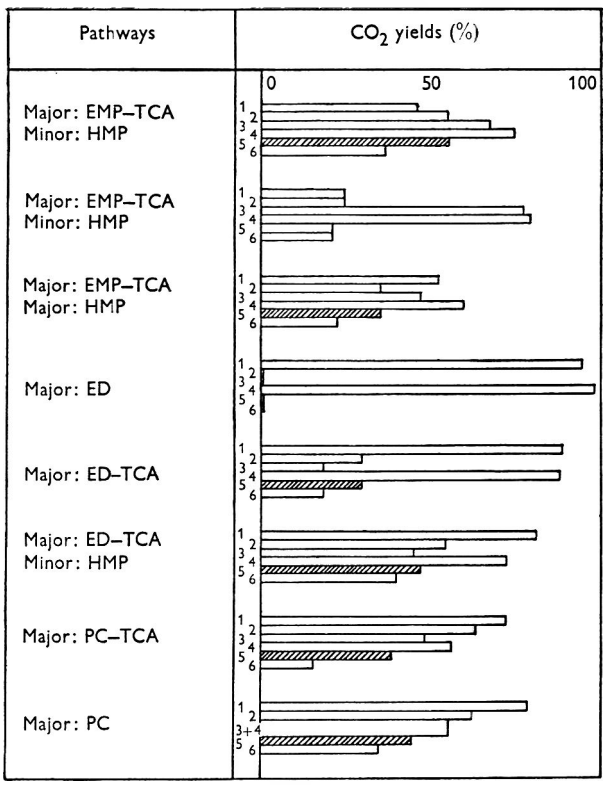

Fig. 5. The relative yield of CO_2 from glucose catabolized by different pathways in micro-organisms. Data taken from references given as follows in the order of the pathway combinations shown in the figure.

Major: EMP–TCA; Minor: HMP (Wang & Krackov, 1962).
Major: EMP–TCA; Minor: HMP (Isono & Wang; unpublished work on baker's yeast.)
Major: EMP–TCA; Major: HMP (Wang & Krackov, unpublished work on *C. utilis*.)
Major: ED (Stern, Wang & Gilmore, 1960).
Major: ED–TCA (Stern, Wang & Gilmore, 1960).
Major: ED–TCA; Minor: HMP (Stern, *et al.* 1960).
Major: PC–TCA (Wang & Bjerre, 1961).
Major: PC (Kitos *et al.* 1958).

▨ Represents values estimated from theoretical considerations.

The numbers in the second column refer to the six carbon atoms of glucose.

slightly greater than that of C-6 suggests that an alternative pathway is also operative in this organism. The nature of the alternative pathway is shown by examination of the radio-respirometric data for gluconate utilization by *A. ureafaciens* grown on gluconate (Table 2). The data can be interpreted on the basis of the concurrent operation of two catabolic sequences, namely the HMP pathway and the Entner–Doudoroff (ED) pathway, since in either case one would find that C-1 and C-4 of gluconate are preferentially converted to CO_2 when compared with other carbon atoms. The exclusive operation of the ED pathway for gluconate assimilation is readily ruled out on the ground that the $^{14}CO_2$ yield from C-1 is considerably greater than that from C-4. The observed gluconate pattern, in fact, points to the operation of the HMP pathway which converts gluconate, upon phosphorylation, to fructose-6-phosphate (F-6-P) which is in turn catabolized in a manner identical with that of substrate glucose, i.e. primarily by the EMP pathway. The conclusion is drawn from a comparison of the observed $^{14}CO_2$ yields from gluconate carbon atoms with that calculated on the basis of the following catabolic sequence:

gluconate → 6-phosphogluconate → pentose phosphate → fructose-6-phosphate →

Predominantly EMP

→ fructose-1,6-phosphate ⟶ triose phosphate → acetyl CoA, etc.

→ glucose-6-phosphate → 6-phosphogluconate → pentose phosphate, etc.

Details of the method of calculation have been reported elsewhere in connexion with studies on the catabolism of glucose and gluconate in *Bacillus subtilis* (Wang & Krackov, 1962).

As shown in Table 3, the calculated $^{14}CO_2$ yields from gluconate carbon atoms are in close agreement with the corresponding values observed experimentally. This fact thus supports the contention that in *Arthrobacter ureafaciens*, glucose is metabolized mainly by way of the EMP-TCA sequence and, to a limited extent, by way

Table 3. *Conversion of gluconate carbon atoms to respiratory* CO_2 *by Arthrobacter organisms*

Gluconate carbon atoms	Calculated yield in respiratory CO_2* (% of substrate)	Observed yield in respiratory CO_2 (% of substrate)
	Arthrobacter ureafaciens	
1	—	79
2	31	26
3	28	27
4	51	59
5	—	—
6	23	23
	Arthrobacter globiformis	
1	—	82
2	35	37
3	24	25
4	44	54
5	—	—
6	17	18

* Calculated on the basis of the catabolic sequence depicted in Fig. 6. Procedures described by Wang & Krackov (1962).

of the HMP pathway depicted in Fig. 6. The participation of each of the two recognized catabolic sequences in the overall glucose catabolism has been estimated according to the method of Wang & Krackov (1962). It appears that in *A. ureafaciens*, substrate glucose is catabolized 93 % by the EMP pathway and 7 % by the HMP pathway.

Arthrobacter globiformis. The radio-respirometric pattern for glucose utilization by this organism resembles to some extent that of *A. ureafaciens* except that in *A. globiformis* C-1 of glucose is converted much more extensively to respiratory CO_2.

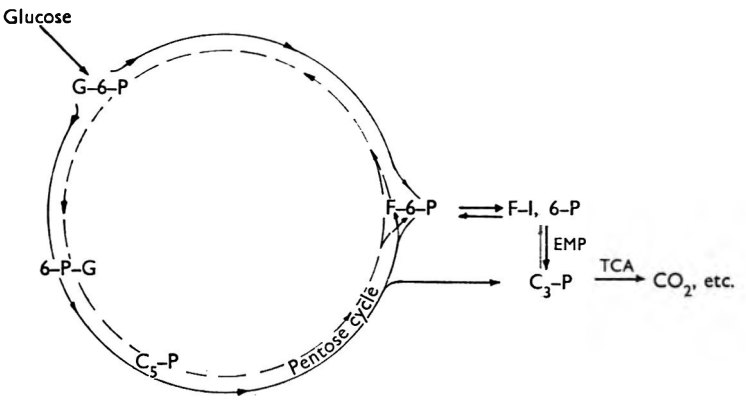

Fig. 6. The catabolic mechanism of glucose in *Arthrobacter ureafaciens* and *A. globiformis*. Primary pathway, ———; secondary re-cycling pathway, - - - - - - - - -.

This can be accounted for on the basis that the HMP pathway plays a more important role in the assimilation of glucose in *A. globiformis*. Data analysis was carried out in the manner similar to that described under *A. ureafaciens* to calculate the yields of CO_2 from gluconate carbon atoms, on the basis that gluconate was catabolized by the HMP–EMP sequence (Fig. 6). Once again the operation of the HMP–EMP sequence is confirmed by the close agreement reached between the calculated and observed values (Table 3). The participation of each of the catabolic sequences for glucose assimilation was then estimated, according to Wang & Krackov (unpublished), on the basis of the foregoing understanding. It appears that 68 % of the substrate glucose was catabolized by the EMP pathway, whereas the HMP–EMP pathway was responsible for the catabolism of 32 % of the substrate glucose. The findings are in good agreement with those reported by Morris (1960) where a different method for pathway estimation was used.

Arthrobacter species of group 2

Arthrobacter simplex. The Arthrobacter species in group 2 appear to metabolize glucose by the intermediary formation of gluconate. The conclusion is drawn from the remarkable resemblance observed when the radio-respirometric pattern for (glucose utilization Fig. 3) is comdared with that of gluconate utilization (Fig. 4). In fact, these patterns remind one of the catabolic behaviour displayed by the pseudomonads (Stern, Wang & Gilmour, 1960). A possible phylogenetic link between the genera *Arthrobacter* and *Pseudomonas*, through the motile plant-pathogenic corynebacteria, has already been suggested by Jensen (1952).

With *Arthrobacter simplex*, the radio-respirometric patterns indicate that the EMP pathway is not operative, or else plays a rather minor role in this organism, since extensive conversion of C-3 or C-4 of glucose to CO_2 was not observed. Instead, the patterns can be best interpreted as representing the occurrence of the Entner–Doudoroff (ED) pathway and the HMP pathway concurrently. This conclusion is drawn from the fact that the metabolic equivalence between C-1 and C-4 of either glucose or gluconate is not realized, thus ruling out the exclusive operation of the ED pathway. The exact nature of the HMP pathway which functions in *A. simplex* cannot be readily recognized by the findings of the present work. Estimation of pathway participation according to Wang *et al.* (1958) indicated that 76 % and 24 % of the substrate gluconate were catabolized by the ED pathway and the HMP pathway, respectively. With glucose as substrate, the ED pathway participated to the extent of 65 % of the total glucose assimilated. The remaining 35 % of the substrate glucose was presumably catabolized by HMP pathway.

Arthrobacter pascens. In close resemblance to the catabolic mechanisms operative in *A. simplex*, glucose was catabolized in *A. pascens* via gluconate by the concurrent operation of the Entner–Doudoroff (ED) and HMP pathways. However, in view of the fact that C-4 of glucose or gluconate was extensively converted to respiratory CO_2 it is reasonable to assume that the ED pathway plays a more important role in the overall carbohydrate catabolism of this organism. An estimation made according to the method described for pseudomonads (Stern, *et al.* 1960) revealed that glucose was catabolized 94 % and 6 %, respectively, by the ED and HMP pathways, whereas gluconate was catabolized 82 % and 18 %, respectively, by the ED and HMP pathways.

Arthrobacter atrocyaneus. Nothing is known about the catabolic mechanisms operative in this recently isolated organism. The radio-respirometric data for the utilization of glucose and gluconate by *A. atrocyaneus* (Table 2) revealed a rather interesting catabolic behaviour. The pattern for glucose utilization resembles basically those of *A. pascens* and *A. simplex*, which suggests that glucose may have been catabolized primarily by the Entner–Doudoroff pathway in conjunction with the HMP pathway in *A. atrocyaneus*. On the other hand, the conversion of C-6 of glucose to CO_2 by *A. atrocyaneus* proceeded at a much slower rate, resulting in an overall recovery of [6-^{14}C]glucose in CO_2 amounting to only 20 % at the end of the experiment. Moreover, the conversion of C-3 of glucose to CO_2 was much more pronounced than that of C-6, leading one to believe that the product of the HMP pathway may have participated more extensively in the pentose cycle pathway than the cases of the other two Arthrobacter species in this group.

With *Arthrobacter atrocyaneus* there were also several significant differences between the radio-respirometric pattern for glucose utilization and that for gluconate utilization (Table 2). With gluconate, the conversion of C-4 of gluconate to CO_2 was not too extensive and the order of CO_2 production from other carbon atoms, i.e. C-1 > C-2 > C-3 > C-6, is reminiscent of the radio-respirometric patterns observed with *Acetobacter suboxydans* (Kitos *et al.* 1958) and *A. melanogenum* (Wang & Bjerre, 1961). It is known that in these two organisms, gluconate is catabolized primarily by the pentose cycle pathway. However, the exact nature of the catabolic mechanism functioning in *A. atrocyaneus* cannot be clearly defined in the present study.

DISCUSSION

One modern trend in bacterial taxonomy endeavours to show natural phylogenetic relationships between groups of micro-organisms. This can perhaps be achieved by a proper evaluation of differential criteria, including morphological and physiological aspects. The relative usefulness of each of these aspects obviously differs from group to group. The emphasis on any given characteristics or set of characteristics in determining the taxonomic position of a particular organism remains largely a matter of personal choice. This accounts for the unsettled situation with regard to the taxonomic status of the genus *Arthrobacter*. The nine recognized species in the genus have been grouped together mainly on account of striking morphological similarities which, for some microbiologists, undoubtedly point to a close taxonomic relationship. Cummins (1959), on the basis of studies on the cell-wall composition of several Arthrobacters pointed out the apparent heterogeneity of the group. On the basis of previous work with other Gram-positive bacteria, Cummins concluded that the amino acid pattern of the cell wall is of significance at approximately the generic level, whereas, the type of sugars and amino sugars present in the cell wall define species within the genus. On these grounds, *Arthrobacter globiformis*, *A. ureafaciens*, *A. citreus*, *A. aurescens* and *A. pascens*, containing alanine, glutamic acid and lysine in the cell wall, would constitute a fairly homogeneous group. On the other hand, *A. simplex* and *A. tumescens*, in which alanine, glutamic acid, glycine and LL-diaminopimelic acid are found in cell walls, should be classified as a separate group. This analysis led Cummins to conclude that the bacteria now classified in the genus *Arthrobacter* are those from a 'mixed origin'. The lack of uniformity among Arthrobacters has repeatedly appeared in the literature, particularly with regard to the marked differences observed in nutritional requirements among these organisms. In our work we noticed such differences which confronted us with difficulties in devising suitable media for growing our organisms.

Recently studies on the biochemical behaviour of different micro-organisms have been more systematic and the information so obtained has been used in the taxonomy of microbial species. A notable example is the work of DeLey (1961) in which a classification of the genus *Acetobacter* has been put forward which takes into consideration the catabolic behaviour of the organisms as well as morphological and physiological characteristics. In the case of Arthrobacter, Jensen (1952) considered this group of micro-organisms as being primitive and versatile in biosynthetic capabilities and accompanied by a catabolic mechanism highly oxidative in nature. Thus, in Jensen's phylogenetic scheme, Arthrobacter, as typified by *A. globiformis*, occupies a position among the coryneform bacteria similar to that of the 'pool of ancestors' as defined by DeLey (1961). Jensen suggested that pseudomonads might have had their origin in the coryneform bacteria, and the Gram-negative characteristic of the pseudomonads was explained on the grounds that the ability to store ribonucleic acid had gradually been lost. This is understandable if it can be recalled that a weak Gram reaction is exhibited by young Arthrobacter cultures. It may be mentioned that one of the outstanding catabolic characteristics of pseudomonads is the fact that the Entner–Doudoroff pathway is known, at the present time, to be operative primarily in this group of organisms.

The diversified aspects of carbohydrate catabolism observed in the single strains of five Arthrobacter species in the present work are in accordance with Jensen's contention (1952) about phylogenetic development. Thus, one finds that on the one hand the Arthrobacter species in group 1 rely on the concurrent operation of the EMP and HMP pathways for glucose assimilation, pointing to a relationship with Propionibacterim, Mycobacterium and animal diphtheroids. On the other hand the Arthrobacters in group 2 appear to be related to the pseudomonads and Acetobacters, as indicated by the fact that glucose and gluconate are catabolized by these organisms primarily by way of the Entner–Doudoroff and the pentose cycle pathways.

It is not suggested that the understanding of catabolic patterns in micro-organisms constitutes by any means the exclusive criteria to be used in microbial taxonomy. However, as suggested by the work of DeLey (1961) and the findings in the present work, the biochemical behaviour of micro-organisms does furnish useful information which may help to devise a better system for the establishment of their phylogenetic relationships. There exists a need for simple procedures for the identification and estimation of catabolic sequences in microbial system; the use of radio-respirometry in this regard is a good example.

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The Physiology of Riboflavin Production by *Eremothecium ashbyi*

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SUMMARY

During the submerged batch cultivation of a riboflavin-producing strain of *Eremothecium ashbyi* three phases were observed. The first phase was characterized by rapid growth of mycelium, rapid utilization and oxidation of glucose and a decrease of pH value caused by accumulation of pyruvic acid. Subsequently acetoin accumulated in the medium. Glucose was oxidized incompletely since only 1·8 μmole oxygen were consumed/μmole glucose utilized. The end of this phase was marked by exhaustion of glucose and cessation of growth. The second phase began with sporulation and was characterized by rapid synthesis of cell-bound riboflavin. Simultaneously a rapid increase in catalase activity and decreases of pyruvate and acetoin were observed. This was accompanied by a marked decrease in Q_{O_2} on glucose while the Q_{O_2} on pyruvate, threonine or acetaldehyde increased to a maximum. Ammonia accumulated in the medium and alkaline pH values were reached. The third phase was characterized by autolysis of mycelium which led to the release of riboflavin and to a decrease of enzymic activities. A comparison of all important physiological parameters was made with two strains of *E. ashbyi* of different riboflavin productivity. On the basis of correlation between riboflavin formation, catalase activity and respiration on acetaldehyde, an hypothesis is proposed to explain over-production of riboflavin by a shift from the initial cytochrome type of terminal respiration to the flavoprotein type which is, however, accompanied by over-production of the flavin prosthetic group.

INTRODUCTION

Although much work has been done in studying the mechanism of biogensis of riboflavin (e.g. MacLaren, 1952; Goodwin & Pendlington, 1954; McNutt, 1954; Plaut, 1954*a*, *b*; Klugsøyr, 1954; Brown, Goodwin & Pendlington, 1955; Goodwin & Jones, 1956; Goodwin & Treble, 1958; Brown, Goodwin & Jones, 1958; Goodwin & Horton, 1960; Katagiri & Imai, 1961) and in examining the nutritional requirements of *Eremothecium ashbyi* (e.g. Schopfer, 1944; Dulaney & Grutter, 1950; Yaw, 1952; Hickey, 1953; Dikanskaya, 1953) little attention has been paid to the physiology of this organism in connexion with riboflavin production. Dikanskaya (1954) found that, under stationary conditions of batch cultivation, in the first days of growth the mycelium was characterized by high respiratory activity and contained cytochromes. When riboflavin biosynthesis began the respiratory activity decreased and the cytochromes disappeared. A strain not producing riboflavin had greater respiratory activity than a producing strain; this difference increased during further

cultivation. The cytochrome system was more active in the non-producing strain than in the producing one. Kramli & Szabo (1956) found that during the submerged growth of *E. ashbyi* the oxidation reduction potential first decreased and later returned slowly to the initial value when riboflavin was synthesized. Holló, Szamel, Nyeste & Tegerdy (1956) described a characteristic pH curve; after a slight decrease on the first day of cultivation the pH rose to about 8. Synthesis of riboflavin began later than growth and the maximal yield of riboflavin was obtained after 6–7 days of incubation.

In the mycelium of *Eremothecium ashbyi* Shimizu, Ohara & Minoura (1952) found not only free riboflavin but also flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD); this observation was confirmed by Stárka (1957). Yagi, Matsuoka, Kuyama & Tada (1956) reported that during the early period of riboflavin production half of the mycelial riboflavin was present as FAD; later FAD was found also in medium. These authors worked out a procedure for preparation of the dinucleotide FAD from *E. ashbyi*.

Dr A. Barth (personal communication, 1959) found that *Eremothecium ashbyi* contained both acid and alkaline phosphatases. While the acid phosphatase had maximum activity during growth, the alkaline phosphatase reached its maximum during riboflavin biosynthesis. The activity curve of acid phosphatase was roughly parallel to the growth curve, while the activity curve of alkaline phosphatase was parallel to the riboflavin formation curve. The specific roles of these enzymes is yet to be considered. On the basis of nutritional studies Dikanskaya (1953) pointed out that growth and riboflavin production were in some sense antagonistic processes: factors which favoured growth retarded riboflavin biosynthesis and vice versa. The same conclusion had already been reached by Schopfer (1944). No attempt was made to elucidate the physiological mechanism of riboflavin over-production by *Eremothecium ashbyi*, i.e. to find why this organism produces such great amounts of this physiologically-active compound. This is the purpose of present paper. Results of our preliminary experiments were published earlier, (Kaprálek, 1957).

METHODS

Organism

Most experiments were carried out with *Eremothecium ashbyi* strain ZA originating from the collection of the Department of Microbiology, Charles University, Prague. For the comparative experiment *E. ashbyi* strain AV was also used; this was derived from a strain from the Centraalbureau voor Schimmelcultures (Baarn, Netherlands) and produces greater amounts of riboflavin.

Growth

Both strains were maintained on medium A (see below) solidified with agar, 20 g./l. A 500 ml. flask with 50 ml. medium A was inoculated from an agar slope. After incubation at 28–30° for 3–5 days on a reciprocal shaker (105 mm. stroke, 96 strokes/min.), 10 ml. of culture were inoculated into a 10 l. flask containing 1000 ml. medium A. This experimental flask was provided with a device for removing samples of culture aseptically during growth which was performed on a reciprocal shaker (50 mm. stroke, 84 strokes/min.) at 28–30°. After the experiment was

finished the volume of culture was not less than 70% of the original volume. In control experiments it was found that this decrease of volume had no significant influence on the physiological parameters. *Medium A* (MacLaren, 1952): glucose, 10 g.; peptone, 10 g.; yeast extract, 1 g.; distilled water to 1000 ml.; pH 6·8 before sterilization for 20 min. at 127°.

Manometric methods

The rates of O_2 uptake and CO_2 production were measured in the Warburg apparatus by the direct methods of Warburg at 28° with air as the gas phase; the respective rates are expressed as Q_{O_2} and Q_{CO_2}, μl. gas/mg. mycelial dry wt./hr. The contents of Warburg vessels will be described later.

Analytical methods

Dry weight of mycelium was estimated by filtration of the culture suspension and drying the washed mycelium at 80° for 24 hr. before weighing.

Riboflavin was determined fluorimetrically on a Klett objective fluorimeter by use of a calibration curve. The *total riboflavin content* was measured as follows. Two ml. of culture suspension + 8 ml. 0·02 N-HCl were autoclaved for 20 min. at 120°; the supernatant fluid was diluted and assayed fluorimetrically. Riboflavin in the medium was determined as follows. Two ml. of culture filtrate + 8 ml. 0·02 N-HCl were autoclaved for 20 min. at 120°; the supernatant fluid was diluted and analysed. The relatively high concentration of riboflavin made it possible to use large dilutions so that the nonspecific fluorescence was eliminated.

pH *measurements* were made with a Philips pH-meter GM 4491 with quinhydrone and antimony electrodes and a saturated calomel electrode.

Oxidation-reduction potential was measured directly in the growing culture. A bright platinum electrode was installed through the cottonwool stopper of the experimental flask together with a bridge of saturated KCl solution in which the saturated calomel electrode was submerged after sterilization.

Paper chromatography. The lower fatty acids were separated according to the method of Brown & Hall (1950) in a mixture of *n*-butanol + 1·5 N-NH_4OH (1 + 1 by vol.) on Whatman no. 4 paper. The keto acids were separated as 2,4-dinitrophenylhydrazones (Cavallini & Frontali, 1954) in a mixture of *n*-butanol + ethanol + water (4 + 1 + 5 by vol.) on Whatman no. 4 paper.

Pyruvic acid was determined by the method of Friedeman (1957). The sample was incubated with the 2,4-dinitrophenylhydrazine at 25° for 25 min. In the control analyses benzene (5 min.) and butanol (25 min.) were used.

Glucose. Analyses were made according to the method of Nelson (Ashwell, 1957).

Acetoin was determined by Westerfeld's method (Krampitz, 1957).

Ammonia was estimated according to the Conway diffusion method with Nesslerization.

The activity of deaminases was measured according to Boyd & Lichstein (1953). The reaction tube contained 2 ml. 0·06 M-solution of substrate, 2 ml. of 0·05 M-phosphate buffer (pH 7·0) and 1 ml. mycelium suspension disintegrated by freezing and thawing. After 1 hr. at 37° 1 ml. of trichloroacetic acid (200 g./l.) solution was added, the mixture centrifuged and the supernatant fluid analysed for ammonia.

The activity was expressed as μmole ammonia liberated by equiv. 1 mg. dry wt. mycelium/hr.

Cytochrome oxidase activity was determined manometrically (Schneider & Potter, 1943): 1 ml. $2{\cdot}3 \times 10^{-4}$ M-solution of cytochrome *c*, 0·3 ml. 4×10^{-3} M-$AlCl_3$, 1 ml. 0·1 M-phosphate buffer (pH 7·4) and 0·4 ml. of cell-free extract of *Eremothecium ashbyi* was put in the Warburg vessel. In the side arm 0·3 ml. 0·114 M-ascorbic acid (adjusted to pH 7) and in central well 0·2 ml. KOH solution (200 g./l.) was placed. The activity of cytochrome oxidase is expressed as μl oxygen consumed/hr./mg. protein, i.e. Q_{O_2} (protein).

The catalase activity was measured iodometrically (Avi-Dor & Yaniv, 1952; Sumner, 1941): 1 ml. washed mycelial suspension (or 1 ml. mycelium suspension disintegrated by freezing and thawing, or 1 ml. cell-free extract, or 1 ml. of culture filtrate) was added to a flask containing 50 ml. of 0·01 N-H_2O_2 in 0·015 M-phosphate buffer (pH 6·8) at 0°. After 3, 6, 9 and 12 min. 5 ml. samples of this suspension were withdrawn and the residual H_2O_2 assayed iodometrically. The velocity constant was determined for each analysis according to the equation $k = \frac{2{\cdot}3}{t} \log_{10} \frac{a}{a-x}$, where a is the initial H_2O_2 concentration and x is the amount of H_2O_2 decomposed in t sec. The value of k_0 was graphically extrapolated for t_0, and a correction for amount of enzyme preparation was used, i.e. the result was divided by $\frac{y}{51}$, where y = mg. dry wt. mycelium, or mg. protein of cell-free extract, or ml. culture filtrate. This corrected value of k_0 was the measure of catalase activity.

Protein in cell-free extracts was determined by the method of Lowry, Rosebrough Farr & Randall, (1951).

Cell-free extracts from mycelium of *Eremothecium ashbyi* were prepared by grinding several ml. of frozen thick mycelial suspension with powdered glass. After centrifugation at 1600 *g* for 10 mins. the supernatant fluid was used. All operations were done at temperatures not exceeding 5°.

Disintegration of mycelium by freezing and thawing. Several ml. of washed suspension (of known dry weight) were submerged in a bath cooled to −70°. After a few minutes the frozen sample was thawed in a water bath at 20°. This procedure was repeated three times. The concentration is expressed as mg. dry wt. original mycelium suspension/ml. This operation led to the complete loss of viability and respiratory activity, indicating complete disintegration of mycelium.

RESULTS

Figure 1 shows the growth and production of riboflavin during submerged batch cultivation. Three phases can be distinguished. The 'phase of growth' was characterized by mycelial synthesis. After growth ceased the culture entered the 'phase of production' in which riboflavin was produced, most of it being in the mycelium. The 'phase of autolysis' was characterized by absence of further riboflavin production, by decrease of mycelial dry weight, and by release of riboflavin into the medium. The boundaries between phases were, of course, not sharp, but for description a simplification may be made; the phases are marked off with interrupted lines (Fig. 1). This overall picture of growth and riboflavin production was accom-

panied by other characteristic changes. The oxidation reduction potential (Fig. 2) decreased rapidly during the lag phase from the initial value of about 0 mV down to −120 mV where it remained during growth. In the phase of production the curve rose to −30 mV but in the phase of autolysis practically no change was observed. The pH curve (Fig. 2) shows that during growth acid was formed, with a decrease to pH 4. This acid disappeared during the phase of riboflavin production and subsequently the culture became alkaline, reaching pH 8, which was higher than the initial value. During autolysis there were usually no changes in pH value; in a few instances the alkalinity increased slightly.

The acid produced was identified by paper chromatography. In the filtrate of 24 hr. cultures much pyruvic acid was found; in older cultures there were only

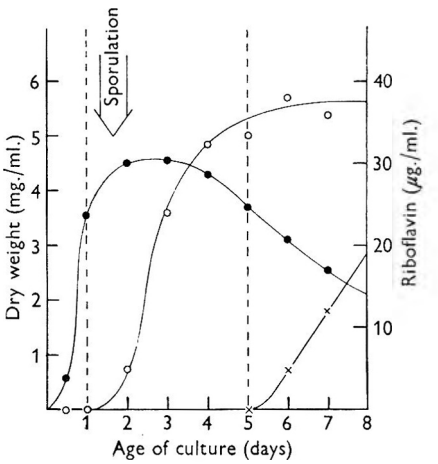


Fig. 1

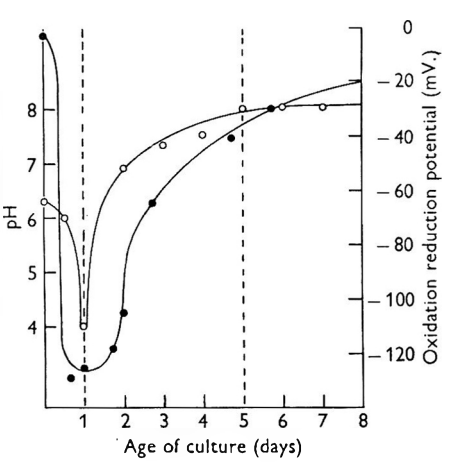


Fig. 2

Fig. 1. Growth and riboflavin production curves of *Eremothecium ashbyi* grown in submerged conditions on glucose-peptone-yeast extract medium. ●——●, growth; ○——○, total riboflavin; ×——×, free riboflavin in medium.

Fig. 2. Changes in pH and oxidation-reduction potential during growth. ○——○, pH; ●——●, oxidation-reduction potential.

traces of pyruvic acid. No α-ketoglutaric acid nor other keto acid was found. These observations were confirmed by analytical methods. Lower fatty acids were not detected by chromatography during growth. Thus the acidity was due only to pyruvic acid. Pyruvic acid accumulated in the medium during the phase of growth and its amount quickly decreased during the production of riboflavin (Fig. 3).

The alkalinity was thought to be due to ammonia arising from the deamination of amino acids of the peptone. Although the concentration of ammonia was low and constant during the phase of growth, a considerable amount went into the medium at the beginning of the phase of production. In the third phase the ammonia concentration increased slightly because of autolysis (Fig. 3). Ammonia liberation into the medium at the beginning of the phase of production was caused by exhaustion of glucose (Fig. 4) since this led to the use of peptone as source of carbon and energy for the growth still perceptible at this stage (Fig. 1). From Figs. 3 and 4 it can be concluded that during the phase of growth the culture dissimilated glucose

quickly but not completely, because from 56 μmole glucose utilized about 25 μmole pyruvic acid were formed i.e. about 25% of utilized glucose accumulated as pyruvic acid. Further oxidation of pyruvic acid was evidently a limiting step in the glucose dissimilation during the phase of growth. A small amount of acetoin also accumulated in the medium (Fig. 4). During further incubation the concentration of pyruvate and acetoin decreased. It seems that during the first two phases the culture was using two different sources of energy and carbon, i.e. glucose in the phase of growth and pyruvic acid in the phase of production.

The catalase activity during incubation was determined since flavin-containing oxidases, supposed to be present during the phase of production, are physiologically coupled with this enzyme. Catalase activity was not detected during the phase of

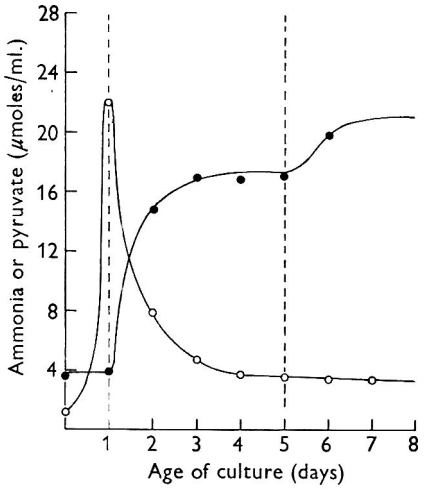


Fig. 3

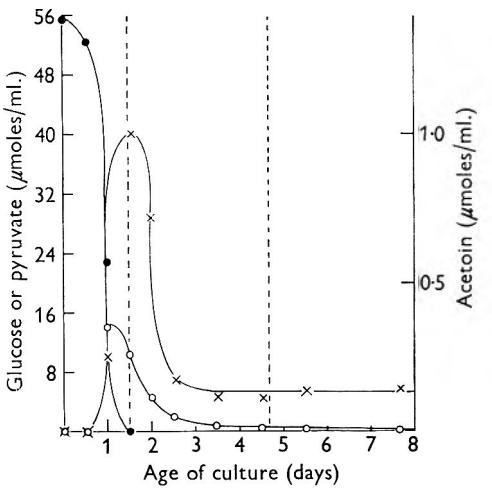


Fig. 4

Fig. 3. The levels of ammonia and pyruvate in the medium during growth. ●——●, Ammonia; ○——○, pyruvate.

Fig. 4. The levels of glucose, pyruvate and acetoin in the medium during growth. ●——●, Glucose; ○——○, pyruvate; ×——×, acetoin.

growth (Fig. 5). In the phase of production catalase activity increased rapidly, together with riboflavin synthesis. There followed a decrease of catalase activity which was typical for the phase of autolysis. Possible effects of permeability factors in this phenomenon were eliminated by using not only intact mycelium but also mycelium disintegrated by freezing and thawing, and a cell-free extract (Fig. 6). The activity of cytochrome oxidase was determined with the cell-free extract (Fig. 6) and the deaminase activities by using disintegrated mycelium (Fig. 7). The deaminase activities for asparagine and serine were consistent with our previous explanation (p. 407) of the alkalization of the medium by ammonia at the beginning of the phase of production.

The respiratory activity of samples of washed mycelium on selected substrates taken during incubation was studied. Warburg vessels contained 1 ml. 0·3 M-phosphate buffer (pH 6·5), 1 ml. 0·06 M-substrate, 1 ml. suspension of washed mycelium and in centre well 0·2 ml. KOH solution (200 g./l.). The results are

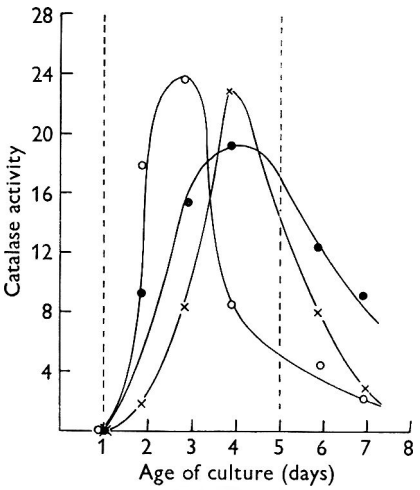


Fig. 5

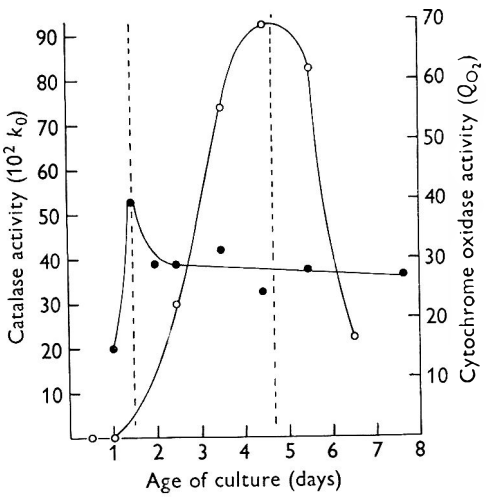
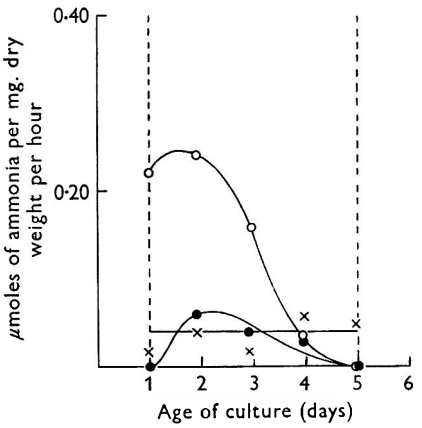


Fig. 6

Fig. 5. The activity of catalase during growth. ●——●, Washed intact mycelium (10^3k_0); ○——○, mycelium disintegrated by freezing and thawing (10^2k_0); ×——×, the filtrate of the culture (10^4k_0).

Fig. 6. The activity of catalase and cytochrome oxidase during growth (cell-free extract). ○——○, Catalase; ●——●, cytochrome oxidase.



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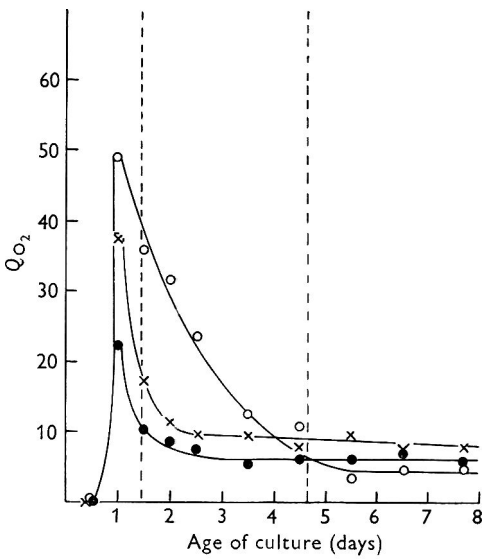

Fig. 8

Fig. 7. The activity of deaminases during growth (mycelium disintegrated by freezing and thawing). ○——○, L-asparagine; ●——●, DL-serine; ×——×, L-threonine.

Fig. 8. The respiratory activity of washed mycelium on glucose and acetate (values are corrected for endogenous respiration); ●——●, Endogenous respiration; ○——○, glucose; ×——×, acetate.

illustrated in Figs. 8, 9 and 10 (values corrected for endogenous respiration). The substrates can be divided into two groups. The first group (glucose, acetate, peptone, DL-serine, L-asparagine; with L-arginine, L-glutamic acid, DL-alanine, vitamin-free casamino acids, in preliminary experiments) showed maximum respiratory activity at the end of the phase of growth. During the phase of production the activity decreased and a low and constant respiration persisted in the phase of autolysis. The second group of substrates (L-threonine, acetaldehyde, pyruvate) had a very low or zero rate of oxidation during the phase of growth. On passing into the phase of production the Q_{O_2} began to rise and reached a peak during production. L-Leucine, L-histidine, glycine, DL-methionine and L-aspartic acid were not oxidized.

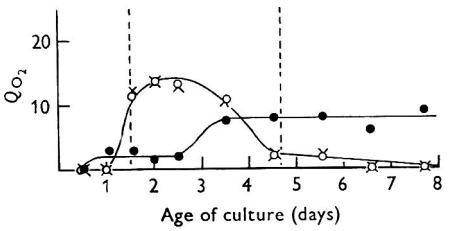

Fig. 9

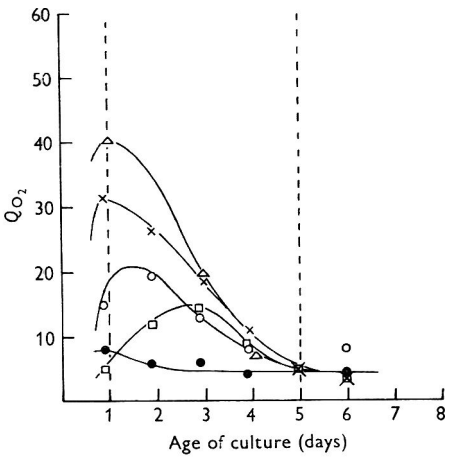

Fig. 10

Fig. 9. The respiratory activity of washed mycelium on pyruvate, L-threonine and acetaldehyde (values are corrected for endogenous respiration which is the same as in Fig. 8). ●——●, Pyruvate; ○——○, L-threonine; ×——×, acetaldehyde.

Fig. 10. The respiratory activity of washed mycelium on L-asparagine, DL-serine, peptone and L-threonine (values are corrected for endogenous respiration). ●——●, Endogenous respiration; ○——○, L-asparagine; ×——×, DL-serine; △——△, peptone; □——□, L-threonine.

The respiratory activity of the whole culture during incubation was also determined by removing 3 ml. samples of culture and immediately measuring Q_{O_2}, Q_{CO_2} and R.Q. in Warburg vessels. From Fig. 11 it is clear that the phase of growth was characterized by high initial values of R.Q., Q_{O_2} and Q_{CO_2} all of which rapidly decreased. During the phase of production the R.Q. curve increased from 0·6 to 0·9, and the rapid decrease in Q_{O_2} and Q_{CO_2} was interrupted. In the phase of autolysis the decrease of Q_{O_2} and Q_{CO_2} continued while the R.Q. value was constant and near to 1·0.

In subsequent experiments the quantitative aspects of oxidation of glucose and pyruvate by 1 day mycelium (phase of growth) and 3 day mycelium (phase of production) were studied. By the direct Warburg method the amounts of O_2 consumed and CO_2 produced per unit of substrate utilized at various concentrations of substrates were determined. At the end of the experiment 1 ml. of 5% (w/v) trichloroacetic acid as added to the Warburg vessel and after centrifugation

the supernatant fluid was analysed for glucose and pyruvate. From Figs. 12 and 13 and Tables 1 and 2 the following conclusions may be drawn for the mycelium from the phase of growth (values of endogenous respiration substracted). The R.Q. value of endogenous respiration was near to 1, suggesting that the endogenous material oxidized was carbohydrate. Glucose was not completely oxidized; about 1·8 μmole oxygen was consumed/μmole glucose. The R.Q. for glucose (20 and 60 μmole/vessel)

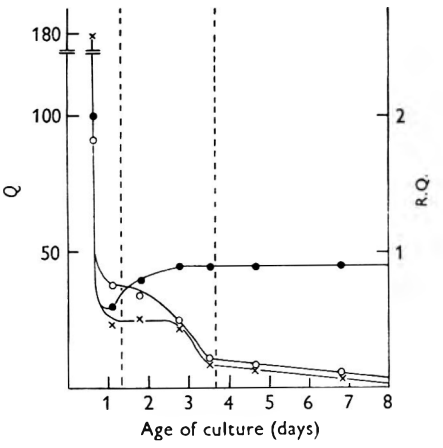

Fig. 11

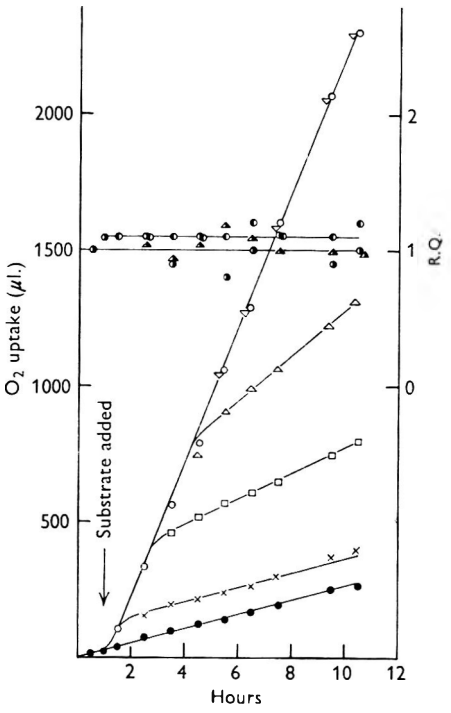

Fig. 12

Fig. 11. The respiratory activity of the whole culture during cultivation. ●——●, R.Q.; ○——○, Q_{O_2}; ×——×, Q_{CO_2}.

Fig. 12. Oxidation of glucose by 1 day-old washed mycelium. Warburg vessels contained: 1 ml of solution of substrate of concentration noted, 1 ml 0·3 M-phosphate buffer pH 6·5 and 1 ml of washed mycelium (4·81 mg dry weight) suspended in 0·8 % (w/v) solution of NaCl. For CO_2 absorption 0·2 ml of 20 % (w/v) KOH was added to the central well. The R.Q. value was determined for each interval separately. ●——●, Endogenous respiration; ×——×, glucose 2 μmole/ml; □——□, glucose 10 μmole/ml; △——△, glucose 20 μmole/ml; ▽——▽, glucose 60 μmole/ml; ○——○, glucose 100 μmole/ml; ◑——◑, R.Q. for endogenous respiration; ▲——▲, R.Q. for glucose 20 μmole/ml; ◐——◐, R.Q. for glucose 60 μmole/ml.

was near to 1. After the glucose (10 and 20 μmole/vessel) was exhausted the curve was not parallel to the endogenous respiration. The possibility exists that a labile endogenous material (pyruvic acid) was made from glucose. This material may then be oxidized very slowly or it may stimulate the endogenous respiration (see below). This would agree with the observation that the endogenous respiration of 1 day mycelium (when glucose is still present in the medium) was greater than that of older mycelium (Figs. 8 and 10).

Pyruvate was oxidized only very slowly, but its concentration decreased more rapidly, suggesting metabolic activity (intracellular accumulation) without O_2 consumption or CO_2 production. The possibility cannot be excluded that pyruvate is not oxidized at all but causes stimulation of endogenous respiration.

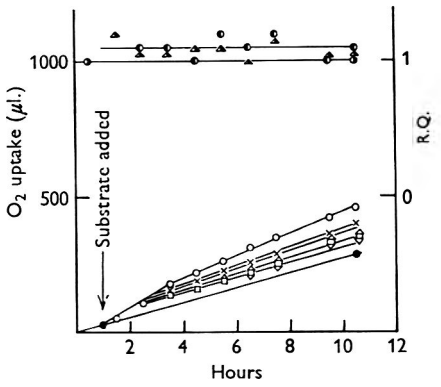

Fig. 13

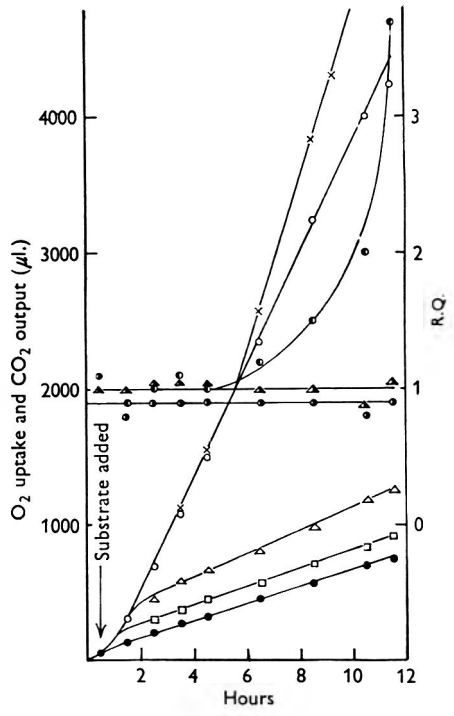

Fig. 14

Fig. 13. Pyruvate oxidation by 1 day-old washed mycelium. The contents of Warburg vessels are the same as in Fig. 12. The R.Q. value was determined for each interval separately. ●——●, Endogenous respiration (the same as in Fig. 12); ▽——▽, pyruvate 40 µmole/ml; □——□, pyruvate 60 µmole/ml: △——△, pyruvate 80 µmole/ml; ×——×, pyruvate 100 µmole/ml; ○——○, pyruvate 150 µmole/ml; ◑——◑, R.Q. for endogenous respiration (the same as in Fig. 12); ▲——▲, R.Q. for pyruvate 80 µmole/ml; ◐——◐, R.Q. for pyruvate 150 µmole/ml.

Fig. 14. Glucose oxidation by 3 days old washed mycelium. The contents of the Warburg vessels are the same as in Fig. 12 with the exception of mycelium concentration: 10·74 mg/ml. The R.Q. value was determined for each interval separately. ●——●, Endogenous respiration; □——□, glucose 5 µmole/ml; △——△, glucose 10 µmole/ml; ○——○, glucose 100 µmole/ml; ◑——◑, R.Q. for endogenous respiration; ▲——▲, R.Q. for glucose 10 µmole/ml; ◐——◐, R.Q. for glucose 100 µmole/ml; ×——×, C_{O_2} production in µl. on glucose 100 µmole/ml.

From Figs. 14 and 15 and Tables 1 and 2 it is apparent that 3 day mycelium also oxidized glucose incompletely: about 1·8 µmole O_2 was consumed/µmole glucose utilized. The R.Q. value was near to 1, except with the high concentration of glucose (100 µmole/vessel). In this case a higher rate of CO_2 production occurred at 4 hr., accompanied by an increase in R.Q. At the end of the experiment an excess of 87 µmole CO_2 had been produced over that expected from the O_2 consumption for an R.Q. of 1. This phenomenon may be explained by anaerobic decarboxylation of the

pyruvic acid arising from incomplete oxidation of glucose; 87 μmole CO_2 corresponds to 87 μmole pyruvate decarboxylated, which corresponds to 43·5 μmole glucose. At the end of the experiment 1·2 μmole pyruvate was found. This means that

Table 1. *Glucose oxidation by* 1 *day old and* 3 *days old washed mycelium (see Figs.* 12 *and* 14)

Glucose added (μmole/vessel)	At the end of experiment: Glucose found (μmole/vessel)	At the end of experiment: Pyruvate found μ(mole/vessel)	Glucose utilized (μmole/vessel)	Oxygen consumed (μmoles)*	Oxygen consumed (μmole/μmole glucose utilized)
		1 day old mycelium			
2	0·0	0·0	2·0	4·5	2·2
10	0·7	0·0	9·3	16·0	1·7
20	2·0	0·0	18·0	31·6	1·8
60	13·3	0·2	46·7	90·9	1·9
100	44·1	0·6	55·9	90·9	1·6
					Mean 1·8
		3 days old mycelium			
5	0·2	0·0	4·8	7·1	1·5
10	0·3	0·0	9·7	22·8	2·3
100	4·3	1·2	95·7	154·0	1·6
					Mean 1·8

* Corrected for endogenous respiration.

Table 2. *Pyruvate oxidation by* 1 *day old and* 3 *days old washed mycelium (see Figs.* 13 *and* 15)

Pyruvate added (μmole/vessel)	Residual pyruvate (μmole/vessel)	Pyruvate utilized (μmole/vessel)	Oxygen consumed (μmoles)*	Oxygen consumed (μmole/μmole pyruvate utilized)
		1 day old mycelium		
40	13·3	26·7	2·2	0·08
60	15·5	44·5	3·8	0·09
80	30·8	49·2	4·9	0·10,
100	35·9	64·1	6·2	0·10
150	56·3	93·7	8·0	0·09
				Mean 0·09
		3 days old mycelium		
60	11·3	48·7	12·5	0·26
80	12·2	67·8	20·1	0·29
150	24·0	126·0	40·6	0·32
				Mean 0·29

* Corrected for endogenous respiration.

from 95 μmole glucose utilized at least 44 μmole (46 %) was transformed to pyruvate. The amount of pyruvate formed might be still greater since intracellular accumulation (see above) and the oxidation (see below) of pyruvic acid might also occur.

The mycelium from 3 day cultures oxidized pyruvate, but very incompletely: about 0·3 μmole O_2 was consumed/μmole pyruvic acid utilized. The observed R.Q. (1·2) is the sum of R.Q.s of endogenous respiration (0·9) and of pyruvate oxidation. The R.Q. for pyruvate oxidation itself can be calculated according to the equation $0{\cdot}9k_1 + Xk_2 = 1{\cdot}2(k_1 + k_2)$ (where k_1 = rate of endogenous respiration, k_2 = rate of O_2 consumption on pyruvate itself and X = R.Q. for pyruvate oxidation). Substituting experimentally obtained values for k_1 and k_2 in this equation yields a value of 1·35 for the R.Q. of pyruvate oxidation. This is greater than the theoretical 1·2 and suggests anaerobic decarboxylation in addition to oxidation of pyruvate.

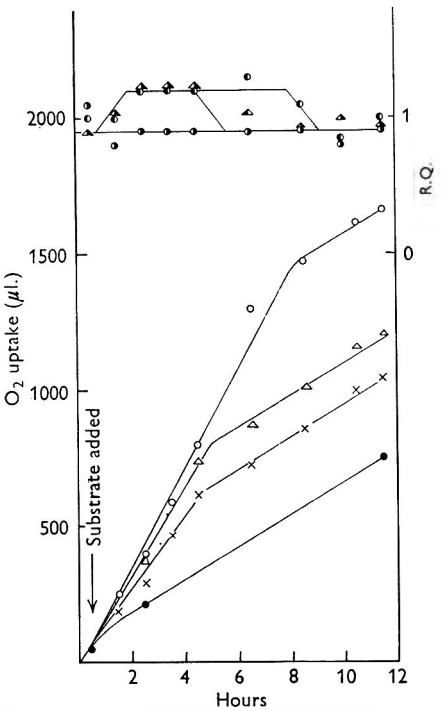

Fig. 15. Pyruvate oxidation by 3 days old washed mycelium. The contents of the Warburg vessels are the same as in Fig. 14. The R.Q. value was determined for each interval separately. ●——●, Endogenous respiration (the same as in Fig. 14); ×——×, pyruvate 60 μmole/ml; △——△, pyruvate 80 μmole/ml; ○——○, pyruvate 150 μmole /ml; ◑——◑, R.Q. for endogenous respiration (the same as in Fig. 14); ▲——▲, R.Q. for pyruvate 80 μmole/ml; ◐——◐, R.Q. for pyruvate 150 μmole/ml.

Comparison of two strains of Eremothecium ashbyi

All the important physiological parameters of the cultivation were compared for two strains: *Eremothecium ashbyi* strain ZA (with which all the hitherto described experiments were made) and *E. ashbyi* strain AV, which produces three times as much riboflavin. The purpose of this comparison was to verify the interrelationships found by using another strain of *E. ashbyi* and to eliminate coincidental phenomena not related to riboflavin over-production and to *E. ashbyi* in general. The other question under study was the quantitative relationships between riboflavin production and other physiological processes. From the comparison it follows that the

results reported here were valid for both strains, so that their pertinence for riboflavin production by *E. ashbyi* is probably general. Both strains showed little significant difference in the physiological parameter except for riboflavin production and Q_{O_2} on pyruvate (Fig. 16).

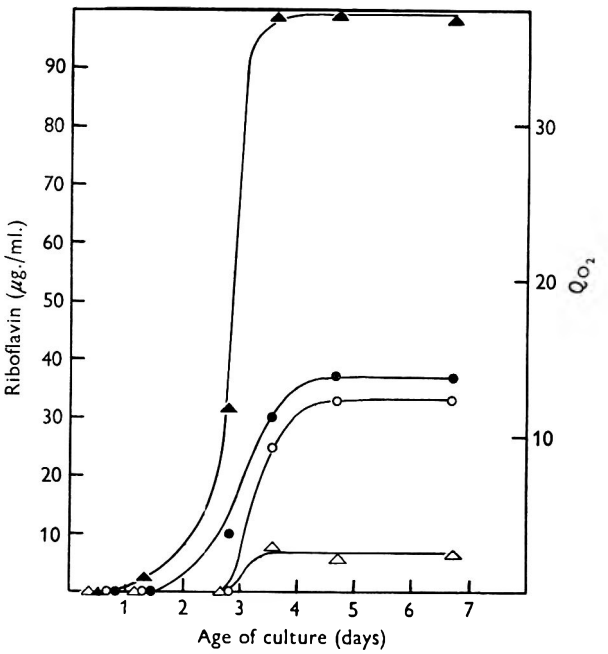

Fig. 16. Comparison of two strains of *Eremothecium ashbyi* of different riboflavin productivities. The lower producing strain *E. ashbyi* ZA: ●——●, total riboflavin in μg/ml; ○——○, Q_{O_2} on pyruvate. The higher producing strain *E. ashbyi* AV: ▲——▲, total riboflavin in μg/ml; △——△, Q_{O_2} on pyruvate. The values of Q_{O_2} are corrected for endogenous respiration and are for washed mycelium.

DISCUSSION

It is apparent that the curves of growth, riboflavin production and pH values of culture differed markedly from those found under static conditions (Dikanskaya, 1954; Goodwin, 1959) not only in that all processes were more rapid in our submerged growth conditions but also that under static conditions no succession was found in the main phases—i.e. of growth, production and autolysis. Our curves of pH value, glucose and total riboflavin production may be compared with the results of Mickelson (1950) and Pfeifer, Tanner & Traufler (1950) with submerged cultivation of *Ashbya gossypii*, an organism very closely related to *Eremothecium ashbyi*. The curves of riboflavin biosynthesis and pH changes are very similar to those found by Holló *et al.* (1956). In our experiments the curve of oxidation-reduction potential as found by Krámli & Szabó (1956) was confirmed.

The most interesting observation seems to be that simultaneously with riboflavin production catalase activity appeared for the first time, although 80% of the mycelium had already been formed. This, together with the finding of Dikanskaya (1954) that the cytochromes of *Eremothecium ashbyi* disappeared during riboflavin production, suggests (Kaprálek, 1957) that riboflavin production is a consequence of

a shift from the cytochrome type of terminal respiration to the flavoprotein type. The latter is physiologically coupled with catalase, but here a disorder in flavin oxidase activity leads to failure in regulation of flavin coenzyme synthesis.

The observations are consistent with this hypothesis. The physiological mechanism of riboflavin production by *Eremothecium ashbyi* may be pictured as follows. Until sporulation the culture grows normally and utilizes glucose (Figs. 1 and 4). Respiration proceeds by the cytochrome system (Dikanskaya, 1954; Fig. 6) but as an intermediate of glucose dissimilation, pyruvic acid, accumulates (Fig. 3), and causes a marked decrease in the pH value of the medium (Fig. 2). The further oxidation of pyruvate is apparently the limiting step in glucose oxidation (Figs. 9, 13; Table 2), i.e. the organism cannot oxidatively decarboxylate pyruvate to acetate, although it has a system for acetate oxidation (Fig. 8). The oxygen consumption of 1·8 μmole/μmole glucose utilized and the R.Q. of 1·0 (Fig. 12; Table 1) can be then explained only by supposing that the pentose cycle operates, allowing oxidation of glucose to CO_2 and H_2O by a mechanism alternative to glycolysis and the Krebs cycle. In the case of *E. ashbyi* the oxidation of glucose is incomplete and the sequence of reactions may be summarized as follows:

$$6 \text{ hexose-PO}_4 + 6\ O_2 + 6\ H_2O \rightarrow 6 \text{ pentose-PO}_4 + 6\ CO_2 + 12\ H_2O$$

$$4 \text{ pentose-PO}_4 \rightarrow 2 \text{ hexose-PO}_4 + 2 \text{ tetrose-PO}_4$$

$$2 \text{ pentose-PO}_4 + 2 \text{ tetrose-PO}_4 \rightarrow 2 \text{ hexose-PO}_4 + 2 \text{ triose-PO}_4$$

$$2 \text{ triose-PO}_4 \rightarrow 2 \text{ triose} + 2\ P_{in}$$

$$2 \text{ hexose-PO}_4 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O + 2\ P_{in} + 2 \text{ triose}$$

The incompleteness of glucose oxidation may be caused by deficiency of aldolase which normally condenses two triose-phosphates to fructose-1-6-diphosphate, from which one phosphate group is split thus completing the cycle. The postulated deficiency of aldolase and the demonstrated low rate of pyruvate oxidation might be the cause of pyruvate accumulation in glucose dissimilation. The fact that the full theoretical amount of pyruvate was not found might be due to utilization of some pyruvate for biosynthesis of mycelium.

Later the cytochromes disappear (Dikanskaya, 1954) for reasons not yet known. Some connexion with sporulation or an effect of some metabolite may be involved. During utilization of peptone as a carbon and energy source the deaminase activities increase (Fig. 7) and ammonia accumulates (Fig. 3) causing the culture to become alkaline, pH 7–8, (Fig. 2). At the same time biosynthesis of riboflavin begins (Fig. 1), catalase activity emerges (Fig. 5) and the amount of pyruvic acid decreases (Fig. 3). The Q_{O_2} on glucose is decreased (Fig. 8) while on the other hand the Q_{O_2} on pyruvate, threonine and acetaldehyde increases (Fig. 9). The riboflavin formed is bound in the cells (Fig. 1) and much is present as FAD (Yagi *et al.* 1956).

These facts could be explained by the appearance of a new enzyme of inducible character, a flavoprotein which reacts directly with oxygen, producing H_2O_2 which is decomposed by catalase. This new enzyme may be involved in the oxidation not only of accumulated pyruvate, but also of L-threonine and acetaldehyde, since the maximum Q_{O_2} values of these all lie in the phase of production. The common denominator of these three substrates is acetaldehyde, which arises from pyruvic acid by decarboxylation and from threonine by the action of threonine aldolase.

The presence of both processes in *Eremothecium ashbyi* was shown by Goodwin & Horton (1960) who pointed out that L-threonine specifically stimulated the biosynthesis of riboflavin. We may therefore conclude that the inducible flavoprotein is an aldehyde oxidase. The well-known aldehyde oxidase contains FAD as coenzyme, has an optimum at pH 7–8 and oxidizes aldehydes to the corresponding acids (in particular acetaldehyde to acetate which is further oxidized) (Fig. 8). This type of respiration is thus used by the organism after the loss of the cytochrome system.

However, the regulation of FAD synthesis breaks down for reasons not yet known and this is the ultimate cause of riboflavin over-production. Perhaps a system is present which continually splits off the prosthetic group of the flavoprotein and this leads to compensation in the form of increased FAD synthesis. The greater this disorder, the lower is the respiratory activity on pyruvate during the phase of production and the greater is the riboflavin over-production (Fig. 16). The opinion that there exists a reserve type of respiration is supported by the results shown in Fig. 11. The rapid decrease in Q_{O_2} during the phase of growth is diminished with the beginning of riboflavin production and a further decrease to the minimal level occurs when production ceases.

The large amount of pyruvate in the medium also causes the rise of acetoin (Fig. 4). From the viewpoint of respiration the formation of acetoin represents a blind alley, but in connection with its possible role in the construction of the riboflavin molecule (Goodwin & Treble, 1958; Goodwin, 1959) this formation might have great importance. This idea is supported by the disappearance of acetoin during the phase of production (Fig. 4). In other words, pyruvate (via acetaldehyde) is not only the substrate of the flavin enzyme but also the building material (via acetoin) for the construction of its prosthetic group. This hypothesis also explains the fact that acetate inhibits riboflavin biosynthesis but not growth on glucose medium (Brown *et al.* 1955). Acetate is the product of the reaction catalysed by the flavin enzyme and may act as a repressor of the synthesis of this enzyme or as a source of energy for which no new enzyme is necessary when this product is added exogenously. When comparing this interpretation of physiological mechanism of riboflavin production by *Eremothecium ashbyi* with the situation in *Ashbya gossypii* (Mickelson, 1950) it seems that the physiological mechanisms of riboflavin production by both organisms are very similar, with the exception that ethanol plays the role in *A. gossypii* which pyruvate plays in *E. ashbyi*.

The interchange of a cytochrome type of terminal respiration with a flavoprotein one, as a result of changes in environmental conditions, is not unknown in microorganisms. Nicholas (1956), with a mutant of *Neurospora crassa* which required riboflavin, found that when riboflavin deficiency occurred the activity of cytochrome oxidase increased to 140% and the activity of nitrate reductase and nitrite reductase decreased to 10% as compared with the control. At the same time the activity of catalase was decreased to 80% of the control. Lenhoff, Nicholas & Kaplan (1956) found that in *Pseudomonas fluorescens* iron deficiency caused an increase in the activity of flavin enzymes and a decrease in the activity of cytochrome system.

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Growth, Cell and Nuclear Divisions in some Bacteria

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SUMMARY

The timing of cell and nuclear division of certain enteric bacteria was determined under conditions of balanced growth. Organisms were grown in a high refractive index medium and photographed at frequent intervals with a phase-contrast microscope. This allowed an estimation of the time between successive divisions of nuclei and cells (interdivision times) and the growth rate of each individual. The interdivision times of cell and nuclear divisions had a similar degree of variation (coefficients of variation of about 20 %). The interdivision times of sister cells and sister nuclei were positively correlated to a significant degree. The correlation between mothers and daughters was negative to a significant degree in some, but not all, experiments. The correlation between interdivision times of a cell and that of its corresponding nucleus was positive in most experiments.

The rate of mass increase of individual cells was estimated by measuring the rate of elongation. Within the limitations of the method of observation, it could be concluded that cells grew exponentially between successive divisions. Different individuals grew with very nearly the same rate constant. The variation in *size of cells* at the time of nuclear and cell division was smaller (coefficients of variation of about 10 %) than that of the interdivision times.

Some observations on the morphological changes of nuclei during growth and division are presented.

INTRODUCTION

The time between successive cell divisions varies significantly among individuals in the same culture of certain species of bacteria commonly studied. For this reason, division synchrony must usually be induced by artificial manipulations. This fact has hampered studies of the structural and chemical changes throughout the life span of bacterial cells.

We will call the effective life span of the cell—the time between two successive divisions—the 'cell interdivision time'.

Powell (1958) has suggested that the variability of the cell-division process may be confined to the last stages of cell separation. He suggested that other components of the cell-division cycle may be less variable. We have attempted to investigate this question by timing the interval required for division of bacterial nuclei (the nuclear interdivision time). We have employed the technique of Mason & Powelson (1956), which permits continuous observation of nuclei in living bacteria. In this technique cells growing in high refractive index media are observed with a

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phase-contrast microscope. Under these conditions, the contrast between the nuclei and the cytoplasm is greatly enhanced.

We have compared the timing of nuclear division to that of cell division and to the pattern of growth of individual cells. These data have been used for the construction of a model for the statistics of division processes in bacteria (Koch & Schaechter, 1962).

MATERIALS AND METHODS

Growth conditions. The chambers used for observation were prepared as follows: a small drop of an exponentially growing culture of density less than 5×10^7 bacteria/ml. was placed on the surface of a thin layer of agar containing Trypticase Soy broth, 1·5 % Ionagar (Consolidated Laboratories), and polyvinylpyrrolidone (hereafter, PVP) in amounts varying between 23 and 27 %. One half to one hour later the drop was spread over a portion of the agar surface with a bent rod. A block of agar about 7 mm. square was cut out, placed on a glass slide, and covered with a 22 mm. square no. 0 cover-glass. Slides and cover-glasses were cleaned by prolonged immersion in fuming nitric acid, rinsing in distilled water, and air drying. The edges of the preparations were sealed with a paraffin-beeswax mixture to prevent drying. All manipulations were carried out at 37° in an incubator room.

Microscopy. All observations were done with a Wild phase-contrast microscope enclosed in a plastic chamber maintained at 37°. As little light as possible was used. Pictures were taken with 35 mm. Kodak High Contrast film which was processed with D-19 developer. Enlargements were made on Kodabromide F-5 contrast paper. Contrast on the prints was considerably higher than by direct observation.

Evaluation of the photographs. The measurements of interdivision time were obtained from photographic prints. Cell lengths were measured with calipers on projections of the photographic films. In most experiments pictures were taken every 1 or 2 min., in some every 2 or 3 min.

The criteria used for establishing when cells and nuclei had divided were, of necessity, subjective. Cell division was considered to be completed when the cells' adjoining ends appeared to be fully hemispherical. It was judged that a nucleus had divided when the two resulting bodies were clearly individualized and no nuclear material could be seen between them. Using these criteria, cell and nuclear division probably terminated before the time assigned by the observer. Such systematic errors in scoring, by themselves, would not lead to errors in the interdivision times. After some experience the time of cell or nuclear division could be estimated with a variation no greater than 2–4 min., that is, within one or two pictures. In less than 10 % of the cases this uncertainty extended itself over as much as 12 min. Despite the subjectivity of the evaluation, the results obtained were quite reproducible. Series obtained earlier in the work were re-evaluated after several months. Close agreement of all measurements was obtained on repeated evaluation by the same or a different observer.

Bacterial strains. Some smooth colonies of *Escherichia coli* strain B/r when illuminated by indirect light showed radial striations characteristic of rough strains. From such a colony a mutant which showed rougher colonies was isolated and designated *E. coli* B/r (rough). *Proteus vulgaris* and *Salmonella typhimurium* LT-2 colonies were smooth.

RESULTS

The growth of single cells. The data employed in the present work were derived from populations undergoing balanced growth, as determined by the following criteria: (1) Exponential increase of the number of cells and nuclei in the field. (2) Constancy of the mean cell size. (3) Close agreement between the mean cell and nuclear interdivision times and the mean doubling time determined independently from the increase in numbers for the entire field. (4) When measured, maintenance of the same rate of elongation by individual cells and their descendants.

Balanced growth was originally defined in a stricter fashion (growth resulting in a proportional increase of every extensive cellular property in the same period of time; Campbell, 1957). We feel that compliance with the criteria employed is a strong indication that growth was indeed balanced.

The period of observation extended over three to four mean doubling times. Thereafter, the microscopic fields became too crowded for careful measurements. About ten cells were present at the beginning of the period of observation in the portion of the field included in the photographic frame. The data reported are derived from scoring two consecutive divisions and include the entire progeny of the original individuals. This procedure avoids the discussed bias due to an arbitrary cut-off of the period of observation, by Powell (1955).

Some experiments were not included because growth was unbalanced. Here cell division either proceeded at a progressively slower rate, or was synchronized to a higher degree than expected from random sampling of small numbers of cells.

The rate of growth (expressed as the mean doubling time in minutes) was estimated from the increase in the number of cells. The mean doubling time for *Salmonella typhimurium* on PVP-agar (31–35 min.) was slower than on agar without PVP (Table 1), but was the same as in aerated Trypticase Soy broth (without PVP) cultures. The mean doubling time of *Escherichia coli* B/r growing on PVP-agar (26–32 min.) was slower than either in broth without PVP or on agar without PVP, where it was 18–20 min. These differences were probably due to the physical conditions of cultivation.

The rate of elongation of *Escherichia coli* B/r and *Salmonella typhimurium* was estimated from measurements of length of individual cells during growth. As can be seen from Fig. 1, cells increased in length at all times during the division cycle, without noticeable discontinuities. Similar plots were obtained when the lengths of the progeny cells were summed over two or three divisions. The shape of the curve varied slightly between individual cells. However, this variation was independent of the size of the cells at division, since large and small cells elongated with approximately the same rate constant. It was very difficult to obtain exact measurements since, in addition to the limitations in the resolution of the microscope, errors were introduced by curvature of the cells or differences in focal plane. For this reason it was not possible to determine the precise mode of elongation between cell divisions. None the less, our measurements were consistent with the hypothesis that elongation was proportional to the length of the cells, i.e. that all cells grew exponentially with the same growth rate constant. It must be kept in mind that an exponential line and a straight line differ only slightly over one doubling. The diameter of the cells was not measured but did not appear to vary perceptibly during growth. Data

Table 1. *Variation of interdivision times*

The mean cell and nuclear interdivision times and the coefficients of variation (standard deviations as fraction of the means) for individual divisions are presented. The growth rate was obtained from a plot of the number of cells in the field *vs.* time. The values given in the last column are the mean doubling times in minutes. The differences between the cell and nuclear mean interdivision times of each experiment are not significant at the P=0·05 level, except for expts. B-5 and C-1, where $P = 0{\cdot}1\ \%$.

			Cell division			Nuclear division			
Organism	Experiment	Growth conditions	No. obs.	Mean time	Coefficient of variation	No. obs.	Mean time	Coefficient of variation	Mean doubling time
E. coli B/r	A-1	Trypticase Soy agar (TSA) with PVP	46	28·50	0·176	71	30·20	0·183	31
	A-2		62	24·95	0·208	87	28·2	0·211	26
	A-3		46	32·89	0·137	60	33·55	0·193	32
S. typhimurium	B-1	TSA with PVP	48	35·70	0·224	42	32·66	0·182	32
	B-2		32	31·80	0·196	29	33·11	0·229	32
	B-3		53	30·48	0·151	64	28·66	0·218	31
	B-4		50	35·28	0·169	37	37·97	0·144	35
	B-5		48	30·92	0·152	36	34·42	0·147	33
E. coli B/r (rough)	C-1	TSA with PVP	62	37·45	0·175	75	33·52	0·219	38
	C-2		62	32·98	0·171	68	34·09	0·204	34
P. vulgaris	D-1	TSA with PVP	45	44·70	0·212	50	47·26	0·172	46
	D-2		25	40·00	0·200	33	42·20	0·256	43
E. coli B/r	E-1	TSA	68	18·85	0·152	—	—	—	20
S. typhimurium	F-1	TSA	63	28·00	0·228	—	—	—	24
	F-2		129	23·60	0·144	—	—	—	24
	F-3		69	23·04	0·179	—	—	—	24

presented by other authors indicate that this dimension remains approximately constant during the development of individual cells (Adolph & Bayne-Jones, 1932; Deering, 1958; Maclean & Munson, 1961). The apparent refractive index of the cells also remained constant during their development. Measurement of the refractive index with the interference microscope has shown little variation among individuals

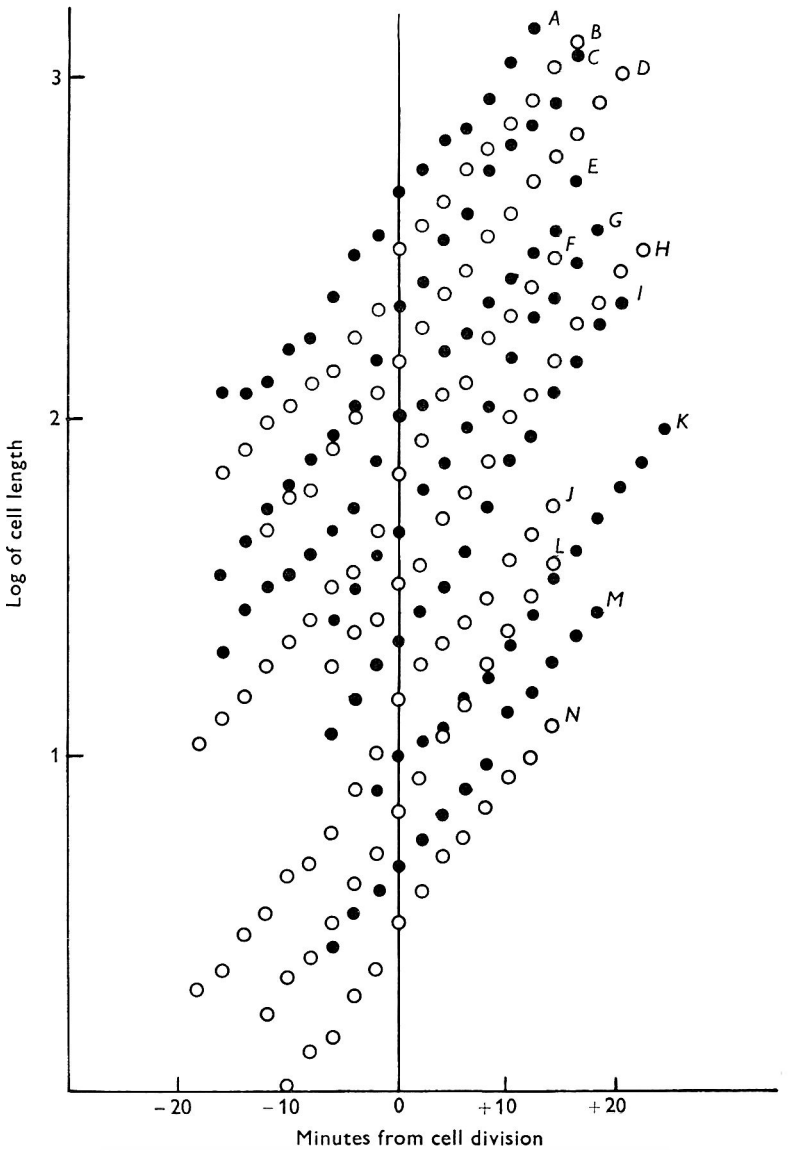

Fig. 1. Elongation of individual cells. The length of *E. coli* B/r cells (Expt. E-1) was measured every 2 min. The length of the resulting daughter cells was added. The measurements are presented in the following manner: the logarithm of cell length in arbitrary units is plotted along the ordinate. Zero on the abscissa represents the time of division. The size at division of individual cells is spaced at equal intervals along the ordinate. The length of the cells at the time of division was (in alphabetical order): 4·1, 4·9, 5·0, 5·1, 5·1, 5·2, 5·4, 5·5, 5·7, 5·7, 5·8, 5·9, and 6·0 μ.

of different ages (Ross, 1957). Therefore, cell elongation was considered to represent a reasonable measure of the increase in cell mass.

Nuclear morphology in growing cells. Plate 1, fig. 1, shows a series of photographs of *Escherichia coli* B/r and *Salmonella typhimurium* taken from sequences used in the evaluation of nuclear interdivision times. These particular pictures were considered typical of the morphological events commonly seen. The changes associated with development and division of the nuclei were similar to those described for stained preparations (e.g. Murray, 1960), and ultra-thin sections (Robinow, 1962). We have not been able to generalize on the morphology of nuclear division because variations in individual nuclei prevented simple interpretations. These variations may be caused by gyrations of the nuclei and divisions along different planes. None the less, we usually observed the following pattern in *E. coli* B/r, *S. typhimurium*, and *Proteus vulgaris*. The most compact and spherical appearance of the nuclei was seen shortly after they had divided and begun to migrate towards opposite poles of the cell. The majority of nuclei remained in this condensed form for about one-third of the interdivision time. Thereafter they assumed a variety of complex patterns with a predominance of elliptical, dumbbell and V-shapes. The first indications of the separation of daughter nuclei were usually given by elongation of the mother structure, followed by narrowing of its central portion. This region became thinner as the daughter nuclei separated and appeared more compact. In some cases the separation process was not along the major but the minor axis of the cell. Here sister nuclei in the later stages of separation were oblong and situated side by side. Final separation occurred when these structures slid past one another towards opposite poles.

With most nuclei, the final stages of separation took place quite rapidly. For this reason, the period of uncertainty in scoring the time at which nuclear division occurred usually covered less than 10 % of the mean interdivision time. However, in about 5 % of the cases the resulting nuclei were connected for a long time by strands of material of the same optical properties as nuclear material.

Plate 2, fig. 2, shows sequences of *Escherichia coli* 15T-. It can be seen that these organisms are considerably larger than those shown in Pl. 1, fig. 1, and that the nuclear configurations are more complex. The compact post-division configuration is seen only rarely, and then for brief times. These nuclei were markedly lobate practically throughout the division cycle. In these cells it was not possible to evaluate the nuclear interdivision times with sufficient accuracy. For this reason, no interdivision time data are presented for *E. coli* 15T-.

Other strains of *Escherichia coli* also showed nuclear morphology which differed from that of the B/r strain. For example, in *E. coli* K-12 (C600, F-) which is composed of short, rounded cells, nuclei almost always divided along the minor axis of the cell. For a considerable portion of the division cycle many cells showed nuclei shaped like coffee beans. These nuclei also separated by sliding past one another towards the poles of the cells.

Distribution of cell and nuclear interdivision times. Each experiment dealt with 20–80 individual nuclear and cell divisions. Despite careful adherence to balanced growth techniques, the growth rate varied somewhat between experiments with the same strain. The coefficients of variation (standard deviation as a fraction of the mean interdivision times) were approximately 0·2 within individual experiments.

Sheppard's correction for grouping decreased the variance by a small amount, ranging from about 1 to 4%. The standard deviations of the interdivision times were considerably higher than the average time for nuclei or cells to become clearly separated. Thus, it is probable that the true coefficients of variation are not much smaller than those estimated.

The distributions of cell and nuclear interdivision times are shown in Fig. 2 for *Escherichia coli* B/r and Fig. 3 for *Salmonella typhimurium*. They represent pooled data from the experiments shown in Table 1. The estimates of the third moment of distribution, g_1, and their standard deviations are presented in Table 2. This estimate is a measure of skewness of the distribution (Fisher, 1948). In all cases

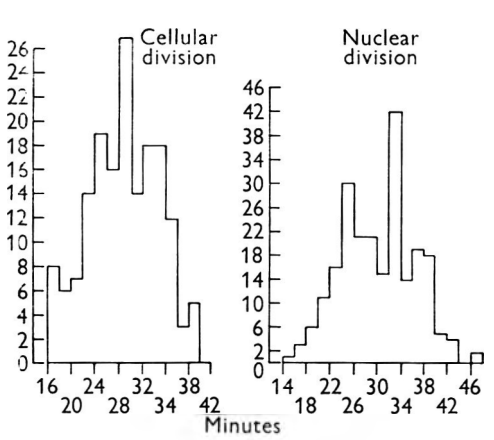

Fig. 2

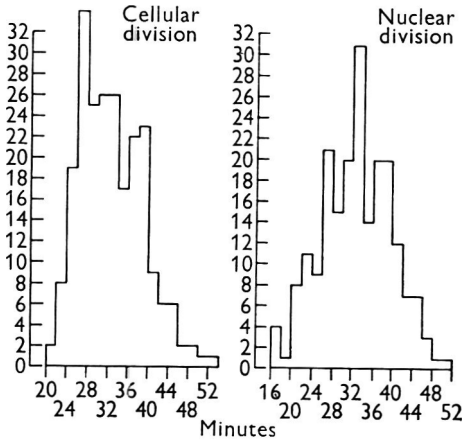

Fig. 3

Fig. 2. Frequency distributions of nuclear and cell interdivision times of *Escherichia coli*.

Fig. 3. Frequency distribution of nuclear and cell interdivision times of *Salmonella typhimurium*.

Table 2. *Skewness statistics of interdivision time distributions*

g_1 is an estimate of the third moment of distribution and is a measure of skewness.

Organism	No. obs.	Mean	Standard deviation	g_1	Standard deviation of g_1
		Cell division			
Escherichia coli B/r	154	28·4	5·93	0·005	0·197
Salmonella typhimurium	231	32·7	6·24	0·566	0·160
Proteus vulgaris	70	43·5	9·62	0·145	0·287
		Nuclear division			
Escherichia coli B/r	218	30·3	6·34	0·145	0·165
Salmonella typhimurium	208	32·8	7·02	−0·039	0·169
Proteus vulgaris	82	45·9	9·61	−0·261	0·266

except cell interdivision times of *S. typhimurium*, g_1 is not significantly larger than its standard deviation. Therefore, with the exception noted, these distributions are not significantly skewed. These computations were performed on data pooled from experiments with different means. However, the same conclusions were reached

by examining pooled individual experiments, or by pooling after normalizing the variation in means and standard deviations of individual experiments.

Since the addition of PVP to the agar resulted in slower growth, we have also estimated the dispersion of cell interdivision times in the absence of this substance. The coefficients of variation (Table 1) are similar to those obtained from growth on PVP. Thus, growth under the conditions used for observation of nuclear divisions did not significantly alter the variability of cell interdivision times.

Length of cells at the time of cell and nuclear division. As can be seen in Table 3, the coefficients of variation of the length of cells at the time of division were of the order of 0·1. In most cases the cells divided into apparently equal halves. Infrequently, a measurable difference between the lengths of the two daughter cells could be seen. However, the resolution of the measurements was insufficient to permit a meaningful estimate of the distribution of this difference.

Table 3 also shows that the coefficient of variation of the length of cells at the time of nuclear division was small and of the same magnitude as that of lengths of cells at cell division.

Correlation between estimates of individual interdivision times. Cells or nuclei engendered by the same division were termed 'sisters' when compared with each other, and 'daughters' when related to the 'mother' structure from which they arose. As shown in Table 4, the interdivision times of sister cells and sister nuclei showed a significant positive correlation (measured by the intraclass correlation, Yule & Kendall, (1948)), according to the following formula:

$$r = \frac{2(s_m^2) - s^2}{s^2},$$

where s_m^2 is the variance of the means of each pair and s^2 the variance of all measurements). The plot of sister cells and sister nuclei interdivision times of a typical experiment is shown in Fig. 4.

Correlation between interdivision times of mother and daughter structures was negative in most experiments. However, in several cases it did not significantly differ from zero. Data from an experiment with *E. coli* B/r are shown in Fig. 5.

As discussed by Powell (1958) and Koch & Schaechter (1962), random errors in the estimation of the times of division tend to make the correlation coefficients between sisters closer to 0·5 and between mothers and daughters closer to $-0{\cdot}5$. It should be noted that many points on the sister versus sister diagrams lie exactly on a 45 degree line. This is due in part to grouping, since observations were not carried out continuously but at definite intervals. Furthermore, there was probably also an artifact of evaluation, since if division of two sisters was nearly simultaneous, the observer was likely to score their division at the same time. Although this bias is small and will not affect the mean values, it tends to make the sister–sister correlation closer to 1·0.

The estimated mother-daughter correlation varied in different experiments with the same strain. Therefore, it was difficult to establish if these correlation coefficients were significantly below zero. The observed values were, in general, more negative than those previously reported (Powell, 1958; Kubitschek, 1962). An example of this correlation is given in Fig. 5.

We have also estimated the correlation between the nuclear and the cell inter-

Table 3. *Length of cells at the time of cell and nuclear division*

The designation of individual experiments is the same as in Table 1.

		Length at cell division			Length at nuclear division		
Organism	Experiment	Number measured	Mean length in μ	Coefficient of variation	Number measured	Mean length in μ	Coefficient of variation
Escherichia coli B/r	A-1	37	5·1	0·085	44	3·9	0·095
	A-3	22	4·3	0·089	45	3·3	0·093
Salmonella typhimurium	B-4	54	3·7	0·099	37	3·2	0·099
	B-5	24	4·3	0·107	22	3·4	0·063
	F-1	73	4·7	0·118	—	—	—
	F-2	69	4·5	0·130	—	—	—
	F-3	19	5·4	0·090	—	—	—

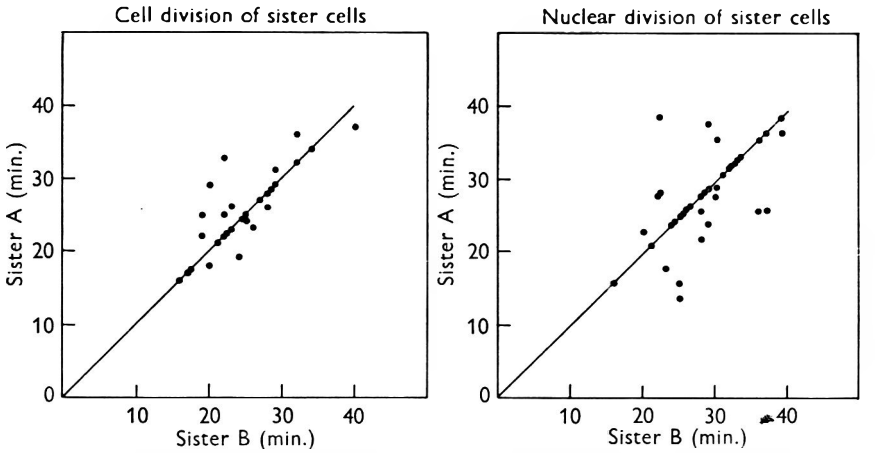

Fig. 4. Correlations of interdivision times of sister cells and sister nuclei. The data were obtained with *Escherichia coli* B/r (Expt. A-2). The coefficients of intraclass correlation were 0·628 and 0·622 for cell and nuclear division, respectively. The line represents the expectation for identical interdivision times of sisters. Possible reasons for the fact that many points appear to lie precisely on this line are given in the text.

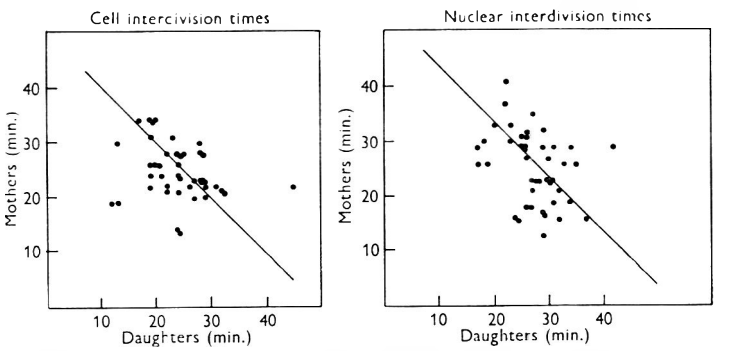

Fig. 5. Correlation of nuclear and cell interdivision times of mothers and daughters. The data were obtained with *Escherichia coli* B/r (Expt. A-2). The coefficients of correlation were −0·542 and −0·336 for cell and nuclear division respectively. The line represents the expectation for a constant time span between inception of a mother and division of daughters.

Table 4. *Correlations of cell and nuclear interdivision times between sisters and mothers and daughters*

The designation of individual experiments is the same as in Table 1.

Organism	Expt. no.	Growth conditions	Cell division correlation coefficient				Nuclear division correlation coefficient				Nuclear *vs.* cell division	
			No. pairs obs.	Between sisters	No. pairs obs.	Between mothers and daughters	No. pairs obs.	Between sisters	No. pairs obs.	Between mothers and daughters	No. pairs obs.	Correlation coefficient
Escherichia coli B/r	A-1	TSA with PVP	23	0·183	16	−0·539	31	0·613	20	−0·179	22	0·173
	A-2		28	0·628	36	−0·542	31	0·622	31	−0·336	49	−0·031
	A-3		22	0·795	30	−0·195	29	0·657	31	−0·499	30	0·503
Salmonella typhimurium	B-1	TSA with PVP	21	0·623	—	—	19	0·566	—	—	27	0·261
	B-3		15	0·677	—	—	22	0·759	—	—	43	0·592
	B-4		25	0·567	32	−0·142	18	0·649	—	—	37	0·566
	B-5		24	0·616	32	−0·401	17	0·506	17	−0·808	36	0·263
E. coli B/r (rough)	C-1	TSA with PVP	27	0·681	—	—	37	0·808	—	—	16	−0·099
	C-2		—	—	28	−0·187	27	0·831	16	−0·174	40	0·445
Proteus vulgaris	D-2	TSA with PVP	21	0·809	—	—	22	0·323	—	—	24	0·609
E. coli B/r	E-1	TSA	33	0·544	46	0·008	—	—	—	—	—	—
S. typhimurium	F-1	TSA	31	0·652	42	−0·102	—	—	—	—	—	—
	F-2		64	0·139	85	−0·259	—	—	—	—	—	—
	F-3		32	0·591	35	0·001	—	—	—	—	—	—

division times of the same individual. As shown in Table 4, the interdivision times of these two processes were positively correlated in all but two experiments. In all the species studied, the interval between cell and nuclear division, as well as that between nuclear and cell division, had coefficients of variation of 0·4–0·6. The standard deviation in minutes was comparable to that of the interdivision times. Despite this variability, cell division took place before the subsequent nuclear division in at least 99 % of the cases. That is, less than 1 % of the cells contained four nuclei at any time.

DISCUSSION

The purpose of the present work was to relate some aspects of cell and nuclear division to the normal growth process. To this end it was necessary to obtain data on the timing of the division processes and on the growth of individual cells. Some results are similar to those previously published. Others are original and obtained by the application of newer methods of observation.

Cell growth was found to take place throughout the division cycle. There have been no indications of deceleration immediately before division, as reported for *Streptococcus faecalis* (Mitchison, 1961). Exponential growth of single cells of several rod-shaped bacteria has been previously reported (Schmalhausen & Bordzilowskaja, 1930; Adolph & Bayne-Jones, 1932; Bayne-Jones & Adolph, 1933). In so far as the technical limitations allow, our data also suggest that growth is exponential throughout the division cycle of the organisms studied. If the rate of growth is indeed proportional to the cellular mass, strong support would be obtained for the previous assertion (Schaechter, Maaløe & Kjeldgaard, 1958; Neidhardt & Magasanik, 1960; Kennell & Magasanik, 1962) that protein synthesizing units perform at constant efficiency. It was found by these workers that the rate of protein synthesis is directly proportional to the amount of ribosomes present in bacteria growing under a variety of conditions. Quantitatively, growth is more directly expressed by the synthesis of proteins than by that of any other class of compounds, since proteins constitute about one half the dry weight of bacterial cells. Continuous exponential growth may then be the result of the fact that ribosomes synthesize proteins at a constant rate at all times in the life of the cell.

Our results on the spread of the distribution of cell interdivision times agree in general with those previously obtained by Kelly & Rahn (1932), Powell (1958) and Kubitschek (1962). The degree of variation of the process is similar in all these reports. However, in contrast to their observations, we have not found that the frequency distributions obtained, with the exception of *Salmonella typhimurium* cell interdivision times, are significantly skewed. This is of some significance since several theoretical models based on the statistics of a small number of molecular events would account for skewness in the distribution (Rahn, 1931; Kendall, 1948). The discrepancy between the shape of the distributions reported by others and that found by ourselves may reflect differences in the strains or in the techniques employed. It is worth emphasizing that unequivocal data cannot be obtained because of the lack of precision in the methods and the relatively small number of observations which can be made within each experiment.

The observations on nuclear division were made with a technique that has not been extensively used and which is not entirely understood. These data must,

therefore, be interpreted with due caution. The technique used may be subject to the following limitations. First, the resolution of the light microscope does not permit precise, unequivocal discernment of the actual time of nuclear division. The extent to which this introduces systematic or random errors is not known. Secondly, the optical relationship between nuclei and cytoplasm may not clearly differentiate these structures at all times. If what is observed at different times during the division cycle is not the whole nucleus or only the nucleus, the scoring of the nuclear interdivision times would be incorrect. However, the similarity between the nuclear morphology observed by this technique and that seen in stained preparations makes it unlikely that our results were greatly influenced by these sources of error. At the present, the timing of nuclear division cannot be independently verified by other methods.

In several respects, the timing of nuclear and cell division is similar. Thus, the following features are comparable in the two processes: the coefficient of variation of the interdivision time, the coefficient of correlation between sisters, and the coefficient of correlation between mothers and daughters. However, nuclear division does not directly trigger cell division, or vice versa, since the interval between the two processes is as variable as the interdivision times. A similar finding was reported by Mitchison (1961) in *Streptococcus faecalis*.

In the bacteria studied, the nucleus commonly divides in the middle third of the cell-division cycle. In this, bacteria differ from many types of animal and plant cells, where karyokinesis is soon followed by cytokinesis. Despite the proportionately long and varied interval of time between them, the two division processes must be related in some fashion since the frequency of two successive nuclear divisions without an intervening cell division, and vice versa, is very small. Indeed, it is less than what would be expected by chance if both processes were normally distributed (approximately 5 %). This is also seen in our finding that, in general, the cell and nuclear interdivision times are positively correlated. Both cell and nuclear division are more closely correlated to the size which the cell has attained than to the time when the previous division took place. This 'critical size' is different for the two division processes, and in both cases is subject to small, uncorrelated random variations. The various statistical correlations between the two processes can be explained on the basis of these variations (as discussed below).

Some strains of bacteria grow in filaments which divide in an almost random fashion. In these cases, nuclear division and cell division are certainly uncorrelated. It is of interest to note that photomicrographs by Mason (Murray, 1960) of a growing *Escherichia coli* filament show that the interdivision times of the individual nuclei contained in a common cytoplasm vary to a significant degree.

In the accompanying report (Koch & Schaechter, 1962), a model of cell division is proposed. It is based mainly on the present experimental findings. The model postulates: (1) that mass growth of individual cells is exponential and proceeds at substantially the same rate that characterizes the whole population; (2) that cell size at division, or some closely related property, is under close physiological control; (3) that the size of cells at division is nearly the same for all individuals, i.e. the coefficient of variation of the size at the time of division is small; (4) that cell division results in daughters which are nearly equal in size. Also inherent in the model is lack of correlation between the size at one division and at the next.

Although no consideration is given in the accompanying paper to nuclear division, the same conclusions should apply to this process as well. This is because nuclear division, much like cell division, seemingly takes place when the cytoplasm has reached a particular size. The other postulates refer to properties of the cell and apply equally to cell and nuclear division.

From these assumptions, a coefficient of variation of the interdivision times of about 0·20 (Table 1) is explained on the basis of the observed 0·10 coefficient of variation of the size at division (Table 3). If cell division results in daughters of precisely equal size, the model predicts that sister-sister interdivision times correlation coefficients be 0·5 and that mother-daughter coefficients be $-0{\cdot}5$. In most experiments (Table 4), the observed values are somewhat more positive for both parameters. Although unequal division of the other cell would render the mother-daughter coefficients more positive, it would also tend to decrease the sister–sister correlations. Therefore, these measurements do not speak for asymmetry of cell division. Another experimental finding which agrees with this conclusion is the lack of gross asymmetry in the interdivision time distribution. It was shown by Koch & Schaechter (1962) that unequal division is one cause of asymmetry in this distribution. Other possible sources of deviation from the expected values have been discussed (Koch & Schaechter, 1962).

The general agreement between the data and the predictions of the theory supports the conclusion that fluctuations in the size at one division are uncorrelated with the fluctuations in size at the previous cell or nuclear division, as well as with fluctuations in the division of the sister structure.

As Powell (1958) has pointed out, the separation of daughter cells may be influenced by biologically trivial factors whose effect would be exerted after more fundamental and more regular processes of replication have taken place. However, for many experimental studies which depend on the actual number of individual cells or nuclei, it is irrelevant if the division processes depend solely on physiologically cogent factors, or are influenced by environmental accidents.

The present work further indicates that the cell-division cycle in certain bacteria is experimentally elusive. Our finding that nuclear division is subject to individual variations further emphasizes these difficulties. From these considerations it follows (as has already been stated by Campbell (1957) and Maaløe (1962)) that synchronization of cell division in bacteria is not usually achieved without the introduction of artificial conditions.

We wish to thank Dr R. E. Hoffman for help in the statistical aspects of this work.

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EXPLANATION OF PLATE

PLATE 1

Fig. 1. Excerpts from photographic sequences of cells growing on PVP-agar. The three rows are (from top to bottom): *Salmonella typhimurium*, *Escherichia coli* B/r, and *E. coli* B/r (rough). The figures above each picture represent the time in minutes since the beginning of observation. Contrast was reversed photographically.

Fig. 2. As in fig. 1. These pictures are of *Escherichia coli* 15T-. The complex morphology of the nuclei in this strain did not permit precise evaluation of the interdivision time.

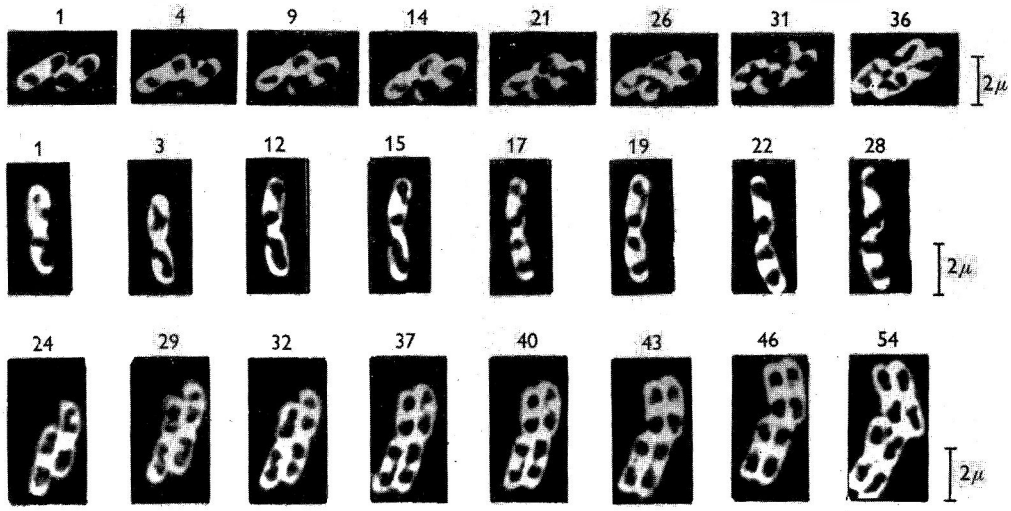
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Fig. 1

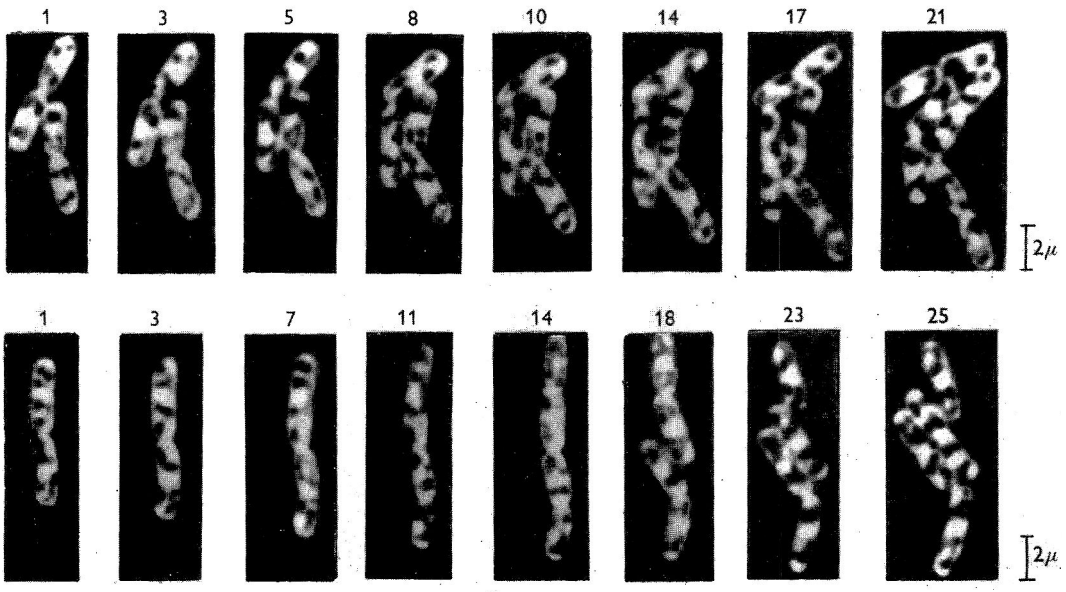
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Fig. 2

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A Model for Statistics of the Cell Division Process

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SUMMARY

A model for the statistics of cell division is proposed. The model assumes: (1) that growth at the cellular level is deterministic; (2) that the mean size of a cell at division is under cellular and environmental control; (3) that the distribution of sizes of cells at division has a small coefficient of variation, and is independent of the size at previous divisions; (4) that the cell divides nearly into equal halves. The observed coefficient of variation of the life-length distribution $f(\tau)$ results from a twofold smaller coefficient of variation in the distribution of cellular mass at division, $g(c)$. The magnification of the coefficient of variation results from the fact that the mass variable enters twice, once in determining the size of cell formed at division, and, secondly, in determining the size of the cell when it in turn divides. In the expression for τ, these sizes enter as the logarithm of their ratio. These mathematical operations contribute to the increased coefficient of variation of the $f(\tau)$ distribution over the $g(c)$ distribution. The skewed nature of the $f(\tau)$ distribution is attributable to a number of causes. We feel that an important source of the skewness is due to deviations from equipartition of cell constituents at division. In this respect the present model is in disagreement with previous models which presume that the skewness results from the statistics of a small number of molecular events taking place inside each cell. The experimentally observed positive sister–sister life-length correlation and the negative mother–daughter life-length correlation are explained by the model. Deviations from the predicted values of $+0{\cdot}5$ and $-0{\cdot}5$, respectively may be explained in part by deviation from equal distribution of cell contents at division. Deviations of the correlations in excess of these, observed in some cases, imply the existence of the other biological processes. The size distribution of cells in balanced growth cultures based on this model is given.

INTRODUCTION

Since the work of Kelly & Rahn (1932) many workers have attempted to interpret the distribution of the times between successive bacterial cell divisions. This has usually been done in terms of the statistics of intracellular molecular events. Thus, Rahn (1931–1932) postulated that cell division ensues when all of a group of cellular elements, pictured as genes, has replicated. He proposed that the replication of each genetic element takes place by a first-order reaction and, therefore, is random in time. This theory was statistically refined by Finney & Martin (1951). Kendall (1948) viewed the process of cell division as resulting from the completion of a succession of necessary steps. He pictured each step to be a random Markov process, i.e. a stochastic process, and that the 'convolution' or concatenation of

these steps determines the distribution of ages that cells will achieve before dividing. Each model thus generates a cell interdivision time or life-length distribution of a particular mathematical form that may be fitted to experimental data. Kendall in 1952 combined both his previous model and Rahn's model in a general theory. For all models we shall refer to the interdivision time distribution as $f(\tau)$, where the argument τ is the span of time between two successive cell divisions.

We should like to explore a radically different type of model, one which postulates that the basic process of biosynthesis of cellular protoplasm is deterministic, i.e. precisely predictable at the level of the single cell. Such a view is much more probable today than in the past because of our increased knowledge in the regulatory biology of micro-organisms. As examples, it appears that deoxyribonucleic acid synthesis at the level of individual bacterial cells is essentially continuous and that ribosomes participate in protein synthesis at a constant rate (e.g. Schaechter, Bentzon & Maaløe, 1959; Neidhardt & Magasanik, 1960).

It is well known that the growth rate of micro-organisms can be controlled by the amount of nutrients supplied in the medium. It is also clear that a large number of enzyme molecules in each cell deal with the nutrient substrates. Therefore, if stochastic processes limited by nutrients are considered to control cell division, it is necessary to postulate that the critical steps involved must be those controlling the *synthesis* of the limiting enzyme. This, however, is unlikely because enzyme synthesis (Jacob & Monod, 1961) is under the genetic and physiological control of both positive and negative repression mechanisms. These tend to regulate the enzyme synthesis very precisely. Thus, one postulates that, in a culture in balanced growth, the bulk of the constituents of each cell increases with the same growth constant that characterizes the cultures as a whole. Our measurements (Schaechter, Williamson, Hood & Koch, 1962) of elongation of individual cells through the growth cycle are in accord with this notion. At the level of the single cell it is difficult to distinguish between an exponential curve and certain other functions (in particular, the linear function). None the less, all cells have nearly the same growth rate constant. There are certainly no measurable major discontinuities in the growth process.

Thus, we take as our fundamental relation that

$$\frac{dx}{dt} = \lambda x, \tag{1}$$

where λ is the growth rate constant of the culture and x is the amount of any cell constituent or some combination of constituents in a cell. In particular, we shall consider the total protoplasmic mass, m.

The second postulate to be introduced is that the mean size of a cell at division is under physiological control; that is to say, on the average, a cell grows until it has reached a critical size $\bar{c}$ dependent on physiological and biochemical parameters; division then ensues. The notion of critical size is inherent in the ideas of Rashevsky (1938), although little credence may be given to the particular mechanism he proposes. This model has been considered and rejected out of hand as applying to higher organisms such as : Colpoda (Adolph, 1929), sea urchin eggs (Swann, 1954) and *Tetrahymena pyriformis* GL (Scherbaum & Rasch, 1957). On the other hand, this idea was tacitly assumed by Hershey (1939) to apply to bacteria. We found that in growing bacteria the size at division is critical, in the sense of having a smaller

coefficient of variation than does the age at division (Schaechter *et al.* 1962). In fact, Hood & Schaechter (unpublished observation) have found that the critical size of bacteria at division changes monotonically, and without increase in its coefficient of variation, throughout the reorganization that accompanies a shift of a growing culture from one medium to another medium in which the growth rate and bacterial size are different. Thus, we propose that the average mass of a cell at division is a well-controlled physiological parameter.

We propose as a third postulate that although the control is effective, the size at division may vary slightly due to random causes. For the present discussion it is immaterial whether one assumes (1) that each cell divides precisely when it reaches its critical size and that the critical size varies from individual to individual, or (2) that every cell of the same genotype in the same environment has precisely the same critical size but that for secondary reasons, the appearance of visible evidence of division may be premature or delayed. In either event we shall assume as an important part of this postulate that this variation for certain cases of interest is small, Gaussian, and uncorrelated with events in previous cell cycles or in sister cells. In analogy with the life-length distribution we shall designate this distribution $g(c)$, where c is the critical mass of the cell at division. Its coefficient of variation will be designated q.

Our last postulate is that cell division results in an equal or nearly equal division of the cytoplasm. We thus introduce the distribution $h(p)$, where p is the proportion of the cytoplasm of the mother, incorporated into one daughter. Obviously, the other daughter has a proportion of $1-p$. In most of the present discussion we shall assume that division is precise, i.e. mean = 0·5, variance = 0. In the remainder we shall assume that the mean is 0·5 but that it has a small coefficient of variation, q_h.

Distribution in balanced growth

We shall first consider the relationships between distributions of certain properties at division with the distribution of these properties in whole cultures. We do this because certain data in the literature must be accounted for by our theory. In balanced growth situations, in particular, it is of interest to be able to calculate the distribution of a property, such as age or mass, for the cells of the entire culture. For the simplest case in which all cells reach the same age $\bar{\tau}$, before dividing, the age distribution $\phi(a)$ is given by

$$\left.\begin{aligned} &\phi(a) = 2\lambda e^{-\lambda a}; \quad 0 \leqslant a \leqslant \frac{\ln 2}{\lambda} = \bar{\tau} \\ \text{and} \quad &\phi(a) = 0; \qquad a < 0, \quad a > \bar{\tau} \end{aligned}\right\} \tag{2}$$

where a is the age of the cell, $\phi(a)da$ the probability that a cell is between a and $a+da$ of age. This distribution has been derived previously by Scherbaum & Rasch (1957), Edwards *et al.* (1960), James (1960), and Stanners & Till (1960).

The situation for mass or length distribution has not been so often studied. For the simplest case, where it is assumed that all cells grow exponentially to a critical mass or length, $\bar{c}$, and then divide precisely in half, the distribution of cell mass in

the culture is readily shown to be (Maclean & Munson, 1961, and Appendix note 1)

$$\left.\begin{aligned} \theta(m) &= \frac{\bar{c}}{m^2}; \quad \frac{\bar{c}}{2} \leqslant m \leqslant \bar{c} \\ \text{and} \qquad \theta(m) &= 0; \quad m < \frac{\bar{c}}{2}; \quad m > \bar{c} \end{aligned}\right\}, \tag{3}$$

where $\theta(m)dm$ is the probability that a cell has mass between m and $m+dm$. Several interesting comparisons between these limiting forms of the $\phi(a)$ and $\theta(m)$ distributions should be noted; these are listed in Table 1.

Table 1. *Comparison of the simplest cases for the $\phi(a)$ and $\theta(m)$ distributions*

	Age distribution $\phi(a)$	Mass distribution $\theta(m)$
Formula:	$2\lambda e^{-\lambda a}$	$\bar{c}/m^2$
Range:	0 to $\bar{\tau} = \frac{\ln 2}{\lambda}$	$\frac{1}{2}\bar{c}$ to $\bar{c}$
Ratio-maximum to minimum frequency:	2	4
Mean value:	$0{\cdot}44\tau$	$0{\cdot}693\bar{c}$
Percent further growth to complete cycle:	39 %	44 %
Coefficient of variation:	64·43 %	20·2 %

The limiting form of the mass distribution, equation (3), is more useful than the limiting form of the age distribution, because the assumptions on which the former is based are more nearly met for normally growing bacteria. Pertinent data are available in the literature. For example, it would be readily possible to fit equation (3) to the data of Kubitschek (1962), Maclean & Munson (1961) and to our own data with *Escherichia coli* and *Salmonella typhimurium* (Schaechter *et al.* 1962). It must be recognized that data fitting equation (3) have not been obtained with the electronic resistance counter: e.g. Coulter Counter. With this instrument a significant proportion of particles many times larger than average is found (Kubitschek, 1958; Lark & Lark, 1960). These results are probably due to coincident entrance of several smaller particles into the sensitive volume (which is not merely that of the orifice) of the counter, to the formation of elongated forms, i.e. filaments, or to the formation of aggregates. Each aggregate would be counted as a single large particle instead of several small particles.

Powell (1956) and Harris (1959) have derived the general solution for the age distribution. The probability distribution is given by

$$\phi(a) = 2\lambda\, e^{-\lambda a}\left[1 - \int_0^a f(\tau)\, d\tau\right], \tag{4}$$

where $f(\tau)\, d\tau$ is the probability that a cell will divide between age τ and $\tau+d\tau$.

A general solution for the mass distribution has not previously been presented, although an empirical calculation has been given (Scherbaum & Rasch, 1957). We have been unable to derive a completely general formula; a somewhat more general distribution is derived in Appendix, note 2 and is

$$\theta(m) = \frac{C}{m^2}\left[\int_0^{2m} g(c)\, dc - \int_0^m g(c)\, dc\right], \tag{5}$$

where $g(c)\,dc$ is the probability that a cell will divide when its mass lies between c and $c+dc$. This distribution, applies only if the distribution $g(c)$ is not abroad. Since for balanced growth cultures of certain bacteria, the $g\,(c)$, distribution does have a small coefficient of variation, it may be hoped that this formula will have a quite broad range of application. It has also been assumed that cell division results in precisely equivalent daughter cells. If this is not true, the distribution in the first integral can be replaced with a broader distribution to correct for inequality of division.

Relationship of mass distribution at division $g(c)$ to life-length distribution $f(\tau)$

We will first consider a case based on the four assumptions given above, with the further restriction that the cell divides evenly in half, i.e. mean of $h(p) = 0{\cdot}5$, variance $= 0$. Designate the mass of the mother cell at division as c_1, and the mass of the daughter cell at division as c_2. Since the growth in mass is deterministic and exponential, we may immediately write

$$c_2 = 1/2\, c_1\, e^{+\lambda\tau}. \tag{6}$$

Solving this equation for the life-length, τ, we obtain

$$\tau = \frac{1}{\lambda} \ln \frac{2c_2}{c_1}. \tag{7}$$

The form of this equation suggests two things. First, that antilog of $\tau\lambda$ be taken as the fundamental variable for statistical analysis. This is considered below. Secondly, that the form of the distribution of $f(\tau)$ will not only depend on the form of $g(c)$, from which both c_1 and c_2 are drawn, but also on the influence of forming a ratio and of taking a logarithm.

In order to see how these mathematical operations influence the distribution, let us consider a variable that is normally distributed and has a mean value of unity. We may write this variable as $1+qx$, where q is the coefficient of variation and x is the normal deviate; i.e. x is a normally distributed variable with zero mean and unit variance. Then, from the statistical principle that 68·26 % of the cases lie between one standard deviation below and one standard deviation above the mean, it follows that 15·87 % of the cases will be below $1-q$ and 15·87 % of the cases will lie above $1+q$. Now the same relationship will hold for the distribution of the reciprocals of those values, i.e. 15·87 % of the cases will lie above $1/(1+q)$, etc. The new distribution is no longer symmetric since $1/(1-q)$ is larger than one by a greater amount than $1/(1+q)$ is smaller than one. Thus the effect of taking the reciprocal tends to skew the distribution by extending the right tail (i.e. positive skewness). For any particular value of q the new distribution is readily calculated by choosing x corresponding to other percentage points on the normal distribution curve and plotting these against the corresponding values of $1/(1+qx)$. In this way a cumulative distribution is obtained. The ordinary distribution can then be computed by substraction.

Accordingly, the ratio of two uncorrelated normal variables will also be positively skewed, but the calculation is more complex. The general solution for the fiducial limits of the ratio of variables has been given previously (Finney, 1952) and is known as Fieller's theorem (1940). We are interested in a very restricted subcase of this theorem. If we take the numerator to be $A = A(1+xq_a)$, and the

denominator to be $B = \bar{B}(1+xq_b)$, the ratio, R, corresponding to given values of the normal deviate, x, may then be written

$$R = \frac{\bar{A}}{\bar{B}} \frac{1+\sqrt{(q_a^2+q_b^2-x^2\,q_a^2q_b^2)}}{1-x^2\,q_b^2}. \tag{8}$$

This equation is derived from general solution by assuming that variations in A are completely uncorrelated with variations in B, and that a large number of single pairs of observations is made. The latter restriction is imposed so that the distribution of ratio of observations and not the ratio of parameters is obtained.

For our immediate purposes we shall assume that the coefficients of variation of both the numerator, q_a, and the denominator, q_b, are the same, since they both are equal to the coefficient of variation, q, of the distribution $g(c)$. From equation 8 and a table of the error function one can readily compute the resultant cumulative distributions. When this is done for a coefficient of variation of $g(c)$ chosen to be 10 or 20%, the resultant cumulative distributions are markedly skewed in the positive sense (Fig. 1).

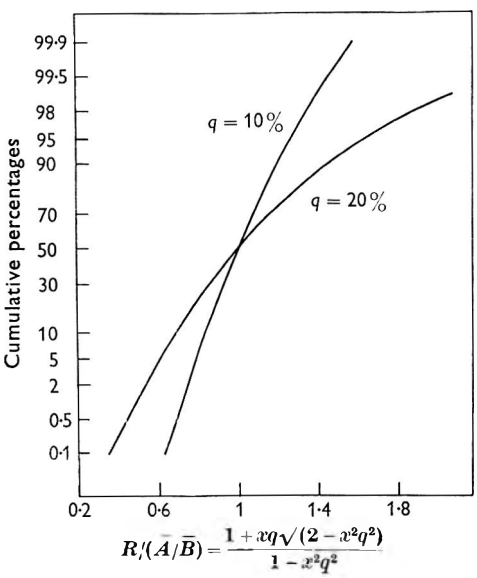

Fig. 1

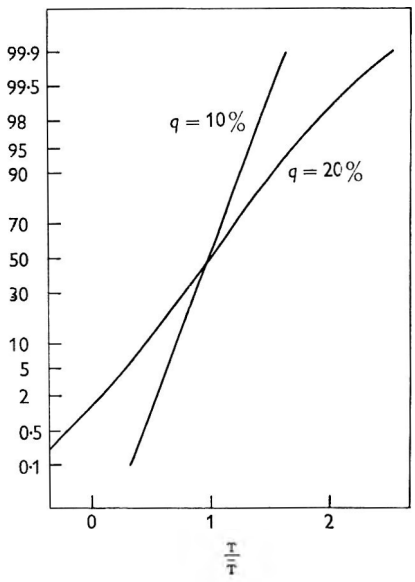

Fig. 2

Fig. 1. Cumulative distribution curves for ratio of two variables having the same value of q.

Fig. 2. Cumulative life-length distribution for cells dividing into two equal halves. $\tau/\bar{\tau}$ is equal to ln (A/B). See eq. 7, p. 439.

However, as shown in Fig. 2, the logarithm of the ratio is nearly normally distributed. Increasing q increases the deviations at the extremes alone, without distorting the central portion. It is not unexpected that the logarithm should be less positively skewed than the ratio since the logarithm function compresses large values. However, the close fit to the normal curve is certainly not obvious from inspection of the equations or their series expansions.

If the coefficient of $g(c)$, q, is small, the coefficient of variation of the ratio is 1·414 q. This follows from the statistical principle that the coefficient of variation of

the quotient is the square root of the sums of squares of coefficients of variation. It may also be deduced from equation (8) by neglecting the third term under the square root sign and the second term in the denominator. However, the empirically calculated value of the coefficient of variation of the *logarithm* of the ratio is considerably larger. It is almost precisely twice that of the distribution $g(c)$, being 2·09 times larger for $q = 10\,\%$ and 2·07 times larger for $q = 20\,\%$ (see Fig. 2).

Thus, the theory derived on the basis of these assumptions can explain the observed coefficients of variation in the age at division (20%) in our experiments (Schaechter *et al.* 1962) entirely on the basis of the observed coefficients of variation in the critical mass (10%) in the same experiments. However, it has been repeatedly stated that $f(\tau)$ has positive skewness. This feature has been emphasized and explained in various ways by previous authors. It does not simply follow from our theory as developed so far. Neither was it found in our experiments (Schaechter *et al.* 1962). In those cases where skew distributions are experimentally observed, they may be explained on the basis of the present model with the following three reasons, alone or in combination. First, $g(c)$ may not be a normal distribution. However, since the same distribution appears in the numerator and the denominator of the ratio c_2/c_1, the quotient tends to have a more normal distribution than that of $g(c)$ (see Appendix, note 3). If this factor be the sole cause of the skewness of the $f(\tau)$ distribution observed in some cases, then $g(c)$ would have to be negatively skewed to quite a high degree. Although this supposition is improbable, it is experimentally difficult to rule out completely in our experiments owing to the small size of the bacteria employed.

The second explanation is simply that environmental fluctuation and experimental vicissitudes are more frequently deleterious than beneficial. Thus a deleterious fluctuation would increase the life length much more than the equivalent beneficial fluctuation would decrease it. This would then distort the distribution in the observed direction. In fact, looking at published work it is quite clear that the skewed character of the curve has decreased as experimental technique has advanced.

The third explanation, which we believe is valid in certain cases, is that the skewness of the $f(\tau)$ distribution arises directly from inequalities of the cell division process. If the division process is not quite precise, the denominator (but not the numerator) of the mass ratio c_2/c_1 contains an additional fluctuation, i.e. the factor $1+x\,q_h$. We have seen that the logarithm of the ratio of two quantities with the same coefficient of variation is nearly normally distributed (Fig. 2). It follows that an increased coefficient of variation in the denominator over that of the numerator will lead to a positive skewness in the distribution of the logarithm of the ratio. This is shown by numerical calculation in Fig. 3 (see Appendix, note 4). On this explanation, the skewness of the $f(\tau)$ distribution is a measure of q_h. On this basis our experimental observations (Schaechter *et al.* 1962) indicate that the division process in the cases and under the conditions employed is quite even, since as mentioned above the $f(\tau)$ distributions in these cases were not significantly skew.

Correlation between sisters

The life-lengths of sister bacteria have repeatedly been found to be positively correlated. A number of explanations may be given: (i) this may represent some common 'trigger' or clock setting in their history (see Appendix, note 5); (ii), it might be caused by a common local micro-environment experienced by both sisters; (iii) it might result from a division synchronization mediated by some chemical stimulus from one cell to the other sister; (iv) it may be the consequence of improperly pooling data from different parts of the same experiment or from different experiments. If the means of the various groups of data are different, a more positive coefficient of intraclass correlation is obtained. However, it may result mainly from the fact that sisters have a common mother, whose critical mass may have been smaller than average, and thus giving both daughters a handicap, or larger than average, thereby giving both daughters a head start.

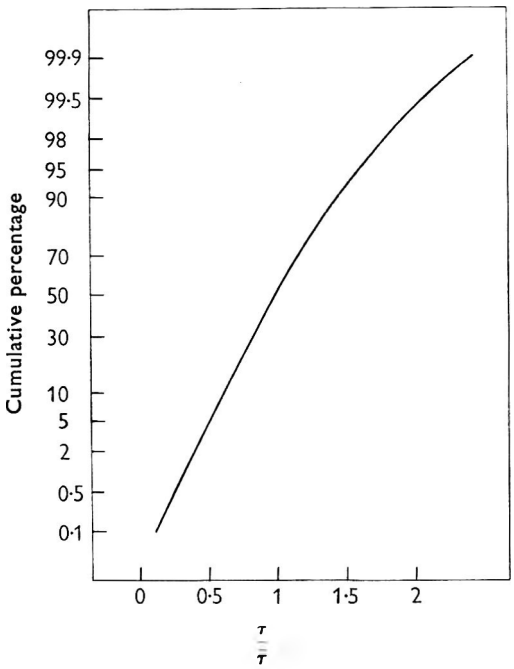

Fig. 3. Cumulative distribution for cell division process in which q for initial critical size of cells is 20 % and q for terminal size of cells is 10 %.

Let us consider the cell interdivision times for the two sisters resulting from the even division of a mother cell. It is seen from equation (7) that the cellular interdivision times will be correlated to the degree that c_{2a}/c_1 and c_{2b}/c_1 are correlated. These ratios will be correlated even if c_1, c_{2a} and c_{2b} are completely uncorrelated. This phenomenon is well known, and was termed by Karl Pearson (1897) 'spurious correlation'. It can be shown (see Appendix note 6), that if the corresponding coefficients of variation, q_1, q_{2a}, q_{2b} are all small, the correlation of these ratios is given by

$$\rho = \frac{q_1^2}{\sqrt{(q_{2a}^2 + q_1^2)}\,\sqrt{(q_{2b}^2 + q_1^2)}}. \tag{9}$$

Thus, if cell division results in an even distribution of cytoplasmic constituents and $g(c)$ has the same dispersion for both generations the three coefficients will be equal and ρ will be $+0{\cdot}5$.

The sister-sister correlation will decrease if the cell division process is not even (see Appendix, note 6). In fact, if $g(c)$ is constant it may be shown (see Appendix, note 8) that

$$\rho = \frac{q^2 - q_h^2}{2q^2 + q_h^2}. \tag{10}$$

Thus, ρ varies from $+0{\cdot}5$ to -1 as q_h becomes large relative to q.

The observed sister-sister correlation of life-lengths is generally a little larger than $+0{\cdot}5$. A proper comparison would require computing the intraclass correlation of the ratios, i.e. the values resulting from multiplying each value of τ by the observed value for the population growth rate λ and taking the antilogarithm. This will certainly alter the correlation coefficient, but, as far as can be deduced from calculations of actual data, the change is slight (0·03 and 0·005 in two examples).

A final possible cause for the correlation between sisters need be considered. If instead of dividing by a common variable, we subtract a common variable from each of two uncorrelated variables, another type of spurious correlation arises. The two cases are similar, and the ratio case becomes the difference case as the coefficient of variation decreases, since then $(1+y)/(1+x)$ becomes $1+y-x$. As in the ratio case, if the three variables are uncorrelated and have equal variance, the correlation coefficient of the derived quantities is $+0{\cdot}5$. Powell (1958) has discussed this difference type of spurious correlation. He has pointed out that it tends to make the intraclass correlation between sisters more positive since a common measurement of time, i.e. the time of the maternal division, is involved in the estimation of the life-lengths and is subject to random experimental error.

Correlation between successive generations

A second kind of correlation has been sought experimentally, namely, that between the life-lengths of mothers and daughters. A formula applicable to this case similar to equation (9) is derived in Appendix, note 6. If c_1, c_2 and c_3 are the critical mass of the grandmother, mother and daughter at division and the coefficient of variations of the three relevant divisions are q_1, q_2 and q_3 respectively, the correlation of the ratios is given by

$$\rho = \frac{-q_2^2}{\sqrt{(q_1^2+q_2^2)}\,\sqrt{(q_2^2+q_3^2)}}. \tag{11}$$

This formula is based on the assumption that division is precisely even. If in addition, it is assumed that the dispersion of the $q(c)$ distribution is the same for the three generations, the correlation coefficient becomes $-0{\cdot}5$. If q_h is not zero although $g(c)$ is constant the relationship becomes (see Appendix, note 7)

$$\rho = \frac{-q^2}{2q^2 + q_h^2}. \tag{12}$$

Thus ρ in this case can vary from $-0{\cdot}5$ to zero, as q_h becomes large relative to q. The need for a negative mother–daughter correlation is self evident in our model. Since we assume mass to increase monotonically in a deterministic manner, a premature division of a cell in one generation will necessarily require a fluctuation

in later generations toward longer life-length. In the present model this recovery is immediate and automatic.

Observed values of the correlation coefficient of mother and daughter bacteria are either zero or slightly negative, although our recent experiments have yielded more negative correlation coefficients than those of other workers. Random experimental error in assessing the division time of the mother would introduce an error in the opposite direction in the life-length of mother and daughter and, for reasons similar to that outlined above, lead to a negative mother–daughter correlation. Combining data from cells with different growth rates will tend to make the correlation coefficient more positive. This can come about in several ways. First, data from different experiments may be improperly pooled. This is not serious because all workers in the field are cognizant of this possibility. Secondly, there may be microheterogeneity in the cultural conditions so that a particular region of the agar is endowed with more or less favourable conditions for bacterial growth. The critical test for this would be the consistency of the mean time for cell division to go through several cycles from one microclone to another; the variance should not be larger than the variance for a single cell division.

This test should also serve to measure fluctuations in growth rate due to physiological changes transmitted through a few generations or to permanent genetic changes. It should be noted that although our observed logarithmic rates of elongation of cells were quite constant from cell to cell and seemingly independent of the division size of the cell, they did fluctuate slightly and the altered rate did continue into the next generation. This phenomenon, regardless of source, will tend to make both the sister–sister intraclass correlation coefficient and the mother–daughter correlation coefficient more positive.

To compare actual measurements to our model, here again it is necessary to perform the transformation, antilog $\tau\lambda$, and recalculate the intraclass coefficient. When this is done, the numerical values are altered slightly. In one case drawn from data of Schaechter *et al.* (1962) the coefficient of the untransformed data was $-0{\cdot}370$ and that of the transformed data, $-0{\cdot}366$. On making the transformation the change in a number of other examples drawn from the data at hand was always less than $0{\cdot}1$. The magnitude of the correction becomes smaller as the magnitude of the correlation coefficient becomes larger. Therefore, we have felt that extensive transformation and recalculation was not necessary.

DISCUSSION

The model proposed here is qualitatively different from those presented by Rahn and by Kendall. Their models are purely statistical in that they presuppose that the elementary events controlling cell divisions are completely random, i.e. as unpredictable as the decay of a single radioactive atom. These models do however explain the fact that the interdivision time of a bacterium is characterized by life-span and not by a half-life. In addition, they interpret the reported magnitudes of the variance and skewness of the interdivision time distribution in terms of the sequential or simultaneous completion of a small number of such random events necessary for the cell to divide. These models predict that the sister–sister and mother–daughter life-length correlations be zero.

The present model assumes four postulates which have been experimentally verified in our laboratory for a few enteric bacteria under particular conditions. Two of these postulates concern source of fluctuations in interdivision time. One of these assumes that quite small, and in some cases very small, random variations take place in determining the amount of cytoplasm in a cell at the time of division. The other postulate makes similar assumptions about the proportion of the cytoplasm distributed to each daughter cell. The sources of variation of these two distributions may arise from the statistics of a small number of fundamental molecular events or from manifold sets of accidental and historical reasons (such as might be imagined to determine the adult weight of a man). For the present, a distinction between these two sources of error is immaterial, This is in part because the variance of the two distributions, $g(c)$ and $h(p)$, is small for the cases we have investigated, so that it is difficult to establish the shape of the distribution by direct observation. The shape of the distribution is, of course, more indicative of the source of variation than is its coefficient of variation. More important, moderate deviation from normality for either distribution would not influence very much the observed parameters of cell division, e.g. the variance of the $f(\tau)$ distribution, the skewness of the $f(\tau)$ distribution, the sister–sister intraclass correlation coefficient, and the mother–daughter correlation coefficient. On the other hand, we believe that the magnitude of these four parameters is directly attributable to the magnitude of the coefficient of variation of the $g(c)$ and $h(p)$ distributions. Thus, we feel that the observable distributions and correlations of τ values are dependent on the second moments and not particularly on the third moments of these two distributions.

Deviations from the predictions of the model must be understood in terms of experimental artifact or in the failure of one or more of the postulates. Only in the case of the experimental systems we have used (Schaechter *et al.* 1962) are enough different kinds of data available to allow testing of the model. In general, the enteric bacteria studied fit the model quite well. Unfortunately, at the present time, not all of the assumptions of the model have been checked experimentally. As noted below, the constancy of λ is a good first approximation, but possibly not exactly correct. Likewise, the assumption that q_h be zero is consistent with most observations, but will be difficult to verify experimentally. Finally, the assumption of the independence of the size attained at division with those at any other division await direct test, though it fits the facts and our expectation.

Next we may consider the generality of the model. The first postulate that protoplasmic growth is continuous and deterministic at the cellular level, appears to apply as well for Paramecium (Kimball *et al.* 1959). It does not appear to be true for a coccus (Mitchison, 1961), a fission yeast (Mitchison, 1957), or an amoeba (Prescott, 1955). Possibly its range of validity only extends to those organisms which have no protein turnover during their growth (Koch & Levy, 1955). As we have mentioned, it can not apply to Colpoda or to sea urchin eggs.

A better reason for employing this postulate is certainly the recent developments in the cellular biochemistry and control mechanisms of bacteria. On this basis it need not and, in fact, would not be expected to hold for higher forms, with more complex control mechanisms.

The even stronger part of the postulate needed to arrive at the conclusions presented here is that the substance of each cell grows with exactly the same growth

rate constant as that of every other cell. As mentioned above, this is very nearly true for the enteric bacteria we have studied. Persistent deviations contribute in a small degree to increasing the sister–sister and the mother–daughter correlation coefficient. They would also tend to broaden the $f(\tau)$ distribution and skew the distribution positively.

The second postulate, that genotype and environment acting through physiological mechanisms regulate the average size of the cell at division, implies the existence of control mechanisms that somehow 'sense' the cell size or some closely related property. This would appear to be an excellent first approximation, since many cell components are synthesized continuously so that the amount of any one is proportional to the amount of any other substance or group of substances more specifically associated with cell or nuclear division. Note, also, that the amount of substance at division is proportional to the amount at an earlier time. Thus, lengths, for example, will serve to describe the statistics as well as quantity or quantities unknown but more intimately involved with division processes.

That cell division is strongly correlated with cell size is an obvious conclusion from the small variation in the size at division. This dependence is emphasized by the unpublished observations from our laboratory showing that the critical size varies monotonically during a shift of growth rate. The same conclusion follows logically from the published finding of Schaechter *et al.* (1958) that the average cell size is precisely dependent on growth rate, together with equations (3) and (5) of the present paper, which requires that the average size in balanced populations be proportional to the mean critical size.

The third postulate, that size of cell at division has only a small coefficient of variation, is valid for the bacteria we have studied. Adolph & Bayne-Jones (1932) observed that the adult size of *Bacillus megaterium* had a coefficient of variation of only 6·5 %. Similar coefficients of variation in the range 6–13 % have been quoted for certain coliform organisms (Bayne-Jones & Adolph, 1933) and for *Tetrahymena pyriformis* GL by Scherbaum & Rasch (1957). In other instances also the coefficient of variation may be very small. For example, this is evident from the fact that the coccus *Lampropedia hyalina* grows in two dimensional sheets which are almost perfectly rectangular (Pringsheim, 1955). In some cocci which grow in chains, all the cells of a chain appear to be in the same stage of cell division. It is not known whether such instances represent precise timing of cell division or an exceptionally high sister–sister correlation as was reported for a yeast by Burns (1956) who found that in yeast cells of certain ploidies and grown at certain temperatures, there was complete positive correlation between the time of division of a cell and the next division of the mother cell from which it was derived. Since cells not attached to each other were not dividing in phase, it seems likely that this synchronization was mediated by chemical or physical interaction. On the other hand, there are some bacteria which tend to grow in filaments which break down almost at random. For these organisms the theory would not apply, since the coefficient of variation would not be small as required by the mathematical development presented here, and the second and fourth postulates would not be obeyed.

Not enough is known about the equality of division of the cytoplasm in bacteria; it has been discussed in reference to yeast (Spiegelman, De Lorenzo & Campbell 1951). From the $f(\tau)$ distribution, the sister–sister correlation, and mother–

daughter correlation, we infer that in the cases studied (Schaechter *et al.* 1962) cell division must divide the cytoplasm quite evenly.

The present model assumes nothing about 'triggers' or a period of time in which cytokinesis is irreversibly preordained, as is the case with sea urchin eggs (Swann, 1954). However, in our proposed model one cell division determined the next. It certainly does not preordain the time of the next division event. There might be more specific triggers, but as such they have not yet been found in bacteria. Of course, finding this type of mechanism would not invalidate the considerations presented here. They would lead to a refined model in which the prime variable(s) had even smaller dispersion than the size at division. The idea that division size is well controlled may be a fundamental biological notion. A search for the mechanism controlling it should be a fruitful area of investigation. Obvious approaches, such as a study of how the critical size changes with the environment are under way and will be extended. The search for genetic, chemical, and environmental agents which affect the control of cell division, rather than the process itself, may be fruitful.

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APPENDIX

Note 1. It appears worthwhile to derive this distribution in a different manner. To do so, we note that the number of cells now of mass m is related to the number of divisions taking place at a time when these cells had mass $\frac{1}{2}\bar{c}$. If the number of cells at that time $-t$, was N_0, it follows from our first assumption that

$$\frac{N_0}{N} = e^{-\lambda t} \equiv \frac{\bar{c}}{2m} \quad \text{or} \quad N_0 = \frac{\bar{c}N}{2m}.$$

The growth rate was then

$$\lambda N_0 = \frac{\lambda \bar{c} N}{2m} = \frac{dN}{dt}.$$

The rate of production of new cells is twice the growth rate, dN/dt, since a division produces two new cells, but only one additional cell. Therefore, we have for dn/dt, the rate of production of new cells

$$\frac{dn}{dt} = \frac{\lambda \bar{c} N}{m}.$$

On eliminating dt by use of the expression $dm/dt = \lambda m$, we obtain

$$dn = \frac{\lambda \bar{c} N dt}{m} = \frac{\lambda \bar{c} N}{m} \frac{dm}{\lambda m} = \frac{N \bar{c} dm}{m^2},$$

and consequently

$$\theta(m) = \frac{1}{N} \frac{dn}{dm} = \frac{\bar{c}}{m^2}.$$

Note 2. The following derivation is patterned after that given by Powell (1956) for the derivation of the age distribution. Define

$$G(m) = \int_m^\infty g(c)dc.$$

Then an organism of mass m has the following chance of achieving a mass of $m(1+k)$ without dividing,

$$\frac{G(m(1+k))}{G(m)}.$$

Hence, of the original $N\theta(m)dm$ organisms, $\frac{N\theta(m)G(m(1+k))dm}{G(m)}$ organisms are still present. At a later time $t = (1/\lambda)\ln(1+k)$ the number of organisms have grown to $Ne^{+\lambda t}$ and the survivors form a fraction of the whole, equal to

$$\frac{\theta(m)G(m(1+k))e^{-\lambda t}}{G(m)}dm = \theta(m(1+k))dm(1+k).$$

This must hold all values of k and the corresponding values of t.

For small k and t,

$$G(m(1+k)) = G(m)+\frac{dG(m)}{dm}(mk),$$

$$\theta(m(1+k)) = \theta(m)+\frac{d\theta(m)}{dm}(mk),$$

and

$$e^{-\lambda t} = 1-\lambda t = 1-k.$$

Therefore

$$\frac{(G(m)+mk(dG(m)/dm))(1-k)}{G(m)} = \frac{(\theta(m)+mk(d\theta(m)/dm))(1+k)}{\theta(m)}.$$

Expanding and neglecting higher powers of k we obtain

$$\frac{mk}{G(m)}\frac{dG(m)}{dm}-k = mk\frac{d\theta(m)}{\theta(m)dm}+k,$$

or

$$\frac{d\ln G(m)}{d\ln m}-2 = \frac{d\ln\theta}{d\ln m},$$

multiplying by $d\ln m$ and integrating, we obtain

$$\ln G(m)-2\ln m = \ln\theta(m)-C$$

$$\theta(m) = \frac{CG(m)}{m^2},$$

$$\theta(m) = \frac{C}{m^2}\left[1-\int_0^m g(c)dc\right].$$

Since the first integral in equation (5) becomes unity for values of m somewhat larger than $m = \frac{1}{2}\bar{c}$, equation (5) is thus derived in part. This method can not be extended to yield the lower end of the distribution. We shall give here a different type of derivation that allows us to calculate the lower as well as the upper ends of the $\theta(m)$ distribution, under conditions where the coefficient of variation of the $g(c)$ distribution is small. We have not yet solved the general case.

From our definition, the number of cells of mass between m and $m+dm$ is $N\theta(m)dm$, when N is the total number of organisms present. At an earlier time

these cells had mass $m' = m - \Delta m$. The total number of cells in the population then was (see note 1) $N(m'/m) = N(m-\Delta m)/m$, and the interval of mass was

$$dm' = \frac{m'}{m}\, dm = \frac{(m-\Delta m)\, dm}{m}.$$

Therefore, these cells contributed to the present class m by an amount equal to

$$N\left(\frac{m-\Delta m}{m}\right) \theta\,(m-\Delta m) \left(\frac{m-\Delta m}{m}\right) dm$$

or

$$N\left(\frac{m-\Delta m}{m}\right)^2 \left(\theta(m) - \frac{d\theta(m)}{dm}\,\Delta m\right) dm.$$

Expanding, and neglecting second and higher powers of Δ we obtain

$$N\left(\theta(m) - \frac{2\Delta m}{m}\,\theta(m) - \frac{d\theta(m)}{dm}\,\Delta m\right) dm.$$

Therefore, the net gain or loss to the class of mass m to $m+dm$ from the corresponding class m' to $m'+dm'$ will be

$$N\Delta m\, dm\left(\frac{-2\theta(m)}{m} - \frac{d\theta(m)}{dm}\right).$$

In balanced growth this need be counterbalanced by the attrition due to cell division in the same interval

$$\frac{N\theta(m)\, dm g(m)\Delta m}{\int_m^\infty g(c)\, dc},$$

and to augmentation by division of cells of double size

$$\frac{-2N\theta(2m)\, d2m g(2m)\, \Delta 2m}{\int_{2m}^\infty g(c) dc}.$$

Equating these sources and cancelling out $N\Delta m\, dm$ we obtain

$$-2\,\frac{\theta(m)}{m} - \frac{d\theta(m)}{dm} = \theta(m)\,\frac{g(m)}{\int_m^\infty g(c)\, dc} - 8\theta(2m)\,\frac{g(2m)}{\int_{2m}^\infty g(c)\, dc}.$$

It is readily noted if the right side is set to equal to zero, equation (3) may be obtained. If just the first term on the right is retained (high end of the distribution), the solution may be obtained by defining $G(m) = \int_m^\infty g(c)\, dc$ and noting that $dG(m)/dm = -g(m)$. The the equation becomes

$$2\,\frac{\theta(m)}{m} + \frac{d\theta(m)}{dm} = \theta(m)\,\frac{dG(m)}{G\,dm},$$

$$\frac{2dm}{m} + \frac{d\theta(m)}{\theta(m)} = \frac{dG(m)}{G(m)}.$$

Integration yields $\quad 2\ln m + \ln\theta(m) = \ln G(m) + \ln C,$

or $\quad m^2\theta(m) = CG(m),$

$$\theta(m) = \frac{C}{m^2}\,G(m) = \frac{C}{m^2}\int_m^\infty g(c)\, dc = \frac{C}{m^2}\left[1 - \int_0^m g(c)\, dc\right],$$

and is thus identical to the results derived above. The lower end of the distribution is derived by substituting this value into the general equation and neglecting the first term on the right hand side of equation. We then obtain

$$2\frac{\theta(m)}{m}+\frac{d\theta(m)}{dm}=\frac{8\theta(2m)\,g(2m)}{\int_{2m}^{\infty}g(c)\,ds}=\frac{8C}{4m^2}\frac{\int_{2m}^{\infty}g(c)\,dc}{\int_{2m}^{\infty}g(c)\,dc}\,g(2m)=\frac{2C}{m^2}g(2m),$$

which on clearing yields

$$2m\theta(m)\,dm+m^2d\theta(m)=2Cg(2m)\,dm,$$

or

$$d(m^2)\theta(m)=2Cg(2m)\,dm,$$

which on integration yields

$$m^2\theta(m)=C\int_0^m g(c)\,dc.$$

Therefore, the entire distribution is given equation (5) where C will have slightly different value than for either end alone, but may be determined from the relationship that

$$\int_0^m\theta(m)=1.$$

C approaches $\bar{c}$ as the coefficient of variation of $g(c)$ approaches zero.

Note that the effects of imprecise division of cell constituents between daughter cells can readily be taken into account by simply employing the correct $g(c)$ distribution for the second integral in equation (5) and an effective broader distribution $g'(c)$ for the first integral. This broader distribution then takes into account both the variability of the critical size as well as the cell distribution variability. Further theoretical work along these lines is in progress.

Note 3. If the variation in the numerator and denominator are small, we may write

$$\frac{\bar{c}_2}{\bar{c}_1}=\frac{(1\pm y)}{(1\pm x)}=\frac{\bar{c}_2}{\bar{c}_1}\quad(1\pm y\pm x\ldots),$$

where y and x are the fractional deviations from the mean. We then simply note that the central limit theorem requires that this ratio will tend towards normality.

Note 4. Experimentally obtained τ distributions obtained previously fit well a number of possible distribution curves, such as the Pearson type III, the log normal, and recently the distribution of the reciprocal of a normally distributed variable with relatively small coefficient of variation (see Kubitschek, 1962). From the numerical calculation presented above it is clear that, over certain ranges of variability, the logarithmic normal curve and the reciprocal are equivalent transformations. Kubitschek's ideas would then be equivalent to the assertion that λ for each cell is normally distributed.

Note 5. Instead of the positive correlation found with bacteria, a negative sister–sister correlation is found with *Tetrahymena geleii* (Prescott, 1959). This suggests that extreme dissymmetry of some cell substance takes place at division of this organism.

Note 6. Both equations (9) and (11) can be derived as limiting forms for the general solution given by Pearson (1897). We shall derive the solution for the mother–daughter ease, and refer the reader to Yule & Kendall (1944; p. 300) for the sister–sister case. The proofs for the two cases are, of course, very similar. Call $R_{12} = c_2/c_1$ and $R_{23} = c_3/c_2$ the ratios whose correlation coefficient ρ we desire. The standard deviation and mean of these ratios are readily derived from equation (8) on the assumption that the errors are small; these are

$$\overline{\left(\frac{c_2}{c_1}\right)} = \frac{\bar{c}_2}{\bar{c}_1}(1+q_1^2); \quad \overline{\left(\frac{c_3}{c_2}\right)} = \frac{\bar{c}_3}{\bar{c}_2}(1+q_2^2); \quad s_{12} = \frac{\bar{c}_2}{\bar{c}_1}\sqrt{(q_1^2+q_2^2)};$$

and

$$s_{23} = \frac{\bar{c}_3}{\bar{c}_2}\sqrt{(q_2^2+q_3^2)}.$$

The correlation coefficient is by definition

$$\rho = \frac{\frac{1}{n}\sum_1^n \left[\frac{c_2}{c_1} - \overline{\left(\frac{c_2}{c_1}\right)}\right]\left[\frac{c_3}{c_2} - \overline{\left(\frac{c_3}{c_2}\right)}\right]}{s_{12}s_{23}}.$$

The numerator may be expanded to yield

$$\frac{1}{n}\sum_1^n \frac{c_3}{c_1} - \left(\frac{1}{n}\sum_1^n \frac{c_2}{c_1}\right)\overline{\left(\frac{c_3}{c_2}\right)} - \left(\frac{1}{n}\sum_1^n \frac{c_3}{c_2}\right)\overline{\left(\frac{c_2}{c_1}\right)} + \overline{\left(\frac{c_2}{c_1}\right)}\,\overline{\left(\frac{c_3}{c_2}\right)}.$$

Note that the last three terms are identical except for sign. Cancellation yields

$$\frac{1}{n}\sum_1^n \frac{c_3}{c_1} - \overline{\left(\frac{c_2}{c_1}\right)}\,\overline{\left(\frac{c_3}{c_2}\right)}.$$

Substituting $c_1 = \bar{c}_1(1+xq_1)$ and $c_3 = \bar{c}_3(1+xq_3)$, where x is the normal deviate, in the first term of this expression yields for this term

$$\frac{1}{n}\sum_1^n \frac{c_3}{c_1} = \frac{1}{n}\frac{\bar{c}_3}{\bar{c}_1}\sum_1^n \frac{1+xq_3}{1+xq_1}.$$

Expanding the argument and neglecting higher powers than second, yields:

$$\frac{1}{n}\frac{\bar{c}_3}{\bar{c}_1}\sum_1^n (1+xq_3-xq_1+x^2q_1^2) = \frac{\bar{c}_3}{\bar{c}_1} + \frac{\bar{c}_3}{\bar{c}_1}q_1^2.$$

The second term may be written

$$\overline{\left(\frac{c_2}{c_1}\right)}\,\overline{\left(\frac{c_3}{c_2}\right)} = \frac{\bar{c}_2}{\bar{c}_1}(1+q_1^2)\frac{\bar{c}_3}{\bar{c}_2}(1+q_2^2) = \frac{\bar{c}_3}{\bar{c}_1}(1+q_1^2+q_2^2+q_1^2q_2^2).$$

Therefore, the numerator becomes (neglecting $q_1^2q_2^2$)

$$\frac{\bar{c}_3}{\bar{c}_1} + \frac{\bar{c}_3}{\bar{c}_1}q_1^2 - \frac{\bar{c}_3}{\bar{c}_1}(1+q_1^2+q_2^2) = \frac{\bar{c}_3}{\bar{c}_1}q_2^2.$$

And the correlation coefficient is then given by equation (11).

Note 7. As in the previous footnote we shall derive the equation for the mother–daughter correlation, and note that the sister–sister equation may be derived similarly. It is simpler to start with the general formulation of Pearson (1897). As before we may designate the ratios $R_{12} = c_2/c_1$ and $R_{2'3} = c_3/c_2$. It will be necessary to distinguish between c_2 and $c_{2'}$ because the coefficients of variation of the masses at division and after division in this case are not equal, and the correlation between

the two, $\rho_{22'}$, is not unity as in the previous case. Pearson's equations with the condition that the other variables are uncorrelated may be written

$$\rho = \frac{-\rho_{22'} q_2 q_{2'}}{\sqrt{(q_2^2 + q_1^2)}\,\sqrt{(q_{2'}^2 + q_3^2)}}$$

which is, of course, extremely similar to the equation 11.

The assumptions for this case, give immediately

$$q_2^2 = q_3^2 = q^2$$

and

$$q_1^2 = q_{2'}^2 = q^2 + q_h^2.$$

The value of $\rho_{22'}$ is readily computed from the correlation of the product cp, with one of the variables, c. p was defined above as the proportion of protoplasm appearing in one daughter cell.

As in the note 6 we write

$$\rho_{22'} = \rho c, cp = \frac{\sum_1^n (cp - \overline{cp})(c - \overline{c})}{n s_c s_c}$$

$$= \frac{\sum_1^n c^2 p - n\overline{cp}\,\overline{c}}{n s_c s_c}$$

$$= \frac{\overline{cp}\,\overline{c}\left(\sum_1^n (1+qx)^2 (1+q_h x')\right) - n\overline{cp}\,\overline{c}}{n s_c s_{cp}}$$

$$= \frac{\overline{cp}\,\overline{c}}{n s_{cp} s_c} \sum_1^n (2qx + q^2x^2 + q_h x' + 2qxq_h x' + q^2 x^2 q_h x'),$$

$$= \frac{\overline{cp}\,\overline{c}}{s_{cp} s_p} q^2 = \frac{q^2}{q_{cp} \cdot q} = \frac{q}{\sqrt{(q^2 + q_h^2)}}.$$

Therefore, we have for the correlation of mass ratio, equation (12)

$$\rho = \frac{\left(\frac{q}{\sqrt{(q^2+q_h^2)}}\right) q\,\sqrt{(q^2+q_h^2)}}{\sqrt{(q^2+q^2+q_h^2)^2}} = -\frac{q^2}{2q^2+q_h^2}.$$

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Sucrose Fermentation by *Proteus hauseri*

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SUMMARY

Sixty-three wild strains of *Proteus mirabilis* were investigated. All 55 strains comprising groups 1 *a* and 1 *b* were found to be cryptic with regard to sucrose fermentation; they possessed competent enzyme systems but did not normally ferment the sugar. No enzyme capable of cleaving this sugar was extracted from the 3 strains of group 2 and the 5 strains belonging to group 3. Partially purified enzyme preparations from two strains of *P. vulgaris* and two cryptic *P. mirabilis* strains were investigated; all four were constitutive β-D-fructofuranosidases capable of splitting raffinose to melibiose and fructose. Sucrose uptake studies showed that strains of groups 1 *a*, 1 *b* and 2 did not accumulate sucrose from 1% (w/v) solution; the strains of group 3 accumulated large amounts of sucrose. None of the *P. mirabilis* strains was permeable to maltose. The permeability barrier for sucrose was overcome by increasing its concentration to 5% (w/v). Under these conditions groups 1 *a* and 1 *b* strains fermented sucrose in peptone water within 36 hr. Sodium deoxycholate also changed the permeability barrier of some of the cryptic strains, enabling them to ferment 1% (w/v) sucrose promptly. After 3–11 days in 1% (w/v) sucrose peptone water all 55 cryptic *P. mirabilis*, as well as the strains of group 3 fermented sucrose. This fermentation was not caused by wild-type organisms, but resulted from the selection of sucrose-positive mutants which arose from the former and were capable of prompt sucrose fermentation. The mutants of strains of group 3 arose at lower rates than those from the cryptic strains. It is concluded that selective permeability to sucrose and β-D-fructofuranosidase activity are genetically distinct properties of Proteus. A scheme for the classification of phenotypes of *P. hauseri* is presented.

INTRODUCTION

Proteus hauseri (Kauffmann, 1951) consists of two types of organisms which differ in a number of biochemical reactions: these are usually named *P. vulgaris* and *P. mirabilis*. Two of the most clear-cut differences between them are that the 'vulgaris' varieties rapidly ferment maltose and produce indole from tryptophan while 'mirabilis' is negative in both respects. The point under discussion centres round the fact that *P. vulgaris* also ferments sucrose promptly, whereas *P. mirabilis* strains ferment it late or not at all. It was shown (Coetzee & Sacks, 1960*a*) that late sucrose fermentation by strains of *P. mirabilis* was brought about by mutants capable of prompt fermentation, which arose in populations of the wild-type. Coetzee & Hawtrey (1962) described a *P. mirabilis* strain which was cryptic (Cohen & Monod, 1957) in regard to sucrose fermentation. This strain did not ferment sucrose and required more than 5 days in 1% (w/v) sucrose peptone water to accumulate enough mutants to ferment the sugar. Sonic lysates of the wild

strain did however contain an enzyme capable of cleaving sucrose to glucose + fructose. It was also found that lysing complexes of this strain + a virulent phage liberated an endolysin. This endolysin modified surface structures of uninfected organisms of the strain so that the latter were then able to ferment sucrose promptly. Permeability factors play a role in lactose fermentation by members of the family Enterobacteriaceae (Lowe, 1960; Li, Barksdale & Garmise, 1961) and it was therefore decided to investigate sucrose fermentation by *P. hauseri* from this point of view.

METHODS

Organisms. Ten *Proteus vulgaris* and 63 *P. mirabilis* strains were examined; they had been isolated from human stools and urine and were maintained on nutrient agar slopes at 4°. Also, a sucrose positive mutant (*suc*$^+$) of *P. mirabilis* strain 57, isolated during the present work, was used in some experiments. The wild strains conformed to descriptions given by Kauffmann (1951); all were in the A phase (Coetzee & Sacks, 1960*b*) and each gave a positive Dienes phenomenon (Krikler, 1953) with all other strains.

Media. All chemicals were A.R. grade and prepared media and other media constituents were Difco products. Saline was 0·85 % (w/v) NaCl in distilled water. Peptone water was made up of 1 % (w/v) peptone in saline with a phenol red indicator (Mackie & McCartney; 1960). Sugars were added to the peptone water as Seitz filtrates to give 1 % (w/v) or 5 % (w/v) solutions. Nutrient agar consisted of nutrient broth with 1·5 % (w/v) agar. Sugar agar media consisted of nutrient agar containing neutral red 0·025 g./l. and Seitz-filtered sugar solutions in distilled water to give concentrations of 1 % (w/v) or 5 % (w/v) at 46°. The sugar bile agar medium contained 1 % (w/v) sodium deoxycholate in addition. Before addition of sugars media were autoclaved at 120° for 15 min. All media were adjusted to pH 7·2.

Procedures. Liquid media were inoculated with one drop of an overnight broth culture delivered from a Pasteur pipette. When isolated colonies were required 0·1 ml. of suitable dilutions in distilled water of an overnight broth culture were spread to dryness with a sterile glass rod. Cultures were incubated aerobically at 37°. Mutation rates to sucrose fermentation were measured by enumerating papillae which arose on colonies on the 1 % sucrose agar medium, according to methods previously used (Coetzee & Sacks, 1960*a*).

Effect of sodium deoxycholate. Overnight broth cultures were diluted 1/4 in fresh broth and bubbled with sterile air for 2 hr. at 37°. Two ml. volumes were then centrifuged at 4000 rev./min. for 10 min. and the deposited organisms (about 2×10^9) resuspended in 5 ml. of different concentrations of sodium deoxycholate in saline. On a few occasions duplicate sets of suspensions were made with the sodium deoxycholate dissolved in 0·6 M-sucrose in saline. This concentration of sucrose was known to keep penicillin-prepared protoplasts of *Proteus hauseri* intact (Coetzee; unpublished). After 30 min. at room temperature the optical densities of the suspensions were read in a Zeiss spectrophotometer at 630 mμ.

Preparation of crude and partially purified cell extracts. Thirty-six hr. confluent growths of organism on nutrient agar or on 5 % sucrose agar were harvested and washed 3 times with distilled water. The packed cell volumes were adjusted to haematocrit values of 35 % and 25 ml. volumes of the suspensions disintegrated by

vibrating in a Raytheon 50 W. 9 kc. sonic oscillator for 30 min., the container being surrounded by a mixture of NaCl and ice. Unbroken organisms and large debris were deposited at 4000 rev./min. at 4°. The opalescent supernatant fluids ('crude extracts') were stored at −10°. These crude extracts were partially purified by adding equal volumes of saturated ammonium sulphate and the precipitates deposited at 4000 rev./min. for 1 hr. in the cold. The precipitates were dissolved in 10 ml. distilled water and stored at −10°. The protein content of these partially purified extracts was determined by a biuret method with ovalbumin as standard (Gornall, Bardwill & David, 1949). On a few occasions 2 ml. benzene (Lowe, 1960) were added to the 25 ml. cell suspensions. The suspensions were agitated for 2 hr. at room temperature and then used as crude extracts. On other occasions 2 vol. of 2% (w/v) sodium deoxycholate in saline were added to 25 ml. of cell suspensions to produce lysis of organisms; after shaking for 2 hr. at room temperature these were used as 'crude extracts'. Extracts were usually diluted 1/5 in distilled water before use.

Chromatography. Descending chromatography was done on Whatman no. 1 paper. The solvent system was ethyl acetate + acetic acid + formic acid + water in (18 + 3 + 1 + 4, by vol.) The solvent was allowed to run for 16 hr. at room temperature and then 5% (w/v) *p*-anisidine hydrochloride in *n*-butanol (Hough, Jones & Wadman, 1950) used as the spray. Appropriate standard markers were used. When the presence of glucose-1-phosphate was investigated the chromatographic techniques of Colvin, Martin & Dearing (1961) were used.

Enzyme assay procedure. Reaction tubes contained: 2·5 ml. 0·05 M-potassium phosphate buffer (pH 7·4) + 1·0 ml. 0·6 M-sucrose + 1·0 ml. of a dilution of crude or partially purified extracts, + distilled water to 6 ml. Extract and substrate controls were included. The extract and other reactants were brought to 37° in a water bath before mixing. Tubes were sampled at 5 min. and 15 min. Reducing sugars in these samples were measured by the method of Haslewood & Strookman (1939) and the enzymic reaction stopped by pipetting the samples directly into the buffer + tungstate mixture of Haslewood & Strookman used. Glucose in the samples was estimated by a glucose oxidase method for which the reagent was obtained from C. F. Boehringer and Son, Mannheim, Germany. With this method 0·1 ml. of sample was pipetted into 1 ml. of 0·05 M-potassium phosphate buffer (pH 7·4) at 80°. The tubes were then placed in a beaker of boiling water for 2 min. This treatment did not hydrolyse sucrose and has been recommended by Bacon (1955) for stopping the enzyme reaction. After chilling, a 0·2 ml. sample from a tube was added to 5 ml. of oxidase reagent and the colour allowed to develop for 30 min. in a 37° water bath, the reaction then being stopped by adding 0·05 ml. 6 N-HCl. The colour was measured in a Zeiss spectrophotometer at 401 mμ. A glucose standard containing 91 μg. glucose/ml. was treated similarly. In experiments with partially purified extracts in a phosphate-free medium the potassium phosphate buffer used in the standard assay procedure was replaced by a sodium barbital buffer at pH 7·4, and the rate of breakdown of sucrose compared with a reaction proceeding simultaneously in phosphate buffer. The rate of the reaction was estimated by sampling the tubes at intervals and estimating reducing sugars. Phosphate in the barbital buffer reaction tubes was determined by the micro method of Berenblum & Chain (1938).

Accumulation of disaccharides in Proteus hauseri. Confluent 36 hr. growths of

organism on nutrient agar or on 1 % or 5 % sucrose agar were harvested and washed 3 times in distilled water. Very thick suspensions of between 114 and 127 mg. dry wt. organism/ml. were made in distilled water and 1 ml. added to 10 ml. of 1 % sucrose peptone water in centrifuge tubes. One ml. lots of the very thick suspensions were also added to 10 ml. peptone water to serve as organism controls, while uninoculated sucrose peptone water served as the sucrose controls. Controls and tests were treated identically and experiments were done in triplicate. Tubes were kept at 37° for 30 min. and the organisms then deposited by centrifuging at 4000 rev./min. for 15 min. at 4°. All the supernatant fluid was carefully removed from each tube and the sucrose in it estimated by treating 1 ml. as substrate in the enzyme assay procedure with 1 ml. of an invertase preparation derived from *P. mirabilis* strain U129. The amounts of glucose liberated in reaction periods of 30 min. were then estimated by the glucose oxidase method. Results were expressed in mg. glucose/total specimen. The three supernatant fluids, each of the experiments done with strain 57 *suc*$^+$ and *P. vulgaris* strains 9 and 15 were also investigated for the presence of extracellular β-D-fructofuranosidases. This was done by the enzyme assay procedure with sucrose as substrate and 1 ml. volumes of the supernatant fluids now treated as the enzyme preparations. Supernatant fluids were used as such and in more concentrated form by adding equal volumes of saturated ammonium sulphate, centrifugation at 4000 rev./min. for 1 hr. at 4°, and dissolving the precipitates in 2 ml. distilled water. The supernatant fluids of these three experiments were also chromatographed to detect the cleavage products (glucose, fructose) of possible extracellular enzymic activity. The deposits of organisms in the centrifuge tubes were carefully washed twice in cold distilled water and then suspended in 15 ml. of cold distilled water. Two of the three suspensions of each test were then disintegrated in the Raytheon oscillator, as described; the remaining test suspension and the control suspension were left intact. The amounts of sucrose present in the intact and disintegrated suspensions were estimated as above. Disintegrated and intact suspensions were also examined chromatographically for sucrose. Identical samples from the intact suspensions which had been exposed to sucrose and the control suspensions were dried and weighed to constant weight *Mackie & McCartney's Handbook*, 1960). Experiments were also done on the uptake of maltose by different strains. These experiments differed from those described in that suspensions were made from 36 hr. growths on 1 and 5 % maltose agar, 1 % maltose peptone water was used and maltose in supernatant fluids and suspensions was determined by the copper reduction method.

RESULTS

Sugar fermentations

The results of sucrose fermentation tests are presented in Table 1. All 10 strains of *Proteus vulgaris* fermented 1 % sucrose peptone water giving a small volume of gas within 24 hr. All members of *P. mirabilis* groups 1*a*, 1*b* and 3 also fermented 1 % sucrose peptone water giving a little gas; the time taken to do this was between 3 and 11 days. When the contents of these fermented tubes were plated on 1 % sucrose agar, pale and many red colonies appeared after overnight incubation. The red colonies arose from mutants in the fermentation tubes capable of fermenting

1 % sucrose peptone water within 24 hr. *P. mirabilis* strains of group 2 produced no indicator change in the sucrose peptone water even after incubation for 25 days. Strains of groups 1*a* and 1*b* fermented 5 % sucrose peptone water giving a small volume of gas within 36 hr. When the contents of these fermented tubes were plated on 1 % sucrose agar all colonies were pale. This proved that the rapid fermentation of 5 % sucrose was not due to mutants capable of prompt sucrose fermentation. *P. mirabilis* strains of group 3 took more than 6 days to ferment 5 % sucrose peptone water while strains belonging to group 2 produced no indicator change after 25 days of incubation. When the contents of the fermented tubes of group 3 were plated on 1 % sucrose agar, pale and red colonies were observed. The red colonies originated from mutants capable of fermenting 1 % sucrose peptone water within 24 hr.

Table 1. *Sucrose fermentation by 73 strains of* Proteus hauseri

Fluid media were inoculated with a drop of overnight broth culture. Solid media were inoculated with 0·1 ml. of a dilution of overnight broth culture in distilled water and rubbed to dryness with a sterile glass rod. Plates with about 10 colonies were examined. Cultures were incubated at 37° and observed daily for 25 days.

Proteus	No. of strains	Fermentation in 1 % sucrose peptone water	Fermentation in 5 % sucrose peptone water	Appearance on 1 % sucrose	
				Agar	Bile agar
'vulgaris'	10, including nos. 9, 15	AG	AG	R	R
'mirabilis'					
Group 1*a*	46, including nos. F25, U123, U129, U183	(AG)	AG*	P+	P+
Group 1*b*	9, including nos. 57, F10	(AG)	AG*	P+	R+
Group 2	3, nos. U18, F20, 40	—	—	P	P
Group 3	5, nos. 12, 13, 34, 55, 63	(AG)	(AG)	P+	P+

AG = acid with a small volume of gas within 24 hr.; (AG) = acid with a small volume of gas after more than 3 days; P = pale overnight; R = red overnight. + = these colonies developed red papillae after more than 3 days; * = acid with a small volume of gas within 36 hr.

Proteus did not swarm on the media used and isolated colonies of all the *Proteus vulgaris* strains were red after overnight incubation on 1 % sucrose agar. *P. mirabilis* strains of groups 1*a* and 1*b* all formed pale colonies on the above medium; after about 3 days of incubation red papillae appeared on the colonies of all these 55 strains. Organisms from the papillae differed from the remainder of the colony in being able to ferment 1 % sucrose peptone water within 24 hr. These papillae were the clonal progeny of mutations which occurred during the development of the colony and were selected by their ability to ferment sucrose (Coetzee & Sacks, 1960*a*). Strains of *P. mirabilis* groups 2 and 3 also formed pale colonies on this sucrose agar medium, but whereas red papillae developed on the colonies of strains of group 3, papillae were never detected on colonies of group 2 even after 25 days of incubation.

A further phenotypic division was possible on the 1 % sucrose bile agar medium. After overnight incubation well separated colonies of the 10 *P. vulgaris* were red

on this medium. Group 1 *a* *P. mirabilis* strains formed pale colonies which eventually developed sucrose fermenting papillae; group 1 *b* strains formed red colonies after overnight incubation. Organisms from these red colonies took more than 3 days to ferment 1 % sucrose peptone water. Papillae also appeared after a few days on the red colonies of these 9 strains. They were small and less numerous than the papillae previously mentioned. Increasing the concentration of sodium deoxycholate up to 2 % in the medium, did not change the appearance of group 1 *a* strains mentioned above. The appearance of strains of groups 2 and 3 on this medium was like that on 1 % sucrose agar.

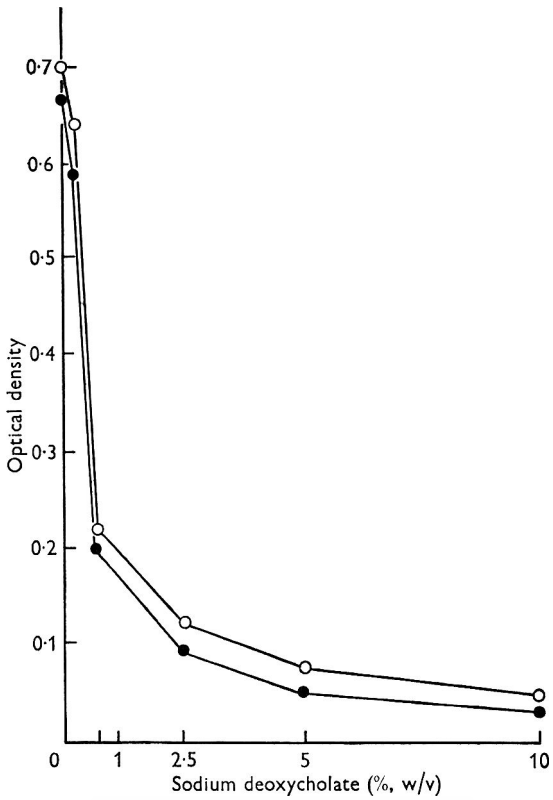

Fig. 1. Effect of sodium deoxycholate on *Proteus mirabilis* strain U 129. An overnight broth culture of *Proteus mirabilis* strain U 129 was diluted 1/4 in fresh broth and bubbled with sterile air for 2 hr. at 37°. 2 ml. volumes were then sedimented at 4000 rev./min. for 10 min. and the deposited organisms (*c.* 2×10^9) resuspended in 5 ml. of various concentrations (w/v) of sodium deoxycholate in saline (○—○). A duplicate set of suspensions was made with the sodium deoxycholate dissolved in 0·6 M sucrose in saline (●—●). Tubes were kept at room temperature for 30 min. before reading densities with a Zeiss spectrophotometer at 630 mμ.

The mutation rates to sucrose fermentation of all strains of group 3, two strains of group 1 *a* (nos. U 129, F 25) and one strain of group 1 *b* (no. 57) were investigated. These varied between 1×10^{-9} bacteria/generation and 2×10^{-9} bacteria/generation in different experiments with group 3 strains. The mutation rates for the strains of groups 1 *a* and 1 *b* ranged between 1×10^{-8} and 5×10^{8} bacteria/generation in different experiments.

All ten *Proteus vulgaris* strains fermented 1 % maltose peptone water within 24 hr. The 63 *P. mirabilis* strains did not ferment maltose, and none of the 73 *Proteus* strains fermented raffinose.

Effect of sodium deoxycholate on Proteus hauseri. Two 'vulgaris' strains, 10 members of group 1*a* and all the members of groups 1*b*, 2 and 3 were used in these experiments. The results were identical in that all strains underwent visible clearing within 30 min. in solutions of sodium deoxycholate down to concentrations of 0·6 % (w/v). Viable counts done after 30 min. from the 1·2 % (w/v) sodium deoxycholate tubes indicated a 75 % killing as compared with the saline control. Experiments conducted in 0·6 M-sucrose in saline showed no effect on the clearing of suspensions or viable counts. Periodic observations on the sucrose suspensions by phase-contrast microscopy showed normal forms with increasing amounts of debris. No protoplast-like structures were seen; this indicated that the mode of action of sodium deoxycholate was not only on the cell wall, but might affect the protoplasmic membrane, as suggested by Salton (1957) and by Pethica (1958) for the mode of action of other surface-active agents on Gram-negative bacteria. The result illustrated in Fig. 1 is representative of all strains tested.

Examination of enzyme effects

Crude enzyme extracts were prepared by the sonic treatment from two of the *Proteus vulgaris* strains (9, 15) and from all 63 *P. mirabilis* strains grown on nutrient agar. These extracts were tested by the enzyme assay procedure for ability to cleave sucrose to fructose and glucose. Extract controls had very small reducing activity which never increased during the 10 min. reaction period; chromatography of extracts and sucrose controls were satisfactory in that they never showed spots moving with glucose and fructose markers. Extracts from the *P. vulgaris* strains and all members of groups 1*a* and 1*b* split sucrose to glucose + fructose as shown by chromatograms. The liberation of glucose and fructose from the non-reducing substrate was accompanied by a marked increase in the reducing activity of the 15 min. sample over the 5 min. sample. No invert sugar was detected in any of the samples from tests with extracts of strains of groups 2 or 3. New extracts of strains from groups 2 and 3 were prepared from organisms grown on 5 % sucrose agar. Despite the fact that the period of sonic treatment produced marked clearing of the suspensions and that very few intact organisms were present as judged by microscopic observations, the results were negative. Crude extracts prepared from these 8 strains by the benzene and sodium deoxycholate methods were also inactive for sucrose, although control extracts produced from other 'mirabilis' strains by these methods yielded invert sugar.

Groups 1*a* and 1*b* comprising 55 of the 63 wild strains of *Proteus mirabilis* are thus cryptic with regard to sucrose fermentation, i.e. although they possess the relevant enzyme system, they do not normally ferment sucrose.

Disaccharide accumulation studies

Strains 57, F10, F25, U123, U129, U183 of groups 1*a* and 1*b*, all members of groups 2 and 3, strain 57 *suc*$^+$ and *Proteus vulgaris* strains 9, 15 were investigated to determine whether sucrose could accumulate in these organisms. With the exception of strains 57 *suc*$^+$ and *P. vulgaris* strains 9 and 15, none of the strains examined

normally metabolize sucrose. Representative results are shown in Table 2. Sucrose was identified chromatographically in the disintegrated suspensions of all members of group 3; none of the other suspensions (disintegrated or intact) showed the presence of sucrose. Despite the fact that recovery of sucrose (as glucose) with experiments on these group 3 strains was never more than about 85%, it was deduced that sucrose from a 1% concentration in the medium, penetrated and was concentrated, by these 5 strains.

Table 2. *Sucrose uptake by suspensions of* Proteus mirabilis

Thirty-six hr. growths on nutrient agar were washed 3 times in distilled water. Dense suspensions were made and 1 ml. added to 10 ml. of 1% sucrose peptone water. 1 ml. was also added to 10 ml. plain peptone water as organism control and uninoculated sucrose peptone water as sucrose control. Experiments were done in triplicate. Tubes were kept at 37° for 30 min., and then centrifuged at 4000 rev./min. for 15 min. at 4°. Supernatant fluids were removed as completely as possible and deposits washed twice in cold distilled water. Deposits were then suspended in 15 ml. cold distilled water. Two of the three test suspensions of each experiment were disintegrated in a Raytheon sonic oscillator for 30 min.; 1 ml. amounts of all tubes were then mixed with 1 ml. volumes of a partially purified extract of Proteus U129 in the presence of 2·5 ml. 0·05 M-potassium phosphate buffer pH 7·4 and 1·5 ml. distilled water. Extract and other reactants were equilibrated at 37° before mixing. Tubes were sampled 30 min. after mixing and the amount of glucose present determined by the glucose oxidase method.

	Proteus mirabilis strains				
	12	U129	57	57 *suc*$^+$	U18
	Sucrose (as mg. glucose/total vol. specimen)				
Supernatant fluid	8·2, 9·4, 8·1	38·7, 39·0, 38·7	37·7, 38·6, 38·5	12·1, 11·8, 10·9	38·5, 38·3, 37·6
Disintegrated supension	22·1, 26·0	0, 0	0, 0	0, 0	0, 0
Intact suspension	0	0	0	0	0
Sucrose control	39·6	38·9	38·7	38·9	38·5
Organism control	0	0	0	0	0

Strain 57 *suc*$^+$ also removed large amounts of sucrose from sucrose + peptone water. Both the unconcentrated and concentrated supernatant fluids in experiments with strain 57 possessed no invertase activity as judged by the results of enzyme assays and by the fact that no spots corresponding to the glucose and fructose markers were seen on chromatograms from unconcentrated supernatant fluids. No sucrose, glucose or fructose was demonstrated chromatographically in the disintegrated or in the intact organism suspensions of strain 57 *suc*$^+$; no glucose was present in these suspensions as determined by the glucose oxidase method. Experiments with *Proteus vulgaris* (strains 9, 15) yielded similar results to those with strain 57 *suc*$^+$. The results with the above three strains may be taken to mean that their sucrose splitting enzymes are intracellular. It appears that the sucrose which disappeared from sucrose peptone water penetrated these organisms in the disaccharide form and once in contact with the enzyme inside the cells was rapidly split and further metabolized (Cohen & Monod, 1957). Sucrose did not enter organisms of groups 1*a*, 1*b* or 2.

The experiments with these 9 strains of groups 1*a*, 1*b* and 2 were repeated with organisms harvested from 36 hr. 1% and 5% sucrose agar plates, with similar results. Four separate experiments were done on dry weights of identical samples

from the suspensions of all members of group 3 and corresponding control suspensions which had been exposed to sucrose. In all four experiments the test suspensions were 11·0–14·8 % heavier than the controls. In similar experiments with the members of group 2 test suspensions were either heavier or lighter than the controls; the differences did not exceed 2 % of the control weight. Although strains belonging to groups 2 and 3 all lacked sucrose splitting enzymes group 2 strains could thus be distinguished on a basis of sucrose uptake.

Table 3. *Maltose uptake by suspensions of* Proteus hauseri

Thirty-six hr. growths on 5 % maltose agar were washed 3 times in distilled water. Dense suspensions were made and 1 ml. added to 10 ml. of 1 % maltose peptone water. 1 ml. was also added to 10 ml. plain peptone water as organism control, and uninoculated maltose peptone water served as maltose control. Experiments were done in triplicate. Tubes were kept at 37° for 30 min. and then centrifuged at 4000 rev./min. for 15 min. at 4°. Superanatant fluids were removed as completely as possible and the deposits carefully washed twice in cold distilled water. Deposits were suspended in 15 ml. cold distilled water and two of the three test suspensions of each experiment disintegrated in a Raytheon sonic oscillator for 30 min. Reducing activity in all tubes was then determined.

	Proteus mirabilis strains				*Proteus vulgaris*
	12	57	57 *suc*$^+$	U129	strain 15
	Maltose (as mg. glucose/total vol. of specimen)				
Supernatant fluid	49·5, 49·6, 49·7	50·2, 49·7, 49·7	48·9, 49·7, 47·9	49·5, 49·0, 48·9	10·1, 9·5, 816
Disintegrated suspension	1·7, 1·9	4·3, 4·0	2·0, 2·4	5·0, 5·0	6·0, 8·7
Intact suspension	1·8	4·4	2·1	4·8	2·8
Maltose control	49·5	49·7	49·5	49·0	49·1
Organism control	1·8	4·3	2·4	4·8	2·1

Table 3 illustrates the results obtained in an experiment on maltose uptake by *Proteus mirabilis* strains 12, 57, 57 *suc*$^+$ and U129 and *P. vulgaris* strain 15, all grown on 5 % maltose agar.

None of the *Proteus mirabilis* strains removed maltose from maltose peptone water and maltose was not found chromatographically in disintegrated suspensions of the strains exposed to maltose. The other *P. mirabilis* strains of groups 2 and 3 tested, yielded similar results. These experiments were repeated with suspensions prepared from organisms grown on 1 % maltose agar and gave similar results. *P. vulgaris* strain 15 removed about 80 % of the maltose from the peptone water; no maltose or glucose was found chromatographically in the disintegrated or intact suspensions of this strain. This and the fact that less than one-third of the input of reducing activity was recovered may be attributed to rapid further metabolism of disaccharide once it was taken up by *Proteus* 15. A measure of selective permeability for disaccharides was thus exhibited by strains of group 3 and *P. mirabilis* strain 57 *suc*$^+$. While impermeable to maltose these organisms took up sucrose from the medium despite the fact that group 3 strains had no means of metabolizing it.

Properties of partially purified enzymes

Extracts of *Proteus vulgaris* strains 15 and 9 and *P. mirabilis* strains 57 and U129 were studied in greater detail. The evidence against a phosphorolytic mechanism of sucrose breakdown in the organisms studied here is that in experiments with partially

purified enzyme extracts the breakdown of sucrose proceeded at the same rate in both barbital and phosphate buffers. The amount of phosphate in the barbital reaction mixture was less than 10^{-6}M. No spot corresponding to the glucose-1-phosphate marker was seen on special chromatograms. These findings taken in conjunction with the fact that glucose was detected in the reaction mixtures by the glucose oxidase enzyme (which does not react with glucose-1-phosphate) and that

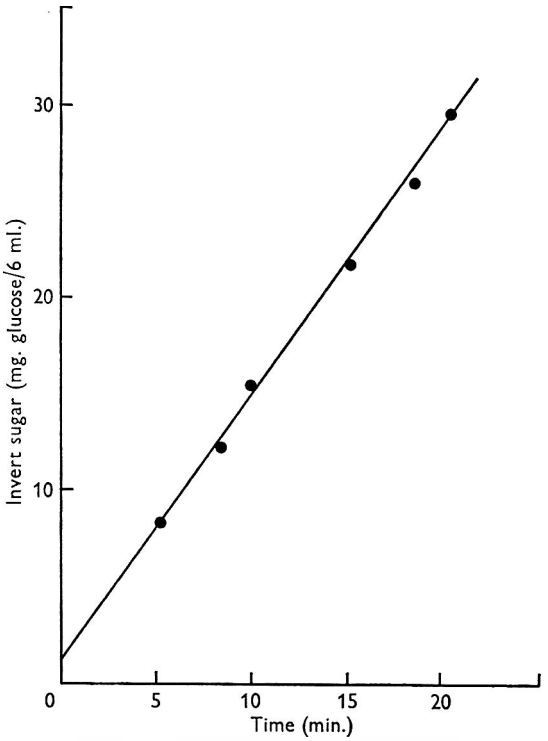

Fig. 2. Relationship between time and quantity of invert sugar produced from sucrose by a partially purified extract of *Proteus mirabilis* strain 57. The reaction mixture contained 1 ml. 0·6M-sucrose, 1 ml. of a 1/5 dilution of a partially purified extract of *P. mirabilis* strain 57 in distilled water, 2·5 ml. of 0·05M-potassium phosphate buffer pH 7·4 and 1·5 ml. distilled water. The extract and other reactants were equilibrated at 37° before mixing. Samples were removed at intervals and reducing activity measured. This was expressed as mg. glucose/6 ml.

glucose and fructose were identified chromatographically in these mixtures, excludes the presence of sucrose phosphorylase, levansucrase and dextransucrase (Hassid, 1951). This labels the responsible enzyme in all four partially purified extracts as an invertase.

Linear relationships were found between the quantities of invert sugar produced and the time periods of reaction and the amounts of extract in reaction mixtures. Figures 2 and 3 illustrate these findings for the partially purified extract of *Proteus mirabilis* strain 57.

The pH optima for invertase activity of all four partially purified extracts was about pH 7·4. Figure 4 shows the results obtained with the extract from proteus

strain 15. The invertase from *Aspergillus oryzae* has an optimum between 6 and 8, but most invertases have pH optima ranging from 4·2 to 7·0 (Neuberg & Mandl, 1951), and the invertase of *Saccharomyces cerevisiae* has an optimum of pH 4·7–4·9 (Bacon, 1955).

The effect of different salts on the invertase activity of partially purified extracts was tested; the results of one experiment with a partially purified extract of *Proteus mirabilis* strain 57 is presented in Table 4.

It is seen that K^+ and NH_4^+ had a stimulatory action while Na^+, Li^+, Mg^+ and

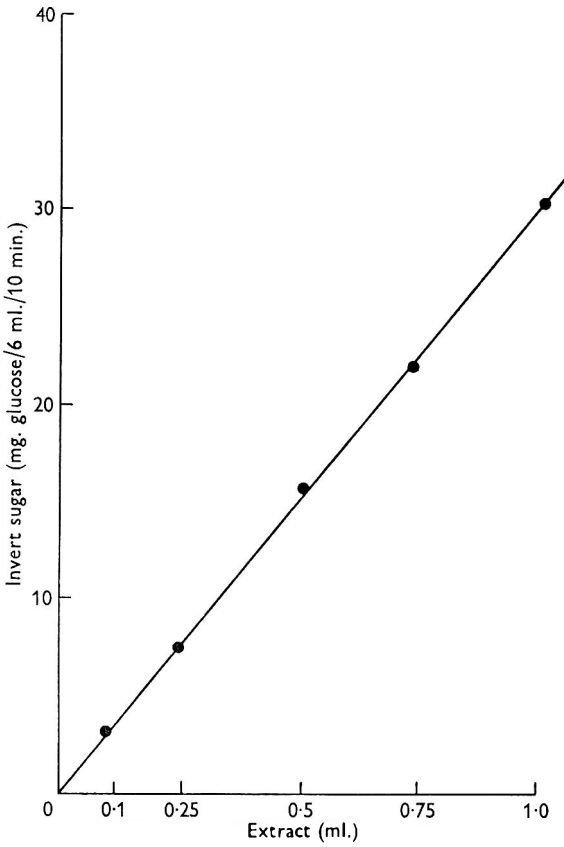

Fig. 3. Relationship between amount of invert sugar produced from sucrose by different quantities of a partially purified extract of *Proteus mirabilis* strain 57. Reaction mixtures contained 1 ml. of 0·6 M sucrose, different volumes of a 1/5 dilution of a partially purified extract of *Proteus mirabilis* strain 57 in distilled water to 1 ml., 2·5 ml. of 0·05 M-potassium phosphate buffer pH 7·4 and distilled water to 6 ml. Extracts and other reactants were equilibrated at 37° before mixing. Samples were removed after 5 min. and 15 min. and reducing activity measured. This was expressed as mg. glucose/6 ml. and the amount produced in the 10 min. interval calculated.

Ca^{2+} were inert; the anions phosphate, chloride, citrate and sulphate also had no effect. The enzymic activity was not influenced by sodium deoxycholate; like other invertases (Neuberg & Mandl, 1951) it was not affected by sodium fluoride. The activities of the other partially purified extracts were like those of strain 57 in the different ionic environments. All four partially purified extracts split raffinose to

melibiose + fructose. These products were identified chromatographically in reaction mixtures containing raffinose as substrate. No glucose was found. This was proved chromatographically and also by testing samples from the reaction tubes by the glucose oxidase method which does not react with either fructose or melibiose.

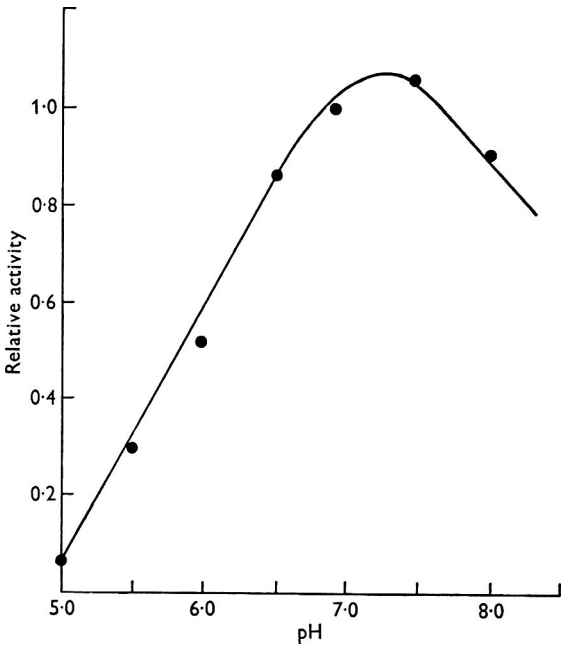

Fig. 4. Effect of pH on invertase activity of a partially purified extract of *Proteus vulgaris* strain 15. Reaction tubes contained 1 ml. of a partially purified extract of *P. vulgaris* strain 15 diluted 1/5 in distilled water, 1 ml. of 0·6 M-sucrose and 3·5 ml. 0·05 M-potassium phosphate buffer at different pH values. Extracts and other reactants were equilibrated at 37° before mixing. Samples were withdrawn after 5 min. and 15 min. and reducing activity measured. These were expressed as mg. glucose/6 ml. and the amount produced in the 10 min. interval calculated. The activity units were arbitrary and were adjusted for unit activity at pH 7.

Table 4. *Influence of salts on invertase activity of extracts of* Proteus mirabilis 57

Reaction tubes contained: 1 ml. 1/5 dilution of a partially purified extract of proteus 57; 1 ml. 0·6 M-sucrose; 2·5 ml. 0·05 M-tris buffer (pH 7·4): 1·5 ml. 0·16 M solution of different salts. The control tube had the 1·5 ml. salt solution replaced by distilled water. Extracts and other reactants were temperature equilibrated at 37° before mixing. Tubes were sampled after 5 and 15 min. and reducing activity (as glucose) measured.

Salt	Reaction rate (mg. glucose/ 6 ml./10 min.)	Salt	Reaction rate (mg. glucose/ 6 ml./10 min.)
Potassium chloride	14·1	Calcium chloride	4·1
Potassium dihydrogen phosphate	13·9	Sodium phosphate	4·8
		Sodium chloride	5·0
Ammonium citrate	11·2	Sodium citrate	4·4
Ammonium chloride	10·8	Sodium fluoride	4·9
Magnesium chloride	4·8	Sodium deoxycholate	4·5
Lithium sulphate	5·2	Distilled water control	4·4

A plot of raffinose concentration and reaction rate is presented in Fig. 5 for a partially purified extract prepared from Proteus U129. Michaelis constants calculated from the slopes and ordinate intercepts according to Lineweaver & Burk (1934) yielded values of 0·114 M, 0·210 M, 0·125 M and 0·245 M for the extracts of Proteus strains U129, 57, 15 and 9, respectively.

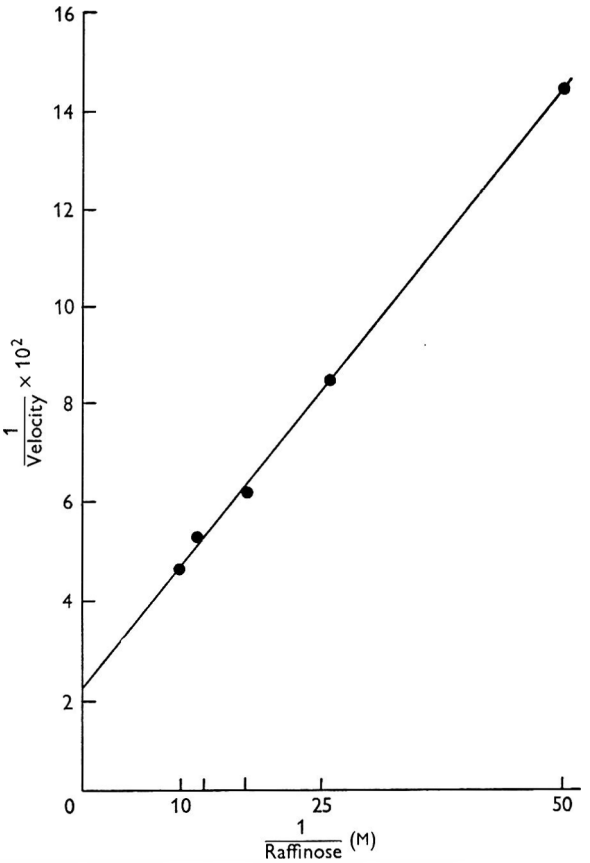

Fig. 5. Breakdown of raffinose by a partially purified extract of *Proteus mirabilis* strain U129. Reaction tubes contained 1 ml. of a partially purified extract of U129 diluted 1/5 in distilled water, different volumes of 0·6 M-raffinose, 2·5 ml. of 0·05 M-potassium phosphate buffer pH 7·4 and distilled water to 6 ml. Extracts and other reactants were equilibrated at 37° before mixing. Samples were removed after 5 min. and 15 min. and reducing activity (expressed as glucose) determined. Reaction velocities were expressed as mg. glucose/6 ml./10 min.

Although only strains 57 and U129 have been investigated it is probable that all 55 strains of groups 1*a* and 1*b* cryptic with regard to sucrose fermentation are thus also cryptic towards raffinose.

The kinetic behaviour of mixtures of raffinose and sucrose in the presence of a partially purified extract of Proteus U129 is presented in Fig. 6. The ordinate intercept of the straight lines indicate that raffinose was a competitive inhibitor of sucrose for the enzyme which may now be termed a β-D-fructofuranosidase. Similar results were obtained with partially purified extracts from the other three strains;

Michaelis constants for the enzyme present in the extracts from strains U129, 57, 15 and 9 with sucrose as substrate are 0·026, 0·031, 0·018 and 0·028 M, respectively. The constants are of the same order as the values reported for yeast and gut saccharases (Baldwin, 1947).

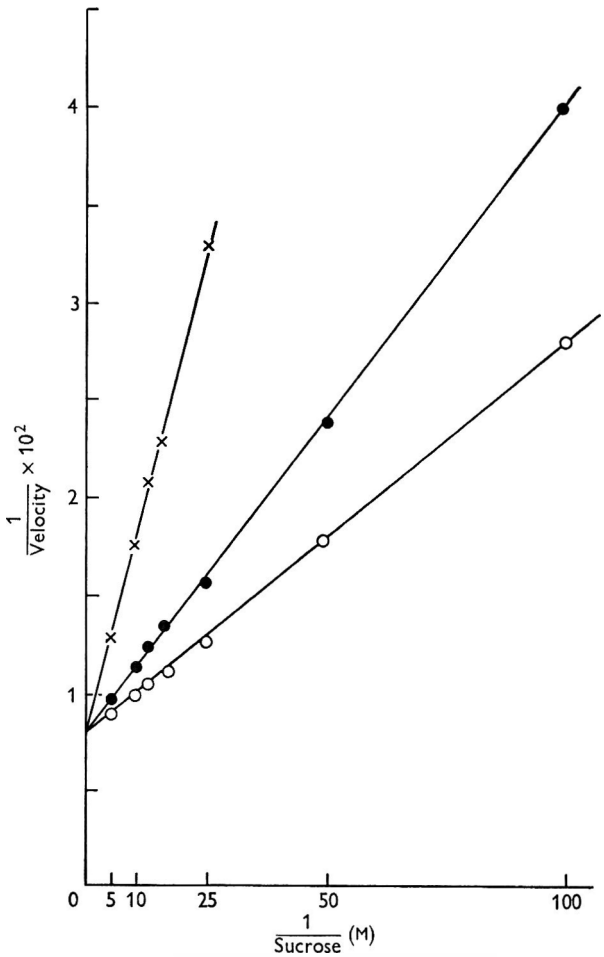

Fig. 6. Competitive inhibition of sucrose by raffinose in the presence of a partially purified extract of Proteus U129. Reaction tubes contained 1 ml. of a partially purified extract of Proteus U129 diluted 1/5 in distilled water, 2·5 ml. of 0·05 M-potassium buffer pH 7·4 and different volumes of 0·6 M-sucrose. Tubes of experiment (○—○) also contained 0·1 ml. of 0·6 M-raffinose and those of experiment (× — ×) contained 1 ml. of 1 M-raffinose. Contents of tubes were made up to 6 ml. with distilled water. The extracts and other reactants were equilibrated at 37° before mixing. Samples were removed after 5 min. and 15 min. and the glucose content determined by the glucose oxidase method. Reaction velocities were expressed as mg. glucose/6 ml./10 min.

Table 5 gives the results of reaction rate experiments with partially purified extracts of *Proteus mirabilis* strains U129, 57 and *P. vulgaris* strains 15, 9 from 24 hr. growth on nutrient agar and 1 % glucose and sucrose agar, respectively. The same amounts of extract proteins were added to the tubes and the results were comparable. The reaction velocities found were very similar for extracts prepared

from any one of these four strains grown on either nutrient agar, 1 % glucose or sucrose agar and implied that the β-D-fructofuranosidase was constitutive in the strains tested.

Table 5. *Reaction rate experiments with partially purified extracts of Proteus grown on different media*

Partially purified extracts were prepared from 36 hr. growths of organism on nutrient agar, 1 % (w/v) glucose agar and 1 % (w/v) sucrose agar. The protein content of extracts was adjusted to 4 mg./ml. Reaction tubes contained: 1 ml. extract + 1 ml. 0·6 M-sucrose + 2·5 ml. 0·05 M-potassium phosphate buffer (pH 7·4) + 1·5 ml. distilled water. Extracts and other reactants were equilibrated at 37° before mixing. Tubes were sampled after 5 min. and 15 min. and glucose estimated by the glucose oxidase method.

	Extracts prepared from organisms grown on		
Extract from Proteus strain	Nutrient agar	1 % (w/v) Glucose agar	1 % (w/v) Sucrose agar
	Reaction rates (mg. glucose/6 ml./10 min.)		
U 129	2·0	2·4	2·0
57	2·7	2·3	2·3
15	3·1	3·2	2·9
9	1·7	2·0	2·0

DISCUSSION

Cohen & Monod (1957) proposed two models to account for the fact that organisms like those of *Proteus mirabilis* group 3 can accumulate large amounts of a substrate. The stoichiometric model explains this unidirectional transfer as a result of the binding of substrate to intracellular macromolecular receptors. No permeable barrier or special permeation mechanism is implicated. The catalytic theory explains the accumulation on the basis of a virtually impermeable osmotic barrier which the substrate (sucrose) can cross only by combining with a specific enzyme, called a permease. The fact that the strains of Proteus mentioned accumulated sucrose up to about 14 % of their dry weight is strongly against the first model. This would demand an impossibly large number of non-diffusable macromolecular (presumably protein) absorption sites. On the other hand, it was found that 55 of 63 wild strains of *P. mirabilis* were impermeable to 1 % solutions of sucrose while they did possess β-D-fructofuranosidases (cryptic strains). Strains of group 3 were selectively permeable to 1 % sucrose but had no invertase; members of group 2 were impermeable and also lacked β-D-fructofuranosidases. The β-D-fructofuranosidases appeared to be confined within the cells. No extracellular invertase activity was detected with suspensions of a sucrose-positive mutant of a cryptic strain and of two *P. vulgaris* strains investigated. The existence of the above phenotypes shows that selective permeability to sucrose and β-D-fructofuranosidase activity are two genetically and functionally distinct properties and that they normally form a metabolic sequence *in vivo*; these observations are strongly in favour of the permease model. In this respect it may be noted that strains of group 3 had lower mutation rates to sucrose utilization than three representatives (strains U129, F25 and 57) of the cryptic group. This is supporting evidence for the contention that factors which control

the β-D-fructofuranosidase and selective permeability to sucrose are genetically distinct. The methods at our disposal for the sucrose uptake studies were crude and devious. For these reasons no kinetic studies on sucrose accumulation were attempted and no sucrose permease was demonstrated. The system which controls the selective permeation of sucrose in *P. hauseri* will consequently be named a sucrose permeability factor.

A number of workers (Kriebel, 1934; Chilton & Fulton, 1946; Lowe & Evans, 1957; Lowe, 1960) have observed that when the concentration of lactose is increased above the usual 1% many paracolon organisms ferment this disaccharide in a shorter time. Lowe (1960) showed that many paracolon strains were cryptic in respect to lactose but when the concentrations were increased to 5% the permeability of the barrier which separates the β-galactosidase from its substrate was increased, permitting the entry of some lactose. Cohen & Monod (1957) assumed that the barrier of the cell was not absolutely impermeable and they invoked 'non-specific leakage' to equilibrate the entry of substrate by the specific permease system. A similar phenomenon was encountered in this work where all 55 cryptic strains of *Proteus mirabilis* were found to ferment 5% sucrose peptone water within 36 hr., while requiring 3–11 days to ferment 1% sucrose. Fermentation at the latter concentration was due to the selection of mutants like those of *P. mirabilis* strain 57 *suc*$^+$ which were permeable to 1% sucrose and thus capable of prompt sucrose fermentation. No organisms capable of fermenting 1% sucrose promptly were isolated from the 5% tubes when these were examined at 36 hr. It may be postulated that the barrier which separates the β-D-fructofuranosidase from external sucrose in the cryptic strains is not wholly impermeable to high concentrations of the latter.

It was demonstrated that all Proteus strains tested underwent marked lysis in the presence of 1% sodium deoxycholate. It was also shown that the action of the bile was not confined to the cell wall. To account for the fact that cryptic strains promptly ferment 1% sucrose in the sucrose bile agar medium it is only necessary to assume that the barrier, which is possibly the protoplast-limiting membrane (Cohen & Monod, 1957), is made more permeable to sucrose by the action of sodium deoxycholate. The problem is why only 9 out of the 55 cryptic strains showed this phenomenon. No difference in bile sensitivity was demonstrated and increasing the concentration of sodium deoxycholate in the sucrose agar had no effect. It was also shown that the β-D-fructofuranosidase of these strains was not affected by similar concentrations of sodium deoxycholate and it must be concluded that subtle differences exist between strains of the cryptic group which were not detected.

From the results obtained a classification of the strains of *Proteus hauseri* investigated is presented in Table 6. Rustigan & Stuart (1945) reported that 197 of 205 strains of *P. mirabilis* fermented sucrose slowly, while the remaining 8 strains failed to ferment it after 21 days of incubation. The fermentation of sucrose by Kauffmann's (1951) 456 *P. mirabilis* strains was also 'late, irregular or negative'. From the information available it is thus impossible to decide to which of the proposed phenotypes the slow sucrose-fermenting organisms in the series of Rustigan & Stuart or Kauffmann belong. The 8 strains of the former authors and the unspecified number of Kauffmann which do not ferment sucrose even after long periods of incubation, are almost certainly however of the phenotype which lacks both β-D-fructofuranosidase and the permeability factor.

Table 6. *A scheme for phenotypes of* Proteus hauseri

1 % sucrose peptone water						
Fermentation	Presence of sucrose fermenting mutants	5 % sucrose peptone water	Appearance on 1 % sucrose bile agar	Fructo-furanosidase activity	Phenotype	Proteus
AG	.	AG	R	+	F^+Pf^+	10 'vulgaris' strains.
(AG)	+	AG*	R^+	+	F^+Pf^-	'mirabilis' group 1*b* strains
(AG)	+	AG*	P^+	+	F^+Pf^-	'mirabilis' group 1*a* strains
–	–	–	P	–	F^-Pf^-	'mirabilis' group 2 strains
(AG)	+	(AG)	P^+	–	F^-Pf^+	'mirabilis' group 3 strains

AG = Acid with small volume of gas within 24 hr.; (AG) = acid with small volume of gas after more than 3 days; R = red overnight; P = pale overnight; * = acid with small volume of gas between 24 and 36 hr.; $^+$, red papillae developed on these colonies after more than 3 days. The presence of β-D-fructofuranosidase and permeability factor are indicated by the symbols F^+ and Pf^+.

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The Genetic Control of Colicinogenic Factors E_2, I and V

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SUMMARY

The state of colicinogenic factors E_2, I and V (*col* E_2, *col I* and *col V*) in *Escherichia coli* K12 was studied. The analysis of the results of conjugation experiments involving different Hfr or F^+ and F^- strains shows that: (1) The frequency of transfer of these colicinogenic factors differs markedly: *col V* is transferred with maximum efficiency, *col* E_2 and *col I*, on the contrary, only at low frequency. (2) Each colicinogenic factor is transferred from different types of Hfr or F^+ strains with similar frequencies. (3) They are not linked with chromosomal markers. (4) The non-colicinogenic character of the donor parent is never transmitted to recombinants. (5) Zygotes that have received *col V*, transmit it to all daughter cells. All these results lead to the conclusion that colicinogenic factors E_2, I and V are in an extrachromosomal state in F^+ and Hfr bacteria. The same conclusion would apply to their state in F^- bacteria, since the results with colicinogenic factor V seem to indicate that this factor replicates in this type of bacteria autonomously and at a faster rate than the chromosome.

INTRODUCTION

Colicines are antibiotic substances produced by certain strains of Enterobacteriaceae that have a lethal action on other strains of the same family. Their synthesis is controlled by hereditary factors called colicinogenic factors.

The genetic behaviour of the colicinogenic factor E_1 in crosses between F^+ and F^- strains of *Escherichia coli* K12 was studied by Fredericq & Betz-Bareau (1953). In crosses involving colicinogenic (*col*$^+$) F^+ and non-colicinogenic (*col*$^-$)F^- bacteria. the *col*$^+$ character was transferred to recombinants; in reciprocal crosses F^+ *col*$^-$ × F^- *col*$^+$ recombinants never received the *col*$^-$ character belonging to the donor parent. From the asymmetric transfer of *col* in these crosses and from the absence of linkage with chromosomal markers they concluded that this factor was of cytoplasmic nature.

The transfer of colicinogenic factor E_1 (*col* E_1) derived from *E. coli* K30 was also studied in Hfr × F^- matings by Alfoldi, Jacob, Wollman & Mazé (1958). Their results showed that the frequency of transfer of the *col*$^+$ character in crosses

between Hfr *col*$^+$ bacteria belonging to different Hfr types and F$^-$ *col*$^-$ bacteria varied according to the type of Hfr used in the cross and led them to conclude that *col* E_1 is localized in the chromosome of the Hfr between *thr leu* and *met thi*. Similar conclusions were drawn from reciprocal crosses Hfr *col*$^-$ × F$^-$ *col*$^+$. In these crosses *col*$^-$ did not appear among recombinants and a lethal zygosis was found to occur with each type of Hfr with a frequency corresponding to that of the transfer of *col*$^+$ in the reciprocal cross. This lethality was assumed to be due to the penetration of *col*$^-$ into the F$^-$ cell.

They also found that once transferred to the zygote, the *col*$^+$ character passed to all daughter cells without segregating. This indicated that *col*$^+$ can multiply in the F$^-$ autonomously and at a greater rate than the chromosome.

Thus, colicinogenic factor E_1, when present in the cell, can exist either in an integrated state (in the chromosome of the Hfr) or in an autonomous state (in F$^+$ and F$^-$ bacteria). These properties pointed to the inclusion of colicinogenic factor E_1 in the category of episomes (Jacob & Wollman, 1958*b*).

The peculiar behaviour of this factor posed, then, the question of whether it could be considered as representative or characteristic of all colicinogenic factors. The study of other colicinogenic factors was undertaken in an attempt to solve this question.

The present paper is concerned with an analysis of the state of colicinogenic factors E_2, I and V in strains of *E. coli* K12. To make the results comparable to those of Alfoldi *et al.* with *col* E_1, similar strains and methods were employed in this study.

MATERIALS

Media. The synthetic medium employed was that of Wollman & Jacob (1959). The minimal medium was supplemented before use with different amino acids, depending on the selective requirements, at a final concentration of 80 μg./ml.

Other media employed were: nutrient broth and nutrient agar (nutrient broth with 2 % of agar).

Streptomycin, when required, was added at a concentration of 200 μg/ml.

Bacterial strains. The strains used in the experiments are described in Table 1 and were kindly supplied by Professor P. Fredericq, Dr E. L. Wollman and Dr E. Balbinder.

Strain 58–161 was utilized as sensitive indicator of colicines, and resistant strains derived from it, as resistant ones.

All strains employed in matings were previously made resistant to the colicine considered, to eliminate the lethal effect of the colicine on sensitive strains.

METHODS

Hfr and F$^+$ strains were made colicinogenic by mixed culture with the original F$^+$ donor strains: 12–317, CA53 and 12–94. The mixture was incubated overnight in nutrient broth at 37°. Aliquots were plated on appropriate selective media and *col*$^+$ colonies detected by the double agar-layer method (Fredericq, 1954). Colicinogenic factors were transferred to the F$^-$ cells by the same method, employing Hfr colicinogenic strains as donors instead of the F$^+$ cells to prevent transmission of the F factor.

Table 1. *Bacterial strains*

Name	Characteristics	Origin	References
E. coli			
CA 53	Prototrophic *str-s col I*$^+$ F$^+$	*	—
E. coli K 12			
12–94	*met*$^-$ *str-r col V*$^+$ F$^+$	†	—
12–317	*cys*$^-$ *his*$^-$ *str-r col* E_2^+ F$^+$	†	—
58–161	*met*$^-$ *str-r* F$^-$	—	Tatum & Lederberg (1947)
Hfr H	*thi*$^-$ *str-s*	—	Hayes (1953)
Hfr type 4	*thr*$^-$ *leu*$^-$ *thi*$^-$ *str-s*	C 600	Jacob & Wollman (1957)
Hfr type 2	*met*$^-$ *str-s*	58–161	Jacob & Wollman (1957)
Hfr AT IIIA	Prototrophic *str-s*	AB311	Taylor & Adelberg (1960)
112	*cys*$^-$ *his*$^-$ *str-s* F$^+$	—	Wollman (1953), Wollman & Jacob (1959)
PA 309	*thr*$^-$ *leu*$^-$ *thi*$^-$ *his*$^-$ *try*$^-$ *arg*$^-$ *str-r* F$^-$	P 678 *str-r*	Jacob & Wollman (1958*a*)

* This strain was sent by Professor P. Fredericq.

† These strains were received from Cold Spr. Harbor and belonged to Professor P. Fredericq.

Abbreviations: Requirement for *thr*: Threonine; *leu*: Leucine; *try*: Tryptophan; *his*: Histidine; *arg*: Arginine; *met*: Methionine; *cys*: Cysteine; *pro*: Proline; *thi*: Vitamin B_1; *str-r*: Resistance to streptomycin; *str-s*: Senstivity to streptomycin; *lac*: Fermentation of lactose.

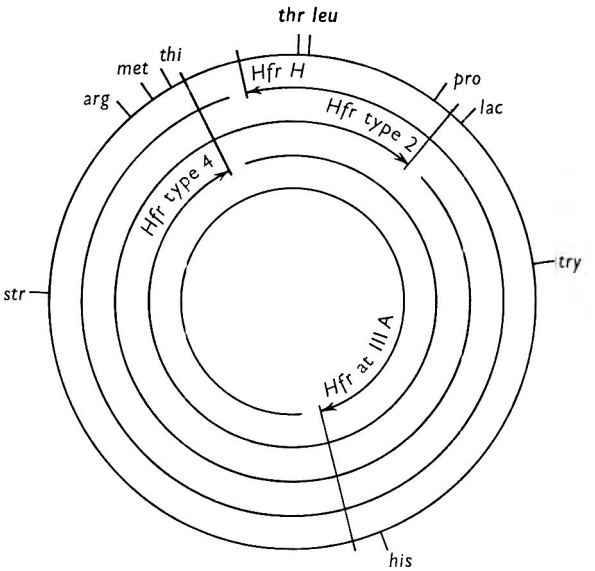

Fig. 1. Genetic map of *E. coli* K 12. Symbols are defined in Table 1. The genetic markers employed are indicated on the outer circle (Wollman & Jacob, 1959; Taylor & Adelberg, 1960). The origin and direction of each Hfr is indicated on the inner ones.

Matings were performed by mixing exponentially growing cultures of donor (Hfr or F$^+$) and recipient (F$^-$) cells in titre of about 2×10^8 bacteria per ml., in proportion of 1 donor:20 recipient cells or 20 donors:1 recipient cell. The mixture, of a total volume of 4 ml., was incubated at 37° in 200 ml. Erlenmeyer flasks. Samples were withdrawn at different intervals, diluted and plated on selective media.

For interruption of mating, samples were treated in Waring Blendor (Wollman & Jacob, 1955) during 1 min.

Recombinants, such as *thr*$^+$ *leu*$^+$ *str-r*, *try*$^+$ *str-r*, *his*$^+$ *str-r*, etc., receiving the ability to synthesize an amino acid or vitamin from the donor and the *str-r* character from the recipient, were selected on synthetic medium supplemented with streptomycin and the corresponding amino acids.

In Hfr *col*$^+$ × F$^-$ *col*$^-$ and F$^+$ *col*$^+$ × F$^-$ *col*$^-$ crosses, the transfer of the *col*$^+$ character was studied by plating a suitable dilution of the mating mixture on agar plates with streptomycin where only F$^-$ cells form colonies; the *col*$^+$ character of these colonies was determined by the halos formed on a sensitive indicator strain.

Unless otherwise stated, the frequency of recombinants was measured at 120 min. after mixing the strains, and is defined as the ratio of number of recombinants to number of the minority parental strain, multiplied by 100.

The transfer of *col*$^+$ was measured, like the frequency of recombinants, by the ratios the number of *col*$^+$ F$^-$ *str-r* cells that received the *col*$^+$ character from the donor parent to the number of the minority parental strain, multiplied by 100.

In every crossing experiment the frequency of transmission to recombinants of an early Hfr marker was measured, as a control of the efficiency of that Hfr as a donor.

Crosses of *col*$^+$ × *col*$^-$ and *col*$^-$ × *col*$^-$ were performed as controls of the corresponding *col*$^+$ × *col*$^-$ and *col*$^-$ × *col*$^-$ crosses.

RESULTS

Matings were generally performed in proportion of 1 Hfr:20 F$^-$ bacteria, except that the proportion of 20 Hfr:1 F$^-$ was specially employed in *col*$^-$ × *col*$^+$ crosses in order to verify if the 'lethal zygosis' observed with *col* E_1 (Alfoldi *et al.* 1958) occurred with *col* E_2, *col I* and *col V*.

In the majority of the experiments where the Hfr was in gross excess over the F$^-$ cells (20 Hfr:1F$^-$) the frequency of recombinants was 1·5 to 5 times less than that obtained in the same crosses in proportion of 1 Hfr:20 F$^-$, but, no decrease in number of the F$^-$ cells was observed, except with Hfr H.

As can be seen from Table 2, in crosses performed with Hfr H as a donor in proportion of 20 Hfr:1 F$^-$, the number of the F$^-$ (str-r cells) showed a decrease ranging from 0 to 92 % referred to the viable count at the time of mixing.

This percentage is not constant in different experiments involving the same strains and sometimes no decrease in number is observed.

The possibility that this decrease in viable count of the F$^-$ cell could be due to some type of agglutination in the presence of a large number of Hfr H cells was tested by treating the mating mixture in a Waring Blendor, before plating. No differences were observed between the treated samples and the control ones.

Although the reasons for this decrease in number of the F$^-$ cells in these crosses as well as of the variability of the decrease, remain unknown, it must be emphasized that it was not found to have any relation to the transmission of *col*$^+$ or *col*$^-$, since it occurs also in control crosses *col*$^+$ × *col*$^+$ and *col*$^-$ × *col*$^-$ (Table 2).

No decrease in fertility due to colicinogenic factors was observed in crosses involving *col* E_2, *col I* and *col V* (Table 3), unlike the results of Fredericq & Betz-

Table 2. *Decrease in number of the F⁻ bacteria measured at* 120 *minutes after mixing, in crosses involving HfrH or strains derived from it, as donors*

Crosses	Frequency (in %) *thr⁺ leu⁺ str-r* recombinants	Decrease (in %) of F⁻ bacteria
Hfr H × PA 309 *str-r*	0·7	92
	5	0
Hfr H *col* I^+ × PA 309 *str-r col* I^-	0·6	79
	3·2	21
	3·4	0
Hfr H *col* I^+ × PA 309 *str-r col* I^+	1·1	79
	6	50
Hfr H *col* I^- × PA 309 *str-r col* I^-	10	42
Hfr H *col* I^- × PA 309 *str-r col* I^+	10	50
	7·2	0
Hfr H *col* E_2^+ × PA 309 *str-r col* E_2^-	6·2	0
	6	50
	2·6	65
Hfr H *col* E_2^+ × PA 309 *str-r col* E_2^+	6·3	40
	1·6	0
Hfr H *col* E_2^- × PA 309 *str-r col* E_2^+	0·8	89
	5	0
Hfr H *col* V^+ × PA 309 *str-r col* V^-	31	0

Crosses were performed with a ratio of 20 Hfr:1 F⁻. All strains were previously made resistant to the colicine involved in each case. The decrease of the F⁻ bacteria was calculated from the comparison of the viable count of F⁻ cells at 120 minutes after mixing with the count at the initial time.

Table 3. *Comparison of fertility in crosses involving colicinogenic factors, with control crosses*

Crosses	Frequency (in %) of recombinants for an early Hfr marker (*thr⁺ leu⁺ str-r*)
Hfr type 2 *col* E_2^+ × F⁻ *col* E_2^-	25
Hfr type 2 *col* E_2^+ × F⁻ *col* E_2^+	30 (control)
Hfr type 2 *col* E_2^- × F⁻ *col* E_2^+	19
Hfr type 2 *col* E_2^- × F⁻ *col* E_2^-	21 (control)
Hfr H *col* I^+ × F⁻ *col* I^-	32
Hfr H *col* I^+ × F⁻ *col* I^+	25 (control)
Hfr H *col* I^- × F⁻ *col* I^+	32
Hfr H *col* I^- × F⁻ *col* I^-	22 (control)
Hfr H *col* V^+ × F⁻ *col* V^-	40
Hfr H *col* V^+ × F⁻ *col* V^+	33 (control)
Hfr H *col* V^- × F⁻ *col* V^+	33
Hfr H *col* V^- × F⁻ *col* V^-	35 (control)

All crosses indicated were performed with a ratio of 1 Hfr:20 F⁻.
Strains were previously made resistant to the colicine employed in each cross.

Bareau (1956) with *col* E_1, *B*, *K* and *S*3. It cannot yet be asserted whether these differences reflect or not true differences in the behaviour of colicinogenic factors, since they could also be assigned to differences in the methods or strains employed.

Colicinogenic factor E_2

This factor is transferred by Hfr and F^- strains with frequencies of about 1 % (Table 4). Crosses were made in proportion 20:1 and 1:20 donor to recipient cells. No lethal zygosis was observed in crosses involving *col* E_2, regardless of which parent carried it. But in crosses with Hfr H performed with a ratio of 20 Hfr:1 F^-, a decrease in number of the F^- (*str-r*) bacteria took place, but, as was explained before, without any relation to *col* E_2 (Table 2).

Table 4. *Crosses between Hfr* col E_2^+ *or* F^+ col E_2^+ *and PA* 309 *str-r* col E_2^-

	Hfr H		Hfr type 2		Hfr type 4		112 (F+)
	20:1	1:20	20:1	1:20	20:1	1:20	20:1
Frequency (in %) of transfer of *col*$^+$	0·3	0·3	0·6	1·3	0·5	3	1·3
Frequency (in %) of recombinants for an early Hfr marker	3·6	44	4·2	18	10	40	—

20:1 indicates crosses performed with a ratio of 20 donors:1 recipient cell. 1:20 indicates crosses performed with a ratio of 1 donor:20 recipient cells. These strains were previously made resistant to colicine E_2.

Early selected markers from Hfr were, *thr*$^+$ *leu*$^+$ for Hfr H and Hfr type 2 and *arg*$^+$ for Hfr type 4. Selected marker from F^-, *str-r*.

Table 5. *Percentage of* col E_2^+ *among recombinants of crosses between Hfr* col E_2^+ *and PA* 309 str-r col E_2^-

			Recombinants			
			thr$^+$ *leu*$^+$ *str-r*	*try*$^+$ *str-r*	*his*$^+$ *str-r*	*arg*$^+$ *str-r*
Donors	Hfr H	20:1	0	0	2	7
		1:20	0	0	0	2
	Hfr type 2	20:1	0·7	1·5	1·2	1·3
		1:20	0	3	1	2
	Hfr type 4	20:1	—	0	0	0·4
		1:20	—	1·5	0	0·3

100 to 400 colonies of each type of recombinants from crosses performed in proportion 20:1 and 1:20 Hfr to F^- cells, were analysed. The order of transfer of the Hfr markers is: Hfr H, *thr leu try his arg*; Hfr type 2, *leu thr arg his try*; Hfr type 4, *arg his try*.

Selected early markers from Hfr were, *thr*$^+$ *leu*$^+$ for Hfr H and Hfr type 2; *arg*$^+$ for Hfr type 4. Selected marker from F^- = *str-r*.

The percentage of col E_2^+ among F^- (*str-r*) cells at 120 min. after mating in crosses performed with a ratio of 1 Hfr:20 F^- cells was less than 0·1 %, with the different donor strains. The percentage of col E_2 among F^- cells, in matings performed in proportion of 20 Hfr:1 F^- cell was: 0·3 % with Hfr H and Hfr type 2 and 0·4 % with type 4 and 112 (F^+).

Col$^+ \times$ *col*$^-$ crosses performed with Hfr strains of different type or with F^+ as donor cells and the frequency of *col*$^+$ *str-r* in each cross is registered in Table 4.

Great differences in frequencies of transfer of *col*$^+$ from Hfr and F^+ cells are not apparent since they vary only within 0·3 and 1·3 % in crosses performed with a ratio of 20 donor:1 recipient cell.

The percentage of *col*$^+$ colonies among recombinants selected from 1:20 crosses (Table 5) is also low and reveals no linkage with chromosomal markers. Similar results were found in crosses 20:1. A direct correlation between the frequency of *col*$^+$ in a recombinant for a given marker and the distance of this marker to the origin seems to exist, at least for Hfr H, since the percentage of *col*$^+$ in a recombinant for a distal marker is greater than that corresponding to a recombinant for a nearer one. In addition the % of *str-r* F$^-$ cells found to be *col*$^+$ at 120 min. is given at the foot of Tables 5, 6 and 8.

Colicinogenic factor I

Colicinogenic factor I is transferred at even lower frequencies than colicinogenic factor E_2. To measure such low frequencies a large number of zygotes had to be analysed and crosses in proportion 20 donor cells:1 acceptor cell were considered most convenient for this purpose.

The crosses performed and the frequency of transfer of *col I*, are shown in Table 6.

Table 6. *Crosses between Hfr* col I$^+$ or F$^+$ col I$^+$ *and PA* 309 *str-r* col *I*$^-$

	Donors		
	Hfr H	Hfr type 4	CA 53 (F$^+$)
Frequency (in %) of transfer of *col*$^+$	< 0·1	0·1	0·5
Frequency (in %) of recombinants for early Hfr marker	4	15	—

These crosses were performed with a ratio of 20 donor:1 recipient cell. These strains were previously made resistant to colicine I. Early selected markers from Hfr were, *thr*$^+$ *leu*$^+$ for Hfr H and *arg*$^+$ for Hfr type 4. Selected marker from F$^-$, *str-r*.

The percentage of col I$^+$ among F$^-$ (*str-r*) cells at 120 min. after mating, in crosses performed with a ratio of 20 Hfr:1 F$^-$ cell was 0·2 % with Hfr H and Hfr type 4 and 0·3 % with CA 53 (F$^+$).

In crosses with Hfr H, a decrease in number of the F$^-$ cells occurred both in *col*$^+$ × *col*$^-$ and *col*$^-$ × *col*$^+$ crosses as well as in *col*$^+$ × *col*$^+$ and *col*$^-$ × *col*$^-$ ones (Table 2).

The low frequency of transmission of the *col*$^+$ character in a cross such as Hfr H *col I*$^+$ × F$^-$ *col I*$^-$ could have been assumed to be due to the localization of *col*$^+$ at the distal end of the Hfr chromosome. But, the fact that such low frequency is also observed with Hfr type 4, where a distal marker of Hfr H is proximal, shows that this is not the case here.

Colicinogenic factor V

The transfer of *col*$^+$ in Hfr *col V*$^+$ × *F*$^-$ *col V*$^-$ or F$^-$ *col V*$^+$ × F$^-$ *col V*$^-$ crosses performed with a ratio of 1 donor:20 recipient cells and measured at 120 min. after mixing the strains, is reported in Table 7.

In contrast to *col* E_2 and *col I*, colicinogenic factor V is transferred with *very high efficiency*, since its frequency greatly exceeds 100 % (of input of minority parent, in this case the Hfr) at the time considered (Table 7).

The time at which the *col*$^+$ factor begins to appear in the F$^-$ cell was determined in conjugations interrupted by Waring Blendor. Similar times of penetration of this factor were found employing different donor parents (Table 7).

The kinetics of transfer of an early Hfr marker and of the *col*$^+$ character of the Hfr was studied in crosses where mating was interrupted. When the number of

col$^+$ *str-r* cells (F$^-$ cells that became *col*$^+$) and *thr*$^+$ *leu*$^+$ *str-r* recombinants is plotted against time in Hfr type 2 *col* $V^+ \times$ F$^-$ *col* V^- cross, performed with ratio 1 donor:20 recipient cells (Fig. 2), it is observed that: (1) The slope of *col*$^+$ *str-r* cells rises rapidly and linearly, soon after mating, similarly to *thr*$^+$ *leu*$^+$ *str-r* recombinants, till about 50 min. after mating, when *col*$^+$ *str-r* cells reach a frequency of 100 % (of input of minority parent). (2) Then, they continue increasing at the same rate as the F$^-$ cells of the population, thus attaining the frequency of 480 % measured at 120 min. (Table 7).

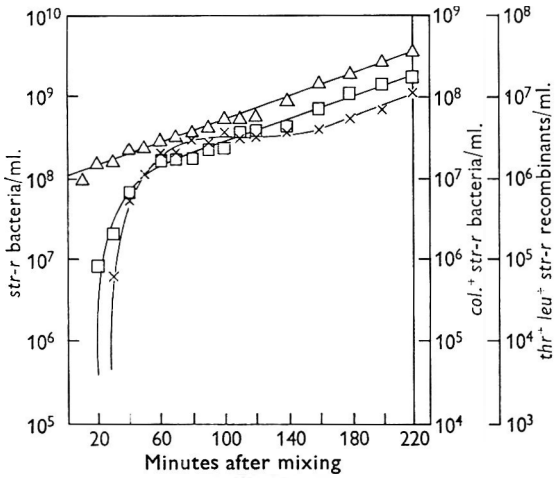

Fig. 2

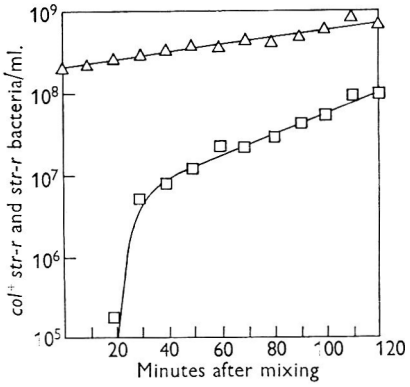

Fig. 3

Fig. 2. The kinetics of transfer of *col*$^+$ and *thr*$^+$ *leu*$^+$ in Hfr type 2 *col* $V^+ \times$ PA 309 *str-r col* V^- cross, performed with a proportion of 1 donor:20 recipient cells. At different time intervals samples were taken, treated in the Waring Blendor, diluted and plated on selective media. To maintain the mixture in logarithmic phase, an aliquot was diluted 1/10 in nutrient broth at 100 min. after mixing and samples were withdrawn from it after 120 min. ×, *thr*$^+$ *leu*$^+$ *str-r* recombinants; △, *str-r* bacteria; □, *col*$^+$ *str-r* bacteria.

Fig. 3. The kinetics of transfer of *col*$^+$ in 112 (F+) *col* $V^+ \times$ PA 309 *str-r col* V^- cross, performed with a proportion of 1 donor:20 recipient cells. Samples taken at different times were treated in a Waring Blendor, diluted and plated on nutrient agar plates with streptomycin. □, *col*$^+$ *str-r* bacteria; △, *str-r* bacteria.

Table 7. *Crosses between Hfr* col V$^+$ *or F*$^+$ col V$^+$ *and PA* 309 str-r col V$^-$

	Donors			
	Hfr H	Hfr type 2	Hfr AT IIIA	112 (F$^+$)
Frequency (in %) of transfer of *col*$^+$	850	480	2100	1100
Frequency (in %) of a recombinant for an early Hfr marker	24	25	41	—
Time (in minutes after mixing) or penetration of *col*$^+$ in the recipient cell	6	13	5–10	8

These crosses were performed with a ratio of 1 donor:20 recipient cells.
The strains were previously made resistant to colicine V.
The time at which the *col*$^+$ character first appears in the F$^-$ cell (time of penetration) was determined in conjugations interrupted by Waring Blendor.

On the contrary, *thr*$^+$ *leu*$^+$ *str-r* recombinants after their initial rise reach a plateau, between 60 and 120 min., and only then begin to multiply at the same rate as the other cells.

In the other crosses reported in Table 7, *col*$^+$ *str-r* cells also reach a frequency of 100 % about 50 min. after mating, that is at the time when the maximum frequency of conjugation for chromosomal recombinants is achieved.

In the 112 F$^+$ *col* $V^+ \times$ F$^-$ *col* V^- cross (Fig. 3) the slope of *col*$^+$ *str-r* bacteria during the first 50 min. is the same as that of this type of bacteria in the Hfr type 2 *col* $V^+ \times$ F$^-$ *col* V^- cross. Later, the slope decreases, although remaining steeper than that corresponding to the division of *str-r* cells. This suggests that the increase of *col*$^+$ *str-r* cells between 60 and 120 min. is due to some additional reason besides that corresponding to cell division.

The analysis of the percentage of *col*$^+$ in recombinants selected in Hfr *col* $V^+ \times$ F$^-$ *col* V^- crosses (Table 8) indicates that: (1) there is no linkage with chromosomal markers; (2) there is a slight increase of the percentage of *col*$^+$ in recombinants for distal markers of the Hfr cells over those for proximal ones.

Table 8. *Percentage of* col V$^+$ *among recombinants of crosses between Hfr* col V$^+$ *and PA* 309 str-r col V$^-$

Donors	Recombinants			
	thr$^+$ *leu*$^+$ *str-r*	*try*$^+$ *str-r*	*his*$^+$ *str-r*	*arg*$^+$ *str-r*
Hfr H	79	91	100	100
Hfr type 2	80	100	100	84
Hfr AT IIIA	100	98	92	100

Crosses indicated were performed with a proportion of 1 donor:20 recipient cells.
100 colonies of each type of recombinants were analysed.
The order of transfer of the Hfr markers is: Hfr H, *thr leu try his arg*; Hfr type 2, *leu thr arg his try*; Hfr AT IIIA, *his try leu thr arg*. (The percentage of *col* V^+ among F$^-$ (*str-r*) cells at 120 min. after mating, in crosses performed with a ratio of 1 Hfr:20 F$^-$ cells was: 6·6 % with Hfr H; 5·2 % with Hfr type 2; 18 % with Hfr AT-IIIA and 12·5 % with 112 (F$^+$).)

All recombinants selected from reciprocal crosses Hfr *col* $V^- \times$ F$^-$ *col* V^+ are *col*$^+$. The frequency of recombinants in these crosses is the same as that in control crosses *col*$^-$ $\times$ *col*$^-$.

DISCUSSION AND CONCLUSIONS

From the analysis of conjugation experiments involving colicinogenic factors E_2, I and V, it follows that:

(1) Each colicinogenic factor is transferred at about the same frequencies from F$^+$ and from Hfr bacteria injecting their chromosomes in various orders. In agreement with this result, similar times of penetration from these different donors were determined for *col V*.

The percentage of *col*$^+$ among recombinants from crosses Hfr *col*$^+ \times$ F$^-$ *col*$^-$ indicates that *col*$^+$ is not linked with chromosomal markers. Moreover, with *col* E_2 and *col V* the transfer of *col*$^+$ seems to be proportional to the duration of conjugation,

since recombinants for distal markers present a higher percentage of *col*$^+$ than those for proximal ones.

From all these results it may be concluded that these colicinogenic factors are not localized on the chromosome in donor cells.

(2) No recombinants have been observed which have received the *col*$^-$ character of the Hfr parent. With the colicinogenic factors studied no lethal zygosis was detected in Hfr or F$^+$ *col*$^-$ × F$^-$ *col*$^+$ crosses, unlike the findings of Alfoldi *et al.* (1958) with *col* E_1; nor was a decrease in fertility due to colicinogenic factors observed.

Therefore, there are no reasons to consider the non-appearance of *col*$^-$ in recombinants as the consequence of some type of lethal zygosis masking its transfer.

(3) The kinetics of transfer of *col V*$^+$ shows that it is transferred in Hfr *col*$^+$ × F$^-$ *col*$^-$ crosses with an efficiency of 100 % of input of the minority parent within the first 50 min. after mixing (Fig. 2). Later, the number of *col*$^+$ *str-r* colonies corresponding to zygotes that have received the *col*$^+$ character, increases at the same rate as the F$^-$ cells of the population. This means that, once transferred to zygotes with maximum efficiency, *col V*$^+$ is capable of multiplying immediately and at a rate that assures its passage to all daughter cells.

The behaviour of *col*$^+$ *str-r* colonies is in striking contrast to that of chromosomal recombinants, such as *thr*$^+$ *leu*$^+$ *str-r*. When chromosomal markers are transferred they must integrate in the genome of the recipient cell in order to reduplicate; the probability of integration being less than $\frac{1}{2}$, the frequency of transmission to recombinants becomes much less than the frequency of transfer to zygotes (Wollman, Jacob & Hayes, 1956). Furthermore, chromosomal recombinants begin to multiply only after integration has been accomplished (Fig. 2).

These results suggest that *col V* divides autonomously in the recipient F$^-$ cell, without requiring integration in the recipient genome, and at a faster rate than the chromosome because more than one copy of *col*$^+$ per cell must be present in order to assure its transmission to all descendant cells.

(4) Notwithstanding that the initial rise in number of *col*$^+$ *str-r* colonies is similar in Hfr type 2 *col V*$^+$ × F$^-$ *col V*$^-$ (Fig. 2) and F$^+$ *col V*$^+$ × F$^-$ *col V*$^-$ cross (Fig. 3) the analysis of their slopes between 60 and 120 min. after mixing reveals some differences. Thus, *col*$^+$ *str-r* colonies in the cross involving the F$^+$, unlike the Hfr type 2 *col V*$^+$ × F$^-$ *col V*$^-$ cross, increase more rapidly than the F$^-$ cells of the population, which suggests that some other factor intervenes, in addition to that corresponding to cell division. As one possible explanation of this added increase it can be assumed that new *col*$^+$ *str-r* cells continue forming in F$^+$ × F$^-$ crosses as the consequence of the transfer of *col*$^+$ from F$^-$ cells that became donors of *col*$^+$ from simultaneous reception of F$^+$ and *col*$^+$ during the first minutes of conjugation. Another assumption would be that a fraction of the original F$^+$ cells are able to reconjugate and so transfer more *col* particles.

Summarizing, the ensemble of results shows that *col* E_2, *col I* and *col V* are in an autonomous state in Hfr and F$^+$ cells. Furthermore, analysis of transfer of *col V* suggests that this same interpretation would be valid also for F$^-$ cells.

Quite striking are the differences in frequency of transfer between colicinogenic factor V, on one hand, which is transferred with an efficiency of 100 % or even higher and colicinogenic factors E_2 and I, on the other, since they are only poorly transferred (1 and 0·1 %) respectively. Nothing is known about the nature of the

forces governing the transfer of genetic material during conjugation; nevertheless, various hypotheses accounting for the differences of transfer of these colicinogenic particles can be considered, such as: (1) different number of particles per cell, (2) different size of the particles, (3) special localization favouring their passage through the conjugation bridge, and (4) capacity of becoming temporarily fixed at any point of the chromosome. The last point seems least probable in view of the fact that the same frequency of transfer was found with F^+ cells which transfer the chromosome at low frequency and with Hfr cells which transfer it at a high one.

All these considerations are concerned with factors that would affect the transfer of these colicinogenic particles; factors affecting the establishment of *col* E_2 and *col I* in a fraction of the recipient population might also be considered.

These colicinogenic factors, like episomes, are not essential constituents of cells and they may be either present or absent from the cell (Jacob & Wollman, 1958*b*). Even though the possibility of their fixation on the chromosome cannot yet be excluded, the evidence obtained so far with these studies indicates their extra-chromosomal state and leads one to consider them as plasmids (Lederberg, 1952; Jacob, Schaffer & Wollman, 1960).

These factors would be among the few recorded in *Escherichia coli* for which chromosomal localization is lacking. Another published example is the transducing phage 363, which is not transferred in matings involving different types of Hfr (Jacob & Wollman, 1959).

Finally, from the studies with colicinogenic factors it would seem that differences in the state of different factors exist, since *col* E_1 behaves as an episome while *col* E_2, *I* and *V* would lack the integrated state, at least for conditions under which *col* E_1 apparently fixes on the chromosome.

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The Summer Air-Spora of Two Contrasting Adjacent Rural Sites

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SUMMARY

The air in a valley near a stream and in an exposed site on a small hill at Silwood Park, near Ascot, Berkshire, was sampled from 14 May to 25 September 1958 at half a metre above the ground by a Hirst automatic volumetric spore trap. Seasonal periodicities of 26 categories of fungus spores and 7 categories of pollen grains are given as 6-day running means of the daily average number of spores per cubic metre of air. Estimated hourly concentrations of spores for 6 consecutive days are given for three fungus spore types and two pollens. The diurnal periodicity is given for these groups. There were 2·6 times more spores at site S near the stream than at the exposed site M. There were 4·9 times more spores of ascomycetes at S than at M, 3 times as many spores of basidiomycetes but only 1·4 times as many from fungi imperfecti. The proportion of the different types of spores at the two sites varied; 14% of spores at S were from ascomycetes, 7% at M; 17·5% of those at S were from fungi imperfecti and 32% of those at M. Tree pollen grains were equal in number at both sites but there was 2·8 times more grass pollen and 6·5 times more weed pollen at S than at M. Urtica pollen was 8·1 times as common at S and made up 55% of the total pollen in that area, but only 24% of the total at M, mainly because of the local abundance of nettle plants. The results suggest that the ecology of an area has a major influence on its air-spora through local flora and microclimate.

INTRODUCTION

The Hirst automatic volumetric spore trap (Hirst, 1952) has been used to study the seasonal and diurnal periodicities of components of the air-spora in several localities. Hirst (1952) and Gregory & Hirst (1957) studied the summer air-spora at 2 and 24 m. above ground at Rothamsted Experimental Station in an area typical of mixed farming in the south of England. The air-spora at Rothamsted was also studied by Sreeramulu (1959) who discussed the seasonal and diurnal periodicity of spores of some plant pathogens, and by Hamilton (1959) who compared it with the air-spora of an urban area in London. A contrasting estuarine area was studied by Gregory & Sreeramulu (1958). These workers all concluded that the air-spora of any locality may come from local sources, but two sites of differing ecological type situated near together have never been compared. In connexion with studies on how environment affects asthma and hay fever (Maunsell, 1958), Dr K. Maunsell suggested that two traps should be run simultaneously, one near a stream and one in an exposed area. The Imperial College Field Station, Silwood Park, Sunninghill,

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near Ascot, Berkshire, provided suitable sites to see whether the air near a stream or river contained spores or pollen differing from that in the drier areas studied hitherto.

METHODS

Two Hirst spore traps were operated continuously at Silwood Park from 14 May to 25 September 1958. One trap (S in Fig. 1) was about 4 ft. from a small stream and 10 ft. from a garden wall running parallel with the stream. The area was in a valley sheltered by the 10 ft. wall to the east and trees and shrubs including elder and

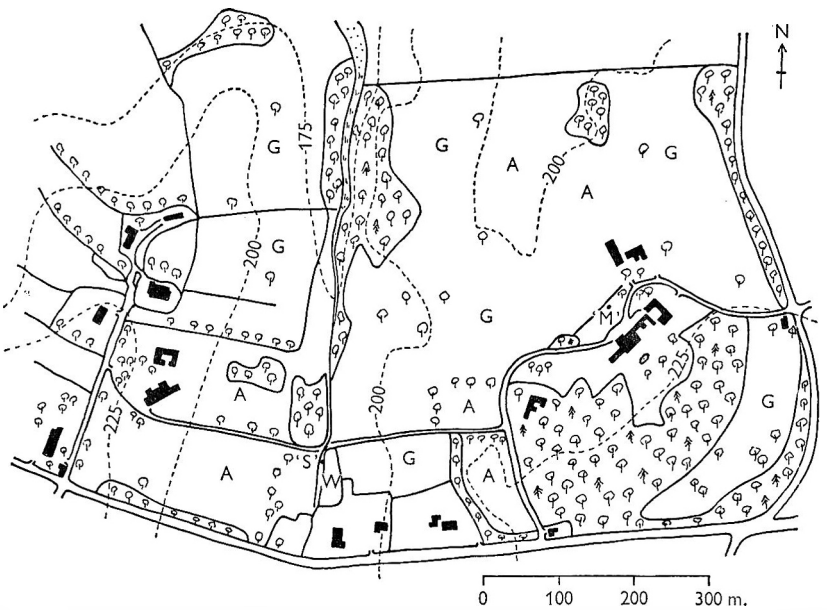

Fig. 1. Map showing position of traps M and S in 1958 at Imperial College Field Station, Silwood Park, Sunninghill, near Ascot, Berkshire. S = trap at stream; M = trap in meteorological enclosure; W = walled garden; A = arable; G = grass. Based on the Ordnance Survey Map with the sanction of the controller of H.M. Stationery Office.

willow on the other three sides; in the nearby wooded area elm and oak predominated; the ground vegetation consisted mainly of grasses, nettles and other weeds. The second trap, M, was in the meteorological enclosure on a closely cut lawn 450 m. E.N.E. of site S, and 12 m. higher on a flat area on a small hill. This site was relatively open and well exposed to winds from the west and north-west; with buildings and ornamental trees on the other sides, the valley to the north-west was grass and arable.

The orifice of each trap was 0·5 m. above ground level. The slides were changed daily at approximately 09.00 hr. G.M.T.; trap M was always changed first. Air was sampled at the rate of 10 l./min. The slides were coated with petroleum jelly with 12·5% paraffin wax as described by Hirst (1953). After exposure they were at once mounted in glycerol jelly under a coverglass, and stored until they were scanned two years later.

Daily mean concentrations of spores and pollen grains/m.^{3} were estimated from counts made on 'long traverses' (Hirst, 1953, p. 378) under a 3·75 mm. oil-immersion

objective and are given uncorrected for variations in trapping efficiency depending on wind speed or particle size. Traverses were 30μ wide for most kinds of fungus spores but 60μ wide for large spores, algal clumps, fern spores and pollen grains. Hourly concentrations were estimated from 'short traverses' on selected days only (see pp. 493 and 496) to study diurnal periodicity and correlation with weather. Many kinds of spores counted in this series are illustrated in Gregory (1961) and of pollen in Hyde & Adams (1958).

Weather. Meteorological records were taken in the enclosure containing trap M. The average daily relative humidity was judged by eye from 24 hr. charts. The summer of 1958 was cool and damp, with July the warmest, sunniest and driest month. June had most rain but the relative humidity remained low at an average of 80%. August and September were wet with average humidity above 85%, as shown in Fig. 2. Separate meteorological data were not obtained from site S, but humidities must have usually been higher.

RESULTS

Fungus spores

Fungus spores were counted in the thirty-two categories listed in Table 1. A few spores were identified as far as species (e.g. *Polythrincium trifolii*) but others were only classified into large, more or less arbitrary, groups. Fig. 2 shows the components of the air-spora as estimated number of spores/m.^{3} of air, and the seasonal trends of weather calculated as 6-day running means but plotted only at 3-day intervals. There were usually many more spores at site S than at M, particularly those that are dispersed in rain or dew, or from species that grow in damp localities, but a few of the dry-spored Fungi Imperfecti and the larger Basidiomycetes were more plentiful in the drier area near trap M.

Seasonal variations in the spore content of the air

With growing knowledge of the air-spora, more spores can be identified than previously. Even though the group described is arbitrary, an attempt is made below to describe those spores included in it. Any spores not classifiable into these categories were included at the end of Table 1 under the heading 'other spores'.

Phycomycetes. Among recognizable Phycomycetes only spores of the Peronospora type were plentiful enough to present separately, although in July there were many of a spore type provisionally attributed to the Entomophthoraceae.

Ascomycetes. The group referred to provisionally as '3-bar ascospores' are very distinctive; they are hyaline and crescent-shaped, with a very thin outer wall contrasting with the three, thick, highly refractile septa (Gregory, 1961, pl. 5, fig. 10). They were 10·5 times more numerous at site S than at M. Nothing is known of their origin, but the fungus is possibly a discomycete most prevalent in damp areas. A large proportion of the ascospores found in the air near a stream in the New Forest (Gregory, 1954; and verbal communication) were spores of this type.

The group termed 'fusiform ascospores' are yellow to brown, septate ascospores mainly belonging to the genus *Leptosphaeria.* Hyaline fusiform spores lacking pedicels or facets of attachment were counted with 'other ascospores', as were spores of Venturia and Chaetomium. The 'Sordaria-type' includes brown unicellular spores such as Xylaria. 'Filiform ascospores' are the long, narrow, generally

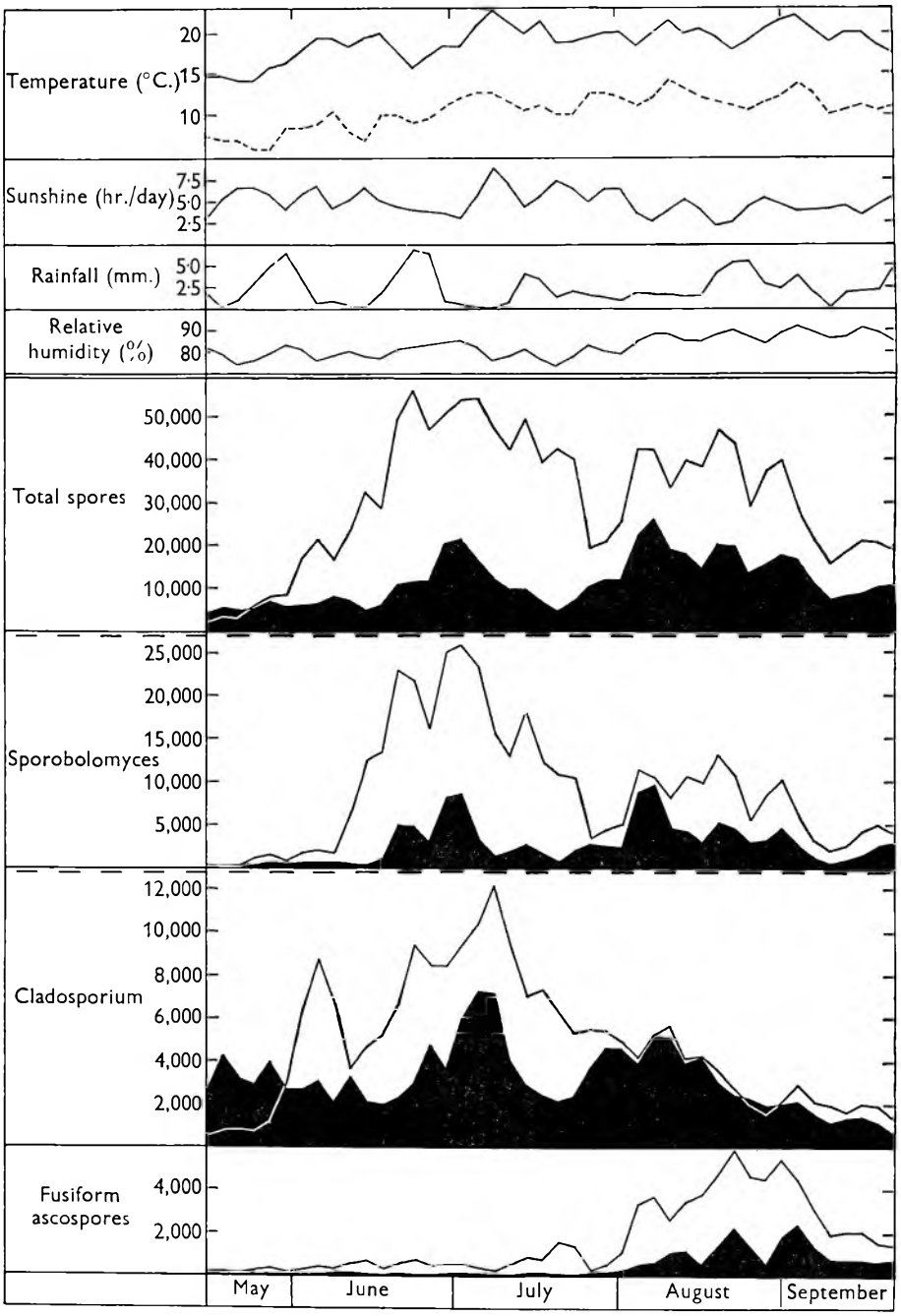

Fig. 2*a*. Weather and seasonal periodicity of fungus spore and pollen grain concentration/m.^{3} air at 0·5 m. above ground level, 14 May to 25 September 1958. Curves are 6-day running means plotted at 3-day intervals. Temperature: solid line—maximum temperature; broken line—minimum temperature. Line—trap S; shaded area—trap M.

septate, hyaline to pale brown spores which are usually released in rain. Ascomycetes as a group grow and release their spores in damp conditions, and it is not surprising that there were 4·9 times more ascospores in the air at site S than at site M: 14% of the spores at site S were classified as ascospores but only 7% at site M.

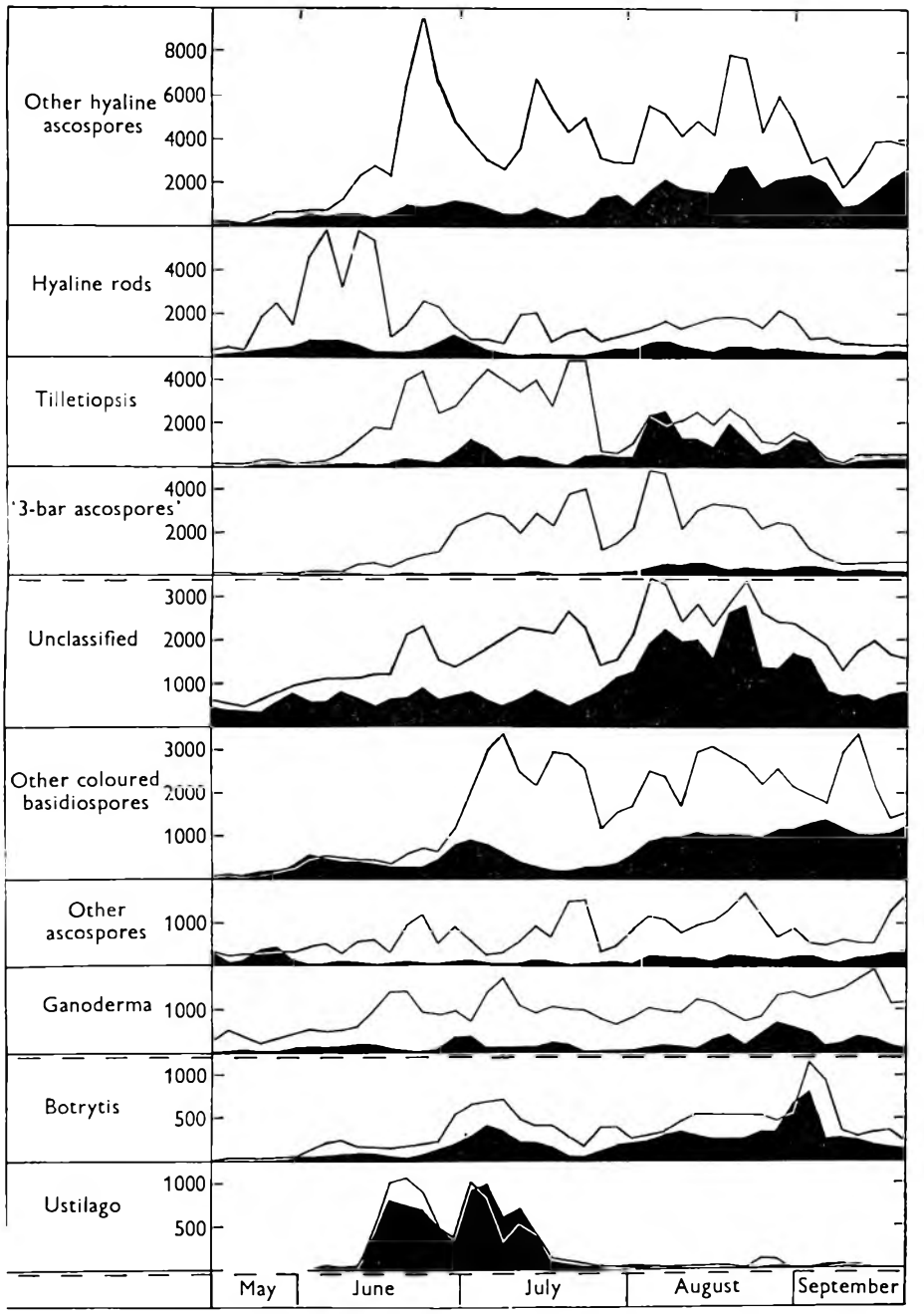

Fig. 2*b*. **For legend see page 488.**

Basidiomycetes. At site S 57 % of the spores, and at site M 49 %, were recognizable as spores of basidiomycetes (ballistospores, uredospores or smut spores). Altogether there were 3 times more basidiospores at S than at M, but more spores of some of the cap fungi occurred at M, e.g. Boletus and *Nolanea staurospora* types. The numbers of smut spores of the *Ustilago avenae* (*perennans*) type were very

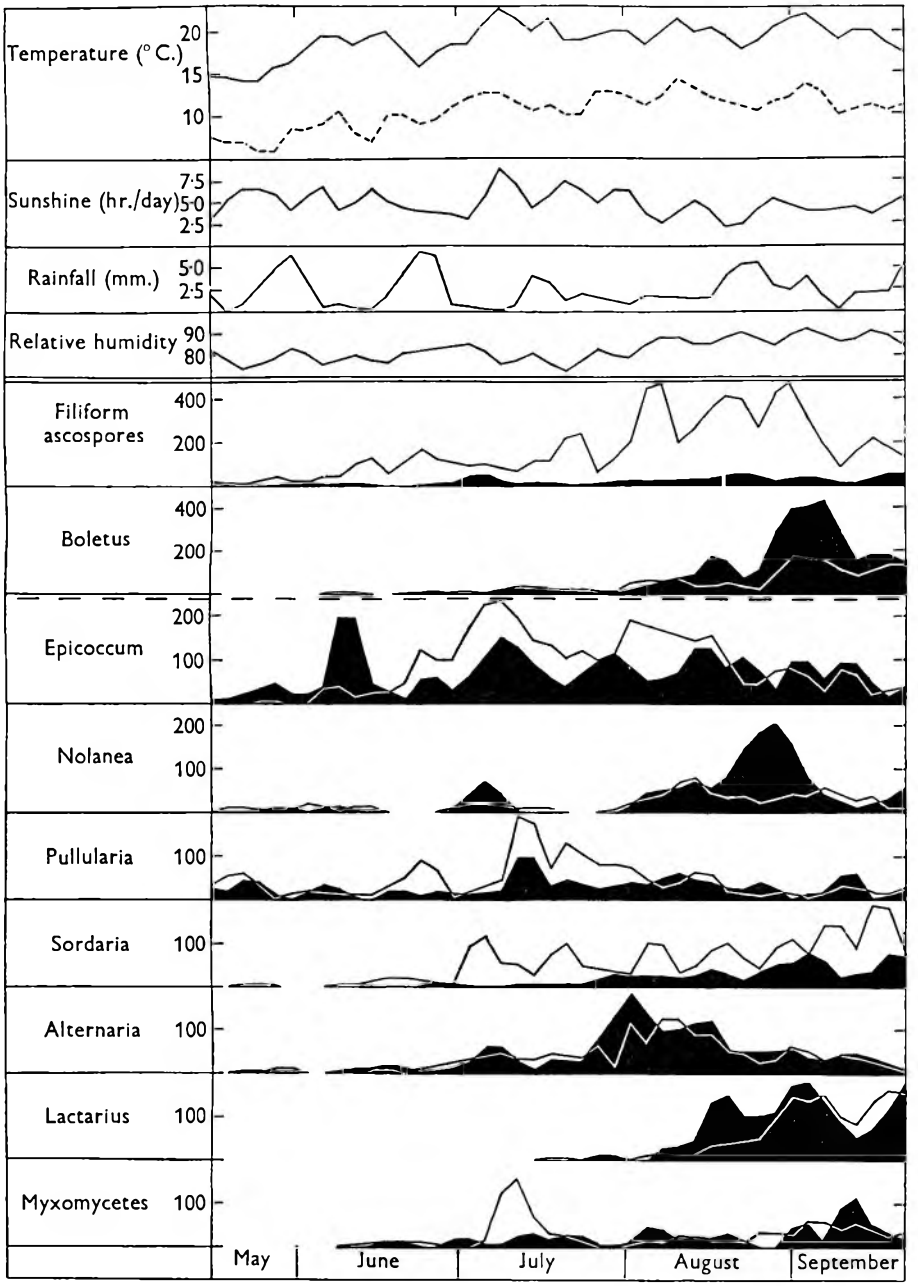

Fig. 2c. For legend see page 488.

similar at both sites during the short season when they occurred; a decrease in numbers in late June coincided with a spell of cool rainy weather. The shadow yeasts, Sporobolomyces and Tilletiopsis, were three times as common at S as at M. The group referred to as 'other hyaline basidiospores' included all the larger hyaline

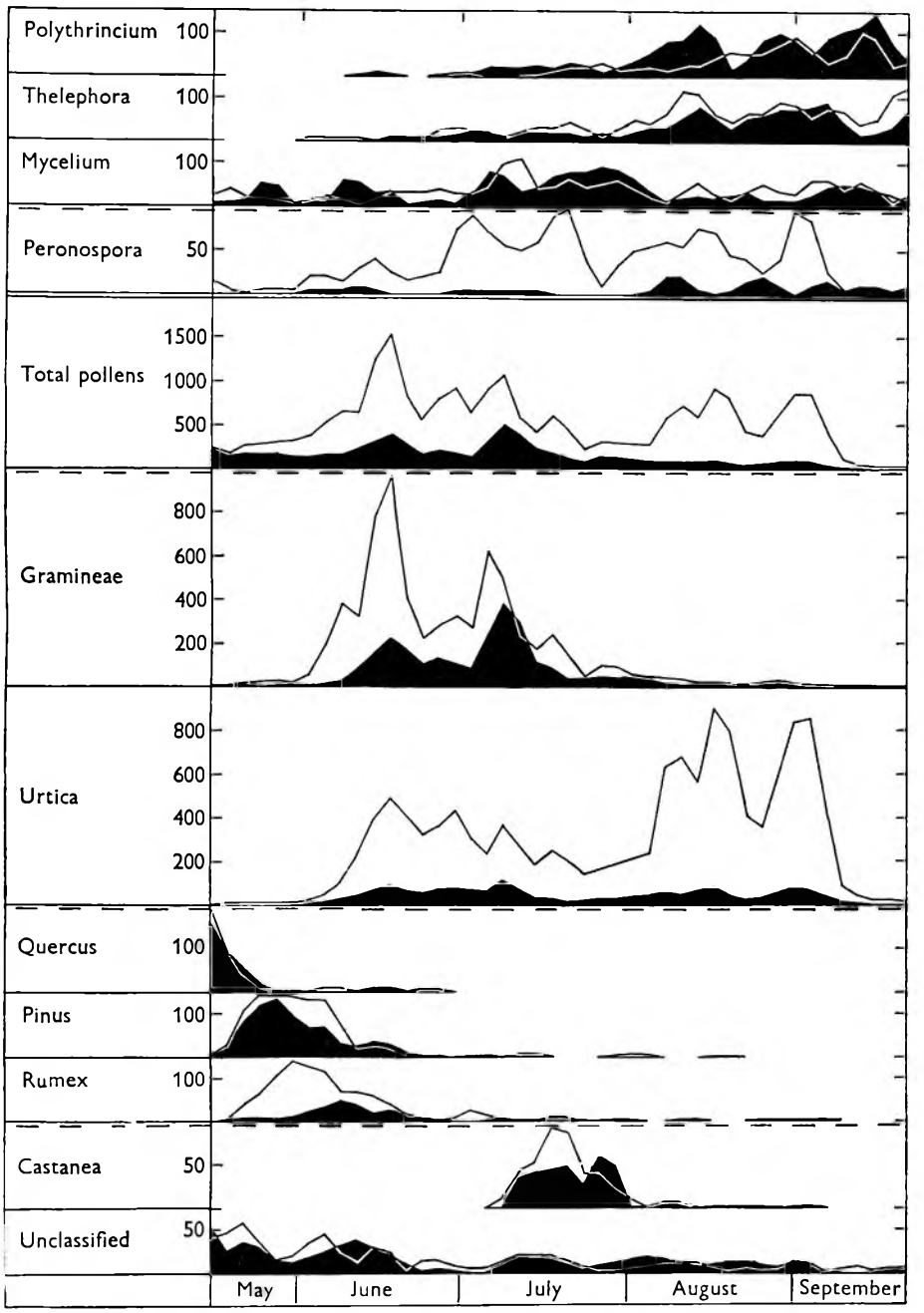

Fig. 2*d*. For legend see page 488.

spores with a typical ballistospore hilum but which lacked the surface markings characteristic of the Lactarius and Russula group. The abundance of Ganoderma at site S was associated with the presence of some large fruit-bodies of *G. applanatum* at about 65 m. west of the trap. The coloured basidiospores counted separately (Psilocybe, Thelephora) are distinctive types. The groups referred to as 'other coloured basidiospores' include a few Coniophora; with more study many further

Table 1. *Contributions of different categories of fungus spores to the air-spora*

	Mean for season no./m.³		Ratio,	Percentage of total caught in area	
	Site S	Site M	S/M	Site S	Site M
Phycomycetes					
Peronospora type	38	5	7·8	0·1	< 0·1
Ascomycetes					
'3-bar ascospores'	1,569	150	10·5	5·3	1·3
Fusiform ascospores	1,614	489	3·3	5·4	4·3
Filiform ascospores	166	20	8·3	0·6	0·2
Sordaria type	52	19	2·7	0·2	0·2
Other ascospores	718	158	4·6	2·4	1·4
	4,119	836	4·9	13·9	7·4
Basidiomycetes					
Sporobolomyces	8,801	2,666	3·3	29·6	23·6
Tilletiopsis	1,645	541	3·0	5·5	4·8
Lactarius/Russula type	32	41	0·8	0·1	0·4
Other hyaline basidiospores	3,599	1,149	3·1	12·1	10·2
Ganoderma	960	172	5·6	3·2	1·5
Boletus	42	76	0·6	0·1	0·7
Nolanea type	20	35	0·6	< 0·1	0·3
Psilocybe type	11	4	2·7	< 0·1	< 0·1
Thelephora type	38	26	1·5	0·1	0·2
Other coloured basidiospores	1,605	639	2·5	5·4	5·7
Uredospores	16	10	1·6	< 0·1	< 0·1
Ustilago (avenae type)	181	170	1·1	0·6	1·5
	16,950	5,529	3·1	57·0	48·9
Fungi imperfecti					
Alternaria/Stemphylium types	35	39	0·9	0·1	0·3
Botrytis	348	181	1·9	1·2	1·6
Cladosporium	4,585	3,197	1·4	15·4	28·3
Epicoccum	86	68	1·3	0·3	0·6
Erysiphae (oidium type)	21	14	1·5	< 0·1	0·1
Helicospores	23	22	1·0	< 0·1	0·2
Helminthosporium type	1	4	0·3	< 0·1	< 0·1
'Penicillium' type	26	33	0·8	< 0·1	0·3
Polythrincium trifolii	22	37	0·6	< 0·1	0·3
Pullularia type	46	29	1·6	0·2	0·3
Torula herbarum	7	4	1·8	< 0·1	< 0·1
	5,200	3,628	1·4	17·5	32·1
Myxomycetes					
Myxomycetes (purple type)	24	18	1·3	< 0·1	0·2
Miscellaneous					
Hyaline rods	1,638	365	4·5	5·5	3·2
Other spores	1,714	890	1·9	5·8	7·9
Mycelial fragments	33	35	0·9	0·1	0·3
	3,385	1,290	2·6	11·4	11·3
Total	29,716	11,306	2·6	100·0	100·0

basidiospore types could have been distinguished. Uredospores were not abundant and many of those caught resembled Melampsoridium.

Fungi imperfecti. At both sites 88% of the spores of Fungi imperfecti were Cladosporium, a common air-borne allergen. Unlike most spore types Cladosporium was more numerous at site M at the beginning of the season, but in June and July there were more at site S; at the end of the season numbers were similar at both sites.

Helminthosporium, 'Penicillium type' and *Polythrincium trifolii* (all 'dry spores') were all more abundant at site M. The 'Penicillium type' includes single, chains or groups of small round spores about 4μ in diameter.

Pithomyces chartarum (syn. *Sporidesmium bakeri*). The occurrence of this fungus in Europe was first indicated by the presence of a few spores on this series of slides (Lacey & Gregory, 1962): at site S 3 spores on 7 July at 17.00–21.00 hr.; at site M 1 spore each on 7 July at about 17.00 hr. and on 21 July at about noon.

Myxomycetes. Those spores of myxomycetes which could be distinguished for purposes of the count were recognized by their subspherical shape, their irregular surface markings and their mauvish-brown colour: they differ from spores of Tilletia which are spherical, and yellowish with distinct honeycomb markings.

Miscellaneous. At both sites 11% of the spores trapped are placed in this category, which includes among others several distinct spores whose origin and affinity are obscure. 'Hyaline rods' is a term given to small, rod-shaped hyaline spores (Gregory & Hirst, 1957), which are possibly ascospores of the Discomycete genus *Orbilia*. 'Mycelial fragments' usually consist of broken pieces of conidiophore of Cladosporium.

Hourly variations in spore content of the air

In an attempt to correlate weather with the number of spores in the air, three types of spores were counted at hourly intervals for the period 19–24 July (Fig. 3). This period was chosen because it included wet and dry days, and the numbers of spores varied greatly from day to day. The same counts were also used to detect any pronounced diurnal periodicity in spore concentration (Fig. 4), by summing the numbers of spores at each hour of the day separately, expressing the highest total as 100% and the totals at other hours as a percentage of this. The spore types were selected for diversity and distinctiveness. The '3-bar ascospore' type is easily recognized and known to be a component of the damp air-spora. Ganoderma is an easily recognizable basidiospore type. Botrytis was chosen as a hyphomycete known to release more spores by day.

'*3-bar ascospores*' were previously thought to be liberated when wetted by rain but, as Fig. 3 shows, very few occurred in the air by day even after rain, but numbers increased consistently at night. This is particularly evident on the diurnal periodicity graph for site S (Fig. 4). Possibly this species can liberate its spores when wetted with rain or even with dew only, but is inhibited by light.

Ganoderma. The diurnal periodicity shown in Figs. 3 and 4 confirms previous reports that spore concentrations are higher at night, but it is not clear from the meteorological records why there were many more spores on some nights than others. Possibly with a point source so near to trap S any differences in wind direction would have a large effect on the concentration reaching the orifice.

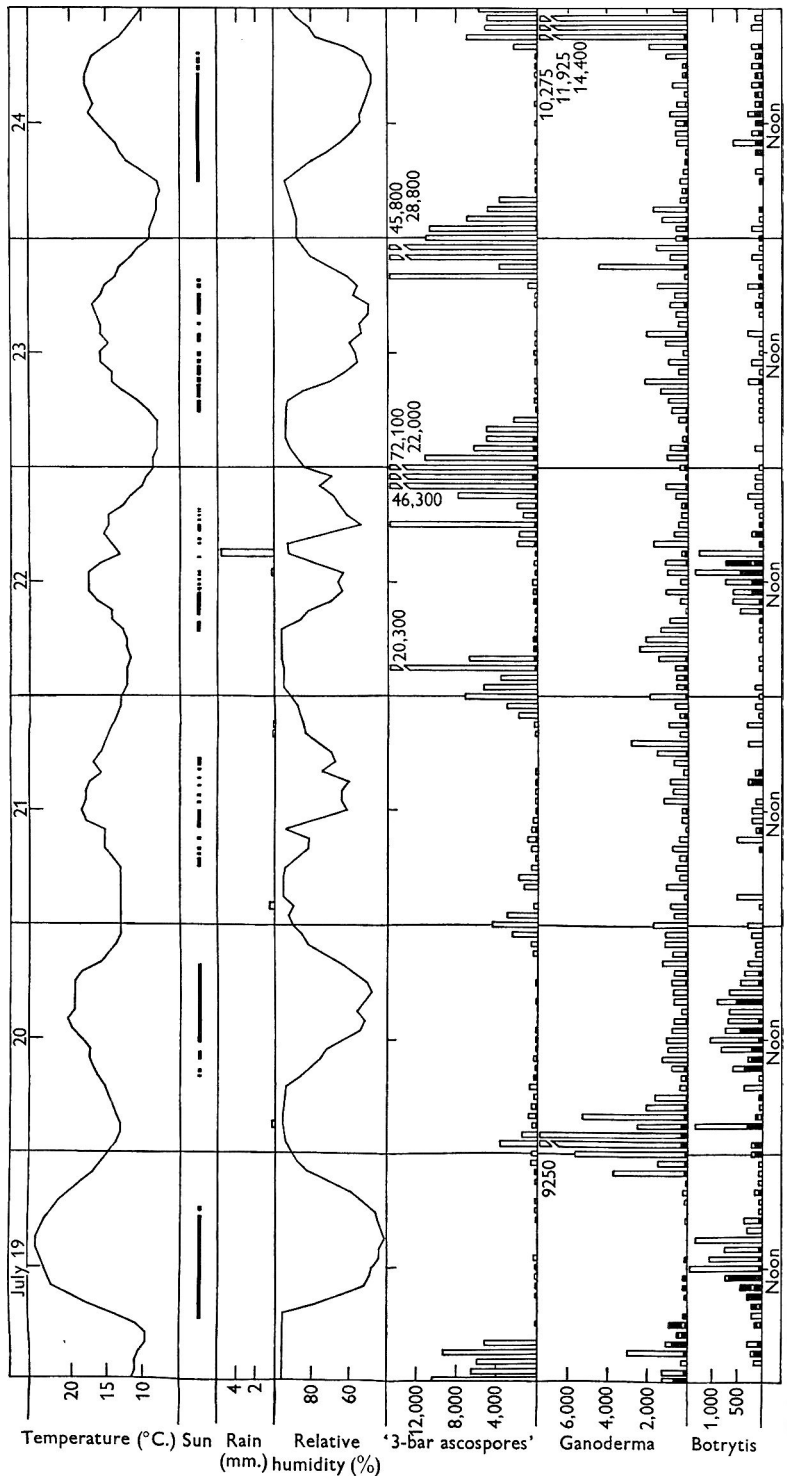

Fig. 3. Meteorological records and number of '3-bar ascospores', Ganoderma and Botrytis spores/m.^{3} air plotted hourly for the period 19–24 July 1958. Both hollow (trap S) and solid (trap M) histograms start from base line.

Botrytis was again in the air more by day than by night (Fig. 4) at both sites, but it is difficult to correlate the numbers of spores with variations in weather (Fig. 3).

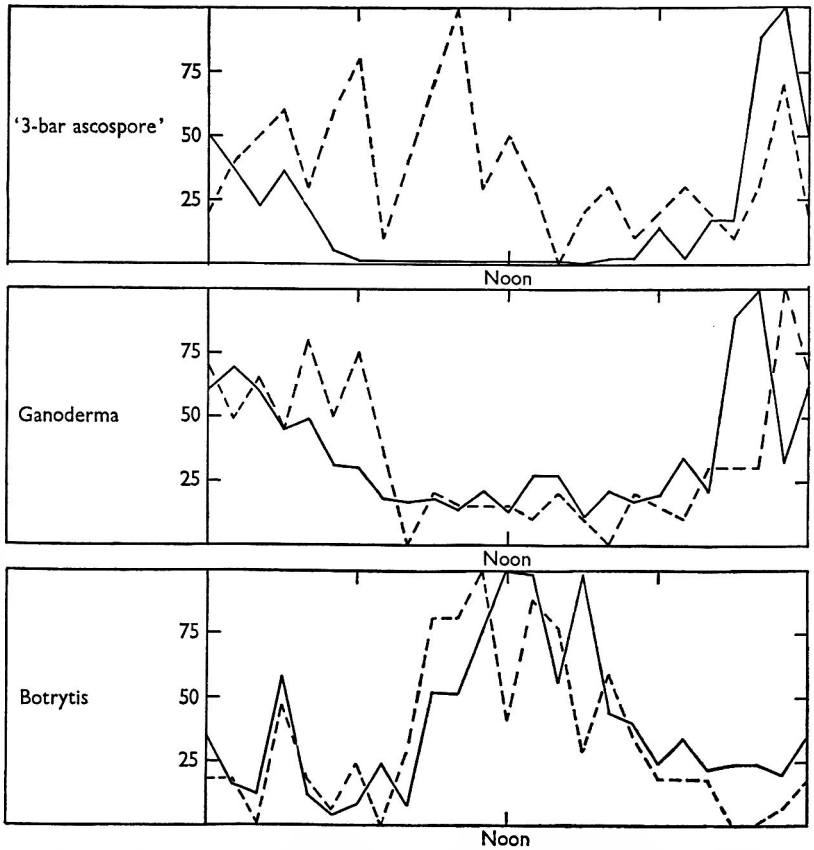

Fig. 4. Diurnal periodicity of '3-bar ascospores', Ganoderma and Botrytis. (Arithmetic mean of data from 19 to 24 July 1958.) — trap S; - - -trap M.

Pollen grains

Pollen grains are relatively easy to identify because of their size. Table 2 gives the peak concentrations and seasonal totals of the most abundant types. Fig. 2*d* gives the seasonal variation plotted as 6-day running means. Tree pollen grains were equally numerous at the two sites, but the two most abundant types, grass (Gramineae) and nettle (Urtica), were three and eight times more numerous near the stream than at the hill site.

Seasonal variations in pollen content of the air

Tree pollens. The numbers of tree pollen grains were similar at both sites, presumably because pollen from a tall tree is easily carried to some distance, so the presence of a local source is less important for traps near ground level than with a ground-level source. Trapping was started at the end of the Quercus season. Pine pollen was most abundant in late May and June, and Castanea in July. 'Other' tree

pollens recorded include Acer, Aesculus, Betula, Fagus, Sambucus, Taxus and Tilia, all of which occurred at times similar to those reported by Hyde (1956).

Grass pollen. The main season for grass pollen is June and the first half of July. The fall in number at both sites in late June was associated with a period of relatively cool, rainy weather.

Weed pollen. Most weed pollens during the season were from Urtica which average 35 grains/m.^{3} at site M and 283 at site S. This type was also remarkable for the long duration of its season, which persisted from mid-June to early September (see Hyde, 1959). Rumex was the only other plentiful weed pollen and occurred mostly in May and June. Other weed pollens included Artemisia and other Compositae, Chenopodium, Plantago, Ranunculaceae and Umbelliferae.

Table 2. *Contributions of different categories of pollens to the air-spora*

	Seasonal totals no./m.^{3}		Ratio	Percentage of total caught in area	
	Site S	Site M	S/M	Site S	Site M
Tree pollen					
Quercus	11	10	1·1	2·1	6·9
Pinus	15	17	0·9	2·9	11·7
Castanea	9	7	1·3	1·8	4·8
Other	5	5	1·0	1·0	3·5
	40	39	1·0	7·8	26·9
Grass pollen					
Gramineae	158	56	2·8	30·8	38·6
Weed pollen					
Urtica	283	35	8·1	55·3	24·1
Rumex	21	7	3·0	4·1	4·8
Other	6	6	1·0	1·2	4·2
	310	48	6·5	60·5	33·1
Unclassified	4	2	2·0	0·8	1·4
Total	512	145	3·5	100·0	100·0

Hourly variations in pollen content of the air

Gramineae and Urtica pollen were counted hourly from 17 June to 22 June inclusive (Fig. 5), a period chosen because both types fluctuated considerably during it. Both types are liberated during the day, the peak for Urtica pollen coming at noon, and that for grass pollen in the afternoon (Fig. 6). The fact that more grass pollen was in the air on 18 June than on 21 June was possibly because 18 June followed a cool rainy day with little pollen shedding. Hyde (1952) noticed that grass pollen is released in great amounts when a sunny day follows a dull one. Over the period 28 May to 6 June inclusive, Pinus pollen increased steadily after 07.00 hr., and most pollen was caught between 09.00 and 13.00 hr. at both sites.

Moss and fern spores and algal clumps

Of these plants moss spores averaged only 0·1/m.^{3} at site S and 0·3 at site M. Fern spores averaged 1·7/m.^{3} at S and 1·9 at M and were mainly Pteridium; none was caught in June and most (average 3·4 at S and 4·4 at M) in August. Algal

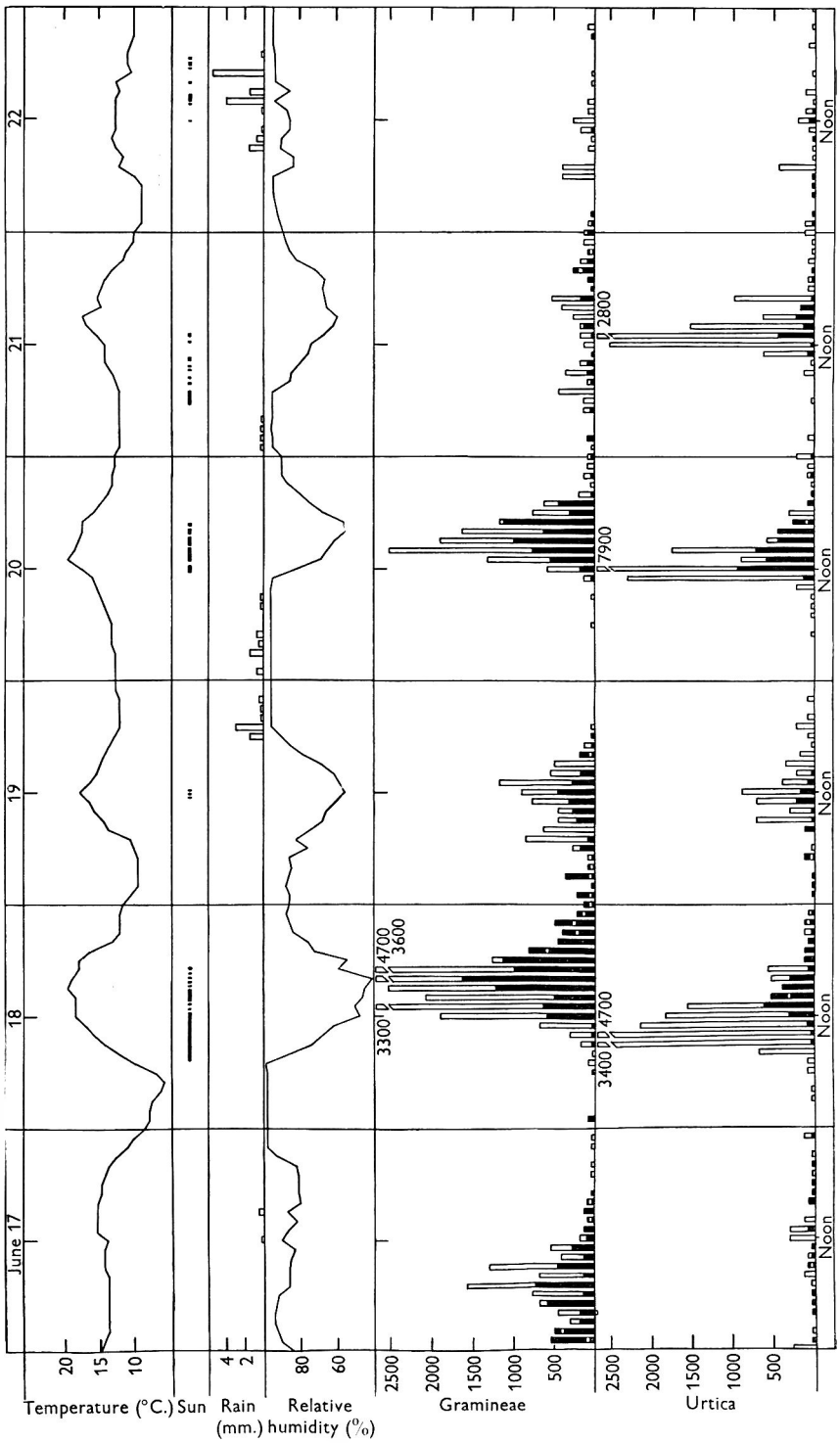

Fig. 5. Meteorological records and number of Gramineae and Urtica pollen grains/m.^{3} air plotted hourly for the period 17–22 June 1958. Both hollow (trap S) and solid (trap M) histograms start from base.

clumps, consisting mainly of small groups of cells of Gloeocapsa type (Gregory, Hamilton & Sreeramulu, 1955), were caught throughout the season and averaged 10·9/m.^{3} at S and 7·1 at M; more were caught in the early part of the season.

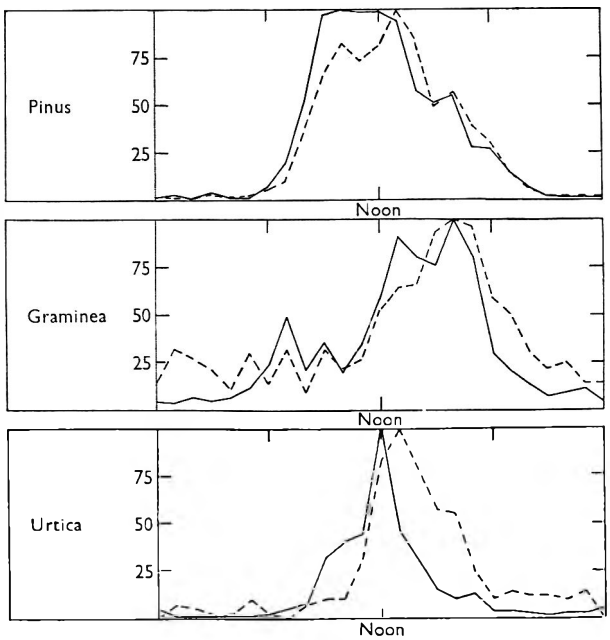

Fig. 6. Diurnal periodicity of Pinus pollen (arithmetic mean of data from 28 May to 6 June 1958), and of Gramineae and Urtica pollen (arithmetic mean of data from 17 to 22 June 1958). — trap S; – – – trap M.

DISCUSSION

The difference between the site S by the stream and the open hill site M is striking: over the whole season there were 2·6 times more spores at site S than at site M and 3·5 times as many pollens. The difference is even greater with components of the 'damp air-spora' (Hirst, 1953) such as Sporobolomyces and '3-bar ascospores', and also with those types which were known to have strong local sources near the stream, such as Ganoderma, nettle and grass pollen (near site M the grass was prevented from flowering by mowing). The seasonal curve for 'total spores' was dominated by Sporobolomyces and to a lesser extent by Cladosporium.

The mean numbers of spores/m.^{3} at site M, with orifice 0·5 m. above ground level, are similar to those caught by a trap 2 m. above ground level at Rothamsted in 1952 (Table 3), but pollens were fewer at both the Silwood Park sites. Comparison between these two places is reasonable, although the two seasons differed considerably, 1952 being dry and 1958 wet. The site used at Rothamsted was on arable land and was more comparable with site M than with S at Silwood Park.

From the differences between the two sites M and S (450 m. apart) it is evident that local ecology of an area is important in determining its air-spora. For example, over the whole season the type referred to as '3-bar ascospores' was 10 times more abundant at S than at M. During the 6 days referred to in Figs. 3 and 4 the difference was 70 times, and the 6347 spores of this type counted at S during this

6-day period, compared with only 91 at M, emphasizes the fact that these spores must be of local origin near the stream. The recorded diurnal periodicity of this fungus at site S is therefore reliable, but not that at M (Fig. 5).

The Ganoderma counts near the stream varied greatly from hour to hour (Fig. 4), an effect attributed to the concentrated source some 65 m. west of the trap—a condition where small changes in wind direction could result in large changes in

Table 3. *Mean numbers of spores and pollen grains/m.³ of air at Rothamsted from 1 June to 25 October* 1952 (*Gregory & Hirst*, 1957) *and at Silwood Park from* 14 *May to 25 September* 1958

	Rothamsted 1952, 2 m. above ground level	Silwood Park 1958, 0·5 m. above ground level	
		Site S	Site M
Total spores	11,368	29,716	11,306
Sporobolomyces, Tilletiopsis, Hyaline basidiospores	3,990	14,077	4,397
Cladosporium	5,837	4,585	3,197
Total pollens	1,184	512	145

concentration of spores reaching the trap. Whether the diurnal periodicity of Ganoderma observed here (Fig. 5), and also found by Sreeramulu (1959), resulted from decreased spore production during the day is not yet clear. Day temperatures of 19–21° are unlikely to inhibit sporulation of this species, which is abundant in warm climates. The amount of daylight reaching the basidia must be small and light is not known to inhibit sporulation in this species. Possibly during the daytime the spore cloud is more diluted by greater wind velocity, and diffused by turbulence through a thicker layer of the atmosphere, than it is at night (Hirst, 1953; Gregory, 1961, p. 119); but all these explanations are speculative.

At both trapping sites, M and S, the catch of *Ustilago avenae* (*perennans*) type was clearly correlated with the grass pollen seasonal curves, and both simultaneously declined temporarily in the last week of June. This agrees with observations of Sreeramulu (1959) on the correlation between barley loose smut spore liberation and the anthesis of the barley plant. Although the mechanisms by which the pollen grains and smut spores are released differ, there is a correlation in timing; the natural entrance of the smut spore to the developing grain is through the open flower at anthesis (Malik & Batts, 1960).

The locality S evidently acted as a strong local source of spores, and near the sheltered site S the spore clouds of local origin remained more concentrated because of lower wind speeds. The trees and shrubs near site S may also have acted as a filter and removed, by catchment on vegetation, some spores of distant origin which easily reached the more exposed trap M; this effect might account for the larger numbers of Boletus and Russula/Lactarius spore types caught at site M, though there may also have been richer sources of these larger fungi near to trap M.

The seasonal mean, Table 1, is an average of all concentrations during the season, from zero up to an instantaneous maximum. When shorter time intervals are considered, higher concentrations will be encountered, as illustrated in Table 4.

The means for the six-day period studied in Figs. 3 and 5 are usually higher than the seasonal means, and the highest concentration per hour is many times higher still. Table 4 illustrates the general principle that there are many high concentrations of short duration; higher values could still, no doubt, be found by further shortening the time interval.

Table 4. *Mean concentrations of spores/m.³ of air, illustrating the occurrence of high concentrations of short duration*

	Data from whole season				Data from 6-day period			
	Mean for season		Highest concentration/24 hr.		Mean for 6-day period		Highest concentration/hr.	
Site at Silwood Park	S	M	S	M	S	M	S	M
'3-bar ascospores'	1,569	150	13,440	1,380	3,248	47	72,150	525
Ganoderma	960	172	4,020	1,290	1,123	84	14,475	1,050
Botrytis	348	181	2,490	780	244	70	1,500	750
Gramineae pollen	158	56	2,055	645	375	165	4,680	1,620
Urtica pollen	283	35	2,115	180	352	73	7,920	945
Pinus pollen	15	17	255	270	116*	125*	1,180*	1,020*

* From 10-day period.

Although some correlations with weather can be surmised from the results in Figs. 2, 3 and 5, it is impossible to account fully for the observed variations in the air-spora at any locality. Maximum and minimum thermometers and surface wetness recorders working at each trap site would obviously be desirable in future work of this type, to help interpretation. The effects of past weather on development of higher plants and fungi, the effects of immediate weather on spore-release mechanisms, effects of wind and turbulence in diluting the spore cloud, and the presence or absence of local sources, are all so interrelated that far more information is needed before firm conclusions can be drawn.

I thank the Asthma Research Council for the loan of a Hirst spore trap; Dr K. Maunsell for helpful suggestions, M. M. S. Malik, J. Sam Raj and E. E. Green for help in field work, J. W. Siddorn for meteorological data, E. D. Hamilton for help in pollen identification, and R. H. Gregory for working out the diurnal periodicity of *Pinus* pollen.

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A Study of the Negative Staining Process

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SUMMARY

The effectiveness of a number of different materials in the negative staining method for electron microscopy of viruses is evaluated. Shadowing was used to study the degree of distortion suffered by the specimen. Changes in pH value which occurred while the negative staining solution was drying were measured in those materials which gave the most satisfactory results in the electron microscope. The ultimate resolution of the method is discussed and demonstrated. It was important to de-grease supporting film: perforated carbon films were found valuable for obtaining good contrast.

INTRODUCTION

Negative staining is used in light microscopy to reveal objects which will not take up conventional stains. The specimen is placed in a solution containing black pigment (nigrosin or aqueous indian ink) and is seen transparent against the dark background. The same principle is used for the electron microscope (Hall, 1955; Huxley, 1956; Brenner & Horne, 1959) but the preparation is in a solid, not a liquid state. The method consists of embedding an electron-transparent object in a structureless electron-dense matrix. The most widely used matrix is potassium phosphotungstate; the normal procedure is to mix a suspension of the specimen in water or ammonium acetate with a solution of phosphotungstate, and to apply this to carbon-coated grids in one of two ways. The mixture can be sprayed (Brenner & Horne, 1959) or spread by allowing a thin film of liquid to dry down on the grid (Bradley & Kay 1960; Huxley & Zubay, 1960). The results obtained with these methods have been valuable and have contributed much to our knowledge of the morphology of small particulate specimens, especially viruses. However, relatively little work has been done on the technique itself, and a number of points require elucidation. First, the physical features are not fully understood. These concern the thickness of the matrix, the degree of three-dimensional preservation it provides, and the conditions under which the matrix spreads. Furthermore, little attempt has been made to use other chemicals, it being assumed that phosphotungstate is the most satisfactory. Great emphasis has been placed on using the correct pH value of solutions at the start of the process, but the fact that this may not remain constant has been overlooked. Negative staining mixtures change their pH values as they dry down (Bradley, 1961*a*); this probably affects the specimen to some degree. Though applied mainly to the study of bacteriophages, the present communication attempts to provide facts of general application relating to these points.

METHODS

The spraying and spreading methods of negative staining have their advantages and disadvantages. For example, quantitative virus counts (Dr D. Watson, personal communication) can only be carried out by using a spray, but with the spray the virus concentration is much more critical than with spreading. On the whole, the spreading method is easier, quicker and uses less virus suspension. This technique was therefore used to obtain the results described here.

The preparation of support films

The physical state of the surface of a support film affects the spreading of the negative-staining material into areas of suitable thickness. Thus, Brenner & Horne (1959) found that phosphotungstate did not spread properly on carbon films prepared in a vacuum unit which included an oil-diffusion pump. It is thus important that, particularly when materials are being tested for suitability as negative-staining agents, the support films used should be free from contaminating oil molecules; if consistent results are to be obtained coated grids should be carefully de-greased by dipping the grid into a solvent, e.g. re-distilled chloroform, immediately before use. Most types of carbon support film are generally suitable, even those which consist of a plastic stabilized with carbon.

It is considered that perforated carbon films (for a suitable method, see Bradley, 1961*b*) are best; many holes are covered with embedding chemical (Huxley & Zubay, 1960) and specimen particles are included in them. The contrast obtained under such conditions has been found to be improved, and the background structure seen significantly diminished. The reason for this is that the presence of a support film not only decreases contrast, but also produces a variable background structure which may be as coarse as 20Å., depending on the nature and condition of the substrate. It has been found that with the spreading method of applying phosphotungstate the thickness of the matrix varies over both holes and grid. This is an advantage because specimen particles embedded under optimum conditions can be readily found and the method used at maximum efficiency.

The preparation and use of negative-staining solutions

Several solutions have been tested as embedding media for negative staining. They were all prepared in the same way except for solutions of ethylenediaminetetra-acetate (EDTA) complexes. Chemicals, analytical reagent grade when available, were used as 1–2% aqueous solutions (concentration not very critical with the spreading method), their pH values being adjusted with potassium or sodium hydroxide for salts of these metals, and with ammonium hydroxide for other salts.

EDTA complex solutions (Van Bruggen, Wiebenga, & Gruber 1960) were prepared as follows: a 1% suspension of ethylenediaminetetra-acetic acid in water was made up. This was mixed with an equal volume of 1% uranyl acetate or nitrate solution. Ammonia was then added until the suspended EDTA disappeared, the pH being finally adjusted to the desired value with ammonia or acetic acid. Solutions containing EDTA were used first, to enable uranyl acetate or nitrate solutions to be raised to alkaline pH values without forming a precipitate, and secondly, to investigate the spreading characteristics of the compound.

Test specimens consisted of various types of bacteriophage (see below) suspended in one of the following solutions (strength 0·1 M, pH about 7·2): ammonium acetate, ammonium carbonate, ammonium benzoate.

The preparation of negative-staining mounts was done as follows. Equal volumes (unless otherwise stated) of virus suspension and negative-staining solution totalling about 0·02 ml. were mixed on a watch-glass or slide by a micropipette. A freshly de-greased support film was then touched on to the surface of the mixture. Excess liquid was removed from the grid with filter paper until only a thin film covered the grid. After drying, the specimen was ready for examination.

Two general practical features are worth mentioning here. First, spreading agents (chemicals which assist the embedding matrix to spread evenly over the support film) in general were found to be unnecessary. However, the following materials have been satisfactorily used in conjunction with phosphotungstate: 0·2–0·4% sucrose + a trace of tryptone (Anderson, 1960); bovine serum albumin trace (Harrison & Nixon, 1960); EDTA (present communication) + uranyl salts only. Secondly, trouble may often be experienced from breaking of the support film, but this may usually be remedied by using the correct support grid. For perforated films, either Smethurst Highlight type A.E.I. or Veco 400-mesh/in. or their equivalents are used. For continuous carbon or plastic + carbon films the standard 200-mesh/in. are generally suitable.

CHARACTERISTICS OF THE SPREADING METHOD

When a thin film of negative-staining mixture dries on a support film, it generally provides a wide variation in concentration of dried material both over the grid as a whole and over individual grid squares. At first sight this may seem to be a disadvantage, but in fact the opposite is the case. A short search invariably shows areas where the staining characteristics are satisfactory, and it is rarely necessary to prepare a fresh grid. The concentration of dried material is usually greater towards one edge of the grid than the other, especially when it has been held vertically during drying. Thus, when it is found that some grid squares are completely 'blacked out' with embedding matrix, it is only necessary to move towards the opposite edge. The distribution of material is usually symmetrical over a grid square, the concentration being highest around the outside and decreasing towards the centre, where the most satisfactory areas are to be found. As Brenner & Horne (1959) stated, satisfactory results depend also on the ratio of specimen particle concentration to embedding material concentration. Where there are no particles, the matrix, depending upon its chemical composition, may aggregate into large dense spheres, and where there are too many particles, contrast may be low. Again, this ratio appears to vary both across grid and grid square, and suitable regions with different particle:matrix ratios can easily be located. These difficulties are encountered mainly with the less successful preparations; extensive areas of the grid are often entirely satisfactory. Thus, though the method is not completely consistent in that the general quality of preparations varies, it is almost certain that good micrographs can be obtained from any one grid. Compared with other electron microscope specimen techniques, this is one of the more reliable, and undoubtedly one of the simplest.

When the spreading method is used, it will be found that on examination in the electron microscope three different embedding conditions are encountered, each of which depends upon local concentrations of specimen particles and embedding medium. First, there is the case when the particle is completely embedded in matrix. Depending on the specimen this may or may not give a good micrograph. Shadowing of negatively stained preparations has revealed that the particles in such areas are often distorted and not always covered completely, but the most usual state of affairs is that shown in the diagram *a* in Fig. 1. This condition is generally considered by most workers to be the most desirable, and also applies when particles are embedded in matrix covering holes. A second condition is when an individual particle is embedded in a small area of matrix as shown in Fig. 1*b*. The contrast-forming conditions differ little from the first case, but it is often found that better

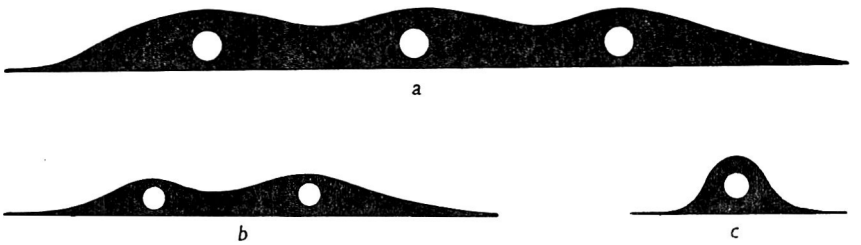

Fig. 1 (*a*). Diagram of particles deeply embedded in a large area of negative staining matrix. (*b*) Diagram of particles embedded in a small area of matrix. (*c*) Diagram of a single 'high and dry' particle with adsorbed layer of matrix.

resolution is obtained. A third condition occurs when particles are left 'high and dry' due to surface tension effects. Here, they generally have a thin film of the matrix adsorbed on their surface as shown in Fig. 1*c*. The resulting contrast is quite different and closely resembles that given by positive staining. Thus it is found that completely different aspects of a particle may be visualized on a single grid square and it is therefore recommended that micrographs should be taken of specimens found under a variety of embedding conditions.

An interesting feature of both spraying and spreading methods has been noted by Dr D. Watson (personal communication). He found that polystyrene latex particles appeared black when not surrounded with phosphotungstate, but white when embedded in matrix on the same micrograph. The difference was real, and could be measured with a densitometer. As yet no explanation has been found for this phenomenon, but Dr Watson has suggested that possibly there might be a phase shift in the electron beam as it passes through the matrix (which may be 500 Å or more thick), producing reversal of contrast. This is a point which must be carefully considered when interpreting micrographs of embedded and non-embedded particles.

Test objects and their structure

The following phages, which were used as test objects, were obtained from the following sources: Phages T2, T5, ØR, 66t- from Dr D. Kay (Sir William Dunn School of Pathology, University of Oxford); phage T4 from Dr and Mrs K. G. Lark (Saint Louis University School of Medicine); staphylococcus phage 70 from Dr Elisabeth Sharpe (National Institute for Research in Dairying, Shinfield, near

Reading). They were all purified by several cycles of centrifugation and used at concentrations between 10^{10} and 10^{11} particles/ml. Phage 1C was isolated in this laboratory.

When investigating a technique it is advisable to use test objects which have been well studied. For this reason, coliphages T2 and T4 were chosen. It was also considered desirable to use a particle similar to small spherical plant and animal viruses which at the same time would provide a severe test for the methods used. Phage ØR was chosen for this (Kay, 1962; Bradley, 1961; Kay & Bradley, 1962).

The general structure of phages T2 and T4 is now well known (Brenner *et al.* 1959). More recently, however, newly discovered features of both phages have aroused interest. On T4 there is a 'thin collar or disc' (Anderson, 1960), and Daems, Van der Pol & Cohen (1961) detected an 'outer jacket'. The present author (in preparation) has studied these features and found that on T4 they consist of an open-mesh outer sheath of protein fibres attached at the top to the collar. Such features are shown in Pl. 1, fig. 6, on a different but morphologically similar phage (66t-). T2 is similar except that the collar is absent and the fibres appear to be attached to the base of the head (Pl. 1, fig. 4). This fibrous structure, together with the familiar cross-striations as well as the relative instability of the extended sheath, provide excellent delicate features to test preservation.

Bacteriophage ØR consists of a core of single-stranded DNA surrounded by a protein coat in the form of an icosahedron 300 Å in size. There are small subunits at its apices, apparently in the form of dimers. The presence or absence of these subunits is a valuable guide to the qualities of preservation provided by a negative stain, since they are small and easily lost.

Coliphage T5 and staphylococcus phage 70 have also been used and will be described in the appropriate section.

The investigation of different embedding chemicals

Potassium phosphotungstate (PTA). Because this chemical has been the most widely used and is considered the most efficient, it was studied under a variety of conditions. Phages T2, T4, 66t- and ØR were used as test objects. The mixtures of the three buffer solutions already mentioned with phosphotungstate were tested under neutral conditions. On the whole, a mixture of 0·1 M-ammonium benzoate and 2 % phosphotungstate provided good spreading, contrast and preservation with phage T4 (Pl. 1, figs. 1, 2), but some sort of chemical reaction appears to have occurred with the T2 sheath protein causing it to contract and disrupt (Pl. 1, fig. 3). The fibrous sheath is broken up to some extent in both cases.

With 0·1 M-ammonium carbonate no such chemical action occurred with phage T2, and preservation seemed, if anything, slightly better. In Pl. 1, fig. 4, the fibrous sheath appears to be intact, albeit lacking a little in contrast. In phage T4 the collar and slightly disrupted fibres are clear in Pl. 1, fig. 5.

With 0·1 M-ammonium acetate, much the same state of preservation is obtained. Phage 66t-, which is morphologically similar to phage T4, was often found perfectly preserved (Pl. 1, fig. 6). Though the phosphotungstate + ammonium acetate mixture appeared to be rather better in this series, it is not considered to be significantly superior to either of the other combinations. However, care must be taken in interpreting micrographs taken when using ammonium benzoate because of the

risk of the chemical action mentioned above. For preparing specimens, therefore, there is little to choose between these three mixtures, as all of them produced excellent results, save for the rather poor preservation of the large heads.

An exceptionally good (Pl. 2, fig. 7) and an average (Pl. 2, fig. 8) micrograph of phage ØR in phosphotungstate + ammonium acetate are shown. The best was taken of a preparation spread over a hole; the advantages of this are immediately obvious. These may be compared with other micrographs obtained when different mixtures described below are used.

The shadowing of phosphotungstate preparations indicates the thickness of the matrix, which is generally about 500 Å. In addition, as shown in Pl. 2, fig. 9, the state of preservation of the heads can be seen; they have completely collapsed. This appears to be the one undesirable feature of phosphotungstate, though it does not always happen. Care should be taken in interpreting asymmetric structures seen in large bodies as real, because there is a very great risk of artifact formation.

Sodium tungstate. Results obtained with sodium tungstate under neutral conditions + ammonium acetate are comparable with results with phosphotungstate. The apical subunits of phage ØR are clear in Pl. 2, fig. 10, though in other micrographs they show a tendency to fall off. With the specimen suspended in ammonium carbonate or benzoate the results were unsatisfactory showing poor definition and crystallization, respectively. Sodium tungstate is considered a reliable addition to potassium phosphotungstate.

Sodium phosphomolybdate (PMA), was tested under neutral conditions. With the phage in ammonium benzoate, there was crystallization; when in ammonium carbonate most phage T4 tails were disrupted (Pl. 2, fig. 11), the fibrous outer sheath lost, but the head shape well preserved. Improved preservation was obtained with ammonium acetate (Pl. 2, fig. 12), but the contrast was rather low and, as a result, the support film background structure was more noticeable. The results with phage ØR were not good (Pl. 2, fig. 13), the apical knobs being scarcely visible. Very good definition has been obtained with phosphomolybdate in the past, and if it could be spread across holes—a difficult thing to achieve at present—excellent results might be obtained.

Sodium molybdate. Under neutral conditions this chemical crystallizes with ammonium benzoate, and rather poor results were obtained with ammonium acetate with phage T4 (Pl. 3, fig. 14) and phage ØR (Pl. 3, fig. 15). However, with ammonium carbonate, preservation and contrast were greatly improved and the matrix spread across holes. This mixture has only been studied with phage T4, but was found as good as any of the phosphotungstate mixtures (Pl. 3, fig. 15).

Uranyl acetate. Unfortunately this important and useful chemical can only be used in acid (pH 5·2) solution. Its spreading characteristics are unreliable but with ammonium acetate it frequently behaves like phosphotungstate. Shadowing revealed incomplete wetting of the phage particles, phage T2 being the example (Pl. 3, fig. 17; see tail of bottom particle). Particles were also found with a thin adsorbed layer of matrix, which often provides high local contrast. This was not the case with phage T2 (Pl. 3, fig. 18) however, where the result was poor. To assess whether any positive staining had occurred, these particles were removed from uranyl acetate solution by centrifugation after treatment for a few minutes. A comparison between Pl. 3, fig. 18, the negative-stained particles, Pl. 3, fig. 19,

particles treated with uranyl acetate, and Pl. 3, fig. 20, untreated particles dried down from ammonium acetate, indicates that there was no positive staining of the sheath, but that the head (presumably the phosphate groups of the DNA) had taken up uranyl ions to become much denser than untreated heads. If necessary this simple procedure could be used in other cases to detect positive staining. Results on phage ØR were also somewhat disappointing (Pl. 4, fig. 21); the apical subunits were almost completely removed and the shape of the phage rather poorly preserved. Nevertheless, it has been possible to obtain information about the structure of this phage by using uranyl acetate (Kay & Bradley, 1962).

A comparative study of a staphylococcus phage with uranyl acetate and phosphotungstate indicated the differences in appearance which are to be expected in preparations made with these materials. A phosphotungstate preparation is shown in Pl. 4, fig. 22; the head is oblong and rounded, the tail is practically structureless, but there is a knob or appendage at the tip. There is a startling difference between this appearance and Pl. 4, fig. 23 (uranyl acetate), which shows a slimmer and more sharply angled head, and a striated tail which has no appendage at the tip. A 'high and dry' head is shown in Pl. 4, fig. 24; it is dense, and doubtless stained positively, but there is a thin electron-transparent line around the dense region which probably represents the unstained protein of the head membrane. A halo of carbonaceous contamination is also visible, indicating that the particle was standing proud of the support film and hence that the three-dimensional preservation was probably good.

On the basis of these observations, results obtained with uranyl acetate should always be compared with those obtained with phosphotungstate. It is also desirable to take micrographs of particles in as many different conditions of embedding as possible. Uranyl acetate is obviously very useful though difficult to use and to interpret.

Uranyl acetate with EDTA. This mixture was tested with phages T2 and T4 under neutral conditions. For phage T2 in ammonium acetate (Pl. 4, fig. 25) it appeared unsatisfactory, though the shape of the head was well preserved. There was some improvement with phage T4 in ammonium carbonate (Pl. 4, fig. 26), preservation being good, but definition poor. These results are virtually useless; but other workers such as van Bruggen, Wiebenga & Gruber (1960) have obtained with this mixture excellent micrographs of such specimens as haemocyanin molecules. Perhaps better results might be obtained by using a different amount of EDTA at different pH values.

Uranyl nitrate at pH 5·2 with phage T2 in ammonium acetate showed the phage tail to be destroyed (Pl. 5, fig. 27), and there was no spreading. The heads were heavily stained with shape well-preserved.

Uranyl nitrate with EDTA in ammonium acetate at neutral pH value, fully embedded particles of phage T2 showed little detail (Pl. 5, fig. 28). Results with 'high and dry' particles resembled those obtained with plain uranyl nitrate (Pl. 5, fig. 29).

Lanthanum acetate. Phage ØR in ammonium acetate mixed with 1 % lanthanum acetate (pH of mixture 5·2) produced high contrast but coarse background, even across holes (Pl. 5, fig. 30). It is possible that a satisfactory lanthanum acetate mixture could be developed; this ought to be of value because of the high contrast obtainable.

Thorium nitrate and chloride. These two chemicals were tested with ammonium acetate and failed for different reasons. The result with the nitrate and phage ØR shows excessive granularity (Pl. 5, fig. 31). With the chloride there were undesirable effects upon the specimen, phage T2 (Pl. 5, fig. 32), though actual negative staining and contrast was reasonable.

Effect of pH *value on some negative stains*

It is reasonable to suppose that the efficiency of a negative-staining material will be changed according to the pH value at which it is used. This has already been noted with phosphotungstate (Bradley, 1961*a*, *b*); at the same time it was found that the pH value of a mixture changed as it dried on the grid. This point will be discussed below. Different staining characteristics have also been obtained on haemocyanin molecules (van Bruggen *et al.* 1960) when using uranyl acetate + EDTA mixtures at different pH values. This matter has not been exhaustively studied here, since the best pH value to choose will depend largely upon the chemical relations between specimen and embedding material. It has been found that in general best results are obtained at near neutral pH values, the background structure of most materials being least obvious under these conditions. It is not, of course, possible to use neutral solutions of chemicals such as uranyl acetate because of precipitation of metal oxide or hydroxide, a value about pH 5·0 is satisfactory here.

Changes in pH *during drying*

There is little doubt that the more delicate specimens examined in the electron microscope will be damaged to a greater or lesser extent by the changes in pH value which occur in a negative-staining mixture while it is drying. These changes were measured on the most satisfactory materials as follows. Negative-staining mixture (1–2 ml.) was placed on a watch-glass and allowed to evaporate at about 50°. The percentage of water which had evaporated was measured at intervals by weighing, and the pH values determined (cold) at the same time with British Drug Houses Ltd. capillators; the accuracy is usually $\pm 0{\cdot}1$ pH unit. Towards the completion of the evaporation of the sample, the salt concentration reaches a high value; at such concentration pH measurements made with glass electrodes are of little value, and dyes are more reliable. In the present experiment, 0·005–0·01 ml. of liquid was removed from the watch-glass, mixed with an equal volume of pH capillator dye solution, and compared with the standard. The results were plotted against the percentage of solution which had evaporated. Though this procedure is analogous to the drying stage of the negative-staining process with spreading, the pH determinations took 2–3 hr., the spreading process 1 min., and the drying of sprayed droplets perhaps a few seconds.

To determine whether the presence or otherwise of phage particles had some effect on the pH curve, 10^{12}–10^{13} particles of phage T4/ml. were added in a few cases. They had little effect on the curve, and none on the pH value reached by the mixture just before drying (see Table 1). The pH range through which a drying mixture passes depends mainly upon the relative volatility of the acids and bases constituting the salts. Results obtained with some of the unmixed solutions are shown in Table 1. When these solutions are mixed in varying proportions, curves of

almost any shape can be produced. Figure 2 shows curves for various mixtures of potassium phosphotungstate and ammonium acetate; the pH value remained virtually unchanged only when it was mixed with an equal volume of 0·025 M-ammonium acetate. This particular mixture was found by trial and error and it should be possible to adjust most negative-staining mixtures in this way. Figure 2 also includes curves for uranyl acetate and sodium molybdate mixtures. Figure 3 gives curves for sodium tungstate and mixtures with ammonium acetate, and again demonstrates how changes in pH value can be controlled. This graph also shows that ammonium acetate and ammonium carbonate follow the same curve. While the pH changes noted here were relatively small (the largest was 1·6 pH units) they might affect delicate specimens and perhaps the staining efficiency to a small extent. The changes would be more important when proteins with a known isoelectric point were being studied and the pH value must be carefully controlled.

Table 1. *Changes in* pH *value of negative-staining solutions during drying*

				pH values (% evaporated)			
Negative stain	Amount	Buffer	Amount	0 %	50 %	75 %	100 %*
2 % PTA†, pH 7·0	1 vol.	Nil	Nil	7·0	7·0	7·2	7·8
2 % PTA, pH 4·2	1 vol.	Nil	Nil	4·2	4·0	3·8	3·4
Nil	Nil	0·1 M ammonium benzoate	1 vol.	7·2	5·9	5·7	5·6
Nil	Nil	Ditto + phage T4	1 vol.	6·7	6·0	5·8	5·6
2 % PTA	1 vol.	0·1 M ammonium benzoate	1 vol.	6·8	6·7	6·5	5·5
2 % PTA	1 vol.	0·1 M ammonium carbonate	1 vol.	7·0	7·2	6·9	6·0
2 % sodium tungstate	1 vol.	Nil	Nil	7·3	7·9	8·2	8·8
1½ % Sodium PMA‡	1 vol.	0·1 M ammonium acetate	1 vol.	7·2	6·6	6·6	6·4
2 % Uranyl acetate	1 vol.	Nil	Nil	5·2	5·5	5·8	6·0
2 % Uranyl acetate, pH 5·2	1 vol.	0·1 M ammonium acetate pH 6·8	2 vol.	5·6	5·8	5·9	6·0
1 % Uranyl acetate, pH 4·0	1 vol.	Nil	Nil	4·0	4·3	4·4	5·0
1 % Uranyl acetate, pH 4·0	1 vol.	0·1 M ammonium acetate pH 7·2	2 vol.	4·4	4·6	4·6	5·2
1 % Uranyl acetate, + EDTA, pH 7·2	1 vol.	0·1 M ammonium acetate pH 7·2	1 vol.	7·2	0·2	6·0	6·0

* This is a theoretical value obtained by extrapolating the curves obtained by plotting pH against % evaporated, and represents the pH attained immediately before drying is complete.
† PTA = K phosphotungstate. ‡ PMA = phosphomolybdate.

The effect of negative staining on the viability of phages

The effect of different negative-staining chemicals on the viability of several phages was examined. In general there was a reasonably high survival rate when phage suspensions were mixed with the various negative-staining chemicals; uranyl

acetate was the most destructive and killed most types of phage; phosphotungstate was the least destructive. However, when phages either in negative-staining mixtures or in plain buffer solutions were dried *in vacuo*, there was no survival at all.

Size measurements

It is desirable to be able to measure the size of specimen particles in negatively-stained preparations with some degree of accuracy and reliability so that dimensions obtained can be compared with those found by techniques other than electron

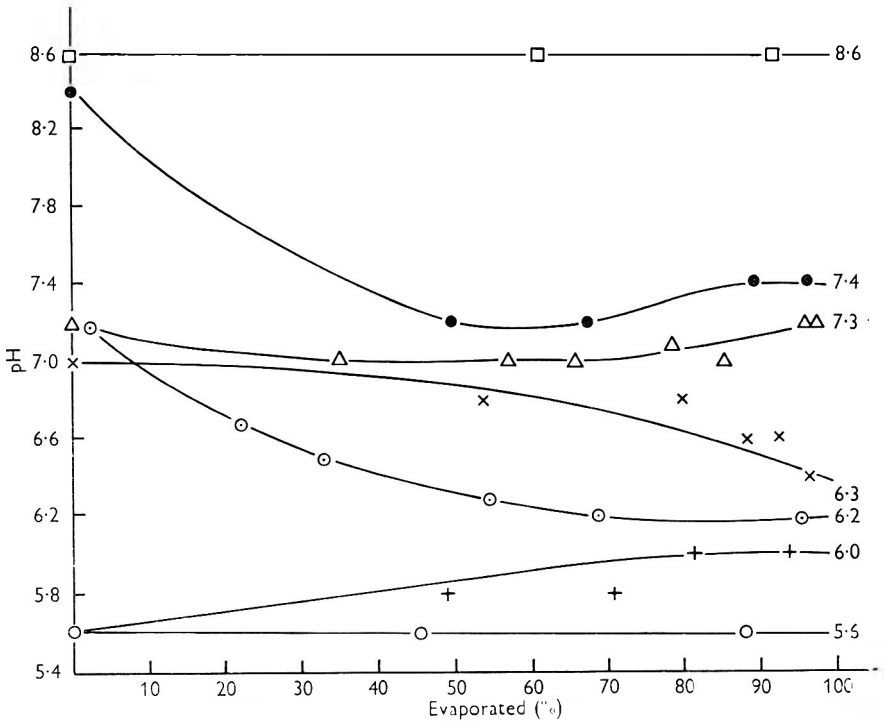

Fig. 2. Curves showing pH changes in suspending media as they evaporate. ●, 2 vol. 2% PTA, pH 8·6; 1 vol. 0·1 M-ammonium acetate, pH 7·2. ○, 2 vol. 2% PTA, pH 4·2; 1 vol. 0·1 M-ammonium acetate, pH 7·2. ×, 1 vol. 2% PTA, pH 7·0; 1 vol. ammonium acetate, pH 7·2. △, 1 vol. 2% PTA, pH 7·2; 1 vol. 0·025 M-ammonium acetate, pH 7·2. □, 2% PTA, pH 8·6. ⊙, 1 vol. 2% Na molybdate, pH 7·2; 1 vol. 0·1 M-ammonium carbonate, pH 7·2. +, 1 vol. 2% uranyl acetate, pH 5·2; 1 vol. 0·1 M-ammonium acetate, pH 6·8.

microscopy. With phage ØR (Kay & Bradley, 1962) different sizes were obtained by different techniques; shadowing gave the highest value and uranyl acetate the lowest. A comparison of the sizes obtained with staphylococcus phage 70 is summarized in Table 2. The discrepancies are considerable, and in the case of the head width, geometrically impossible. In examining the micrographs to determine other differences it can be seen that with phosphotungstate the head was rounded with no sharp angle; with uranyl acetate the angles were very sharp. Shadowed preparations (Pl. 2, fig. 9) have shown that heads in phosphotungstate tend to collapse and hence have a greater apparent size. But the observed discrepancy can only be

partly accounted for on this basis. The only alternative explanation is that contraction has occurred with the uranyl acetate. The following observation indicated that this was, in fact, the case. When particles of phage T2 embedded in uranyl nitrate + EDTA mixture were being examined (Pl. 5, figs. 28, 29) a difference of about 15% in head width was noted (taking into account the carbonaceous contamination on the non-embedded heads). This contraction was seen to occur in the microscope as the non-embedded particles were moved into the electron beam; it took place over a period of $\frac{1}{2}$–1 sec.

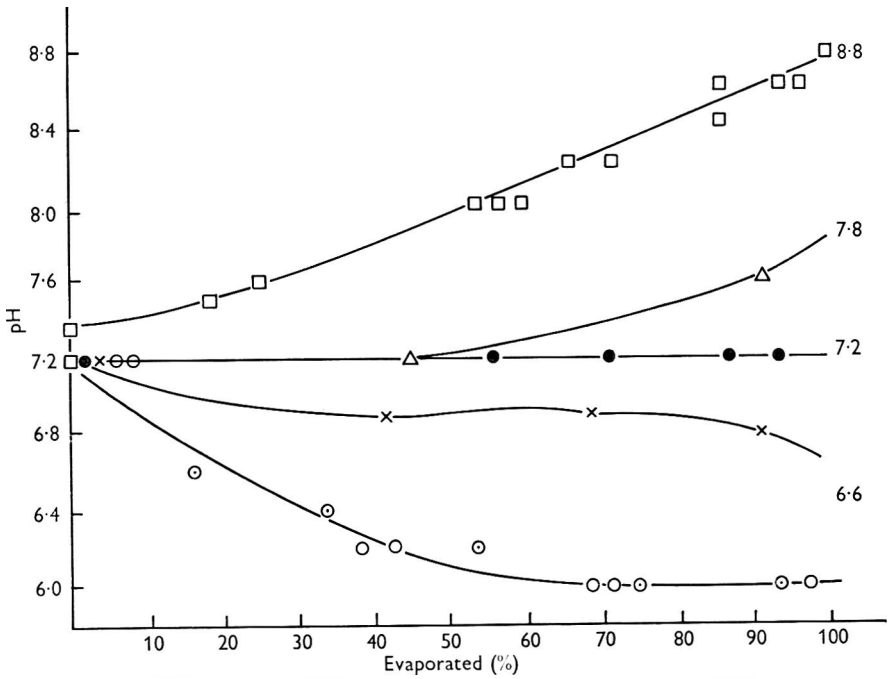

Fig. 3. Curves showing pH changes in suspending media as they evaporate. □, 2% sodium tungstate. △, 2 vol. 2% sodium tungstate, pH 7·2; 1 vol. 0·1 M-ammonium acetate, pH 7·2. ●, 1 vol. 2% sodium tungstate, pH 7·2; 1 vol. 0·1 M-ammonium acetate, pH 7·2. ×, 1 vol. 2% sodium tungstate, pH 7·2. 2 vols. 0·1 M-ammonium acetate, pH 7·2. ○, 0·1 M-ammonium carbonate, pH 7·2. ⊙, 0·1 M-ammonium carbonate, pH 7·2.

Table 2. *Dimensions of staphylococcus phage* 70

Negative stain	Head length	Head width	Tail length	Tail width
	(A°)			
Potassium phospho-tungstate	980	530	3000	95
Uranyl acetate	750	300	2800	85

Dimensions quoted are averages of ten measurements.

There is no doubt that the question of size measurement requires further investigation. At present the most reliable estimate must be an average of results obtained by various preparation methods. In some cases, however, the three-

dimensional preservation may be obviously good, for example in Pl. 5, fig. 33. This shows an octahedral phage head where the facets are clearly shown without any distortion. Any measurement from such a head would undoubtedly be accurate. These observations presuppose an accurate calibration of the instrumental magnification. Care must be used with polystyrene latex suspensions since the spheres are liable to appreciable shrinkage (personal observation) under electron bombardment.

Resolution

The efficiency of a technique is often judged by the resolution obtainable with it, and it is usually difficult to quote an exact figure. Brenner & Horne (1959) claimed 15 Å, but showed no pictures to support this. In the experience of the present author, it has been found that the average micrograph resolves only about 30 Å, though, on occasions when conditions are exactly right less than 15 Å can be attained. It is a fortunate coincidence that the apical subunits of phage ØR (Pl. 2, figs. 7, 10) provide a conclusive test object; in the micrographs shown they can be seen to consist of two portions, about 15 Å in size and separated by a gap of 10–15 Å. Thus, on the basis of the particle separation definition of resolution, better than 15 Å was obtained here; but it must be emphasized that this micrograph is an exception rather than the rule.

CONCLUSION

Though it is basically a very simple technique, the negative-staining method has many variations. As with replica processes, each worker appears to have his own special handling procedure, but the ultimate result is the same. Many of the real variations have been described here and they involve different combinations of materials. These often appear to give complementary information. For example, while phosphotungstate preserves fine detail better than most other reagents, the three-dimensional shapes of larger structures usually become badly distorted. These are, on the other hand, well preserved by uranyl acetate though they may have shrunk slightly. It is clear that it is dangerous to rely only on one staining material. The information given here should provide a basis for the selection of reagents for most types of specimen, whether delicate, or stable.

The author is grateful to those listed above who provided phages, and Miss M. McCulloch for technical assistance. The author would like to thank Dr D. Kay (Sir William Dunn School of Pathology, University of Oxford), Dr P. Wildy and Dr D. Watson (Institute of Virology, University of Glasgow) for valuable criticism and advice and also Professor M. M. Swann (Zoology Department, University of Edinburgh) for his help and interest.

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EXPLANATION OF PLATES 1–5

PLATE 1

Figs. 1, 2. Phage T4 in ammonium benzoate and phosphotungstate, × 330,000.
Fig. 3. Phage T2 in ammonium benzoate and phosphotungstate, × 333,000.
Fig. 4. Phage T2 in ammonium carbonate and phosphotungstate × 333,000.
Fig. 5. Phage T4 in ammonium carbonate and phosphotungstate × 333,000
Fig. 6. Phage 66t- in ammonium acetate and phosphotungstate, × 333,000.

PLATE 2

Figs. 7, 8. Phage ØR in ammonium acetate and phosphotungstate, × 333,000, by courtesy of *Virology*.
Fig. 9. Phage T2 in ammonium acetate and phosphotungstate, shadowed at $\tan^{-1}\frac{1}{4}$, × 150,000.
Fig. 10. Phage ØR in ammonium acetate and sodium tungstate, × 333,000.
Fig. 11. Phage T4 in ammonium carbonate and sodium phosphomolybdate, × 333,000.
Fig. 12. Phage T4 in ammonium acetate and sodium phosphomolybdate, × 333,000.
Fig. 13. Phage ØR in ammonium acetate and sodium phosphomolybdate; × 333,000.

PLATE 3

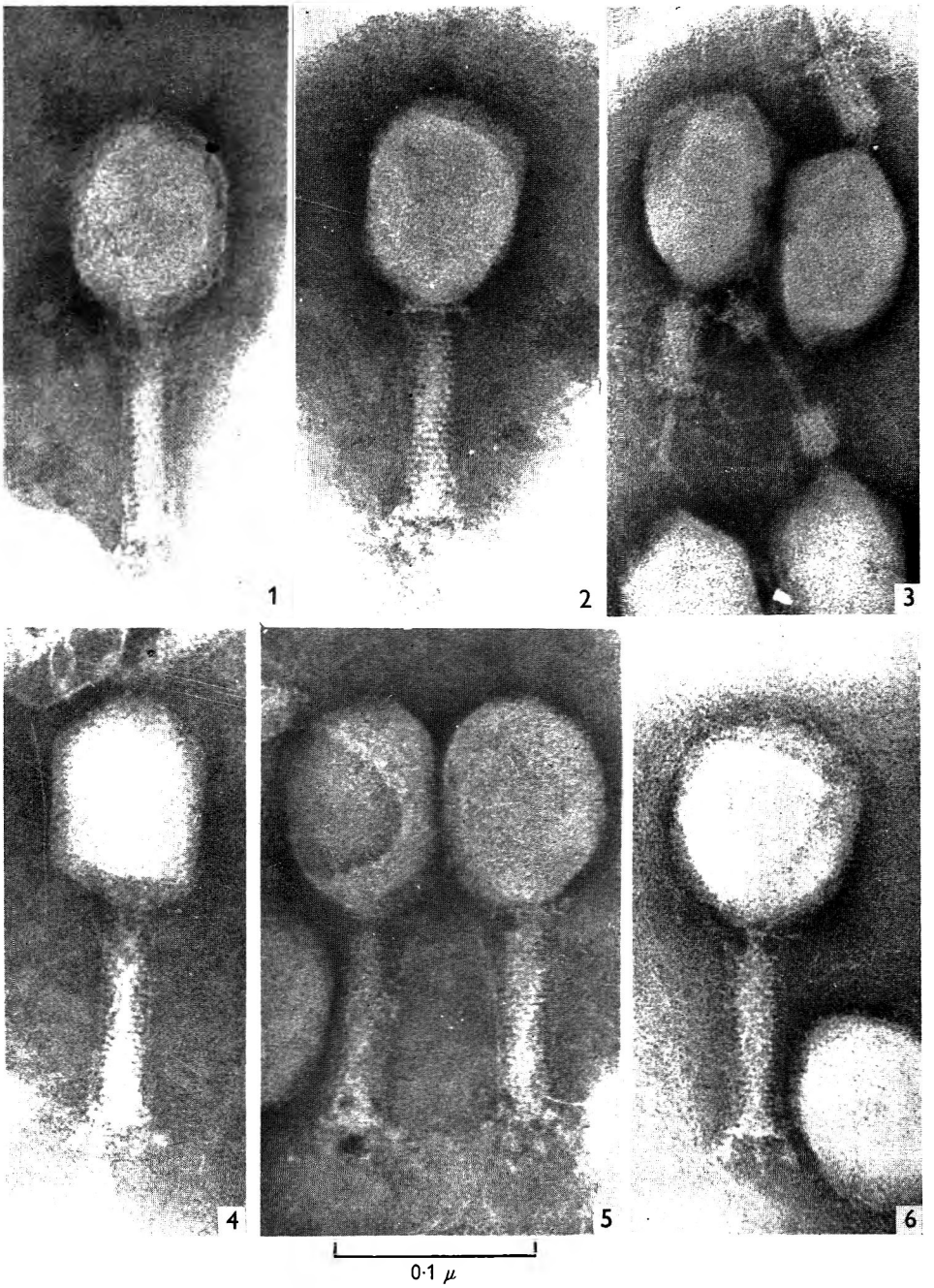
Fig. 14. Phage T4 in ammonium acetate and sodium molybdate, × 333,000.
Fig. 15. Phage ØR in ammonium acetate and sodium molybdate. × 333,000.
Fig. 16. Phage T4 in ammonium carbonate and sodium molybdate, × 333,000.
Fig. 17. Phage T2 in ammonium acetate and uranyl acetate, shadowed $\tan^{-1}\frac{1}{4}$, × 150,000.
Fig. 18. Phage T2 in ammonium acetate and uranyl acetate, × 333,000.
Fig. 19. Phage T2 treated with uranyl acetate, × 330,000.
Fig. 20. Phage T2 suspended in ammonium acetate only, × 333,000.

PLATE 4

Fig. 21. Phage ØR in ammonium acetate and uranyl acetate, × 333,000.
Fig. 22. Staphylococcus phage 70 in ammonium acetate and phosphotungstate, × 333,000.
Figs. 23, 24. Staphylococcus phage 70 in ammonium acetate and uranyl acetate, × 333,000.
Fig. 25. Phage T2 in ammonium acetate and uranyl acetate with EDTA, × 333,000.
Fig. 26. Phage T2 in ammonium carbonate and uranyl acetate with EDTA, × 333,000.

PLATE 5

Fig. 27. Phage T2 in ammonium acetate and uranyl nitrate, ×333,000.
Fig. 28. Phage T2 in ammonium acetate and uranyl nitrate with EDTA, ×333,000.
Fig. 29. The same 'high and dry', ×333,000.
Fig. 30. Phage ØR in ammonium acetate and lanthanum acetate, ×333,000.
Fig. 31. Phage ØR in ammonium acetate and thorium nitrate, ×333,000.
Fig. 32. Phage T2 in ammonium acetate and thorium chloride, ×333,000.
Fig. 33. Phage 1C in ammonium acetate and phosphotungstate, ×333,000.



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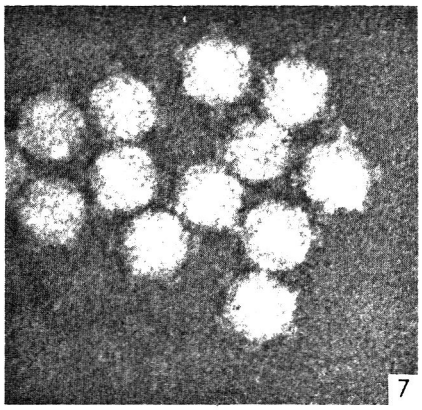
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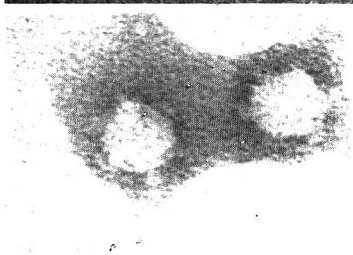
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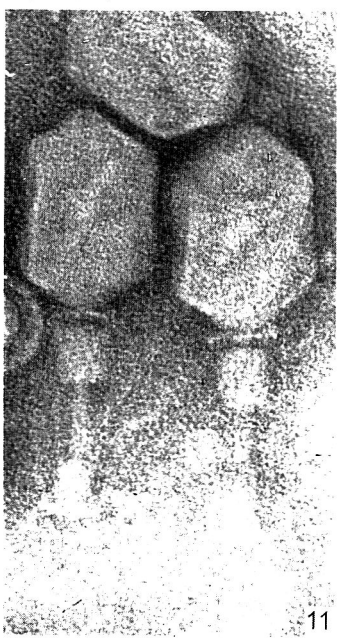
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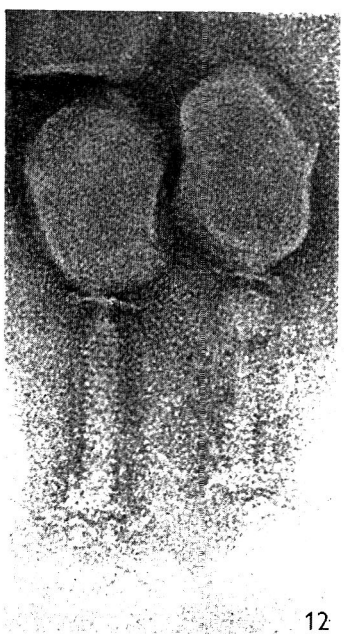
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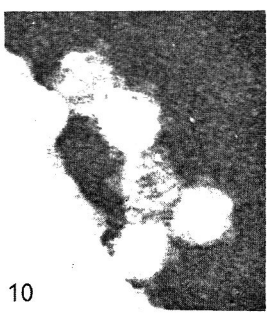
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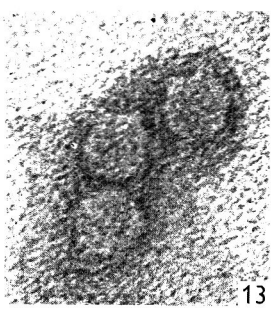
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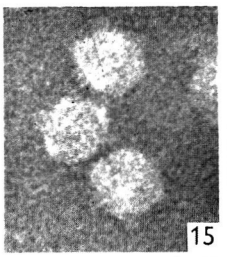
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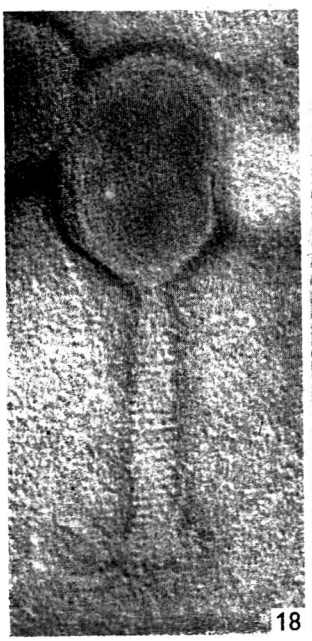
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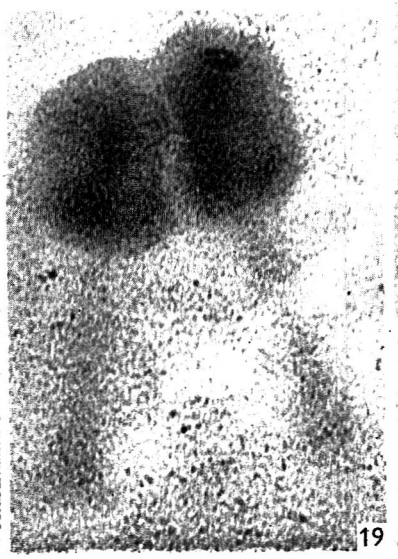
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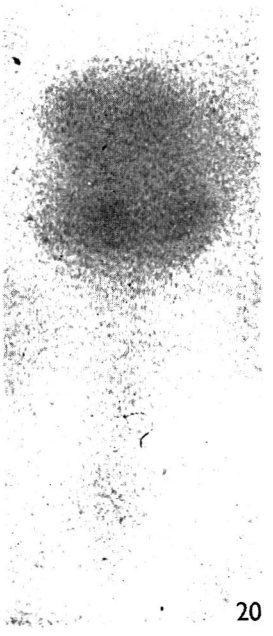
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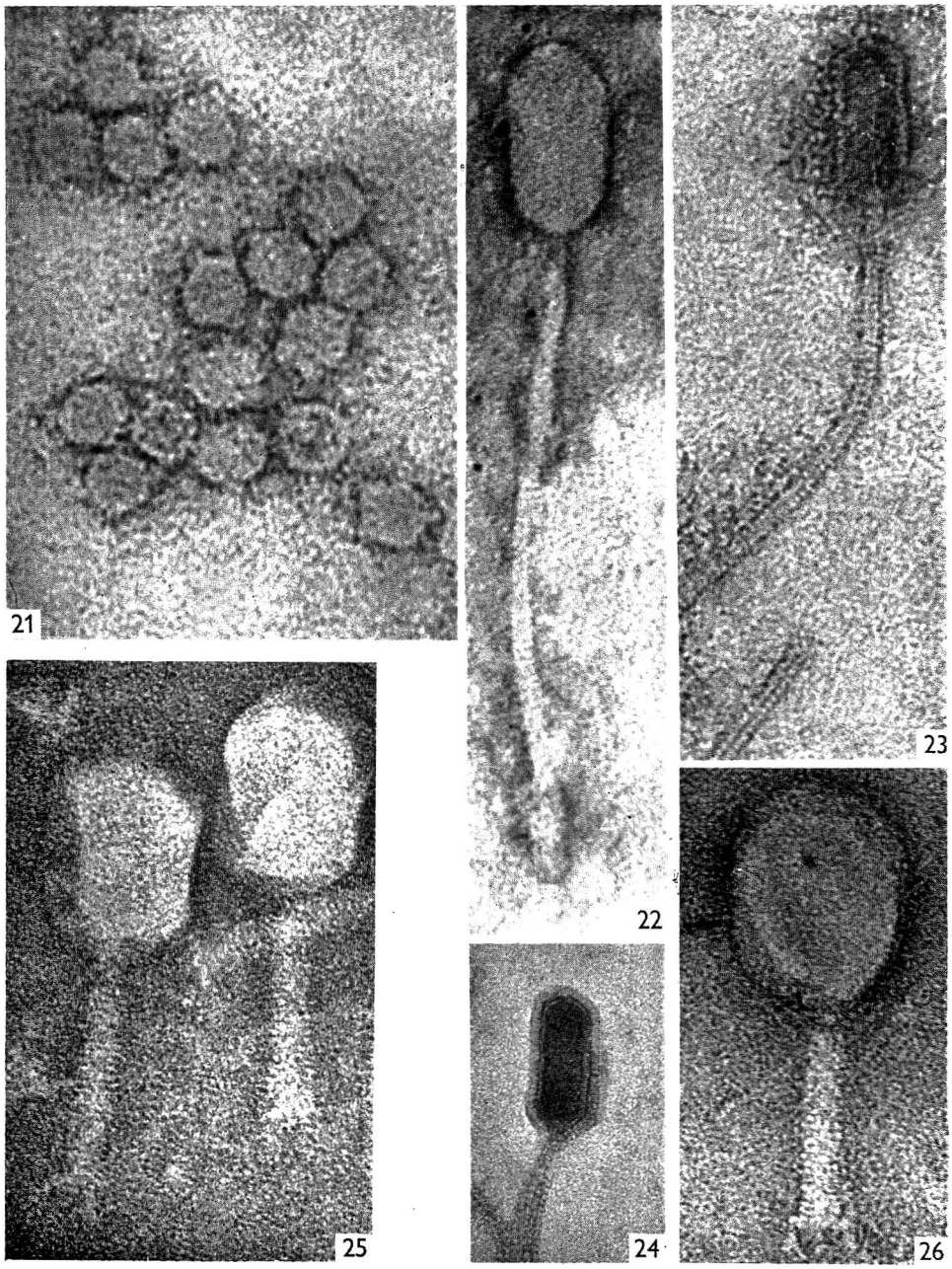

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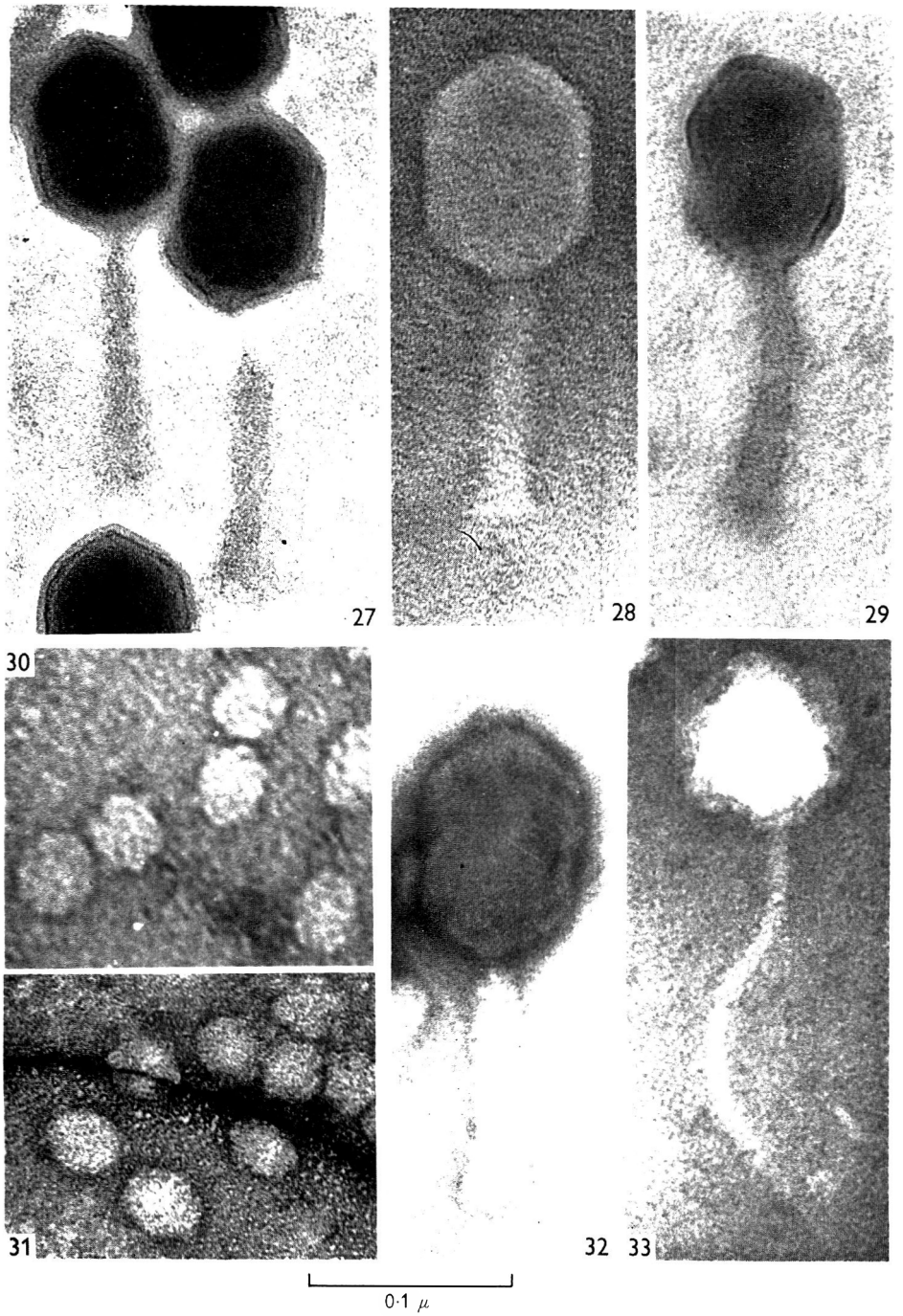
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Purification of the Third Factor of Anthrax Toxin

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SUMMARY

Factor III of anthrax toxin, which increases the lethality of mixtures of factors I and II for mice and decreases their capacity to produce oedema in the skin of rabbits, has been purified. The final preparation showed a single peak in the ultracentrifuge and a single band on paper electrophoresis, but it might still contain more than one serological component. The final preparation was a protein (N = 15·1 %) containing all the usual amino acids but no carbohydrate, phosphorus, lipid or ash.

INTRODUCTION

Bacillus anthracis produces a specific lethal and oedema-forming toxin which was first recognized *in vivo*, and then produced *in vitro* (Smith, Keppie & Stanley, 1955; Harris-Smith, Smith & Keppie 1958; Thorne, Molnar & Strange 1960). At first the toxin appeared to consist only of two components, factor I and factor II, which acted synergistically (Smith *et al.* 1956; Thorne *et al.* 1960; Stanley, Sargeant & Smith 1960; Sargeant, Stanley & Smith 1960). During the further purification of factor I (Stanley & Smith 1961) a third component, factor III, was recognized in the following way. The final preparation of factor I was not toxic when injected alone; as expected a mixture of factor I with purified non-toxic factor II evoked oedema in the skin of a rabbit and killed mice. However, the concentration of this mixture which killed mice formed a much larger skin reaction in rabbits than did a comparable dose (based on mouse LD 50 dose) of either crude toxin or a mixture of crude factors I + II. An investigation of this observation led to the demonstration of a factor III, present in crude factor I or II, which (*a*) increased the lethality of mixtures of factors I and II for mice and decreased their capacity to produce oedema in rabbits; (*b*) was different serologically from factors I or II; (*c*) was non-toxic when injected alone; (*d*) was lethal for mice when mixed with factor II but not with factor I; (*e*) was present in the anthrax toxin produced *in vivo*. The present paper describes the purification and properties of factor III.

METHODS

Crude factor III from toxin produced in vitro. Crude anthrax toxin produced by the method of Thorne *et al.* (1960) was separated into crude factor I and crude factor II by filtration through sintered glass filters. Factor III was present in the crude factor I (absorbed on glass filters and eluted as described by Stanley & Smith 1961, except that the volume of saturated Na_2CO_3 used to elute the filters was increased to 8 ml.) and in the crude factor II (the filtrate). The relative amounts of factor III

in the eluate from the filters and in the filtrate varied with the particular batch of filters used, but the eluate from a filter always contained more than half the factor III in a volume (about 8 ml. from one filter) considerably less than that of the filtrate (1 l. from one filter). Therefore factor III was purified from the filter eluate (crude factor I).

Detection and estimation of factor III. Factor III was present in a solution which was non-toxic when injected alone but which formed a mixture lethal for mice when added to an otherwise non-lethal mixture of purified factor I (4μg.)+purified factor II (40μg.; see Stanley & Smith 1961, table 5). However, it was more convenient (especially with solutions which contained large amounts of factors I and II which affected the mouse test) and less expensive of materials, to fractionate factor III by its reaction in serological diffusion plates which used the system and antiserum ('spore' H533) described by Sargeant *et al.* (1960) and Stanley & Smith (1961). Preliminary fractionations (Stanley & Smith, 1961) had showed that under these conditions the factor III activities of solutions were associated with their ability to form a precipitation line which was different from that formed by purified factors I and II, and which appeared to be single, although there was some evidence (see later) that it might have been multiple. Fractionation of factor III was followed by comparing the null-points (the first dilution which did not form the factor III line) of different fractions. Finally, the toxicity (in association with factors I and II) for mice of the final preparation of factor III was compared with that of the original crude eluate, to check that the recovery of activity (mouse-toxicity) in the final product was in fact equal to the recovery indicated by the serological titrations (see later).

Purified factors I and II of anthrax toxin were prepared as described by Stanley & Smith (1961).

Analytical and physico-chemical methods. These were as described by Stanley & Smith (1961).

Biological tests for lethality in mice and oedema production in rabbits. These were described by Stanley & Smith (1961).

RESULTS

Purification of factor III

A preliminary fractionation of factor III from crude factor II (i.e. culture filtrate) was described by Stanley & Smith (1961), and this provided a basis for the first part of the purification of factor III from crude factor I (eluate from the filters).

Chromatography on diethylaminoethyl cellulose (DEAEC). Crude factor I (containing factor III; 320 ml., 'protein' 0·06%, null point of factor III line 1/64) was diluted with 0·005*I* phosphate buffer (pH 7·4; 2880 ml.) and applied to a column (4·5 cm., diam., 10 cm. long) of DEAEC (25 g.) which had been equilibrated with 0·1*I* phosphate buffer (pH 7·4); the flow rate was about 20 ml./min. The column was eluted with 0·12*I* phosphate buffer (pH 7·4; 500 ml.), 0·2*I* phosphate buffer containing 0·01*I* NaCl (pH 7·4; 2·5 l.) and 0·2*I* phosphate buffer containing 0·2*I* NaCl (pH 7·2; 600 ml.) in 8×75 ml. fractions. The latter buffer eluted the factor III (in the last six fractions, 450 ml.; null point of factor III line 1/16 to 1/32; 0·005% 'protein') which was contaminated with some factor I (about 5% of the 'protein' by comparative titration with purified factor I in serological gel-diffusion

plates). This fraction was concentrated and the contamination with factor I decreased by the following procedure. After dialysis (overnight, 0–2°) against 0·01 *I* phosphate buffer (pH 7·4), it was applied to a column (2 cm. diam, 10 cm. long), of DEAEC (3 g.) which had been equilibrated with 0·1 *I* phosphate buffer (pH 7·4); the flow rate was about 8 ml./min. The column was eluted with 0·2 *I* phosphate buffer containing 0·01 *I* NaCl (pH 7·4; 250 ml.) followed by 0·2 *I* phosphate buffer containing 0·2 *I* NaCl (pH 7·2; 10 fractions of 10 ml.). Fractions 3–7 of the latter buffer contained the factor III (50 ml.; 'protein' 0·036 %, null point of factor III line 1/160). Although contamination with factor I had been decreased (less than 2 % of the 'protein'), examination of the preparation of factor III in the ultracentrifuge showed some smaller and larger molecular material in addition to the main component. The material was then fractionated on hydroxylapatite.

Chromatography on hydroxylapatite. The material (50 ml.; 'protein' 0·036 %; null point of factor III line 1/160) was dialysed overnight at 0–2° against 0·1 M-potassium phosphate buffer (pH 7·0) and applied to a column (2 cm. diam., 10 cm. long) of hydroxylapatite (3 g.) which had been equilibrated with 0·1 M-potassium phosphate buffer (pH 7·0). A slight positive pressure was used to produce a flow rate of about 1–2 ml./min. The column was eluted with 0·1 M-potassium phosphate buffer (pH 7·0; 30 ml.) followed by 0·4 M-potassium phosphate buffer (pH 7·0; 50 ml.) in 10 × 5 ml. fractions. Factor III was removed by the second buffer and concentrated in 3 of the later fractions which were bulked and concentrated as described below.

Concentration by precipitation with ammonium sulphate. The preparation of factor III (15 ml., 0·1 % 'protein', null point of factor III line 1/320) was mixed with ammonium sulphate (7·5 g.). After standing overnight (0–2°) the precipitate was collected by centrifugation (12,000 *g*; 30 min.; 0°), dissolved in 0·05 *μ* phosphate buffer (1·0 ml.; pH 7·4) and dialysed for 48 hr. at 0–2° against frequent changes of 0·05 *μ* phosphate buffer (pH 7·4). The preparation was stored at −20°.

Recovery of factor III activity. In the assay by serological precipitation the final solution (1·5 ml., 'protein' 0·85 %, null point of factor III line 1/3200) contained 25–30 % of the factor III originally present in the crude eluate (320 ml., 'protein' 0·06 %, null point of factor III line 1/64). This recovery of factor III activity was also indicated by the following toxicity tests. When added to a mixture of factor I (4 *μ*g.) and factor II (40 *μ*g.) which alone killed only 10/26 mice, the final preparation of factor III in doses of 0·0005 ml. and 0·00025 ml. killed 22/28 and 10/20 mice, respectively. Since 1·5 ml. of the final preparation came from 320 ml. of crude eluate, (about 25 % recovery of activity was indicated) about 0·025 and 0·0125 ml. of crude eluate should have produced similar death rates in mice as the above doses of purified preparation when tested under similar conditions. For this, the content of factor I in the crude eluate (which is crude factor I − crude factor III) was estimated (by serological precipitation; see Stanley & Smith 1961) and adjusted to 4 *μ*g./dose by addition of purified factor I before adding purified factor II (40 *μ*g./dose) and injecting the mixture into mice. Under these conditions, 0·025 and 0·0125ml. of crude eluate killed 21/30 and 18/30 mice, respectively; these are death rates similar to those produced by the corresponding doses of purified preparation.

The final product was used as prepared (i.e. in 0·05 *μ* phosphate buffer at pH 7·4) for ultracentrifugal, electrophoretic and serological analyses and for biological tests; for chemical analysis the solution was thoroughly dialysed and freeze-dried.

Criteria of purity

Ultracentrifugation. The final preparation showed a single peak in the ultracentrifuge (Fig. 1).

Paper electrophoresis. The final preparation of factor III was concentrated by dialysis against Carbowax. When examined in 0·2 μ barbitone buffer (pH 8·6) the material (0·5 mg. dry wt.) migrated, as a single band (9 cm.; in the system described by Stanley & Smith 1961), towards the anode. It was not examined at an acid pH value since the concentrated solution precipitated at pH 5·9.

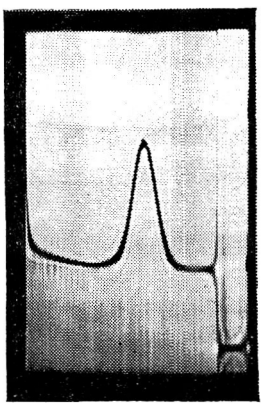

Fig. 1. Ultracentrifuge diagram of the best preparation of factor III (0·85 %, w/v). Sedimentation in 0·05 μ phosphate buffer (pH 7·4), field 100,000 *g*. Picture taken 48 min. after reaching maximum speed. Sedimentation from right to left.

Serological precipitation in gels. Against spore antiserum (H.533) the final preparation of factor III (70 μg.) formed a strong and a faint line; the latter was due to factor I. Appropriate comparative titration with purified factor I indicated that contamination with this factor was less than 2 %. The strong line appeared to be single (and remained so on dilution) and was different from the lines formed by purified factors I and II. However, when certain concentrated fractions containing mixtures of antigens from *Bacillus anthracis* were diffused side by side with the final preparation of factor III, two of the lines formed by these mixtures joined with the single line from factor III. Furthermore, when concentrated solutions were allowed to diffuse for long periods (23 days), on plates having a long distance between the diffusion wells, two closely associated lines were sometimes apparent. Hence the factor III line might be composite (compare purified factor II; Sargeant *et al.* 1960). Attempts to separate two possible components by fractionation on DEAEC and on hydroxylapatite, by fractional precipitation with ammonium sulphate and by precipitation at different acid pH values failed; all the fractions obtained from these procedures showed the same single line on serological diffusion plates.

Properties

Chemical analysis. The final preparation of factor III had N 15·1%; 'protein' (as ovalbumin) 90%; carbohydrate (as glucose) less than 0·5%; P less than 0·05%; lipid less than 1%; sulphated ash, less than 0·2%. Paper chromatography of a hydrolysate (6 N-HCl, 100°, overnight) showed all the usual amino acids and no others. The ultraviolet absorption spectrum was typical of a protein, and showed no evidence for the presence of nucleic acid.

Biological properties. The final preparation of factor III was not lethal for mice when injected (60 μg. per mouse) alone; when added to a mixture of purified factor I (4 μg.) and factor II (40 μg.) which alone killed only 10/126 mice, it (8, 4, 2 μg.) produced many mouse deaths (49/66, 22/28, 10/20 respectively). This activity of factor III was not decreased by allowing it to stand for 24 hr. at 0°, 18° or 37°. Addition of the final preparation of factor III to mixtures of purified factors I and II decreased the capacity of the latter mixtures to produce oedema in the skin of rabbits. Skin lesions of size 16:60 produced in rabbits by a mixture of factor I (1·6 μg.) + factor II (16 μg.); (compare Table 5, Stanley & Smith, 1961) were decreased to 7:23, 9:40, 11:50 by addition of 3·2, 1·6, 0·8 μg., respectively, of the best preparation of factor III. In serological diffusion plates 0·2 μg. of the best preparation of factor III still formed a line against spore antiserum (H 535). Against antigen antiserum (H 25), high concentrations of factor III formed only a hazy line which easily dispersed in antigen excess, indicating a low content of factor III antibody in this antiserum.

We are indebted to Mr F. C. Belton for producing the anthrax toxin and to Mr R. Blake for excellent technical assistance.

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With 1 *plate*
Printed in Great Britain

Purification of Pox Viruses by Density Gradient Centrifugation

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SUMMARY

Rabbit pox virus grown in tissue culture cells and vaccinia virus grown on rabbit skin have been purified as follows. The virus was sedimented and washed once by centrifugation to provide a tenfold virus concentrate. This semi-purified material was layered on top of a sucrose density gradient and centrifuged to produce a zone of virus separated from zones of impurity. Sucrose was removed from the purified virus suspension by thorough washing in the centrifuge. About 40 % of the original infectivity was recovered in the purified material which consisted almost entirely of virus particles when examined in the electron microscope. Control experiments indicated a maximum of 5 % protein impurity. Almost complete separation of vaccinia haemagglutinin and virus was obtained.

INTRODUCTION

Viruses of the pox group are generally purified by the technique of differential centrifugation based on the work of Craigie (1932) and Hoagland, Smadel & Rivers (1940) with vaccinia virus. Dawson & McFarlane (1948) found that vaccinia virus prepared by differential centrifugation was impure and that subsequent flocculation of the virus with M-NaCl eliminated the impurity. More recently, Gessler, Bender & Parkinson (1956), used 'fluorocarbon' (trifluorotrichloroethane) treatment to purify vaccinia virus but Epstein (1958) showed that centrifugation after fluorocarbon treatment sedimented impurity as well as the virus although a certain region of the sediment contained only virus. The haemagglutinin and infectious virus particles of vaccinia have been separated on columns of diethylaminoethyl-cellulose (DEAE-cellulose; McCrea & O'Loughlin, 1959) but the amount of virus recovered was not reported. Dumbell, Downie & Valentine (1957) found that the infectivity titres of crude cowpox virus preparations were increased by treatment with trypsin although the infectivities of both purified cowpox virus and crude vaccinia virus were decreased. We attempted to purify rabbit pox virus, grown in cultivable cells, by the technique of differential centrifugation but the product was substantially contaminated with non-viral particles. The present work shows briefly that other published methods were also unsatisfactory for purifying rabbit pox virus and describes a method of purification by centrifugation in a sucrose density gradient.

METHODS

Crude rabbit pox virus. Confluent monolayer cultures containing about 5×10^7 ERK1 cells (Westwood, Macpherson & Titmuss, 1957) were infected with rabbit pox virus (Utrecht strain, which produces no haemagglutinin) and incubated with 50 ml.

medium for 26 hr. at 37°. The medium (Westwood, Macpherson & Titmus, 1957) contained 5% calf serum. The cultures were shaken to complete detachment of the cells from the glass and the cells separated from the medium by centrifuging at 200 *g* for 10 min. The cells (4×10^7 cells/ml.) were resuspended in phosphate buffered saline (pH 7·3) and disrupted to liberate the virus by ultrasonic treatment with a 500 W. Soniclean Generator at 40 kcyc./sec. (Dawe Instruments Ltd., London). Large particles of cell debris were removed by centrifuging at 800 *g* for 5 min.

Crude vaccinia virus. Vaccinia virus was grown on the skin of rabbits for 48 hr. as described by Hoagland *et al.* (1940). Coloured rabbits were as satisfactory as white rabbits. The skin scrapings of each rabbit were collected into 25 ml. of 0·004 M-McIlvaine buffer (pH 7·8) containing 0·1% sodium azide to inhibit growth of contaminating organisms (buffer + azide). The suspension of crude virus was shaken with glass beads and then centrifuged at 800 *g* for 10 min. to sediment the largest particles. The deposit was re-extracted by shaking with half the original volume of buffer + azide, centrifuged as before and the two supernatant fluids combined.

Protein estimation. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) and expressed as mg./ml.

Virus infectivity. Virus was titrated on chicken embryo chorioallantoic membranes as described by Westwood, Phipps & Boulter (1957) and expressed as pock-forming units (pfu)/ml. or pfu/mg. protein.

Vaccinia virus haemagglutinin. Haemagglutinin was titrated in calcium magnesium saline containing 1% normal rabbit serum (Fenner, 1958); 0·1 ml. volumes of serial twofold dilutions and 0·5% suspension of vaccinia sensitive fowl cells were mixed and left at room temperature 1 hr. Results were expressed as the reciprocal of the final dilution producing haemagglutination.

Purification process

Preliminary centrifugation. The virus was sedimented from the crude suspension by centrifugation in an angle rotor (Spinco Model L Ultracentrifuge) at 35,000 *g* for 30 min. and the supernatant fluid, containing most of the protein, discarded. The deposit was resuspended in a volume equal to the original volume of buffer + azide and centrifuged in the same way. This washed deposit was resuspended in one-tenth of the original volume of buffer + azide and is referred to as semi-purified virus. Large volumes of crude vaccinia virus were satisfactorily centrifuged in the Spinco batch rotor no. 1600 at 30,000 *g* for 60 min.

Centrifugation in sucrose density gradients. Lusteroid tubes (3 × 1 in.) were filled by layering successively from pipettes 6 ml. each of 60, 50, 40 and 30% (w/v) sucrose in buffer + azide followed by 6 ml. of semi-purified virus. The tubes were centrifuged at room temperature in the Spinco swinging bucket rotor no. SW 25 at 39,000 *g* for 20 min. and the rotor decelerated by using the brake. Purified virus formed an opalescent zone in the 50% (w/v) sucrose layer with zones of impurity above and below.

Separation of zones in density gradients. The technique used for separating and removing zones from the centrifuge tube was a simplified and reversed procedure of the method described by Hogeboom & Kuff (1954). The centrifuge tube was held in a clamp which could be slowly elevated by means of a screw. A perforated Perspex disk (thickness $\frac{1}{16}$ in., diameter slightly less than the centrifuge tube and with

106 conically shaped holes of diameter 0·02 in. and 0·06 in. at the upper and lower surfaces of the disk, respectively) was clamped by a central stainless steel rod so that the disk entered the rising centrifuge tube. The level of the disk in the tube was successively adjusted to coincide with the boundaries of the required zones which were then easily removed from above with a pipette. Corresponding zones from all the gradients of one batch of semi-purified virus were then combined.

Final washing to remove sucrose. The purified virus in about 50 % (w/v) sucrose was diluted with 2 vol. of 0·004 M-McIlvaine buffer (pH 7·8) and centrifuged at 35,000 *g* for 60 min. The deposited virus was washed twice with buffer and three times with water using a volume equal to the original sucrose suspension and centrifugation at 35,000 *g* for 30 min. for each wash. The final deposit of virus was suspended in water and stored at −60°.

RESULTS

Application of published methods to purification of rabbit pox virus

Flocculation with NaCl. Salt was added to a suspension of rabbit pox virus, previously purified by centrifugation, to make M-NaCl. A precipitate formed slowly at 4°; it was collected after 3 days, washed with water and suspended in 0·004 M-phosphate buffer (pH 7·6) by ultrasonic treatment. Only 10 % of the virus infectivity was recovered in the precipitated fraction. Of two samples of vaccinia virus treated similarly, one retained its infectivity but the other lost 99·5 %.

Table 1. *Fluorocarbon treatment of crude rabbit pox virus*

No. of treatments	Infectivity recovered (%)	Protein recovered (%)	pfu/mg. protein ($\times 10^{-8}$)
0	100	100	1·7
1	86	51	2·9
2	56	28	3·5
3	16	20	1·5
4	7	12	0·9

Treatment with fluorocarbon. After removing large particles of cell debris, crude rabbit pox virus suspension was homogenized with a half-volume of trifluorotrichloroethane (Arcton 113, I.C.I. Ltd., London) for 1 min. The aqueous phase was separated by centrifugation and treated in the same way thrice more. Table 1 shows that a little purification of the crude virus was obtained at first but repeated treatment removed both virus and protein impurity from the material. When a single treatment was carried out by homogenizing a similar mixture for 20 min. with ultrasonic vibration, only 1 % of the infectivity and 12 % of the protein was recovered. The fluorocarbon method would have appeared more effective if the initial virus suspension had contained large particles of cell debris since these would have sedimented during the centrifugation following the fluorocarbon treatment. However, the large particles were easily removed by preliminary centrifugation of the crude virus and it was concluded that little advantage would be gained by including fluorocarbon treatment as a preliminary step in the purification of this material.

Adsorption on DEAE cellulose. Rabbit pox virus was retained by columns of DEAE-cellulose, but only about 5% of the infectivity could be eluted using NaCl up to 3 M at pH 6, 7 or 8. When the DEAE-cellulose was subsequently removed from the column and shaken with buffer, a further 10% of the infectivity was recovered suggesting that the virus had been retained mechanically in the column of DEAE-cellulose.

Treatment with trypsin. Crude rabbit pox virus preparations were treated with 0·2% Difco 1:250 trypsin or 0·01% Armour crystalline trypsin for 30 min. at 20°. The effect on infectivity titres of the preparations varied from no change to a three-fold increase. However, when the treated virus was sedimented and washed in the centrifuge, up to 99% of the infectivity was lost as compared with untreated control samples. Purified rabbit pox virus and purified vaccinia virus both lost infectivity when treated with 0·01% crystalline trypsin.

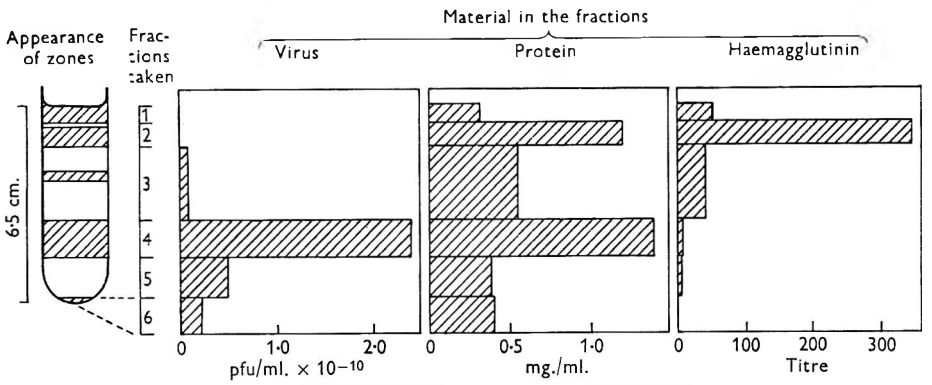

Fig. 1. Separation of vaccinia virus, haemagglutinin and protein impurity after centrifugation of semi-purified virus in a sucrose density gradient.

Purification of rabbit pox and vaccinia viruses by density gradient centrifugation

Separation in the sucrose density gradient. Essentially similar results were obtained with rabbit pox and vaccinia viruses but the latter provides a more detailed example since it was associated with haemagglutinin. Figure 1 illustrates the separation obtained after centrifuging a density gradient of semi-purified vaccinia virus. The majority of the virus was present in a well-defined opalescent zone separated from zones of protein impurity which contained relatively little virus. Almost complete separation of haemagglutinin and infective virus occurred. In some early experiments a large proportion of the virus sedimented below the typical virus zone into the deposit at the bottom of the density gradient. This appeared to be due to aggregation of the virus and did not occur when the following three conditions were adhered to: (1) the use of buffer at pH 7·8 to suspend the virus and to buffer the sucrose solution; (2) dispersion of the virus in the semi-purified suspension by ultrasonic treatment (5 min. with Dawe apparatus) just before placing it on the density gradient; (3) processing the semi-purified virus as soon as possible.

Yield from the purification process. Tables 2 and 3 show the range of results

obtained in several purifications of rabbit pox and vaccinia virus. Most of the original infectivity was recovered in the semi-purified virus and about 50 % in the virus zone of the sucrose density gradients. During the subsequent washing of purified virus to remove sucrose some infectivity was lost, probably because of aggregation of virus. The final washes with water caused pronounced macroscopic aggregation so that the virus would not remain suspended but settled out of the water on standing. The washed virus could be largely disaggregated by dilution into buffer and ultrasonic treatment so that stable suspensions were formed again. However, the infectivity/mg. protein was usually less than before washing, sometimes as much as threefold with vaccinia virus.

Table 2. *Purification of rabbit pox virus; range of virus infectivity and protein recovered*

Virus suspension	Relative volume	Infectivity recovered (%)	Protein recovered (%)	pfu/mg. protein ($\times 10^{-8}$)
Initial Crude*	120	100	100	0·5–2
Semi-purified	12	75–100	5–13	10–20
Purified—in sucrose	13	40–70	0·6–2·5	31–61
Purified—washed	5	30–50	0·5–1·8	33–45

* Initial crude virus suspensions had 4×10^8–1×10^9 pfu/ml. and 5–10 mg. protein/ml.

Table 3. *Purification of vaccinia virus; range of virus infectivity and protein recovered*

Virus suspension	Relative volume	Infectivity recovered (%)	Protein recovered (%)	pfu/mg. protein ($\times 10^{-8}$)	Haemagglutinin/mg. protein
Initial crude*	120	100	100	6–20	5–10
Semi-purified	12	60–100	6–16	80–250	30–100
Purified—in sucrose	14	30–70	2–6	100–240	0–8
Purified—washed	10	15–60	1·5–4	50–150	0–8

* Initial crude virus suspensions had 3×10^9–1×10^{10} pfu/ml. and 5–7 mg. protein/ml.

Smadel, Pickels & Shedlovsky (1938) demonstrated an osmotic effect of sucrose solutions on vaccinia virus by detecting changes in the virus sedimentation rate. The possibility that osmotic shock caused loss of virus infectivity during the washing to remove sucrose was tested. However, no loss of infectivity was detected when suspensions of virus in 60 % (w/v) sucrose solution were rapidly diluted. It was concluded that the osmotic changes which occurred when the purified virus in sucrose was diluted and centrifuged to wash away the sucrose did not cause the loss of infectivity but that aggregation of virus particles during the washing was the most probable cause.

Purity of the virus preparations

Electron microscopy. Plate 1 shows electron micrographs of typical purified samples of the two pox viruses. The samples were sprayed on to the support film and shadowed without further treatment (e.g. draining, washing, dialysis) in order that all impurities should be detectable. Dense areas containing clumps as well as

isolated particles were selected for photography in preference to less dense areas, since the former were found to constitute a more exacting test for purity. In the rabbit pox preparations, apart from flattened particles resembling virus ghosts, non-viral debris was readily detectable but, nevertheless, constituted only a small proportion of the total material present. In the vaccinia preparations much less non-viral material was present.

Processing of control material. When uninfected tissue culture cells were disrupted and submitted to the virus purification process, the yield of protein in the fraction corresponding to rabbit pox virus was 0·025 %. Comparison with the yield of protein in the purified virus fraction from infected cells indicated a contamination of up to 5 % of non-viral protein in the rabbit pox preparations. It was not possible to make a similar comparison for vaccinia virus grown on the skin of rabbits because comparable starting material could not be scraped from uninfected skins.

DISCUSSION

Centrifugation in density gradients has been used for purification of several viruses, notably those obtained from plants (Brakke, 1960). It has been customary to purify the relatively large pox viruses by differential centrifugation and it is clearly a refinement to employ the greater resolution obtainable by using a density gradient. In the method described here, sucrose was chosen to prepare the density gradients rather than a salt because the latter at high concentration aggregates and precipitates pox viruses. For the centrifugation it was essential to use a swinging bucket rotor since in an angle rotor the material could not sediment through the density gradient and was deposited on the outer side of the centrifuge tubes.

The infectivity/mg. protein of crude vaccinia virus was about ten-fold greater than that of crude rabbit pox virus. During purification, the infectivity/mg. protein of rabbit pox virus was increased more than that of vaccinia virus, but purified vaccinia virus in sucrose was still about four times more infective than similarly purified rabbit pox virus. In the final washed preparations, the difference was only about twofold since vaccinia virus suffered a greater loss of infectivity during the washing. Examination in the electron microscope and processing of uninfected control material both showed that the lower infectivity of purified rabbit pox virus was not due to the presence of large amounts of impurity. Presumably it was due to greater aggregation or to the presence of a higher proportion of 'non-infective' virus particles which did not give pocks in the infectivity assay used.

While treatment with reagents, such as fluorocarbon and trypsin, which act on protein are advantageous with very crude pox virus preparations, they are detrimental to the virus when large excesses of protein impurity have been removed. Suspensions of purified virus were susceptible to attack by contaminating bacteria and moulds, probably due to protease action in a similar manner to trypsin. Growth of contaminating organisms and consequent possible loss of virus infectivity was minimized by incorporating 0·1 % sodium azide in the virus suspensions during purification and by storing the purified material at −60.

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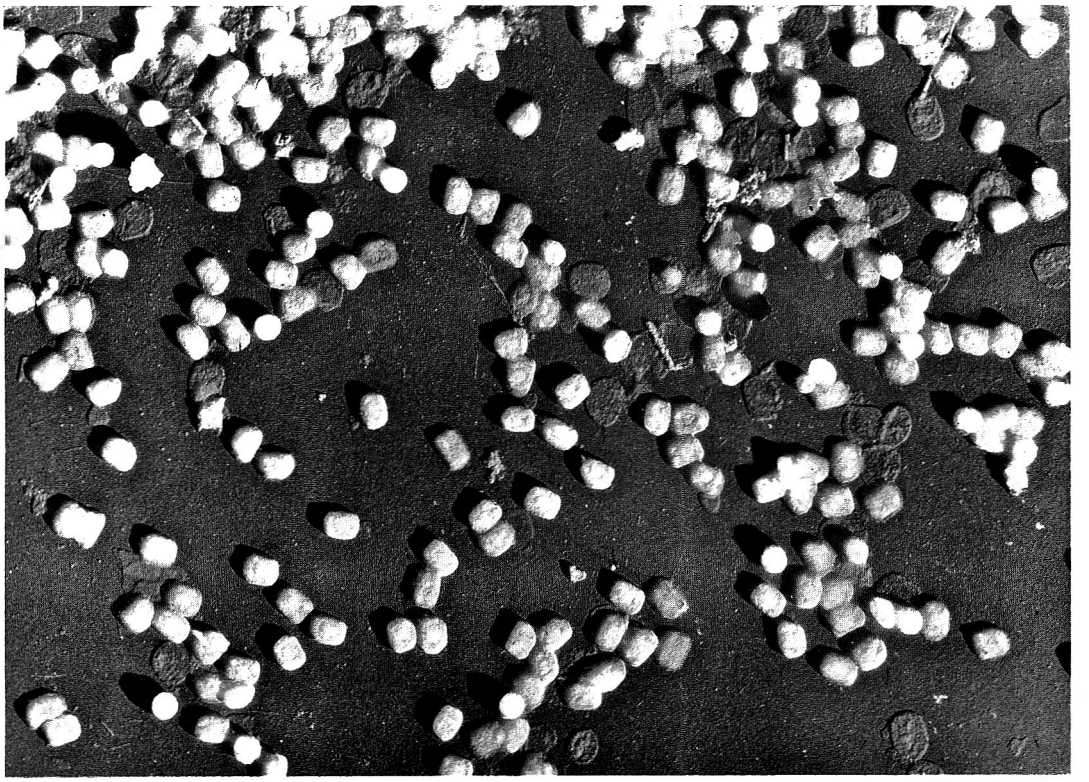

Fig. 1

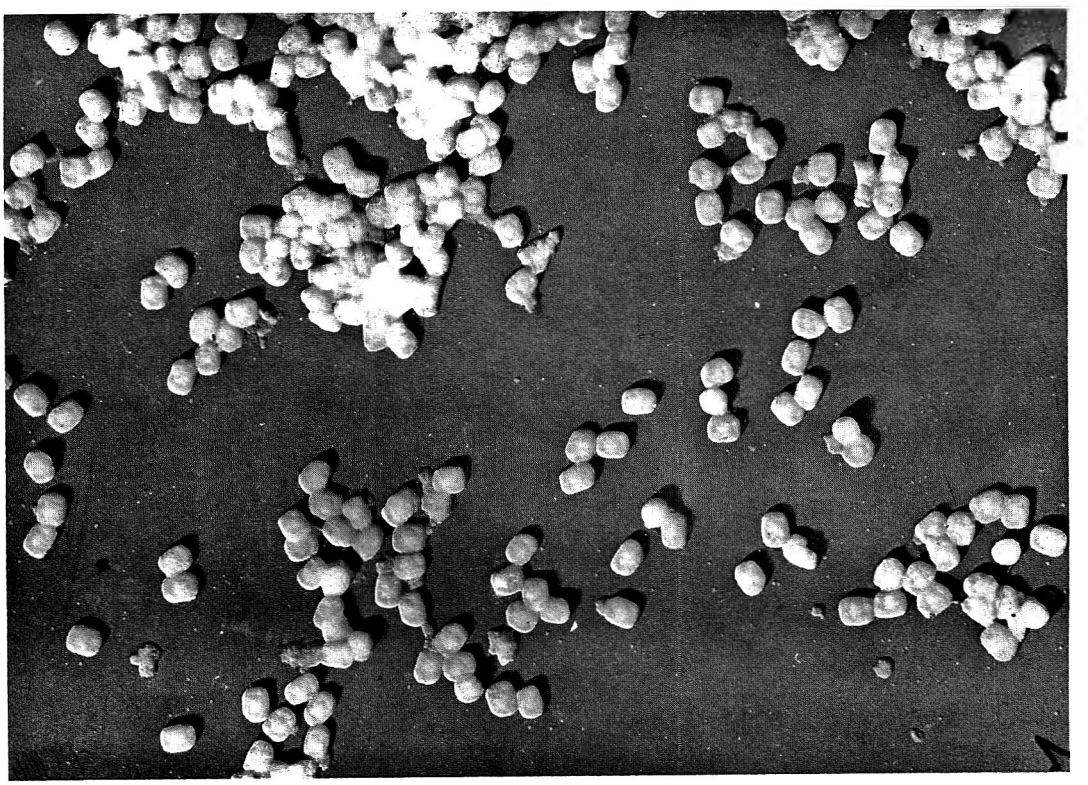

Fig. 2

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EXPLANATION OF PLATE 1

Electronmicrographs of purified pox viruses. Specimens were sprayed on to collodion supports and shadowed with gold-palladium. × 17,000.

Fig. 1. Rabbit pox virus from tissue culture cells.

Fig. 2. Vaccinia virus from rabbit skin.

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The Effect of Growth Conditions on Oxidative and Dehydrogenase Activity in *Staphylococcus aureus*

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SUMMARY

Suspensions of *Staphylococcus aureus* grown aerobically on nutrient broth oxidized glucose, acetate and intermediates of the tricarboxylic acid cycle, but glucose-grown organisms oxidized glucose only. The inability of glucose-grown staphylococci to oxidize intermediates of the tricarboxylic acid cycle was correlated with diminished succinate and isocitrate dehydrogenase activity in cell-free extracts as compared with extracts from organisms grown without glucose. Suspensions of aerobically-grown staphylococci fermented glucose anaerobically at only about one third the rate observed with anaerobically-grown organisms. The nicotinamide-adenine dinucleotide-linked lactate dehydrogenase activity in extracts of the anaerobically-grown organisms was about ten times higher than that in extracts of aerobically-grown staphylococci.

INTRODUCTION

Previous work with *Staphylococcus aureus* showed that suspensions of glucose-grown organisms oxidized glucose to acetate but the latter substance was not metabolized further (Gardner & Lascelles, 1962). However, there are reports in the literature that suspensions of staphylococci grown in nutrient broth are capable of oxidizing acetate and tricarboxylic acid cycle intermediates although these substrates are not oxidized to completion (Goldschmidt & Powelson, 1953; Stedman & Kravitz, 1955). Goldschmidt & Powelson (1953) also observed that organisms grown with glucose failed to oxidize acetate. In the present work the effect of growth on glucose upon the ability of suspensions to oxidize intermediates of the tricarboxylic acid cycle was examined and the oxidative capacity has been correlated with the degree of activity of certain enzymes of the cycle. The effect of aerobic growth on the ability of suspensions to ferment glucose and upon the activity of lactate dehydrogenase in cell-free extracts was also examined.

METHODS

Organisms. The parent strain of *Staphylococcus aureus* (SG 511 A) was that described previously (Gardner & Lascelles, 1962). The streptomycin-resistant strain used was isolated following growth of the SG 511 A strain on a streptomycin gradient agar plate (Szybalski & Bryson, 1952); it grew in the presence of streptomycin 200 μg./ml. Stock cultures of both strains were maintained on nutrient agar slopes grown for 18 hr. at 37°.

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Growth of organisms and preparation of suspensions. Cultures were grown in Oxoid nutrient broth (Oxoid Ltd. London) supplemented where shown with 0·04 M-glucose (added after autoclaving). Inoculation was with suspensions from nutrient agar slopes, the initial concentration being equivalent to about 0·01 mg. dry wt./ml. The cultures were grown aerobically on a gyrorotatory shaker or anaerobically in completely filled glass-stoppered bottles. Incubation was at 37° for 18 hr. The organisms were harvested and suspensions prepared as described previously (Gardner & Lascelles, 1962).

Manometry. Conventional Warburg techniques were used; details of vessel contents and atmosphere are given in the text. Incubation was at 37°.

Preparation of extracts and assay of enzymic activity. The preparation of cell-free extracts, the determination of protein and the assay of lactate dehydrogenase were described previously (Gardner & Lascelles, 1962). Isocitrate (NADP as acceptor) and succinate dehydrogenase activities were assayed spectrophotometrically by the methods of Ochoa (1955) and Redfearn & Dixon (1961), respectively. The activity of all three enzymes is expressed as μmoles substrate oxidized/min./mg. protein. The assays were carried out with an Optica recording spectrophotometer, Model CF4DR (Optica Ltd., Gateshead, Co. Durham).

RESULTS

Effect of glucose on oxidative and enzymic activities

Oxidative activities of suspensions. Suspensions of the parent strain of *Staphylococcus aureus* grown aerobically in broth without glucose consumed oxygen in the presence of glucose, acetate or dicarboxylic acids at rates in excess of that observed without substrate (Table 1). In contrast, organisms grown with glucose did not oxidize acetate or the dicarboxylic acids, though the rate of oxygen uptake with glucose was similar to that of organisms grown without this sugar (Table 1).

The effect of growth with glucose on the respiratory activity might have been due (*a*) to acid production which impaired the ability of organisms to oxidize sub-

Table 1. *Oxidation of substrates by a streptomycin-sensitive and a streptomycin-resistant strain of* Staphylococcus aureus *grown without and with glucose*

The organisms were harvested after 18 hr. aerobic incubation in broth, supplemented where shown with 0·04 M-glucose. Suspensions were incubated in air at 37° in Warburg vessels containing: organisms, equiv. 2·5–5·0 mg. dry wt.; potassium phosphate buffer (pH 7·4) 250 μmoles; substrate (added from side arm at zero) 40 μmoles; water to 2·5 ml. The centre wells contained 0·2 ml. 5 N-NaOH. The sodium salts of the organic acids were used. The Q_{O_2} values are calculated from the rates observed during the first 30 min. incubation, after subtraction of the endogenous value.

	Parent strain grown		Streptomycin-resistant strain grown	
Substrate	Without glucose	With glucose	Without glucose	With glucose
	Q_{O_2} values			
Nil	16	8	20	10
Glucose	52	47	49	40
Acetate	13	0	3	0
Succinate	39	0	17	0
Malate	28	0	7	0

strates other than glucose, or (*b*) to a faster growth rate with glucose present resulting in the organisms being physiologically 'aged' at harvest. These possibilities were eliminated by testing the activity of organisms grown in glucose broth buffered with 0·04 M-potassium phosphate (pH 7·4) and harvested during the exponential phase of growth; the culture at harvest was at pH 7·1. Such organisms again showed no ability to oxidize substrates other than glucose.

The streptomycin-resistant strain behaved qualitatively in the same way as the parent organism, but when grown without glucose it oxidized acetate and the dicarboxylic acids less actively, though the Q_{O_2} with glucose as substrate was similar to the parent (Table 1). Growth with glucose completely suppressed the ability to oxidize all substrates except glucose.

Suspensions of the parent-strain grown on galactose behaved similarly to those grown on glucose. The situation in *Staphylococcus aureus* differs, therefore, from that in *Saccharomyces cerevisiae* which exhibits enhanced oxidative activity when grown on galactose instead of glucose (Strittmatter, 1957).

Activity of succinate and isocitrate dehydrogenases in cell-free extracts. The inability of glucose-grown organisms to oxidize acetate and intermediates of the tricarboxylic acid cycle suggested that one or more enzymes of this sequence might be lacking. Assay of two enzymes of the cycle, namely succinate and isocitrate dehydrogenases, showed them to be present in extracts from organisms grown aerobically without glucose; but in extracts from glucose-grown organisms isocitrate dehydrogenase was not detected and the activity of succinate dehydrogenase was considerably decreased (Table 2). Lactate dehydrogenase activity was not influenced by growth with glucose. The enzymic activities in extracts of the streptomycin-resistant staphylococcus were similar to those of the parent grown under comparable conditions (Table 2).

Table 2. *Succinate and isocitrate dehydrogenase in extracts of staphylococci*

Dehydrogenase activity was assayed as described in the Methods section in extracts prepared from organisms grown aerobically on nutrient broth alone or with 0·04 M glucose. Results are expressed as units of enzyme/mg. protein and are the mean of at least three determinations with different extracts showing less than 20 % variation.

Strain	Glucose in growth medium	Succinate dehydrogenase	Isocitrate dehydrogenase	Lactate dehydrogenase
		Units of enzyme/mg. protein		
Parent	−	0·13	0·16	0·92
	+	0·02	< 0·01	0·68
Streptomycin-resistant	−	0·09	0·15	—*
	+	0·02	< 0·01	—

* Not tested.

Effect of growth under aerobic and anaerobic conditions on the fermentation of glucose and on lactate dehydrogenase activity

Suspensions of the parent staphylococcus grown aerobically with glucose, fermented glucose anaerobically at only about 30 % of the rate observed with organisms grown anaerobically (Table 3). In these experiments acid production from glucose

in a bicarbonate+CO_2 buffer was taken as an index of fermentative activity. Previous work had shown that lactic acid was the main end-product of glucose fermentation by this organism (Gardner & Lascelles, 1962).

Assay of lactate dehydrogenase in extracts of organisms grown aerobically and anaerobically showed that this activity was about ten times higher in preparations from anaerobically-grown staphylococci (Table 3); the enzyme was specific for the L(+)isomer. Thus, there was a correlation between the rate of glucose fermentation and lactate dehydrogenase activity.

Table 3. *Fermentation and lactic dehydrogenase activity of staphylococci grown aerobically and anaerobically*

The parent strain *Staphylococcus aureus* (SG 511 A) was grown aerobically or anaerobically in nutrient broth + 0·04 M glucose. Fermentation by washed organisms was followed in manometric vessels containing: organisms, equiv. 2·5–5 mg. dry wt.; $NaHCO_3$, 25 μmoles; glucose (added from side arm at zero), 100 μmoles; water to 2·5 ml.; pH 7·6. Incubation was in an atmosphere of 5 % CO_2 in N_2 at 37°. The results are the mean of three experiments, the results differing by less than 10 %. No correction made for acid production without glucose as this was negligible. Results are expressed as μl. CO_2 liberated/hr./mg. dry wt. organism. Lactate dehydrogenase was measured in extracts as described in the Methods section. The results are the mean of at least three assays with different extracts, showing less than 20 % variation.

Growth conditions	Fermentation of glucose (Q_{CO_2})	Lactate dehydrogenase (units/mg. protein)
Aerobic	35	0·58
Anaerobic	119	4·6

DISCUSSION

There are many examples in the literature of repression of enzyme synthesis by glucose. In particular, the presence of glucose prevents the formation of inducible enzymes necessary for the utilization of alternative energy sources (Magasanik, Magasanik & Neidhardt, 1959; Mandelstam, 1961). The results presented here suggest that in *Staphylococcus aureus* glucose represses the development of some of the enzymes of the tricarboxylic acid cycle. Since staphylococci are normally grown on complex media containing preformed amino acids the cycle is not required to provide precursors of these essential metabolites. When glucose is also provided, sufficient energy for growth is presumably gained by incomplete oxidation of the sugar to the acetate stage, and the operation of the cycle is again not necessary. In the absence of glucose the energy requirements of the organism are probably satisfied by oxidation of amino acids via mechanisms which probably involve the tricarboxylic acid cycle.

Lactic acid is the main end-product of glucose fermentation in anaerobically grown *Staphylococcus aureus* (Gardner & Lascelles, 1962). Thus the main outlet for substrate hydrogen is by reduction of pyruvate mediated by the NAD-linked lactate dehydrogenase. The importance of this enzyme in the anaerobic energy-yielding mechanism of the organism is stressed by the observation that it is about ten times more active in organisms grown anaerobically than in those grown aerobically. Also, there is a correlation between the activity of this enzyme and the ability of suspensions to ferment glucose; when grown aerobically, the organisms are low both in

lactate dehydrogenase and in fermentative activities as compared to the anaerobically-grown organisms. The importance of another NAD-linked enzyme, alcohol dehydrogenase, in anaerobic metabolism has been suggested by the observation that this enzyme is ten to twenty times more active in *Aerobacter aerogenes* grown anaerobically on glycerol than when it is grown aerobically (McPhedran, Sommer & Lin, 1961).

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Transformability of *Haemophilus influenzae*

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SUMMARY

A method is described for the preparation of highly competent (transformable) bacteria of *Haemophilus influenzae*. After three successive treatments, i.e. aerobic growth, anaerobic incubation and incubation in saline containing a few per cent broth, transformation frequencies close to 5 % have been obtained.

Competent bacteria absorb DNA very fast. Untransformable ones lack this ability. Absorption is inhibited by dinitrophenol or arsenate; it is not inhibited by chloramphenicol. This suggests the participation of an enzyme system.

Development of competency is blocked by dinitrophenol, arsenate or chloramphenicol. It is strongly dependent upon temperature. It is not influenced by the absorption of one or two DNA molecules per bacterium.

INTRODUCTION

Bacterial transformation is that process in which bacteria acquire new genetic characteristics by means of absorption and incorporation of homologous deoxyribonucleic acid (DNA, usually derived from a mutant clone) (Avery, MacLeod & McCarty, 1944). Bacteria are called competent if they can undergo transformation. It is not understood what makes them competent. However, it must involve adsorption of the DNA on to the cell wall, its transport through the bacterial membrane to the nuclear aggregate and some way of incorporation into the bacterial genome. Hotchkiss (1954) studied it in *Diplococcus pneumoniae* and showed that it might be related to its normal growth cycle. Chloramphenicol inhibited the development of competency (Fox & Hotchkiss, 1957). S. H. Goodgal & R. M. Herriott (pers. comm. 1958) obtained competent cultures of *Haemophilus influenzae* through interruption of the aerobic growth of a culture by a period of more or less anaerobic incubation. This was recently described in detail (Goodgal & Herriott, 1961). Stuy (1960), however, pointed out that holding aerobically grown bacteria (in vigorously shaken suspensions) under conditions excluding oxygen nearly completely, was merely a 'pretreatment'. It had to be followed by incubation in media which support growth poorly at best (such as saline plus a few per cent broth) in order to obtain transformation frequencies greater than one per cent. This process has been studied by us in more detail, the results of which are reported below.

METHODS

Growing of cultures, preparation of media and of DNA, and transformation techniques (Stuy, 1961*a*, *b*) were modified as follows. Stock cultures were maintained at room temperature as stab-cultures in Levinthal agar. Plating agar consisted of one volume each of Levinthal Stock (Difco Brain Heart Infusion supplemented with hemin and DPN) and Eugon broth (Baltimore Biological Laboratories) mixed with 1·75 % Bacto agar (Difco). This agar is very clear, facilitating colony counting after 20 hr. of incubation. Competent bacteria were exposed to DNA (2×10^8 viable centres/ml. in a total of 2 ml.) while gently shaken at room temperature. The time of exposure was generally limited to 5 min. when the DNA was present in excess (more than 0·1 μg./ml.). The bacteria were in those cases diluted into Eugon broth containing 5 μg./ml. of DNase (Worthington Laboratories) and incubated for at least 5 min. Transformants were scored by seeding proper dilutions in a total of 5 ml. of agar, followed by 2 hr. of incubation at 37° (which period allows complete expression of the DNA incorporated) and then relayered with antibiotic-containing agar.

Transformation frequencies were calculated by dividing the numbers of transformants by the numbers of colony-forming units. Unless stated otherwise, the latter was only occasionally determined as it was always found to be about 2×10^8 per ml. The actual frequencies are estimated to be lower by about 30 % due to the fact that competent cultures do not exclusively contain single bacteria.

The wild type strain Rd of *Haemophilus influenzae* (obtained from Miss Grace Leidy, New York) was used as the recipient test strain. It is sensitive to 3 μg. streptomycin/ml., 0·3 μg. cathomycin/ml. (novobiocin) and 1 μg. erythromycin/ml. The genetic markers employed: SM, Ca and Ery 6, conferred resistance on the recipient bacteria to, respectively, more than 2 mg. streptomycin/ml., 5 μg. cathomycin/ml. and 6 μg. erythromycin/ml. (Stuy, 1961*a*). DNA carrying these markers was extracted from the strains Rd/SM, Rd/Ca and Rd/Ery 6 and is designated DNA(SM), DNA(Ca) and DNA(Ery 6), respectively.

Standard saline contained 0·15 M-NaCl. Chloramphenicol was used at a concentration of 10 μg./ml.

Aerobic growth of cultures was carried out by vigorously shaking 15 ml. at 37° in a 125 ml. flask having a side-arm. When the turbidity of the culture at 5900 Å (measured in the 18 mm. side-arm) had reached a value of 0·35–0·40 (1×10^9 viable centres/ml.) the 15 ml. of culture were poured in the side-arm (length of column about 6 cm.) and incubation at 37° was now continued without shaking. After 75 min. 0·2 ml. of the culture were added to 1·6 –1·8 ml. of saline in a 25 mm tube and incubated at 30°. Nearly all bacteria can be shown to have become competent after about 60 min. at this temperature.

RESULTS

Absorption of DNA

As will be shown, highly competent (transformable) bacteria are obtained only after three successive treatments: aerobic growth to about 10^9 colony-forming centres per ml., more or less anaerobic incubation in the same broth (anaerobically

held or sensitized) and incubation in a 'non-growth' medium. We will describe the effects of variations in either step.

The actual process of DNA absorption by the bacteria can be of great importance with respect to drawing of sound conclusions. We presumed that the observed responses to exposure of test cells to genetically marked DNA (numbers of transformants detected) can under certain conditions be used to study the kinetics of the absorption proper. This was done first.

Kinetics. Competent bacteria (2×10^8 per ml., see below) were mixed with DNA and gently shaken at room temperature (23°) in a gyratory shaker. Samples were withdrawn at intervals, exposed to DNase and transformants scored. Figure 1 demonstrates that DNA absorption (leading to transformation) is virtually completed by the tenth min. in the presence of excess DNA. Limiting amounts take more time to be absorbed completely, depending upon concentration of bacteria and DNA. The rate of DNA absorption is very high and this justifies the short exposure time of 5 min. generally employed in cases of excess DNA.

Inhibition. Competent bacteria were spun down and resuspended in the media to be investigated. The rate of DNA absorption (excess DNA, 5 min. exposure time) was not or little influenced by saline, saline plus 10 % Levinthal broth, Levinthal broth, Eugon broth, saline containing chloramphenicol, ammonium chloride or sodium acetate (both 1 g./100 ml., buffered at pH 7·0 with Tris); pH variations from 6·5 to 7·5 had little effect. From 50 to 90 % inhibitory were 10^{-4} to 10^{-3}M-2,4-dinitrophenol, sodium arsenate in ratios to phosphate present from 1 to 10 (see controls in Table 3) and ammonium acetate as the only electrolyte (tested from 0·5 to 2·0 g./100 ml.). Inhibition by dinitrophenol was readily reversed by diluting it out. It was not reversed by 10^{-3} or 10^{-2}M-adenosine triphosphate.

Incubation in non-growth medium

Development of competency. The development of competency in such a medium was demonstrated in the following experiment. Anaerobically held bacteria (see below) were diluted ten fold into buffered saline (with phosphate at pH 7·0). A series of duplicate tubes was incubated at 30°. At the times stated, excess DNA was added to one culture and the transformants scored after 5 min. Chloramphenicol was added to the other culture and incubation was continued. At 60 min. all remaining cultures were exposed to excess DNA and transformants scored. It can be seen in Table 1 that at time zero the cultures contained very few competent (transformable) bacteria but that they became highly competent upon incubation. Chloramphenicol halted the development completely (the small drops in transformants are probably due to the observed 30 to 50 % killing by the antibiotic) except in the zero-time culture where it allowed some increase. It did not at all interfere with the absorption of the DNA by the bacteria. Nor did it reduce the rate of marker expression in the 2 hr. period at 37° at the proper concentration as was established in a separate experiment.

Influence of temperature. A more refined experiment is presented in Fig. 2. An anaerobically held culture was diluted ten-fold in ice-cold saline. Samples (1·8 ml.) were put in variously thermostated water baths at time zero. Chloramphenicol and excess DNA(SM) were added together at indicated times. Trans-

formants were scored after 10 min. of exposure at room temperature. Since chloramphenicol stops the development of competency, a set of proper curves was obtained which describe this development at various temperatures. It is shown in Fig. 2 that anaerobically held bacteria become competent at rates which depend strongly upon temperature. At a low temperature of 14·5° there is no development at all. It should be pointed out here that turbidity measurements showed some growth at 35° and at 37° but none at 30° or lower temperatures. The decreased numbers of viable centres at the lower temperatures are probably due to the death of some of the (cold-sensitive) bacteria.

Table 1. *Development of competency at* 30°

An anaerobically held culture was diluted ten-fold into tubes containing 0·15 M-saline. Chloramphenicol was added at a final concentration of 10 μg./ml. after 0, 15, 30 and 60 min. In the first series of tubes, transformants were scored immediately (after exposure during 5 min. to 0·8 μg./ml. (excess) of DNA(SM)). In the second series of tubes, transformants were scored after incubation at 30° had been continued up to 60 min.

Time of incubation at 30° (min.)	Transformants per ml.	Viable count per ml.
0	$2{\cdot}5 \times 10^3$	—
0 to 60 plus chl.	$8{\cdot}0 \times 10^3$	$1{\cdot}5 \times 10^8$
15	$5{\cdot}5 \times 10^5$	—
15 to 60 plus chl.	$2{\cdot}7 \times 10^5$	—
30	$1{\cdot}9 \times 10^6$	—
30 to 60 plus chl.	$9{\cdot}3 \times 10^5$	—
60	$3{\cdot}1 \times 10^6$	$2{\cdot}0 \times 10^8$
60 plus chl.	$2{\cdot}8 \times 10^6$	$2{\cdot}0 \times 10^8$

Chemical requirements. The 'non-growth' medium can be varied in a number of ways. We have studied the influence of decreasing broth concentrations (Table 2). Below a 300-fold dilution, the transformation frequency drops. These results are in good agreement with those by Grace Leidy (in the press). She established that the chemical requirements for the development of competency were aspartate or glutamate in phosphate-buffered saline plus calcium and magnesium ions. We confirmed this (Table 2) except that Mg^{2+} was not required. However, it may have been present as an impurity in the calcium chloride used. In addition to the above chemicals, we found that oxygen is also needed.

Metabolic inhibitors. An anaerobically held culture was diluted ten-fold into saline containing increasing amounts of the poisons studied and incubated at 30° during 60 min. before addition of excess DNA. The effective inhibitors were active at concentrations which just blocked normal aerobic growth. At the highest concentrations used, roughly 50 % of the viable centres had been killed. The results are presented in Table 3. It is pointed out that the observed effects are several orders greater than those found on DNA absorption.

Influence of DNA itself. Absorbed DNA might in some way block the subsequent absorption of more DNA. This phenomenon could then partly explain why excess DNA has a saturating effect. We have studied this problem in two series of experiments. First, anaerobically held bacteria were diluted ten-fold into saline (to make them competent) containing increasing amounts of DNA(Ery 6). The second culture contained one DNA molecule per Rd bacterium (0·006 μg./ml.), the third

culture two, the fourth culture four and the fifth culture eight. The first culture contained no DNA. It was assumed that upon preparation of the DNA from the donor cells, it was broken up into some 100 different particles, one of which carried the Ery 6 marker (Goodgal & Herriott, 1957; Stuy, 1961*a*). After 60 min. at 30°, excess DNA(SM) was added. Ery 6- and SM-transformants were scored after 5 min. of exposure. It is clear from Table 4 that the predictably absorbed DNA(Ery 6) had little or no effect on the subsequent response to the exposure to DNA(SM).

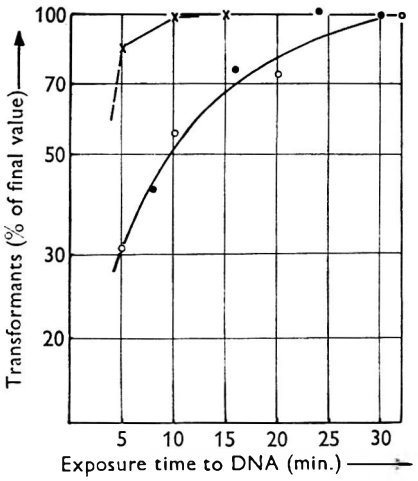

Fig. 1

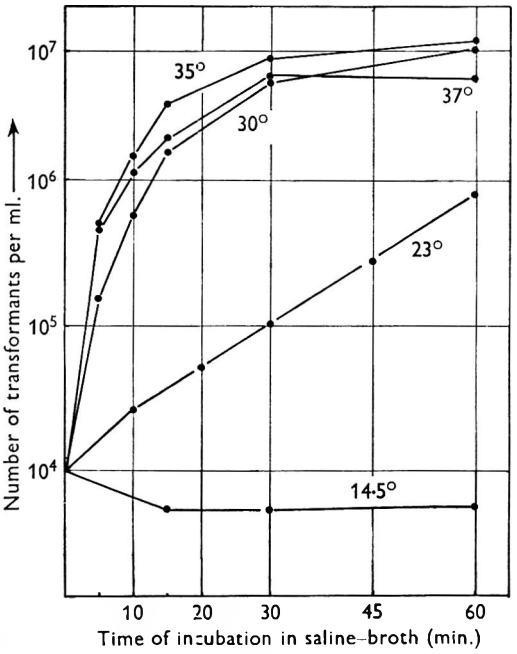

Fig. 2

Fig. 1. Rate of DNA absorption by competent bacteria of *Haemophilus influenzae* strain Rd (2×10^8 per ml.). Medium: saline plus 10 % broth. DNA was added to the suspensions and the mixtures gently shaken at room temperature. Transformants were scored at intervals. It is presumed that the absorption of the DNA is the rate-limiting step of the effect observed. Crosses: excess DNA, 0·4 μg./ml.; final value, $5{\cdot}0 \times 10^6$ transformants per ml. Open circles: limiting amount of DNA(SM), 0·004 μg./ml.; final value, $9{\cdot}0 \times 10^5$. Filled circles: limiting amount of DNA(Ery 6) in the presence of chloramphenicol, 0·001 μg./ml.; final value, $1{\cdot}7 \times 10^5$.

Fig. 2. Development of competency in *Haemophilus influenzae* strain Rd during incubation in saline plus 10 % broth at various temperatures. An anaerobically held (sensitized) suspension was diluted ten-fold in series of tubes with saline pre-warmed at the indicated temperatures. Chloramphenicol and 0·8 μg./ml. (excess) of DNA(SM) were added after intervals and transformants scored after 10 min. Viable counts times 10^{-8}; after 90 min., 14·5°; 1·3 and 23°: 1·8; after 60 min., 30°: 2·2, 35°: 2·7 and 37°: 2·9. Transformants at 23° after 90 min.: $1{\cdot}5 \times 10^8$.

Excess amounts of DNA were used in the second series of experiments. At time zero, 0·47 μg./ml. of DNA(Ca) was added to a series of highly competent Rd cultures. Transformants were scored at intervals after which 0·8 μg./ml. of DNA-(SM) was added. Ca-, SM- and (SM-Ca)-transformants (doubles) were assayed after 5 min. DNA(SM) alone and a mixture of two DNAs were also added to two

Table 2. *Chemical requirements of the development of competency in phosphate-buffered saline*

Anaerobically held bacteria were incubated at 30° in the media given below during 60 min. after which 0·8 μg./ml. of DNA(SM) was added. Time of exposure was 5 min. The transformation frequencies were actually determined. Viable centres had dropped to about one-half in broth dilutions lower than 1/1000. Anaerobic conditions during incubation at 30°: bacteria were diluted in cold saline in a tube carrying a side-arm containing chromochloride. The tube was evacuated in the cold and then incubated at 30° during 60 min. Hereafter, the culture was chilled again and air was admitted. Chloramphenicol was added and then DNA. A similarly treated aerobic culture (no air evacuation) served as a control.

Saline dilution	Addition	Transformation frequency
10	None	$2{\cdot}1 \times 10^{-2}$
30	None	$2{\cdot}3 \times 10^{-2}$
100	None	$1{\cdot}7 \times 10^{-2}$
300	None	$9{\cdot}0 \times 10^{-3}$
1,000	None	$6{\cdot}8 \times 10^{-4}$
3,000	None	$2{\cdot}3 \times 10^{-4}$
10,000	None	Less than 10^{-4}
10,000	0·03 volume of broth	$1{\cdot}3 \times 10^{-2}$
10,000	0·01 M-Mg, 0·01 M-Ca	$1{\cdot}6 \times 10^{-3}$
10,000	0·5 % aspartate, Mg	$5{\cdot}5 \times 10^{-3}$
10,000	Aspartate, Ca	$1{\cdot}3 \times 10^{-2}$
10,000	Aspartate, Mg, Ca	$1{\cdot}5 \times 10^{-2}$
10	None, anaerobic	$1{\cdot}8 \times 10^{-3}$

Table 3. *Effect of cell poisons on the development of competency and on DNA absorption*

Anaerobically held bacteria were diluted ten-fold into tubes containing saline and the indicated poisons. They were incubated at 30° during 60 min. after which 0·8 μg./ml. of DNA(SM) was added. Transformants were scored after 5 min.

Cell poison	Transformants per ml.	Viable centres per ml.
None	$5{\cdot}2 \times 10^{6}$	$2{\cdot}2 \times 10^{8}$
Chloramphenicol 0·1 μg./ml.	$5{\cdot}1 \times 10^{6}$	—
1·0 μg./ml.	$7{\cdot}8 \times 10^{5}$	—
10 μg./ml.	$1{\cdot}0 \times 10^{4}$	$1{\cdot}1 \times 10^{8}$
added with DNA,* 10 μg./ml.	$5{\cdot}6 \times 10^{6}$	—
Dinitrophenol 10^{-5}	$5{\cdot}3 \times 10^{6}$	—
10^{-4}	$4{\cdot}0 \times 10^{6}$	—
10^{-3}	$3{\cdot}4 \times 10^{3}$	$9{\cdot}3 \times 10^{7}$
Added with DNA,* 10^{-3} M	$9{\cdot}0 \times 10^{5}$	—
Arsenate: phosphate 0·1†	$3{\cdot}7 \times 10^{6}$	—
1·0	$4{\cdot}7 \times 10^{5}$	—
10	$4{\cdot}6 \times 10^{4}$	$1{\cdot}1 \times 10^{8}$
Added with DNA,* 10	$1{\cdot}4 \times 10^{6}$	—

* The poisons were added together with the DNA in a control tube in order to determine their effect on the DNA absorption proper.

† Arsenate concentration: 12 μg./ml.

Table 4. *Effect of exposure of competent bacteria to limiting amounts of DNA on the subsequent response of exposure to excess DNA*

An anaerobically held Rd culture was diluted ten-fold in saline containing increasing amounts of DNA(Ery 6). 0·8 μg./ml. of DNA(SM) was added after 60 min. at 30°. Transformants were scored after 5 min.

Amount of DNA(Ery 6)	Transformants scored	Number per ml.	Total
No DNA(Ery 6)	SM	$5{\cdot}2 \times 10^6$	$5{\cdot}2 \times 10^6$
1 molecule per bacterium (0·006 μg./ml.)	Ery 6	$8{\cdot}1 \times 10^5$	$6{\cdot}3 \times 10^6$
	SM	$5{\cdot}5 \times 10^6$	
2 molecules per bacterium	Ery 6	$1{\cdot}9 \times 10^6$	$7{\cdot}0 \times 10^6$
	SM	$5{\cdot}1 \times 10^6$	
4 molecules per bacterium	Ery 6	$3{\cdot}3 \times 10^6$	$7{\cdot}4 \times 10^6$
	SM	$4{\cdot}1 \times 10^6$	
8 molecules per bacterium	Ery 6	$4{\cdot}7 \times 10^6$	$8{\cdot}2 \times 10^6$
	SM	$3{\cdot}5 \times 10^6$	

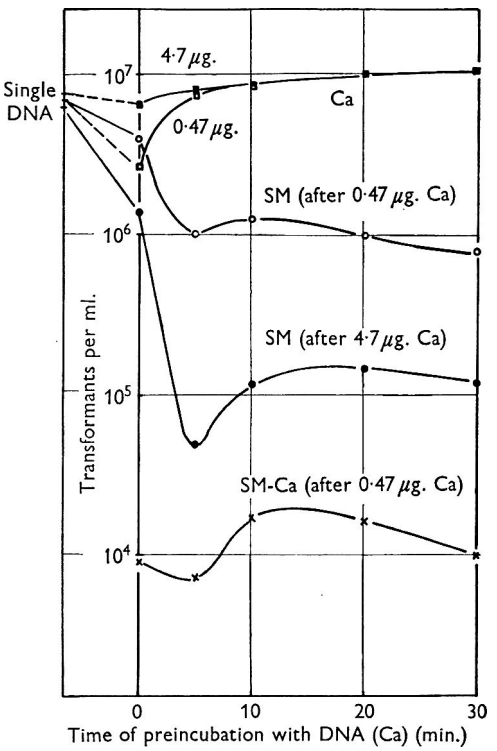

Fig. 3. Influence of preincubation of competent bacteria of *Haemophilus influenzae* strain Rd with excess DNA(Ca) on subsequent transformation with excess DNA(SM). DNA(Ca) was pipetted at time zero into a series of tubes containing the competent bacteria. Ca-transformants were scored and DNA(SM) was added at the indicated intervals. SM-, Ca-, and (SM + Ca)-transformants were assayed 5 min. later. Zero-time data were obtained by adding a mixture of the two DNAs to the bacteria. The competition effect was demonstrated by adding only DNA(SM) to a control suspension.

competent cultures. The results of this experiment and of another one in which the DNA(Ca) concentration had been increased ten times are shown in Fig. 3. The numbers of Ca-transformants scored before addition of DNA(SM) were always equal to those determined after addition of the DNA(SM). The data warrant the following conclusions. (1) Absorbed DNA is fixed in less than 5 min., i.e. it can no longer be competed against. (2) A competent culture has a given capacity with regard to the absorption of DNA. (3) Under the conditions employed, the remaining transformability of the bacteria after addition of DNA drops little with time.

Anaerobic period at 37°

So far, a competent bacterium has been defined as one which can undergo transformation when brought in contact with the proper DNA. It is clear from the preceding sections that a sizable fraction of anaerobically held bacteria develop competency during as little as 5 min. at 30–37° when diluted into saline. Since exposure of recipient bacteria to DNA routinely occurs under conditions which permit the development of competency, we had to block the latter process. This was done by first adding 10 μg./ml. of chloramphenicol to the bacteria. In the following sections, then, we consider a bacterium competent if it can undergo transformation in the presence of chloramphenicol.

Length of anaerobic period. We should point out first, that in this phase oxygen is not completely excluded from the bacteria as the 15 ml. of culture are incubated in a standing 18 mm. tube plugged with cotton wool. The cell concentration being over 10^9, we believe, however, that oxygen is used up much faster in most of the suspension than its supplementation by diffusion into the culture. This justifies in our opinion the term anaerobic.

The anaerobic period is necessary for the subsequent development of competency, but during the period itself very few bacteria become transformable. Perhaps one can best describe what happens as a sensitization. Logarithmic-growth-phase bacteria (15 ml.) were incubated in an 18 mm. tube at 37°. At indicated intervals, 0·2 ml. samples were added to: (1) 1·6 ml. of saline and incubated at 30° to make them competent and (2) 1·8 ml. of saline containing chloramphenicol and excess DNA(SM) to measure the transformable bacteria present. To the first series of tubes, the antibiotic and DNA were added after 60 min. The transformants scored after 5 min. are plotted in Fig. 4. The lower curve gives the numbers of competent bacteria present in the anaerobically held suspension while the upper curve gives the corresponding numbers of sensitized bacteria, i.e. bacteria which became competent after a further incubation in saline-broth.

Loss of competency and of sensitization. Competency and sensitization seem to be two different cell conditions. This is further demonstrated by two experiments designed to measure the loss of either one upon growth. First, sensitized bacteria (anaerobically held for 75 min.) were diluted four times with fresh broth and shaken at 37°. At intervals, 0·2 ml. samples were diluted ten-fold in saline, incubated during 60 min. at 30° to make all sensitized bacteria competent and exposed to excess DNA(SM) in the presence of chloramphenicol. Secondly, competent bacteria, derived from the originally sensitized culture, were diluted ten-fold with fresh broth and shaken at 37°. At intervals, 0·2 ml. amounts were added to 1·8 ml. of saline

containing chloramphenicol and excess DNA. Both cultures grew with the normal division rate of once every 30 min. Transformants were scored and their numbers plotted relative to the initial ones (Fig. 5). It can be seen that sensitization is reduced ten-fold over a 60 min. period while competency drops much faster. The dilution itself into fresh broth did not cause any significant loss of either condition. There is no indication that competency or sensitization is related to the normal growth cycle of the bacteria.

Development of competency in broth. The number of competent bacteria in an anaerobically held suspension is usually very small, i.e. transformation frequencies range from 10^{-5} to 10^{-4}. Upon renewed shaking, sensitization is lost but competency increases relatively much. This is shown in Table 5. A sensitized (anaerobically

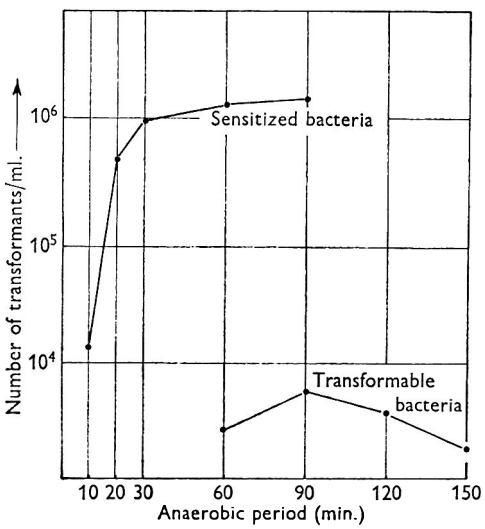

Fig. 4

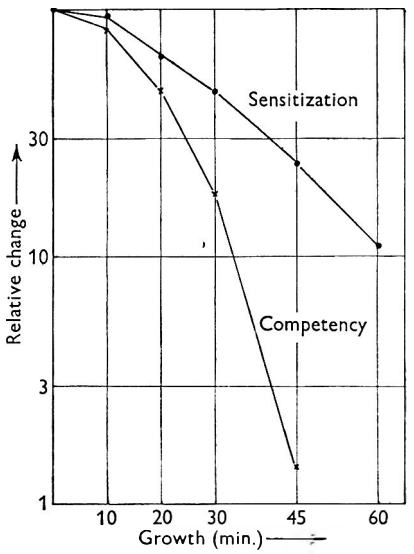

Fig. 5

Fig. 4. Development of competency and of sensitization in *Haemophilus influenzae* strain Rd during the anaerobic period. 15 ml. of logarithmic-growth-phase bacteria were incubated in a standing 18 mm. tube immersed in water at 37°. At times, 0·2 ml. samples were withdrawn and added to (1) 1·8 ml. of saline containing chloramphenicol and excess of DNA(SM) (determination of competent bacteria) and (2) 1·6 ml. of saline followed by incubation at 30° during 60 min. after which 0·1 ml. of chloramphenicol solution and 0·1 ml. of DNA(SM) solution were added (determination of sensitized bacteria). Transformants were in both cases scored after 5 min. Competent and sensitized bacteria at time zero: less than 10^3 per ml.; transformants at times 30 and 180 min.: less than 10^3 per ml.

Fig. 5. Loss of sensitization and of competency upon growth of a sensitized, respectively, competent culture of *Haemophilus influenzae* strain Rd. A sensitized (anaerobically held) culture was diluted in fresh broth and shaken at 37°. 0·2 ml. amounts were diluted at the indicated times into 1·6 ml. of saline and incubated during 60 min. at 30° after which chloramphenicol and excess of DNA(SM) were added. The transformants scored after 5 min. give the loss in sensitization. A highly competent culture derived from the sensitized one (in fact, the zero-time one) was also diluted in fresh broth and shaken at 37°. 0·2 ml. samples were at times added to 1·8 ml. of saline containing chloramphenicol and excess DNA(SM). The transformants scored after 5 min. give the loss of competency of the culture. Initial transformation frequencies (after dilution); sensitization, $3{\cdot}9 \times 10^{-2}$ and competency, $3{\cdot}0 \times 10^{-2}$. Competent bacteria at 60 min. had dropped to less than 0·01 %.

held) suspension was again shaken at 37°. 0·2 ml. amounts were added at indicated times to (1) saline plus chloramphenicol plus excess DNA(SM) to measure the competent bacteria present, and (2) saline alone to measure the sensitized bacteria present. The latter tubes were incubated at 30° during 60 min. after which chloramphenicol and DNA were added. The numbers of competent bacteria increased to more than 10^6 per ml. but the actual transformation frequencies did not exceed 5×10^{-3} since the total number of viable centres had also increased. This is a low value compared to the ones routinely obtained. The second series of tubes gave the loss in sensitization (compare with data in Fig. 5).

Table 5. *Development of competency and simultaneous loss of sensitization (presented as transformation frequencies calculated) during renewed growth of an anaerobically held (sensitized) suspension of* Haemophilus influenzae *strain Rd*

The suspension was again shaken at 37° The numbers of transformable bacteria were determined by adding 0·2 ml. of culture to 1·8 ml. of saline containing chloramphenicol and excess DNA(SM). Transformants were scored after 5 min. The numbers of sensitized bacteria were determined by adding 0·2 ml. of culture to 1·6 ml. of saline followed by incubation at 30° during 60 min. after which chloramphenicol and excess DNA were added. Transformants were scored after 5 min. The total numbers of viable centres per ml. were also determined so that the results can be given as frequencies.

Time of growth (min.)	Competency (transf. frequ.)	Sensitization (transf. frequ.)
0	$4{\cdot}2 \times 10^{-4}$	$3{\cdot}6 \times 10^{-2}$
15	$4{\cdot}3 \times 10^{-3}$	$1{\cdot}9 \times 10^{-2}$
30	$4{\cdot}5 \times 10^{-3}$	$9{\cdot}5 \times 10^{-3}$
45	$1{\cdot}9 \times 10^{-3}$	$2{\cdot}9 \times 10^{-3}$

Aerobic period

The bacterial concentration at which aerobic growth is stopped is not very critical between 6×10^8 and 2×10^9 per ml. We have found optimal transformation frequencies when aeration was stopped at about 10^9 viable centres per ml. During the following anaerobic period turbidity increases by about 50 % while the viable count goes up a little more. We have also grown bacteria in Levinthal stock instead of broth and obtained nearly equal results.

Absorption of DNA

Competent bacteria obviously absorb DNA but do growth-phase or sensitized ones? We found out that they do not. Growth-phase bacteria and sensitized ones (0·4 ml.) were added to 1·6 ml. of saline containing chloramphenicol. To eight tubes in each series, 0·001 μg./ml. of DNA(SM) was added and the suspension shaken at room temperature. Eight competent cultures (0·2 ml. of sensitized bacteria diluted into saline and incubated at 30° after which chloramphenicol was added) were also exposed to this amount of DNA. Two tubes of each series were each time analysed for transformants after 10, 20, 30 and 40 min. and then centrifuged. The supernatants were stored overnight at −25°. They were analysed the following day for remaining DNA by adding one volume each from both duplicate tubes to 2 vols. of competent Rd bacteria which had been concentrated four times by centrifugation and resuspending into saline. Transformants were scored after 20 min.

The numbers of markers (activity of the DNA) remaining in the supernatants are presented in Table 6. We conclude from these data and from similar ones obtained in repeated experiments, that little, if any, DNA is absorbed by either growth-phase or sensitized bacteria.

This was confirmed by transforming (1) a pure suspension of competent bacteria 2×10^8 per ml. (2) the same suspension with 2×10^8 per ml. of sensitized bacteria added, and (3) the same suspension with 4×10^8 per ml. of sensitized bacteria added; all with 0·001 μg./ml. of DNA(SM) during 20 min. and in the presence of chloramphenicol. The numbers of transformed cells observed were $2{\cdot}9 \times 10^5$, $3{\cdot}2 \times 10^5$ and $3{\cdot}2 \times 10^5$ per sample, respectively. Clearly, the sensitized bacteria did not influence the response to DNA by the competent ones.

Table 6. *Absorption of limiting amounts of DNA in the presence of chloramphenicol by growing bacteria, by sensitized ones and by competent ones*

DNA concentration: 0·001 μg./ml. Bacteria concentration: growth-phase and sensitized: 4×10^8; competent: 2×10^8 per ml. The bacteria were mixed with the DNA in saline containing 10 % broth and chloramphenicol. They were spun down after intervals after transformants had been scored. The supernatants were mixed with known numbers of competent bacteria, shaken during 20 min. and thereafter transformants were scored. Their numbers are given as markers per ml. (activity of the DNA) in the fourth column. A fresh solution containing 0·001 μg./ml. of DNA served as a control (no DNA absorbed).

Culture	Exposure time (min.)	Transformants per ml.	Markers/ml. in supernatant	Absorbed DNA (% of control)
Control (no cells)	—	—	$2{\cdot}7 \times 10^6$	—
Growth phase	10	Less than 10^3	$3{\cdot}2 \times 10^5$	−18
	20	Same	$2{\cdot}2 \times 10^5$	19
	30	Same	$2{\cdot}4 \times 10^5$	11
	40	Same	$2{\cdot}4 \times 10^5$	11
Sensitized	10	Less than 10^3	$2{\cdot}8 \times 10^5$	− 4
	20	Same	$2{\cdot}5 \times 10^5$	8
	30	Same	$1{\cdot}9 \times 10^5$	30
	40	Same	$2{\cdot}6 \times 10^5$	4
Competent	10	$2{\cdot}2 \times 10^5$	$5{\cdot}5 \times 10^4$	79
	20	$2{\cdot}0 \times 10^5$	$5{\cdot}3 \times 10^4$	80
	30	$2{\cdot}4 \times 10^5$	$7{\cdot}0 \times 10^4$	74
	40	$2{\cdot}8 \times 10^5$	$1{\cdot}8 \times 10^4$	93

DISCUSSION

It may seem confusing to speak of highly competent (transformable) cultures which show transformation frequencies of ('only') 1–5 %. But the point is that purified DNA solutions contain 'broken up' bacterial DNA. Estimates in this respect are that one bacterial DNA unit splits into 50–100 particles with a molecular weight of 15–30 million (Goodgal & Herriott, 1957; Stuy, 1961*a*). We believe that only one of those particles carries a given marker. Consequently, bacteria which have picked up genetically unmarked DNA, may be transformed, i.e. they may incorporate this DNA. Since they do not change genetically as a consequence, there is no simple way of telling whether this happens. By employing two or more unlinked markers and by measuring the transformation frequencies of single markers and their doubles, one can compute the fraction of bacteria which would have been

transformed after absorption of marked DNA. This fraction is close to one in highly competent cultures, i.e. cultures showing transformation frequencies of one per cent or more (Stuy, 1961*a*; Goodgal & Herriott, 1961). As a consequence, we feel confident to state that the actual number of competent bacteria is roughly 100 times the number of transformants observed in cases where their frequencies are lower than one per cent.

We have tried to make clear that it takes three successive treatments in order to obtain highly transformable cultures of *H. influenzae* strain Rd. This observation seems to disagree with a report by Goodgal & Herriott (1961) who state that the anaerobically held bacteria (in our study: sensitized) are competent. We believe that in their experiments bacteria may have become competent during the relatively long exposure to DNA. To prevent this, we added chloramphenicol.

The development of competency by sensitized bacteria shows surprisingly few requirements, as was first established by Grace Leidy and co-workers (in the Press). It is not so surprising that it is blocked by growth inhibitors. Unfortunately, this reveals very little about the nature of the process.

Absence of competency goes along with incapability of absorbing DNA. We would like to know whether this incapability is in fact the reason why most bacteria are not transformable. Absorption of DNA seems to be energy-requiring as it is blocked by dinitrophenol or arsenate. This suggests the participation of an enzyme system. Untransformable bacteria, perhaps, lack such a system.

Competency and sensitization are retained quite well by the bacteria in the absence of growth. One may wash the bacteria without destroying much of either condition. The absorption of small amounts of DNA has little or no effect on the competency of the bacteria involved. Excess amounts of DNA still leave 'some room' for subsequently added DNA. The extent of this is dependent upon the amount of the first DNA which fact indicates that we are indeed dealing with a relatively simple DNA saturation effect. We wondered about the observed speed with which the first DNA was fixed by recipient bacteria, i.e. it could no more be competed against after 5 min. or less. Recent experiments (unpublished) have revealed that the DNA had not yet been crossed in (recombination with the bacterial chromosome) by that time. We tend to believe, therefore, that the absorbed DNA is quickly and firmly bound by the available intracellular sites. In a later stage, it recombines with the bacterial DNA.

I wish to record my appreciation to Miss Joke Bloemendal who ably assisted in carrying out the experiments.

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Some Features of the Fine Structure and Chemical Composition of *Rhizobium trifolii*

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SUMMARY

The conspicuous, large, high refractive index, sudanophilic granules of *Rhizobium trifolii* appeared to be aggregations of polymeric β-hydroxybutyric acid, probably closely associated with the cytoplasm. They became more conspicuous as the organism aged, provided that carbohydrate was in excess. A well-grown culture contained 40–50% polymer, based on cell dry weight. Relatively large cytoplasmic granules (50–80 mμ) were a feature of this organism whether in fixed and sectioned cells or in material shadowed after mechanical disintegration.

Classical 'double' (? lipoprotein) membranes were demonstrated both for the cell wall and the cytoplasmic membrane. Carefully fixed and embedded material often showed an accumulation of material between the two double membranes, especially at one or both ends, without any evidence of gross damage in the sectioned organisms.

INTRODUCTION

Rhizobium trifolii consists of motile Gram-negative rods, approximately 2 μ × 1 μ, which characteristically stain unevenly with the usual basic dyes. Burdon (1946) noted that Rhizobium possessed more sudanophilic granules than most Gram-negative genera. Others (Forsyth, Hayward & Roberts, 1958; Smithies, Gibbons & Bayley, 1955) have reported rhizobium as one of many organisms that contain a polymer of β-hydroxybutyric acid. This polymer was first described in *Bacillus megaterium* by Lemoigne (1925), and was found by Képès & Peaud-Lenoel (1952) to consist of β-hydroxybutyric acid molecules linked through the carboxyl and hydroxyl groups into chains of various lengths.

We were made particularly aware of this polymer in our attempts to prepare cell walls of rhizobium by usual procedures. In this we were frustrated by the large amounts of sticky material which formed as globules when the cells were disintegrated and which could not be separated from the walls by differential centrifugation. Dr M. J. Salton's suggestion that this material might be the polymer led to a modified procedure giving us a clean preparation and the means of analysing the walls of normal and abnormal calcium-deprived cells (Humphrey & Vincent, 1962).

This paper reports some features of the rhizobial cell revealed by the electron microscope, and observations on the polymeric β-hydroxybutyric acid which so much affects the appearance of the organisms under the light microscope.

METHODS

Organism. Rhizobium trifolii SU 297/31.

Cultural conditions. It was grown in a defined medium (Vincent, 1962) having a total concentration of divalent cation of 1 mM. Cultures were aerated by shaking and grown for 3–4 days at 25°.

Light microscopy. Phase equipment was used for the examination of unstained cells. Otherwise normal staining methods were used, including the procedure of Burdon (1946) for Sudan Black B.

Electron microscopy. The organism was fixed with 2% $KMnO_4$ using 2% uranyl nitrate as a post-fixative (North, 1961) to obtain better definition of membranes, embedded in araldite, and sectioned and examined in the Siemens electron microscope. Shadowed preparations were by normal techniques.

RESULTS

Structure

Appearance under the light microscope. The larger cells of a well grown culture contained several conspicuous refractile granules, clearly seen under the phase microscope, which corresponded in position to areas which remained unstained with simple basic dyes, but which were strongly sudanophilic. In shorter, and presumably younger cells, on the other hand, the more refractile material was restricted to the ends in 'polar caps', but this material appeared to be different from that of the granules (see below). In cells of intermediate length there was a conspicuous central granule, generally in cells still having 'polar caps'. Table 1 summarizes the distribution of these three main conditions in relation to cell length. When the culture as a whole was younger (16 hr.), the refractile granules were smaller and less frequent.

Table 1. *Relationship of cell structures to cell length*

The organism was grown in the defined medium containing Ca^{2+} for 85 hr. at 25°.

	Distribution of cells according to length (μ)					
Cell condition	0·8–1·3	1·4–1·8	1·9–2·4	2·5–3·0	3·0	Total
Without granules but with 'polar caps'	17	5	1	0	0	23
With central granule, generally with 'polar caps'	10	22	8	3	0	43
With multiple granules	0	6	14	11	3	34

When the organism was mounted in media of higher refractive index (R.I.), it was found that the central and multiple granules had R.I. = 1·41, compared with about 1·38, the R.I. of the rest of the cell. Extraction with chloroform removed the most refractile granules, leaving areas having a lower R.I. than the rest of the cell and which could now be stained, though lightly, with methylene blue and other basic dyes. 'Polar caps' were not extracted by chloroform and were further distinguishable from the other granules in that formation of the latter was prevented when the carbon source was limiting.

The properties of the granules *in situ* (i.e. high R.I., ability to stain with Sudan

Black, failure to stain with basic dyes, and solubility in chloroform) were compatible with those of polymeric β-hydroxybutyric acid. This substance was obtained in quantity from this organism (see below), and these facts, considered along with those of Lemoigne (1946) and Williamson & Wilkinson (1958) for other bacteria, led to the conclusion that the high R.I. granules did, in fact, contain a large amount of the polymer. This probably constituted a food reserve formed under conditions of abundant carbon supply (Macrae & Wilkinson, 1958; Doudoroff & Stanier, 1959; Merrick & Doudoroff, 1961).

Fine structure. Two sets of double membranes were observed in ultra-thin sections under the electron microscope (Pl. 1, figs. 1, 2). The outer, which we took to be the cell wall, had structure and dimensions in agreement with those described by Kellenberger & Ryter (1958) for *Escherichia coli*. Two 30 A° dark lines were separated by a 30 Å clear space so that the whole could represent the classical lipoprotein double layer. Additionally the outer layers appeared to have associated with them granular, electron-dense material that gave the wall a rather more substantial appearance than the underlying cytoplasmic membrane.

The inner membrane was also clearly resolved in these preparations. It had the same over-all dimensions (about 80 Å) as what appeared to Kellenberger & Ryter (1958) to be a single-layered structure in *Escherichia coli* but which was probably not resolved in their case. Beer (1960) recently reported a double-layered cytoplasmic membrane in *Alcaligenes faecalis*. The dimensions we observed for the cytoplasmic membrane were similar to the double-layered unit membrane of higher organisms (Robertson, 1955, 1958). We, like Beer (1960), often found a large area at one or both poles of the cell that separated the cell wall and the cytoplasmic membrane (Pl. 1, fig. 3). This might have represented shrinkage, but it was by no means empty of electron-dense material and both the cytoplasmic membrane and the cell wall enclosing the area appeared to be well organized and relatively undamaged. Its location and dimensions were, moreover, the same as the terminal areas of high R.I. ('polar caps') seen regularly in the living organism with phase illumination. It is possible that these areas were real structures and represented material excreted into the inter-membrane space from the cytoplasm, or synthesized at the outer surface of the cytoplasmic membrane.

The cytoplasm enclosed by the inner membrane contained many electron-dense and roughly spherical bodies, about 50–80 mμ diam. (Pl. 1, fig. 2). In this respect the cytoplasm of rhizobium differed from the more finely granular cytoplasm of other bacteria. Similar spherical bodies were liberated from disrupted cells (Pl. 1, fig. 4) and a concentrated fraction of this material showed vigorous succinoxidase activity. The chemical nature of the granules has not yet been fully investigated. Because they appeared so electron-dense in permanganate-fixed preparations, and because of their enzymic activity, they were likely to contain a considerable amount of protein. On the other hand, they were also obvious with osmium tetroxide fixation.

Composition

Isolation and identification of polymeric β-hydroxybutyric acid. Washed freeze-dried cells were shaken with chloroform for 24 hr. at 25° and separated from the liquid by filtration under pressure. When the chloroform was evaporated, this highly viscous solution left a thin 'plastic' film clinging to the glass surface. It was also refractile

and sudanophilic, and dried in the polygons observed by Lemoigne, Sanchez & Girard (1943). The chloroform solution, when poured into three volumes of ether, produced a white flocculent precipitate adhering in strings to the stirring rod. This material melted sharply at 179°, compared with 160–179° as variously reported for this polymer. This value is probably dependent on the length of the polymer chain. It was soluble in phenol, pyridine and N-NaOH, but insoluble in ether, acetone and dilute mineral acids.

Heating the dry polymer in a tube caused it to melt and fume, and the plate-like crystals which condensed on the cold part of the tube had a melting point of 69°. These crystals decolorized $KMnO_4$ (the test for unsaturation in the molecule) as would be expected of crotonic acid (m.p. 70°), the sublimation product of β-hydroxybutyric acid.

The polymer was saponified by N-NaOH in 30 min. at 100° to give a 'soapy' solution. Na^+ was removed by Amberlite 120R H^+ and the resulting solution used for chromatography by the method of Duncan & Porteus (1953). The main spots of the chromatogram corresponded to the markers of authentic β-hydroxybutyric acid and crotonic acid. Hydrolysis of the polymer for 48 hr. in 6N-HCl at 100° did not dissolve the material completely, but the soluble portion contained mainly β-hydroxybutyric acid. Amino-acids and sugars could not be detected in the hydrolysate.

Table 2. *Increase of poly β-hydroxy-butyric acid with age of culture*

Age of culture (hr.)	% polymer in cell dry wt.
16	39
37	41
86	50

As the cells aged in the presence of sufficient carbohydrate and the refractile granules became more conspicuous, the percentage of polymer increased (Table 2). Cultures growing in limiting amounts of carbohydrate produced considerably less polymer and showed a decline in opacity with age, probably an indication of the polymer being utilized as a food reserve.

DISCUSSION

In our electron micrographs we seldom saw the conspicuous inclusions of polymer found by Schlegel, Gottschalk & von Bartha (1961) in Hydrogenomonas. It may be that the polymer was completely or largely removed during our preparation for fine sectioning. More probably, the difference between Hydrogenomonas and our organism rests with a non-polymer electron-dense matrix that persisted in the electron micrographs of rhizobium. Indications of the stainability of areas from which the polymer was removed by chloroform extraction support the indication that in rhizobium the polymer was closely associated with the cytoplasm (possibly the small granules noted in this paper) and did not simply displace it.

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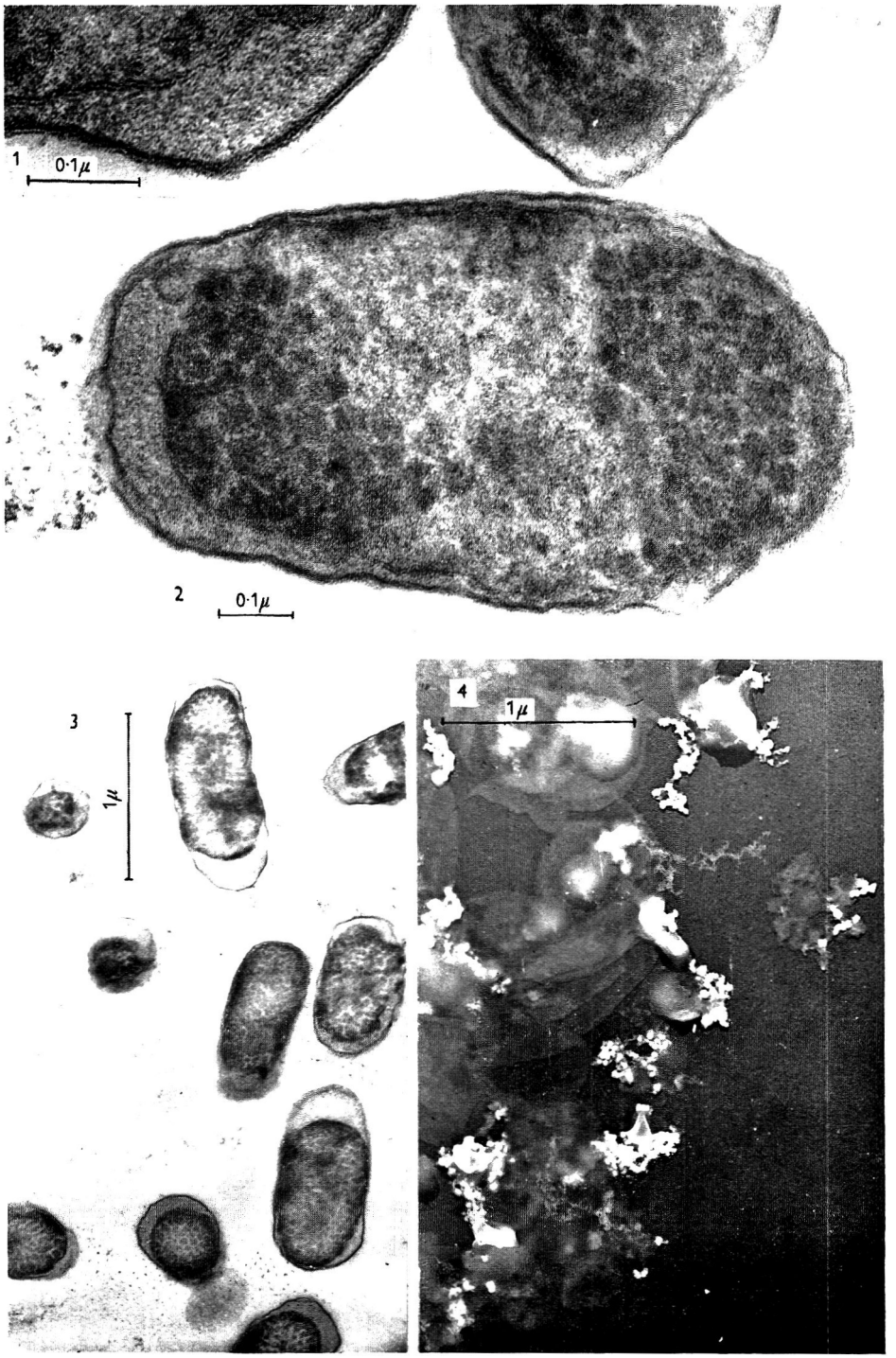

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EXPLANATION OF PLATE 1

Fig. 1. *Rhizobium trifolii* section: details of cell wall and cytoplasmic membrane.

Fig. 2. *R. trifolii* section: cell wall, cytoplasmic membranes and granules in cytoplasm.

Fig. 3. *R. trifolii* section: Inter-membrane areas; granules in cytoplasm.

Fig. 4. *R. trifolii*: Disintegrated cells to show granules and residual walls.

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Calcium in Cell Walls of *Rhizobium trifolii*

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SUMMARY

The cell walls from *Rhizobium trifolii*, grown under both 'normal' and 'calcium-deprived' conditions, were analysed in an attempt to detect a chemical cause for the apparent weakness or looseness of the walls in calcium-deprived organisms. The organic components typical of Gram-negative cell walls were present in normal and calcium-deprived cells. The latter concentrated most of the small amount of available calcium in the walls, which, however, contained only 60 % of that present in the walls of normal organisms. Magnesium was not able to substitute for calcium as a wall component.

INTRODUCTION

Calcium-deprived cells of *Rhizobium trifolii* developed an abnormal morphology (Vincent & Colburn, 1961). They became swollen and much more spherical in form, apparently distorted by a huge vacuole. Although a double-layered wall structure was seen in the calcium-deprived as well as in the calcium-sufficient cells, it appeared to be appreciably weakened in its sectioned appearance. The effect could be one of a loosening of wall structure, and in this paper we have looked for some chemical evidence of this. We have particularly considered the possibility that calcium may be structurally involved.

METHODS

Organism. Rhizobium trifolii SU 297/31.

Cultural conditions. The organism was grown in a defined medium (Vincent, 1962) having a total concentration of divalent cations of 1 mM. This was made up of either 0·5 mM-Ca^{2+} + 0·5 mM-Mg^{2+} ('normal') or 1 mM-Mg^{2+} ('calcium-deprived'). All salts were A.R. grade. Cultures were harvested after 72 hr. incubation, with shaking, at 25°.

Preparation of cell walls. Early attempts at a clean cell-wall preparation were frustrated by the presence of up to 50 % poly-β-hydroxy-butyric acid in the cells (Vincent, Humphrey & North, 1962). It was found best to remove this polymer first, by lyophilizing the organism and extracting with chloroform for 24 hr. at room temperature. Such extracted organisms gave a satisfactory cell-wall preparation by the usual method of Mickle disintegration, trypsin digestion and differential centrifugation.

Identification of wall components. For the identification of sugars, walls were hydrolysed at 100° for 20 hr. in 2N-HCl. Chromatography was in butanol:acetic acid:water, 6 + 1 + 2 descending, and the papers were developed by *p*-anisidine-HCl.

For the identification of amino acids and amino sugars, walls were hydrolysed

for 20 hr. at 100° in 6 N-HCl. Chromatograms were developed with ninhydrin after two-dimensional chromatography in pyridine + water (4 + 1) and butanol + acetic acid + water (3 + 1 + 1).

Calcium and magnesium analyses. These were carried out by the method of atomic absorption spectrophotometry. Five mg. whole cells or cell walls were ashed overnight at 450° in an electric muffle furnace in covered platinum crucibles. Two ml. of 3 N-HNO_3 were added to the residue and evaporated to dryness on a water bath. The crucibles were again placed in the muffle furnace overnight, after which no visible ash remained. The residue was taken up in 0·1 ml. 6 N-HCl and diluted to 5 ml. with water containing 1500 p.p.m. strontium to suppress phosphate interference. The atomic absorption of the solution was measured and read against a standard curve for Ca^{2+} prepared at the same time. Values lay within the range of 0·2–1·2 p.p.m. calcium. Duplicates were within 10 %, as were different batches of the organism. The same solution, sometimes with further dilution, could be used for Mg^{2+} analyses within the range of 0·1–1·5 p.p.m.

RESULTS

Composition of cell walls

Removal of the poly-β-hydroxy-butyric acid by extraction with chloroform enabled us to obtain satisfactorily clean preparations of cell walls, free from high refractive index material as judged by phase and electron microscopy. Walls prepared in this way contained the 'R' layer components (Weidel, Frank & Martin, 1960)—glucosamine, muramic acid, glutamic acid, alanine and diaminopimelic acid, as well as the usual wide range of amino acids found in Gram-negative walls; in this case, lysine, aspartic acid, glycine, serine, valine, methionine, leucine and tryptophane were present, together with glucose and rhamnose.

Cell-wall composition in relation to calcium nutrition

The peculiarly swollen appearance of cells of *Rhizobium trifolii* (Vincent & Colburn, 1961) led us to believe that the shape-determining rigidity of the cell wall had been lost. The 'R' layer components were, however, found to be qualitatively alike, whether the organism had been grown in the presence or absence of Ca^{2+}, and all the other amino acids and sugars were also present in the calcium-deprived walls. It seemed therefore that the deficiency might lie in the Ca^{2+} itself, or in the organization of the components.

Calcium and magnesium analyses of whole cells and cell walls

Table 1 summarizes the results of Ca^{2+} and Mg^{2+} analyses of cells and cell walls. Those grown in the presence of calcium were thereafter deprived of Ca^{2+} by ethylene diamine tetra-acetic acid (freeze-dried normal material was exposed to M/10 EDTA in M/200 tris buffer, value pH 7·0, at 0° overnight); those grown in the absence of calcium were then exposed to Ca^{2+} (freshly harvested deprived cells were exposed either to normal medium containing 0·5 mM-Ca^{2+} for 1 hr. at 25° or to 10 mM-$CaCl_2$ in M/100 tris buffer, value pH 8, at 0° for 72 hr.). In each case, where organisms were exposed to Ca^{2+}, they were washed five times with water, and a cell-wall preparation was then made.

The following conclusions could be drawn: (1) In 'normal' organisms the Ca^{2+} concn. in the cell wall was 25% greater than that in the cell as a whole. (2) The total Ca^{2+} in deprived cells was very low (about 10% of normal) and this was probably almost all accounted for by the amount retained in the walls (approximately 60% of the normal wall Ca^{2+} content). (3) The deficiency of Ca^{2+} in the whole cell was compensated by additional Mg^{2+}. This did not apply to the walls. Those of deprived cells still had less than 75% of the total divalent cations of normal walls. (4) EDTA removed most of the cations, Mg^{2+} as well as Ca^{2+}, from normal whole cells and walls. The small part resistant to removal would seem to be more strongly bound in the structure of the cell and wall. (5) Exposure of the deprived cells and walls to Ca^{2+} (in normal medium and as dilute $CaCl_2$) led to its uptake, more strikingly in the whole cells than in the walls. The concentration of Ca^{2+} in the walls remained appreciably below the value of walls of cells grown in the presence of Ca^{2+}.

Table 1. *Calcium and magnesium content of cells grown in the presence and absence of calcium*

\+ = normal cells grown in 0·5 mM-Ca^{2+} + 0·5 mM-Mg^{2+}. − = calcium deprived cells grown in 1 mM-Mg^{2+}.

Fraction	Calcium status and treatment	mM/g. dry wt. of cells		
		Calcium	Magnesium	Total
Whole cells	+	0·062	0·064	0·126
	−	0·007	0·154	0·161
Cell walls	+	0·081	0·016	0·097
	−	0·047	0·024	0·071
Whole cells	+ EDTA treated*	0·008	0·005	0·013
Cell walls	+ EDTA treated	0·010	0·005	0·015
Whole cells	− Exposed 1 hr.* to normal medium	0·019	.	.
Cell walls	− Exposed 1 hr. to normal medium	0·054	.	.
Whole cells	− Exposed 72 hr.* to M/100 $CaCl_2$	0·058	.	.
Cell walls	− Exposed 72 hr. to M/100 $CaCl_2$	0·063	.	.

* See text for details.

Observations by phase and electron microscopy of the EDTA-treated organisms did not reveal the striking morphological abnormalities seen in cells deprived of Ca^{2+} during their growth. The electron micrographs of cells shadowed with platinum did, however, indicate some loss of structure in that those treated with EDTA had lost their smooth contours and had the walls wrinkled and collapsed in an angular manner over the cytoplasm. Phase observations of deprived cells exposed to Ca^{2+} failed to reveal any convincing evidence of morphological recovery.

DISCUSSION

Calcium deprivation could affect the cell wall in various ways:

(1) Calcium may have a catalytic role in the manufacture of the rigid-layer components. However, in the case of rhizobium, the walls of cells grown in the absence of added Ca^{2+} had the same 'R' layer components, amino acids and sugars, as those grown in its presence. It seems therefore that dependence of 'R' layer precursors on Ca^{2+} is not the explanation of the observed condition.

(2) Calcium may be needed for organizing the 'R' layer or other components into a rigid unit. Primosigh, Pelzer, Maass & Weidel (1961) pointed out that the peptide side chains of the 'R' layer which contain diaminopimelic acid would carry an excess negative charge which would need to be neutralized before they could be packed into a tight, rigid structure. It is feasible to visualize the specificity of Ca^{2+} being associated with its having, unlike Mg^{2+}, the dimensions that permit it to fit structurally into such a network. Recent growth observations by one of us (J.M.V.) have shown that Sr^{2+}, but not Ba^{2+}, could replace Ca^{2+}, though less efficiently, to support the growth of cells of normal morphology. If the dimension of the ion is involved, this observation fits with the respective place of Mg, Ca, Sr and Ba in the group of alkaline earths. There is some evidence that Ca^{2+} may be involved structurally in bacterial cell walls, apart from that presented in this paper. Keerer & Gray (1960) found up to 70% of radioactive Ca^{2+} incorporated into the cell-wall fraction of *Listeria monocytogenes*. Murti (1960) prepared spheroplasts of *Vibrio cholerae* and *Escherichia coli* by suspending the cells in EDTA with lysozyme. Lysozyme attacked the polysaccharide backbone of the 'R' layer (Salton & Ghuysen, 1960) and the function of the EDTA could be to withdraw Ca^{2+} from the structure.

(3) Calcium may affect the stability and permeability of proteins present in the wall. Takahashi & Gibbons (1959) found that Ca^{2+} and Mg^{2+} protected the cells of *Micrococcus halodenitrificans* from swelling under unfavourable salt concentrations. Nermut (1960) found that *Proteus* large bodies became permeable to Alcian blue after treatment with lauryl sulphate and EDTA, suggesting that removal of Ca^{2+} has altered the permeability of the cell membrane. Brown (1961) found that the ionic strength of the medium, and particularly the concentration of divalent cations, determined the amount of cell envelope protein formed in a marine pseudomonad. Organisms growing on media of low ionic strength contained low concentrations of cell-wall protein and became spherical. Levy & Slobodiansky (1950) suggested that under conditions of unfavourable ionic strength the amount of protein synthesized in a system was reduced since proper hydrogen bonding did not occur, and the entropy of the system was too high for synthesis to continue. In rhizobium the effect of Ca^{2+} and Sr^{2+} was more specific since the swollen cells were seen in the presence of excess Mg^{2+}, sufficient to keep the total molarity of divalent cation constant. It is possible, however, that here the Ca^{2+} was specifically involved in stabilizing cell wall protein and that in the absence of Ca^{2+}, this wall protein was present in reduced quantity.

We were not able to simulate the morphological appearance of calcium-deprived cells by treatment of normal cells with EDTA, nor to restore normal morphology by exposure of deprived cells to Ca^{2+}. These observations, combined with the difficulty in restoring normal Ca^{2+} content to walls of deprived cells, compared with the

relative ease with which the whole cell took up the ion, suggested that there was an active incorporation of Ca^{2+} into the wall during growth, and that this was what was required for normal morphology. Alternatively, the Ca^{2+} could have been required to assist the normal formation of wall protein during cell growth. Cells grown in insufficient Ca^{2+} could not then be restored to normal by later addition of Ca^{2+}, nor would the removal of Ca^{2+} at that stage have any effect on a wall already synthesized. Noller & Hartsell (1961) studied the effect of pre- and co-lytic treatment of *Escherichia coli* and *Aerobacter* with lysozyme. They suggested that the action of EDTA and other agents (butanol, heat) in potentiating the action of lysozyme was by dissociation of the lipoprotein rather than the 'R' layer component of the wall. It is possible that in rhizobium also, Ca^{2+} may be concerned as much with the stability of the lipoprotein as with that of the 'R' layer portion of the wall.

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