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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

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"Easy writing's curst hard reading."—*Richard Brinsley Sheridan*

"Easy reading's curst hard writing."—*The Editors, J. gen. Microbiol.*

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in English and should in general be divided into the following parts in the order indicated: (*a*) Summary: brief and self-contained; (*b*) Introduction; (*c*) Methods; (*d*) Results (illustrative protocols only should be included); (*e*) Discussion (if any), and general conclusions; (*f*) acknowledgements; (*g*) References.

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Symbols and Abbreviations. Authors should refer to current issues of *The Journal of General Microbiology* for information in this connection. Attention is particularly drawn to the following points: *c.* = circa or approximately; degrees Centrigrade are written, e.g. 100°, not 100° C.; hr., min., sec. (singular and plural); M = molar; m (milli-) = 10^{-3} and μ (micro-) = 10^{-6}; ml. (millilitre) should be used instead of c.o., and μg. (microgram) instead of γ; N = normal (of solutions); No. or no. = number. Ratios should be written 1:10; dilutions, 1/10.

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Nomenclature of Amino acids and Vitamins. The rules published in the *Biochemical Journal* (1952), **52**, 1–2, should be followed.

Nomenclature and Descriptions of Micro-organisms. Binomial Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with International Rules of Nomenclature; in full at the first mention in each paragraph and in the Summary but in subsequent mention with the generic name abbreviated. Single initial letter abbreviations are used where they are not ambiguous. Binomials should be underlined in the typescript. Scientific epithets or trivial names are not underlined and should be without capitals.

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

The word 'generation' should not be used synonymously with 'subculture'. For an agreed use of terms like strain, type, variant, phase, etc., see the International Bacteriological Code of Nomenclature, Section **1**, Rules 7 and 8.

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

MICROFUNGI. Ainsworth & Bisby's *A Dictionary of the Fungi*, 1954, 4th ed. (Kew: Imperial Mycological Institute.)

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1946). *Rev. appl. Mycol.* **24**, 513–56.

BACTERIA. Author's references in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired a synonym may be added in brackets when a name is first mentioned.

J. gen. Microbiol. (1961), **24**, 1–14
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A Study of Radiosensitive and Radioresistant Mutants of *Escherichia coli* strain B

By RUTH F. HILL and EVA SIMSON
Radiological Research Laboratory, Columbia University, N.Y.C., N.Y.

(*Received* 16 *May* 1960)

SUMMARY

Relative differences in radiosensitivity between *Escherichia coli* strain B and the mutant strains, B/r (more resistant than B) and two types of B_s (more sensitive), are maintained with respect to inactivation by ultraviolet radiation, X-rays and decay of incorporated ^{32}P. The differences cannot be ascribed to variations in number of nuclei or in content of DNA or RNA. The effectiveness of various post-irradiation and post-decay treatments in preventing inactivation varies for each strain. With the exception of reactivation by light, strain B/r is less reactivable than B and B_s.

The strains do not differ in their rates of mutation to resistance to bacteriophage T_1. However, the rates of mutation of the B_s mutants to the first step of furacin-resistance are considerably lower than the rate for the parent strain B. From one of the B_s mutants, new strains were obtained whose properties are mixtures of those of B and B/r.

INTRODUCTION

Among bacteria *Escherichia coli* strain B and its radioresistant mutant B/r have been frequently employed in studies concerned either with the mechanism of inactivation by radiation or with the genetic basis of radiation sensitivity (Witkin, 1947). It has been established, for instance, that B and B/r differ not only in the lethal effect of X-rays and ultraviolet radiation but also in such related properties as the shapes of their radiation survival curves, the severity of their cytological response to irradiation and their reaction to various post-irradiation treatments (Payne, Hartman, Mudd & Phillips, 1956; Anderson, 1951; Roberts & Aldous, 1949). With respect to the genetic aspects, it appears that B and B/r differ by a single gene.

A recent report described briefly the isolation of another radiological mutant of *Escherichia coli* strain B—called B_s—which is more radiosensitive (Hill, 1958*a*). It is of interest both to microbial radiobiology and to microbial genetics to inquire which properties other than radiosensitivity are affected by these contrasting mutations in strain B. The present paper will give the results of an investigation designed to elucidate the basis for differences in radio-sensitivity by studying the following points:

(1) The relationship between the type of mutation and the effectiveness of various post-irradiation treatments in preventing lethality.

(2) The nuclear multiplicity, DNA and RNA contents of strains B, B/r and B_s.

(3) The relative response to decay of incorporated ^{32}P, another lethal agent which is reported to act directly on the DNA of bacteria (Fuerst & Stent, 1956).

(4) The relative response to the antibiotic furacin. Mutation to the first step of furacin resistance is invariably accompanied by increased radioresistance and vice versa (Szybalski & Nelson, 1954). The effect of mutation to radiosensitivity on furacin sensitivity was therefore studied.

(5) The relative ability of strains B and B_s to mutate spontaneously to the B/r form. In order to compare this with the relative ability to mutate at other genetic loci, the spontaneous rates of mutation to resistance to bacteriophage T_1 were also determined.

METHODS

Bacterial strains. Strains B, B/r and B_{s-1} (this strain was called B_s in the previous report) have been described previously (Hill, 1958*a*). Strain B_{s-2} was isolated more recently in the same way as B_{s-1}. Strain B_{s-1} was found among twelve colonies of strain B originating from survivors of a high dose of u.v. radiation (5×10^{-4} survival); B_{s-2} among 100 such colonies. The frequency of B_s mutants after u.v. irradiation is therefore of the order of 1–2 % and comparable to the frequency of B/r mutants after u.v. irradiation (Witkin, 1947).

Ultraviolet irradiation. The experimental set-up has been described previously (Hill & Rossi, 1952). The strains were grown by aeration in 0·8 % nutrient broth for 24 hr. and then diluted 1/50 into physiological saline. Separate 3 ml. samples were irradiated with each dose (unfractionated irradiation). One tenth ml. samples of appropriate dilutions were plated by the agar layer technique commonly used for bacteriophage assays (Adams, 1950). Colonies were counted after 24 hr. incubation at 37°.

For the diagnosis of colonies as consisting of cells of B, B/r or B_s, a modification of Witkin's method was used (1947). Each colony was suspended in 1 ml. broth, diluted further in broth, grown to saturation and subcultured twice more. A loopful was streaked over a small area on a nutrient agar plate, which was then u.v. irradiated with a dose sufficient for 10 % survival of strain B. After 3 hr. incubation at 37° the spread areas were examined microscopically. Cells of strain B show 'snake' formation; cells of B/r form short filaments and are collected into micro-colonies; the cells of B_{s-1} and B_{s-2} remain small, with a few short filaments appearing in areas containing B_{s-2}. After examination, the plates were re-incubated overnight and the spread areas re-examined without a microscope. Areas containing B or B/r show heavy confluent growth; those containing B_{s-2} show many small, separated colonies; those containing B_{s-1} are either blank or show at most one or two colonies. In cases of doubt, the colony was subcultured once more and given a test dose of u.v. radiation, after spreading an appropriate dilution on a nutrient agar plate. The test dose (20 sec. of u.v. under these conditions) gave the following surviving fractions for our bacterial stocks: B_{s-1}—1×10^{-7}, B_{s-2}—1×10^{-5}, B—1×10^{-2}, B/r—8×10^{-1}.

X-irradiation. The X-ray technique has been described (Hill, 1958*b*). Cultures were grown by aeration in nutrient broth for 24 hr. and 5 ml. undiluted aliquots irradiated.

Inactivation by decay of incorporated ^{32}P. This procedure has also been described (Fuerst & Stent, 1956; Hill, 1960). Each culture was grown for 1½ hr. in non-radioactive medium so that the organisms were in the logarithmic phase. They were then

diluted 1/10 into ^{32}P-containing medium and growth continued for another 4 hr. The cultures were diluted into cold non-radioactive medium and samples frozen and stored at −78°. All four strains were grown in samples of the same batch of ^{32}P-labelled medium on the same day.

'*Reactivation*'. Heat reactivation (HR) was determined by preparing two sets of plates containing spread bacteria and incubating one set at 37° and the other at 45°. The numbers of colonies appearing after 19–22 hr. were then compared. In the experiments involving reactivation by the plating medium (PMR), the numbers of colonies appearing on nutrient broth agar plates were compared with those on synthetic M9-agar plates (Dulbecco, 1950). Photoreactivation (PHTR) was obtained in the following manner: Two ml. of the u.v.-irradiated cultures or of radioactive samples (after dilution into physiological saline) were put into test tubes supported in a specially constructed stainless-steel bath. (This apparatus was built by the Laboratory Glass and Instrument Corp., 514 W. 147 St, N.Y.C., N.Y. and was generously lent to us by S.A. Ellison.) One face of the bath consisted of quartz. The bath contained a clear lucite inner tank filled with 0·05 M-$CuCl_2$ and a test-tube rack. The space between the outer walls of the bath and the inner tank and rack was filled with water maintained at 37° by means of a heating coil and thermoregulator. The lamp used for illumination (Hill & Rossi, 1952) was placed 15 cm. from the quartz face of the bath. After an exposure of 1 hr., the cultures were diluted appropriately and plated for colony count.

Nuclear multiplicity. Twenty-four hr. aerated broth cultures were grown and samples spread on nutrient agar plates. After 15 min. and after 2 hr. of incubation at 37°, stained impression smears were prepared (Robinow, 1944; Witkin, 1951). Photomicrographs of representative fields were then made and used to count the average number of 'nuclei' per cell.

DNA and RNA contents. These chemical analyses were performed by Dr Herman Shapiro of the Cell Chemistry Laboratory, Department of Biochemistry, Columbia University. The DNA and RNA contents of 24 hr. aerated broth cultures were determined colorimetrically by using the diphenylamine and orcinol reactions, as described by Dische (1955).

The total number of bacteria was determined by a modification of the Millipore filter method (Ecker & Lockhart, 1959). One hundredth of a ml. of each culture was diluted into 5 ml. of physiological saline and diluted further 1/10 with 1 % picric acid. Ten ml. of the picric-acid dilution were filtered; the filtered cells were stained with 0·1 % acid fuchsin and then counted.

Inhibition by furacin. The antibiotic, furacin (5-nitro-2-furaldehydesemicarbazone) was obtained from the Eaton Laboratories, Norwich, N.Y. The inhibiting concentration was determined by the gradient plate technique (Szybalski, 1952).

Mutability. Spontaneous mutation rates were estimated from the average numbers of mutants appearing in series of cultures grown in parallel (Luria & Delbrück, 1943). The double-irradiation technique for the detection of B/r mutants in cultures of strain B could not be applied to cultures of B_{s-1} and B_{s-2} (Witkin, 1947). This technique is based upon the formation of snake formation by irradiated cells (Payne *et. al.* 1956). Attempts to induce snake formation by irradiating cells of B_{s-1} and B_{s-2} have been unsuccessful so far. Therefore mutants of the B/r type in cultures of B, B_{s-1} and B_{s-2} were detected indirectly by selecting for first-step furacin-resistant mutants.

In the first set of experiments involving mutation to furacin resistance, the cultures were grown in nutrient broth without aeration. One tenth ml. of each culture and of a 1/10 dilution were spread on the surface of nutrient agar plates containing 1·5 μg. furacin/ml. This concentration is inhibitory for all bacteria of strains B, B_{s-1} and B_{s-2} under our conditions and slightly less than the concentration required to inhibit the majority of bacteria of type B/r (see below). In later experiments, a pour-plate technique was used. The cultures were grown by aeration and used to determine mutation rates both to furacin resistance and to phage T_1 resistance. Cultures of strain B were diluted 1/100 and 0·1 ml. was added to a tube containing 10 ml. of nutrient agar plus 1·5 μg. furacin/ml. at 45°. The contents of the tube were then poured into a Petri dish, allowed to harden and then incubated. In the case of B_{s-1}, 1 ml. of the undiluted cultures was added to 9 ml. of the furacin-containing agar. One tenth ml. of the remainder of the cultures of each strain was then spread on plates which had been previously spread with 5×10^9 T_1 phages.

RESULTS

Survival after u.v.-irradiation, X-irradiation and ^{32}P decay

Figure 1 *a* shows the u.v. survival curves of B, B_{s-1} and B_{s-2} and for comparison, the beginning of the sigmoid curve characteristic of B/r (Witkin, 1947). It can be seen that although both B_{s-1} and B_{s-2} are more radiosensitive than the parent strain B, they are not identical.

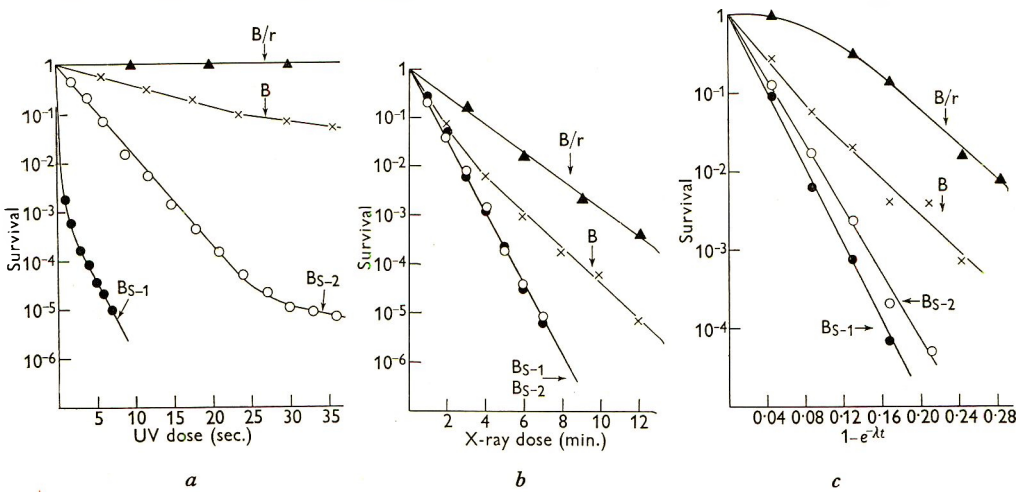

Fig. 1. Survival of *Escherichia coli* strain B and its mutants after (*a*) u.v.-irradiation, (*b*) X-irradiation and (*c*) decay of incorporated ^{32}P. The dose rates were 5·6 ergs/mm²/sec for u.v. and 3000 roentgens/min for X-rays; the specific activity of the ^{32}P in each strain was 20 mc./mg total P.

The survival curves for B, B_{s-1} and B_{s-2} have the same shape, i.e. a decreased slope at higher doses. In the case of strain B, this change in curvature cannot be fully explained by the selective survival of B/r mutants and is usually interpreted as due to the presence of bacteria which are phenotypically but not genetically radioresistant (Cavalli & di Modrone, 1949; Gunter & Kohn, 1956; Buzzell, 1956). This situation was investigated for mutants B_{s-1} and B_{s-2} by testing 100 colonies of each

stemming from cells surviving high doses, corresponding to the final portions of the curves. In the case of B_{s-2}, the final slope was about the same as the final slope for B (and B/r). In one experiment, 84 out of 100 colonies corresponding to 6×10^{-6} survival exhibited the well-defined snake formation characteristic of strain B. Two of these colonies were subjected to a test dose of u.v.; their survivals were 2·4 and 1·5 %, in agreement with the 1·9 % survival of strain B used for comparison. However, in another experiment, only 1 out of 100 colonies corresponding to 2×10^{-6} survival of B_{s-2} showed snake formation. Therefore it may be concluded that, although a form resembling strain B can be isolated after large doses of u.v. to B_{s-2} cultures, the change in curvature of the B_{s-2} survival curve cannot be wholly accounted for by the presence of this form in the unirradiated population.

In the case of B_{s-1}, the final slope of the survival curve was considerably greater than the final slopes of the curves for the other strains. Of 100 colonies corresponding to 1×10^{-5} survival, all gave a cytological response typical of B_{s-1}. Thus with B_{s-1}, as with B and B_{s-2}, the change in slope of the survival curve cannot be wholly due to a genetically resistant fraction. If the change is due to a phenotypically resistant fraction, the phenotype is not that of the presumed resistant phenotype present in either the B or B_{s-2} populations.

Although the u.v. survival curves for B_{s-1} and B_{s-2} were clearly separated, the X-ray survival curves coincided (Fig. 1*b*). Furthermore, the X-ray curves, unlike the u.v. curves, were exponentials. After X-irradiation, the survival curve for the parent B still had the shape characteristic of u.v.-irradiation, i.e. concave upward.

The survival curves corresponding to inactivation by decay of incorporated ^{32}P were also clearly separated and in the same relative positions as for u.v.-inactivation (Fig. 1*c*). It may be noted that in the case of all three inactivating agents, the survival curve for strain B has the same shape—concave upward. The curves for B_{s-1} and B_{s-2} have this shape only for u.v.-irradiation; for X-irradiation and for ^{32}P decay, the curves are exponentials. On the other hand, the curve for B/r is an exponential only for X-irradiation and is sigmoid for u.v. and ^{32}P decay (Witkin, 1947; Fuerst & Stent, 1956).

Prevention of lethal effects ('reactivation')

Figure 2 shows the effectiveness of HR, PMR and PHTR after u.v.-irradiation of the four strains. Certain differences are readily apparent. In the case of the parent strain B, all three reactivation mechanisms were equally effective. PHTR was effective for B/r but HR had no effect and PMR caused an increase in mortality after high doses of u.v. (Roberts & Aldous, 1949). In the case of the two radiosensitive mutants, all three reactivation methods were effective but not to the same extent. For both mutants, PHTR was the most efficient method. In the case of B_{s-1}, HR was more effective than PMR but the reverse was true for B_{s-2}.

Similar differences in reactivability were observed with X-irradiation and decay of incorporated ^{32}P. For example, Fig. 3 shows that after exposure to X-rays, HR was more effective for strain B than for B_{s-1}. HR was ineffective for B_{s-2} and lethal for B/r. In the case of ^{32}P decay also, HR was effective only for B (Hill, 1960) and to a smaller extent for B_{s-1}.

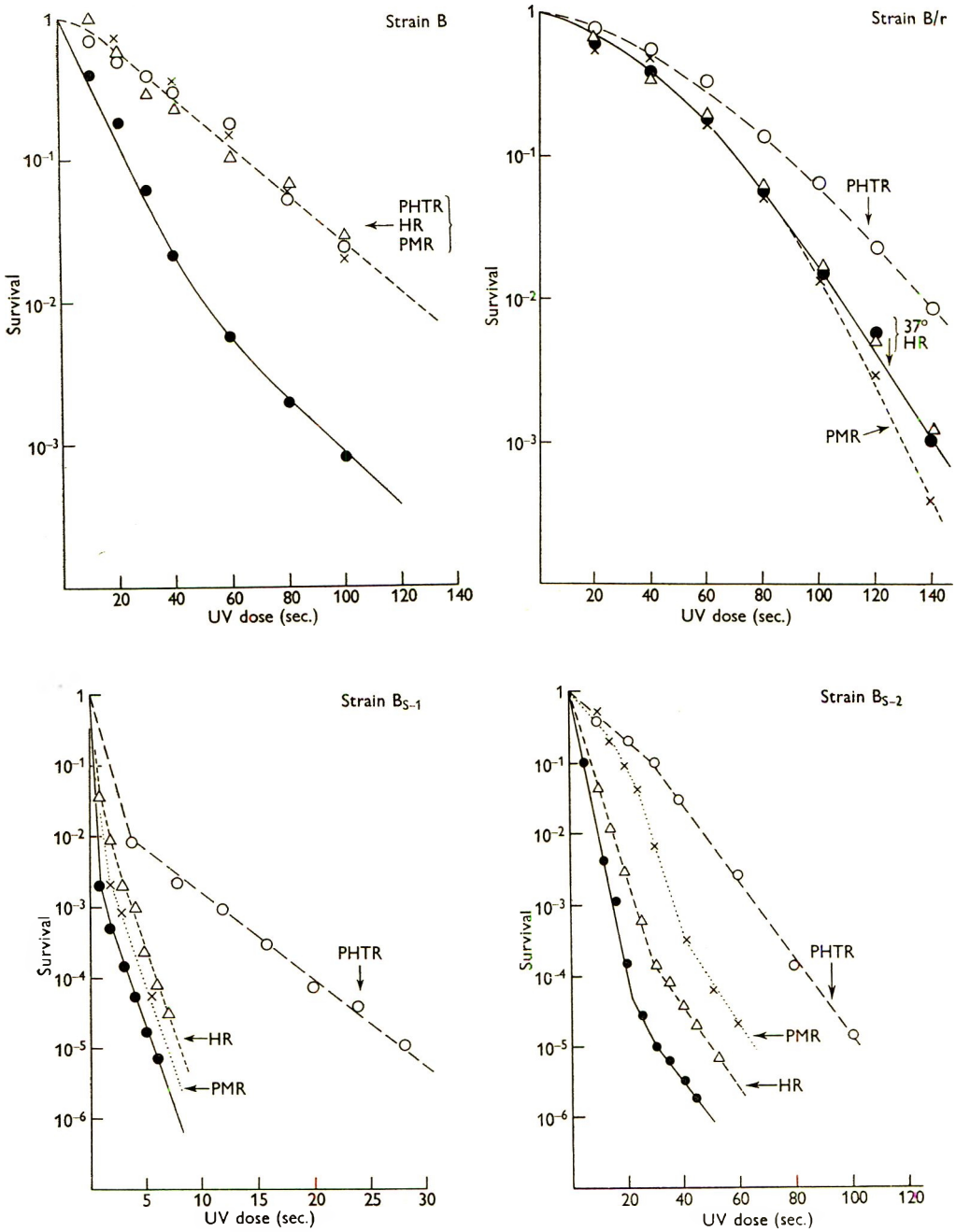

Fig. 2. Effect on survival of various treatments after u.v.-irradiation. PHTR = photoreactivation (illumination with wavelength 3650 Å); HR = heat reactivation (incubation at 45°); PMR = plating medium reactivation (incubation on M9 medium).

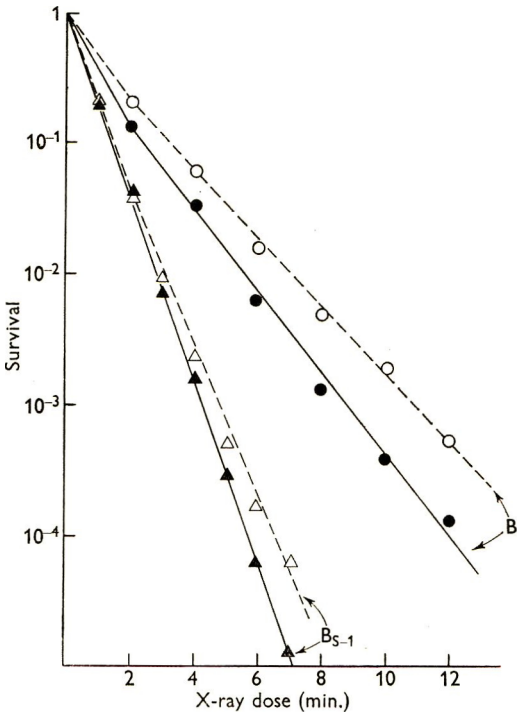

Fig. 3. Effect of incubation at 45° on survival of strains B and B_{s-1} after X-irradiation. ● and ▲, incubation at 37°; ○ and △, incubation at 45°.

Furacin sensitivity

The inhibitory concentrations of furacin (μg./ml.) were as follows: B/r—2·3, B—0·25, B_{s-1}—0·23 and B_{s-2}—0·53. As expected, B/r was markedly more resistant than the other strains. The value for B was the concentration inhibitory for a majority of B cells; the gradient pattern for B did not show a sharp break, but at concentrations greater than 0·25 μg./ml. the density of bacterial growth decreased (Payne *et al.* 1956). The gradient patterns for B_{s-1} and B_{s-2} showed sharp breaks at the above furacin concentrations. It was concluded that the furacin sensitivity of B_{s-1} was the same as that of a majority of B cells, while that of B_{s-2} corresponded to that of a minority of B cells.

Nuclear multiplicity, DNA and RNA contents

No significant differences between the four strains were observed with respect to these properties (Table 1).

In addition to these studies, the DNA of strains B, B_{s-1} and B/r was extracted by S.M. Beiser of the Department of Microbiology, Columbia University. Chromatographic fractionation on Ecteola-cellulose columns was performed by A. Bendich of the Sloan-Kettering Institute (Bendich, Pahl & Beiser, 1956). No obvious differences in profile between the strains were observed.

Table 1. *Nuclear multiplicity, DNA and RNA contents*

Strain	Average nuclear no.* of cells growing on agar for 15 min.	Average nuclear no.* of cells growing on agar for 2 hr.	DNA (μg./cell)	RNA (μg./cell)
B_{S-1}	1·23	2·00	$6·5 \times 10^{-9}$	$3·3 \times 10^{-8}$
B_{S-2}	1·20	1·92	$6·3 \times 10^{-9}$	$3·2 \times 10^{-8}$
B	1·27	1·85	$7·5 \times 10^{-9}$	$4·2 \times 10^{-8}$
B/r	1·25	1·83	$6·2 \times 10^{-9}$	$2·9 \times 10^{-8}$

* These numbers are the averages of values obtained by three observers, each of whom counted 200 cells per strain.

Spontaneous mutation

The spontaneous mutation rate to T_1-resistance was essentially the same for the four strains (Table 2). On the other hand, mutation to the B_S forms produced a striking change in the subsequent ability to mutate to furacin-resistance (Table 3). The parent strain B mutated to furacin resistance at the rate of 10^{-5}/bacteria/generation, in agreement with the reported value for the rate of mutation to radio-resistance (Witkin, 1947). However, in a total of approximately 4×10^{11} plated bacteria of strain B_{S-1}, no furacin-resistant mutants were detected. It seemed possible that such mutants might have been produced but unable to survive under conditions of growth in the presence of the parent furacin-sensitive B_{S-1} bacteria. This was tested by growing mixtures containing known initial percentages of B_{S-1} and B/r bacteria

Table 2. *Spontaneous mutation to T_1-resistance*

Vol. of each culture = 10 ml. Vol. of samples = 0·1 ml.

Culture no.	Strain: Numbers of mutants in culture B_{S-1}	B_{S-2}	B	B/r
1	169	26	170	319
2	61	123	77	29
3	57	102	22	564
4	165	47	24	38
5	66	28	80	17
6	34	3	4	43
7	153	31	18	38
8	79	32	32	37
9	29	19	28	101
10	72	18	78	197
11	33	61	18	23
12	15	65	42	39
13	98	84	18	54
14	23	194	182	14
15	82	431	36	72
16	100	297	21	15
17	88	149	24	112
18	35	100	55	13
19	415	64	29	46
20	29	30	33	11
Aver. no. mutants/culture	9000	9500	4950	8910
Bacteria/culture	$1·9 \times 10^{10}$	$1·9 \times 10^{10}$	$9·6 \times 10^{9}$	$1·4 \times 10^{10}$
Mutation rate	$4·8 \times 10^{-8}$	$5·2 \times 10^{-8}$	$5·5 \times 10^{-8}$	$6·4 \times 10^{-8}$

Table 3. *Spontaneous mutation to furacin resistance*

Method 1: cultures grown without aeration; samples spread on surface of agar containing furacin.

Method 2: cultures grown with aeration; samples added to melted agar containing furacin; agar poured into Petri dish.

	Strain				
	B_{s-1}		B_{s-2}	B	
Method	1	2	1	1	2
Vol. culture (ml.) ...	3	10	3	3	10
Vol. sample (ml.) ...	0·01	1	0·01	0·01	0·001
	No. of mutants in culture				
Culture no.					
1	0	0	0	554	35
2	0	0	0	16	15
3	0	0	0	11	30
4	0	0	1	10	41
5	0	0	0	432	36
6	0	0	0	9	6
7	0	0	0	7	90
8	0	0	1	46	17
9	0	0	0	374	23
10	0	0	2	2	32
11	0	0	0	6	19
12	0	0	0	4	29
13	0	0	3	947	3000
14	0	0	0	17	30
15	0	0	1	7	12
16	0	0	0	17	21
17	0	0	0	8	24
18	0	0	0	9	30
19	0	0	0	40	14
20	0	0	3	16	13
Aver. no. mutants/culture	0	0	0·55	$3·8 \times 10^4$	$1·8 \times 10^6$
Bacteria/culture	$7·32 \times 10^8$	$1·9 \times 10^{10}$	$4·8 \times 10^9$	$3·8 \times 10^8$	$6·9 \times 10^9$
Mutation rate	0?	0?	$5·4 \times 10^{-8}$	$9·0 \times 10^{-6}$	$1·25 \times 10^{-5}$

and then determining the final composition. The results showed that instead of failing to compete successfully with B_{s-1} bacteria, the final percentages of B/r were somewhat higher than expected (Table 4). Therefore it appears that either B_{s-1} mutates to furacin resistance, i.e. to the B/r form at a greatly reduced rate compared to the rate for strain B, or does not mutate at all.

Table 4. *Survival of strain B/r when grown with strain* B_{s-1}

The strains were grown separately in nutrient broth, diluted and then mixed in various ratios. The total bacterial concentration in each mixture was $9·2 \times 10^4$ per ml. The mixtures were grown to saturation and tested for the percentage of each component by diluting and plating on nutrient agar with and without 1·5 μg. furacin/ml.

Initial % B/r	50	10	1·0	0·10
Final total titre	$3·0 \times 10^8$	$2·8 \times 10^8$	$3·1 \times 10^8$	$2·1 \times 10^8$
Final titre as determined by plating on agar containing furacin	$2·6 \times 10^8$	$7·3 \times 10^7$	$8·6 \times 10^6$	$3·5 \times 10^7$
Final % B/r	86	26	2·8	0·17

In the case of B_{s-2}, eleven furacin-resistant colonies appeared in a total of 10^3 bacteria plated. This gave a mutation rate of $5{\cdot}4 \times 10^{-8}$—considerably less than that for strain B. These eleven colonies were tested for (*a*) sensitivity to phage T_1 in order to eliminate possible contaminants; (*b*) cytological response to u.v. irradiation; (*c*) survival to a test dose of u.v. radiation; (*d*) inhibitory concentration of furacin. No contaminants were found. According to criteria (*b*), (*c*) and (*d*), one colony consisted of typical B/r bacteria. However, the remaining ten colonies (not all independent) were peculiar. Their cytological response to u.v. was not entirely characteristic of B_{s-2}, since many, instead of a few, short filaments, were formed after 3 hr. incubation and in addition they showed confluent growth after 24 hr. Furthermore, these colonies resembled B/r in furacin resistance, but their survival to the test dose of u.v. was like that of B (Table 5).

Table 5. *Properties of colonies originating from strain B_{s-2} plated in the presence of furacin*

	% surv. to test dose of u.v.	Snake formation	Inhibiting concn. furacin (μg/ml.)
Strain B control	1·9	+	0·25
Strain B/r	77	0	2·8
Colony no.			
1	1·2	0	2·4
2	1·5	0	2·3
3	1·7	0	2·3
4	17*	0	> 5
5	0·91	0	2·6
6	1·1	0	2·6
7	2·4	0	2·5
8	1·2	0	2·6
9	0·90	0	3·0
10	0·64	0	3·0
11	0·95	0	3·3

* Although this % survival is lower than the value for the particular strain of B/r used in this experiment, it agrees with values for three other strains of B/r in our laboratory.

DISCUSSION

Several investigators have reported that strains of B/r characterized generally by increased radioresistance (compared to that of the parent strain B) constitute a heterogeneous group when examined more critically (Witkin, 1947; Kohn & Gunter, 1956; Adler & Haskins, 1960). Although only two mutants showing increased radiosensitivity were studied here, these also were found to be different and distinguishable by their response to u.v.-irradiation and to ^{32}P decay. The coincidence of the survival curves for X-irradiation may have been due to the choice of experimental conditions. Strain B_{s-1} could be distinguished from B_{s-2} since the survival after X-irradiation of the former could be increased by incubating for colony formation at an elevated temperature.

The present results show that neither the nuclear multiplicity nor the nucleic-acid content are responsible for the differences in sensitivity between strain B and the two types of radiological mutants, Br and B_s. Similar conclusions regarding

B and B/r have also been drawn by Gillies & Alper (1960). Evidently these differences are due to changes at the gene level. The present failure to find corresponding differences in the chromatographic profiles of the DNA of these strains is disappointing but probably due to an inadequate resolving power (A. Bendich, personal communication).

Since the function of the gene is to determine metabolic processes in the cell, changes in radiosensitivity reflect changes in these processes. For a given strain, the radiosensitivity may change if experimental conditions are varied, for example, by the application of different post-irradiation or post-decay treatments. The present results show that PHTR, HR and PMR were all effective in increasing the survival of B, B_{s-1} and B_{s-2} after u.v.-irradiation, although the relative effectiveness of these three kinds of reactivation was different for each strain. However, only PHTR was effective for strain B/r. After X-irradiation and after ^{32}P decay, HR was effective for B and B_{s-1} but not for B_{s-2} and B/*r*. This failure to increase the survival of B/r by post-treatments (other than PHTR) is also evident in the work of Alper & Gillies (1960) in which it was shown that B and B/r were equally radiosensitive under certain conditions. This equality resulted from a decreased resistance for B/r and an increased resistance for B when the strains were u.v.-irradiated during the rapid growth phase and then plated on defined medium instead of the usual broth. Similarly, Okagaki showed that post-u.v. treatment with chloramphenicol increased the resistance of B but was lethal for B/r (1960). Thus although the mutation of B to the B_s form confers an obvious disadvantage as far as inactivation is concerned, it is interesting that mutation to the B/r form can be disadvantageous with respect to reactivation.

The finding that the four strains did not differ in their spontaneous mutation rates to T_1-resistance can be interpreted as further evidence that the genetic differences between them do not involve the *whole* genetic apparatus but only a limited part, probably one or a few genes. This is corroborated by the very different rates of mutation of B, B_{s-1} and B_{s-2} to furacin resistance, i.e. mutation at a locus which is related to increased radioresistance.

Since, as mentioned above, strains of B/r have been found to be heterogeneous, it seems possible that strain B contains a number of loci such that mutation at any one confers increased radio and furacin resistance. Only two studies to date have been reported involving recombination between radioresistant strains. Bryson & Tuttle (private communication) crossed *Escherichia coli* B/r and *E. coli* K12, which is also radioresistant as compared to B. Some of the recombinants were radiosensitive in that they were snake formers like B. However, this could have resulted either from the presence of a single radioresistance gene in non-identical positions in B/r and K12, which are unrelated strains or from the presence of multiple loci in either or both strains. More recently, Zelle & Ogg reported recombination data involving three strains of B/r, one of which was characterized by increased cell size and increased radioresistance as compared to the other two B/r types (1957). The large cell variant is believed to be diploid or polyploid so that recombination might involve whole chromosomes. Thus the data concerning this strain do not illuminate the question of the possible multiplicity of radioresistance loci in the usual, presumably haploid, B/r. Unfortunately, the presence of the B phenotype was not looked for among the recombinants of the other two B/r strains.

In the absence of this kind of data, it can be assumed at present that strain B does

contain a number of 'radioresistance' genes. Since the present findings also indicate heterogeneity of B_s types, it might be also assumed that strain B contains a number of 'radiosensitive' genes. The evidence with respect to B_{s-2} suggests that there is at least one such gene. In particular the finding of B-like forms in irradiated populations of B_{s-2} indicates that reversion at this presumed locus can occur. If B_{s-2} were to mutate at one of the radioresistance loci, the resulting strain might have just the hybrid properties found for the ten colonies on furacin plates, i.e. such strains might be furacin-resistant like B/r, but since there has been mutation of two genes, one of which confers *increased* and the other *decreased* radioresistance, the resultant radiosensitivity might be something in between that of B_{s-2} and that of B/*r*, namely, that of B.

Strain B_{s-2} also yielded one mutant which was typically B/r—both furacin- and radioresistant. This could have arisen in at least one of two ways. The extemely low frequency of this B/r—one in a total of 10^{10} bacteria plated—suggests that there may have been two mutations in B_{s-2}. These might both have occurred at radioresistance loci. The second such mutation might raise the radioresistance from that found for the putative hybrid strains to a level much more like that of a typical B/r. Alternately, this B/r mutation might have arisen from a reversion at the B_{s-2} locus itself followed by a mutation of the resulting B-type cell to B/r. It was mentioned that in one culture of B_{s-2}, 85 % of the colonies obtained after a large dose of u.v. were like strain B (snake formers) whereas in another culture, only 1 % such colonies were found. This variation in frequencies suggests that the B-like forms were surviving spontaneous revertants. If so, then the mutation of B_{s-2} to B would have to occur spontaneously at a fairly high rate. Since the mutation rate of B to B/r is of the order of 10^{-5} (Witkin, 1947), an over-all frequency of 10^{-10} for the mutation B_{s-2} to B to B/r does not seem unreasonable. A choice between these two possible modes of origin of B/r from B_{s-2} must await attempts to detect selectively B in B_{s-2} populations and consequently to measure the mutation rate of B_{s-2} to B.

The findings with respect to B_{s-1} do not quite fit in with the idea that B contains a number of radiosensitive loci. In this case, neither furacin-resistant mutants nor B-like forms were detected. The simplest explanation is that the radiological loci are missing in this strain, i.e. that it results from a deletion in strain B. The recent report that u.v.-irradiated phages T_1, T_3 and T_7 show a considerable decrease in plaque count with B_{s-1} compared with B and B_{s-2} is in agreement with this hypothesis (Ellison, Feiner & Hill, 1960). It might be supposed that the chromosomes of B, B_{s-1} and B_{s-2} contain loci necessary for the replication of normal phages of these three types but that the chromosome of B_{s-1} alone is missing a piece necessary for the growth of u.v.-damaged phage. This piece might also contain the radiological loci.

A last point to be considered is the finding that the mutation of B to B/r was accompanied by increased furacin resistance, whereas mutation to either of the B_s forms did not increase furacin sensitivity. Apparently only the radioresistance genes can alter furacin sensitivity. This suggests that both properties result from the same alteration in cellular metabolism. Therefore it would be interesting to determine whether, under conditions in which the radioresistance of B/r is markedly decreased, its furacin resistance is decreased also and conversely whether, when the radioresistance of B is increased, there is an increase in its furacin resistance. One possible set of conditions is that described by Alper & Gillies (1960).

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The Measurement of Bacterial Viabilities by Slide Culture

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SUMMARY

A procedure is described for measuring the viabilities (% viable/total organisms) of populations of *Aerobacter aerogenes* by short-term incubation on agar films followed by differential counting under the microscope. The only special apparatus needed is a supply of metal annuli. The procedure estimates viability with 95% fiducial limits of ±9%; it appears applicable to other aerobic bacteria, to yeasts and to spore suspensions.

INTRODUCTION

The viability of a microbial population may be defined pragmatically as the proportion of its members that are capable of multiplication when provided with optimal conditions for growth. In this usage an organism may be called dead if it does not multiply in those conditions, and certain philosophical difficulties in the concept of death as applied to micro-organisms (see, for example, Valentine & Bradfield, 1954; Powell, 1956) may be avoided. Many procedures are available for determining the viability in this sense, the most popular being that of obtaining simultaneous total and viable counts on the population. Other procedures include 'vital staining', based on the assumption that death is accompanied by an altered permeability to dyes, which has been used with yeasts (e.g. Townsend & Lindegren, 1953) and bacteria (e.g. Knaysi, 1935; Knaysi & Ford, 1938; White, 1947). Strugger (1948) modified this technique by using a fluorescent dye with preparations of soil bacteria and Razumovskaya & Osipova (1958) applied Strugger's method to pure cultures of *Acetobacter* spp. Techniques based on the belief that death is associated with inability to reduce a redox dye such as a tetrazolium have been reported (e.g. Greenberg, Eidus & Diena, 1958). Koch (1959) assumed that leakage of labelled purines from populations of labelled bacteria measured the extent of death in such populations. Barer, Ross & Tkaczyk (1953) proposed that immersion refractometry should distinguish dead from living cells since their refractive indices were often different; this technique was used by Firkhman (1959). Mager, Kuczynski, Schatzberg & Avi-dor (1956) showed that populations of live Gram-negative bacteria had a greater optical density in solutions than in distilled water, but that dead ones did not, and suggested the exploitation of this 'optical effect' for viability determination. Wade & Morgan (1954) developed a staining procedure which made use of the accumulation of ribonucleic acid that takes place before a viable organism divides; Valentine & Bradfield (1954) measured viability by incubating populations in 3% urea, which permitted growth but not division, and determined the ratio of giant to ordinary cells with an electron microscope. Powell (1956) grew bacteria on a cellophan grid and determined the ratio of the individual count before incubation

to the group count after incubation; Taubeneck (1957, 1959) proposed modifications of Powell's procedure for slide cultures.

A critique of the methods available is not appropriate to the present paper, but their existence and the fact that none is widely accepted as both satisfactory and convenient perhaps justifies the publication of yet another one. The procedure reported below is a modification of the slide culture used by Knöll (1944) and others. The cellophan or Formvar grids needed for Powell's procedure or Taubeneck's modification are dispensed with; the method requires no special apparatus beyond microscope slides, metal annuli and a microscope set up for phase contrast or dark ground illumination. It has proved satisfactory as a routine procedure for studying the viability of microbial populations, provided these fall in the range 5–100 %.

METHODS

Preparation of agar cultures on slides. Brass annuli of 20 mm. internal diameter, 22 m. external diameter and 1 mm. depth were cut from piping; these support circular coverslips (nominally $\frac{7}{8}$ in., actually less) without overlap. They were cleaned, chemically blackened to facilitate observation of the sealing operation (below) and stored in 98 % (w/v) ethanol in water ready for use. Clean microscope slides 0·8–1 mm. thick were kept in ethanol, and before use both were burned dry and the annulus placed on the slide. A 0·22–0·24 ml. portion of hot agar medium (see below) was spread inside the annulus, thus sticking it to the glass, and the whole was stood under a Petri plate lid for 5 min. A portion of the population to be studied was diluted to *c* 100 million organisms/ml., spread on the surface of the agar film and allowed to stand for 10 min. If the surface of the agar was not dry by then, excess fluid was wiped off with a piece of clean filter paper.

A clean coverslip kept in ethanol was wiped with a cloth and placed on the annulus over the agar. The annulus-coverslip interface was touched with a loop of distilled water and capillarity caused water to flow between the two surfaces, thus sealing the agar under an air space. In spite of evaporation of the distilled water, the seal remained effective for many hours. The slide was incubated at a suitable temperature and for an appropriate period (37° and 2–4 hr. with populations of *Aerobacter aerogenes*) cooled and inspected under phase contrast either after cooling to room temperature or after storage for up to 18 hr. at 4°. Two stages in the preparation of these slides are illustrated in Pl. 1.

Preparation of agar. Commercial agar preparations and broths brewed from butchers' meat frequently contain solid debris and dead bacteria, both of which interfere with counts. We have usually found it desirable to filter agar media hot through a Millipore filter before use, though occasional batches have been obtained with which this expedient was not necessary. Blood agars were unsatisfactory because the corpuscles interfered with the phase contrast used for microscopic examination of the slides.

Inspection of slides. For bacteria and yeasts we have used a binocular microscope (magnification of ×360; field diameter: 0·29–0·32 mm.) with a 4 mm. objective arranged for phase contrast in green light. A condenser with a working distance sufficient to focus through the slide and agar film is necessary; we have successfully used microscopes supplied by Messrs Cooke, Troughton and Sims Ltd. and by

Messrs Watson Ltd. Mr R. E. Strange and his colleagues in this Establishment have used dark ground illumination satisfactorily. An objective having a long working distance is an advantage, since the slide may then be inspected without removing the coverslip, but fogging of the coverslip can be a nuisance and routinely we removed it for counts. Differential slides of pathogenic bacteria were sterilized by exposure to formaldehyde vapour before inspection.

Fields containing forty objects (dead bacteria or microcolonies) were usually chosen for counting, though with populations of low viability a larger number of objects per field was permissible. The microcolonies and dead organisms per field were counted separately. Sufficient whole fields, preferably adjacent, were counted to give a total of over 300 objects per slide studied. A tally counter or electric counting device is a great help in routine work of this kind. The viability was derived directly from the ratio of the number of microcolonies to the total number of microbial objects counted.

RESULTS

Aerobacter aerogenes (NCTC 417) obtained from Professor Sir Cyril Hinshelwood's laboratory was grown in continuous culture at 40° and at a dilution rate of 0·2–0·3 vol./hr. in a glycerol + salts medium resembling that used for *Cloaca cloacae* by Herbert *et al.* (1956) in which glycerol limited growth. Organisms were harvested in the logarithmic phase of growth, washed twice in a mixture of 0·137 M-NaCl (9 parts) + 0·048 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH 7·15 ± 0·15; 1 part) and incubated in this buffer at 40° with gentle aeration to obtain moribund populations. In some experiments, for reasons unconnected with this paper, 0·316 mM ethylenediaminetetra-acetic acid (EDTA) was present. Data quoted refer to organisms harvested between the 250th and 1000th generations in continuous culture.

Growth on slide cultures

As a preliminary, some fresh or moribund populations were watched from shortly after inoculation until considerable multiplication of the viable bacteria had taken place. Records were kept of the behaviour of the individual organisms. The following points were established:

(i) When a large proportion of the bacteria were viable, microcolonies tended to over-grow the non-viable organisms. Hence fields containing more than 30–40 objects should be avoided.

(ii) In populations showing a high viability, all organisms that were going to divide had done so by the time the earliest starters had undergone two or three divisions. With moribund populations (less than 30 % viability) the scatter of individual division lags was wider and more prolonged incubation was desirable.

(iii) Moribund populations contained a small proportion of organisms that appeared to attempt growth but to fail. They 'died' as drumstick-shaped or spherical bodies of low contrast. Such organisms were encountered most often in populations of intermediate (70–20 %) viability.

(iv) Loss of organisms owing to lysis was rare (usually a recognizable 'ghost' could be seen) and restricted to populations of less than 20 % viability.

(v) Loss of organisms owing to lysis or overgrowth occurred most often when the average division lag was long. Hence media rich in nutrient supplements were

desirable. In the case of *Aerobacter aerogenes* the medium of choice was that used for continuous culture of the organism supplemented with 0·1 % (w/v) Difco yeast extract, 0·1 % (w/v) Difco Casamino acids, 1/10th volume of home-brewed tryptic meat broth and Difco agar to 1·5 % (w/v). On this medium satisfactory differential counts were obtained after 2–4·5 hr. at 37°; moribund populations required the longer period of incubation.

Evaluation of the method

The reproducibility of the method with a population of bacteria dying 'naturally' was studied in the following manner. Organisms harvested from continuous culture at 40° were washed twice in saline + tris buffer, suspended in distilled water to their original concentration and 1 ml. of this suspension added to four 50 ml. portions of saline + tris buffer. The population was then *c.* 5×10^7 organisms/ml. The tubes were incubated at pH $7{\cdot}0 \pm 0{\cdot}2$ and 40° with gentle aeration; one tube (A) was sampled at fairly frequent intervals during 10 hr., single differential counts being performed on the population. On some of these occasions tube A was tested in quadruplicate and then single differential counts on the other three tubes were also performed, thus providing blocks of seven tests on populations that should have had identical viabilities. These blocks of seven tests were performed in closely spaced pairs intended to bracket a high, a medium and a low viability of the population.

The counts were performed by three operators, none of whom 'specialized' in counting samples from a given suspension; each had his own microscope. On one occasion (9 hr.) the operators re-counted each others' slides; Fig. 1 includes all the counts then obtained. The death curve of this population was essentially linear (Fig. 1); apparent tendencies to a sigmoid form suggested by inflexions during the first two and last hours fell within the 95 % fiducial limits of the regression line and the death curve has been drawn without them. (Other experiments on this population indicated that the later inflexion was probably real.) Some of the data from which Fig. 1 was plotted are given in Table 1. A χ^2 test on the actual counts from which these data were obtained showed excellent agreement within each set of seven counts; the usual 95 % fiducial limits of the regression line were $\pm 9{\cdot}2$ %; the viabilities of the four suspensions did not tend to appear in any order during the 10 hr. of the experiment.

Comparison with a conventional viability determination. A second death curve was obtained by using ordinary plating-out procedures, combined with fourteen separate total counts by three operators on the initial population using two Thoma counting chambers of 0·03 mm. depth. This procedure gave a mean population of $5{\cdot}9 \times 10^7$/ml. but in our hands the standard deviation of the technique was $\pm 1{\cdot}44 \times 10^7$ (counts by individuals showed greater reproducibility). Hence the error of the absolute viability from this source was 25 % without taking account of errors in the plating-out procedures. The experiment is illustrated in Fig. 2. The death curve obtained by the conventional procedures resembled that obtained by the differential procedure, but the viabilities were systematically lower. This difference is most likely due to errors in the total count; when viabilities were expressed as percentages of the initial plate count (taken as 97 %, the value indicated by the differential count), the death curves followed very similar courses.

Table 2 illustrates some data obtained with a population which survived for a considerably longer period; there is close agreement between the differential count and the colony count, based on the assumption that the initial colony count represented *c.* 100% viability. These data were obtained in this laboratory by Mr R. Strange and his colleagues; as indicated in the legend, they refer to stationary phase *Aerobacter aerogenes* grown in conditions different from those used by ourselves.

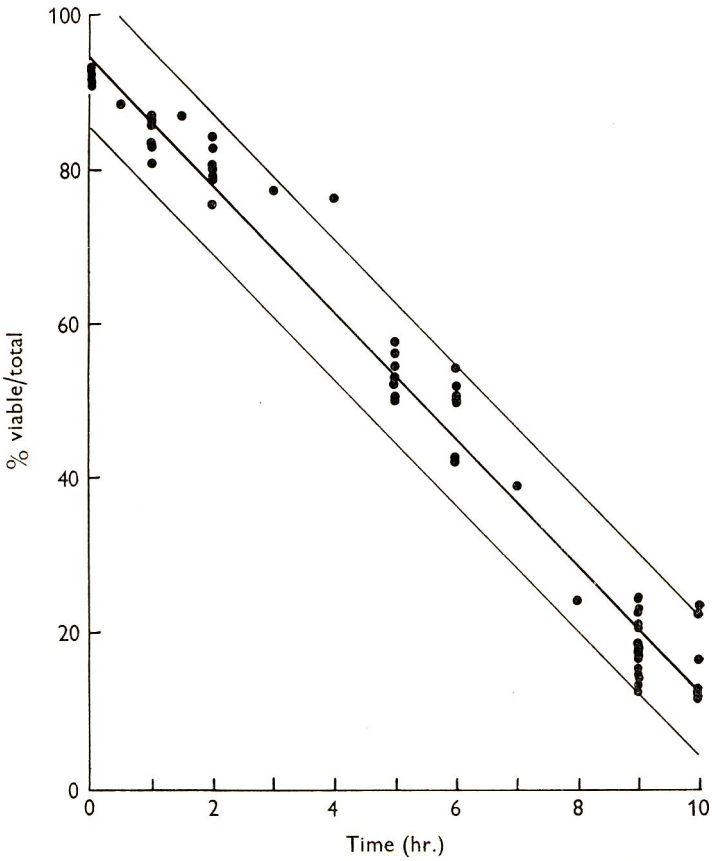

Fig. 1. Death curve of log phase *Aerobacter aerogenes* in aerated saline + tris buffer at 40°. Viabilities of four similar populations of *A. aerogenes* were determined by three operators using the differential counting procedure. For methods and sampling plan see text; for selected data see Table 1.

Various applications of the method

Use with bactericides. The procedure provides a rapid method of determining the proportion of a population killed by a bactericide and may be used to follow the killing rate of such a compound, provided the kill can be expressed in percentages rather than powers of ten. Table 3 shows titrations of the immediate bactericidal effects of methylene blue, aureomycin and proflavine on *Aerobacter aerogenes* harvested in the logarithmic phase; Fig. 3 shows a death curve in which methylene blue exerted a delayed bactericidal effect even though its concentration was insufficient to show an immediate effect.

Use with freeze-dried material. The procedure is useful for assessing the viability of suspensions before and after freeze-drying. A population of log phase *Aerobacter aerogenes* had a viability of 95·1 %; after freeze-drying its viability was 4·4 % by a combination of conventional total and viable counts; 4·1 % by the differential counting procedure.

Use with other micro-organisms. Several microbes have been grown on slides in the manner described to see whether their microcolonial appearance was suitable for use by the method. Those that were successful are listed, with the temperature of incubation and the incubation periods at which the differential count was clear and

Table 1. *Representative sample of experimental material from which Fig.* 1 *was obtained*

Viabilities of four similar dying populations of *Aerobacter aerogenes* were determined by three operators using the differential counting precedure. For methods and sampling plan, see text.

Time of incubation (hr.)	Population tested (A, B, C or D)	Micro-colonies	Dead organisms	Viability (%)	Person counting	Time slide culture was incubated (hr.)
0	A_1	292	26	91·8	JRP	2
0	A_2	280	21	93	JRH	2
0	A_3	289	21	93·2	JRH	2
0	A_4	282	20	93·4	JRH	2
0	B	285	29	90·8	JEC	2
0	C	285	21	93·1	JEC	2
0	D	301	24	92·6	JRP	2
0·5	A	273	33	89·2	JRH	2
1	A_1	300	44	87·2	JRH	2·5
1	A_2	260	51	83·6	JRP	2·5
1	A_3	261	52	83·4	JRH	2·5
1	A_4	269	43	86·2	JRH	2·5
1	B	247	57	81·2	JEC	2·5
1	C	279	44	86·4	JRP	2·5
1	D	292	40	86·6	JRH	2·5
1·5	A	274	40	87·2	JRP	2·5
6	A_1	166	153	52	JRP	2·5
6	A_2	144	196	42·3	JRH	2·5
6	A_3	156	150	50·9	JEC	2·5
6	A_4	152	148	50·6	JEC	2·5
6	B	144	194	42·6	JRH	2·5
6	C	158	158	50	JRP	2·5
6	D	172	143	54·6	JRP	2·5
7	A	120	187	39·1	JEC	2·75
8	A	75	236	24·1	JEC	2·7

constant recorded in parentheses. *Pseudomonas ovalis* (30°; incubated for 2, 3½ hr.), *Serratia marcescens* (37°; 1¼, 2 hr.), *Pasteurella pestis* (28°; 4½, 6 hr.), *Salmonella typhimurium* (37°; 2, 3 hr.), *Micrococcus* 'M 9' (37°; 3½, 5 hr.), *Bacillus subtilis* (37°; 2, 3 hr.), *Alkaligenes metalcaligenes* (18–20°; 17, 19 hr.), bakers' yeast (18–20°; 18, 22 hr.). All bacteria were tested in home-brewed tryptic meat agar; that for *P. pestis* was supplemented with haematin. 'Viability' of the micrococcus was obtained as percentage viable units in the population because the strain showed many pairs and tetrads before incubation; hence the relatively long incubation periods. Bakers' yeast (pressed yeast from Distillers Co. Ltd.), which was grown on a medium containing malt extract, peptone, yeast extract and glucose, showed

91 % viability on slide culture and 90 % viability by the methylene blue vital staining procedure (Townsend & Lindegren, 1953) when fresh; after 14 days in buffer at pH 7 ± 0·3 and room temperature the slide procedure gave a viability of 62 % compared with 18·5 % indicated by the methylene blue procedure. The procedure provided a rapid spore germination test with suspensions of *B. subtilis*, *B. megaterium* and *B. cereus* spores.

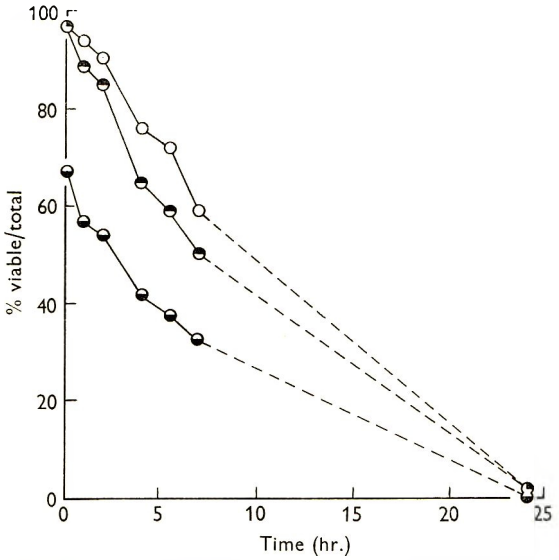

Fig. 2. Death curves of a population of log phase *Aerobacter aerogenes* obtained by conventional procedures and by slide culture. Logarithmic phase *A. aerogenes* were washed by centrifugation and incubated in saline + tris buffer containing a small amount of EDTA at 40°, pH 6·8 ± 0·05. Samples were removed at intervals for viability determinations (see text). ◒ Conventional procedure; ○ differential procedure; ◓ colony counts as % initial colony count.

Table 2. *Comparison between colony and differential counts on a population of* Aerobacter aerogenes

A. aerogenes grown in tryptic meat broth and harvested after the population density had become stationary for 0·5 hr. were incubated with aeration at 37° in NaCl (0·13 M) + KH_2PO_4/K_2HPO_4 (0·02 M), pH 6·50 ± 0·05.

Incubation period (hr.)	0	16	24	40	48	64	72	88
Colony count/10^{-8} ml.	98	95	90	96	95	75	73	60
Differential count (%)	97·5	98	96	90	87	78	68	59

Organisms for which the method was not suitable were *Escherichia coli* type B (37°; 1¼–3½ hr.) and *Bacillus megaterium* (*c.* 37°, *c.* 2½ hr.), both of which formed a mixture of ordinary cells and long ramifying filaments which obscured the slide.

DISCUSSION

The determination of viability directly by slide culture has certain obvious limitations. The method as described is limited to non-filamentous aerobes; bacteria with division lags longer than the incubation period are counted as dead; all organisms that have divided once are counted as live, even though both may occasionally be dead. The method is at its most exact at 50 % viability, and is highly

Table 3. *Bactericidal effects of three inhibitors on* Aerobacter aerogenes

Bacteria were harvested from continuous culture at 40°, washed twice and populations of *c.* 6×10^7 organisms/ml. exposed to bactericide solutions in saline + tris buffer (pH 7), centrifuged and re-suspended in saline + tris buffer. The viable populations were then determined by slide culture. Exposure to inhibitor and centrifugation took about 10 min. at room temperature. Controls without dye were also centrifuged.

Bactericide	μg./ml.	Viability (%)
Methylene blue	—	90·7
	5	82·3
	10	55·9
	20	39·1
	40	7·6
	80	1·3
Proflavine	—	92·9
	10	87·4
	20	89·3
	40	82·7
	80	62·4
Aureomycin	—	96·5
	10	91·7
	20	94·7
	40	86·7
	80	56·1
	100	39·3

insensitive at viabilities below, say, 5 %; errors due to overgrowth of dead organisms by microcolonies or to lysis on the agar film exist and may assume importance with organisms other than *Aerobacter aerogenes*. Moribund cultures often yield morphological aberrants and experience is needed to decide whether the organism is living,

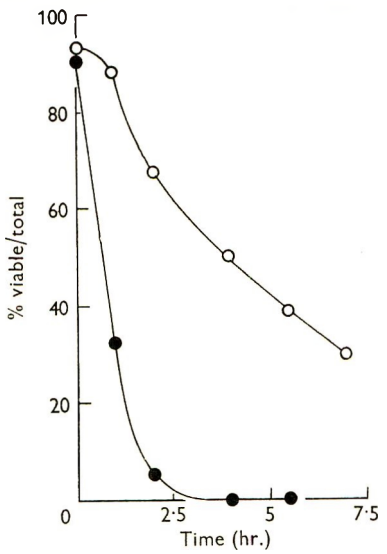

Fig. 3. Progress of bactericidal action of methylene blue on log phase *Aerobacter aerogenes*. Suspensions of *c.* 5×10^7 washed organisms/ml. saline + tris buffer containing EDTA aerated at pH 7·05 ± 0·05 and 40°. 1 ml. samples were removed at intervals, washed once by centrifuging to remove inhibitor and the viabilities determined by slide culture. ○ Control; ● methylene blue, 5 μg./ml.

dead, or a contaminant. The method has no obvious use in the counting of anaerobes; it cannot be used with populations totalling less than *c.* 10^7 organisms/ml. Within these limitations, however, the method has one or two advantages. The operator has added confidence in the method because he actually sees the viable and non-viable organisms instead of deducing their presence from altered staining properties, permeability, dehydrogenase activity or refractive index. The ordinary determination of the ratio of total to colony counts is slow as well as being beset by two sets of experimental errors; it is probable that the differential count described here gives a considerably more accurate ratio provided the two counts fall within a tenfold range of each other. Finally, though it is probably inferior in accuracy to other methods based on direct observation of growth of individual organisms (Valentine & Bradfield, 1954; Powell, 1956), it requires considerably less specialized apparatus.

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EXPLANATION OF PLATES

PLATE 1

Slide cultures for differential counts in preparation. The right-hand annulus contains agar but has not been covered. The left-hand annulus has been covered with a coverslip and is in process of being sealed with water from a loop.

PLATE 2

Photomicrograph of the surface of a slide culture of a moribund population of *Aerobacter aerogenes*. The population from which this slide was prepared had been harvested in the logarithmic phase of growth, washed by centrifugation and aerated for 1 hr. in saline + tris buffer with Versene at 40°, pH 7·05 ± 0·05. A portion was then incubated as a slide culture for 3·5 hr. Viability: 68 %. Phase contrast; magnification × 800.

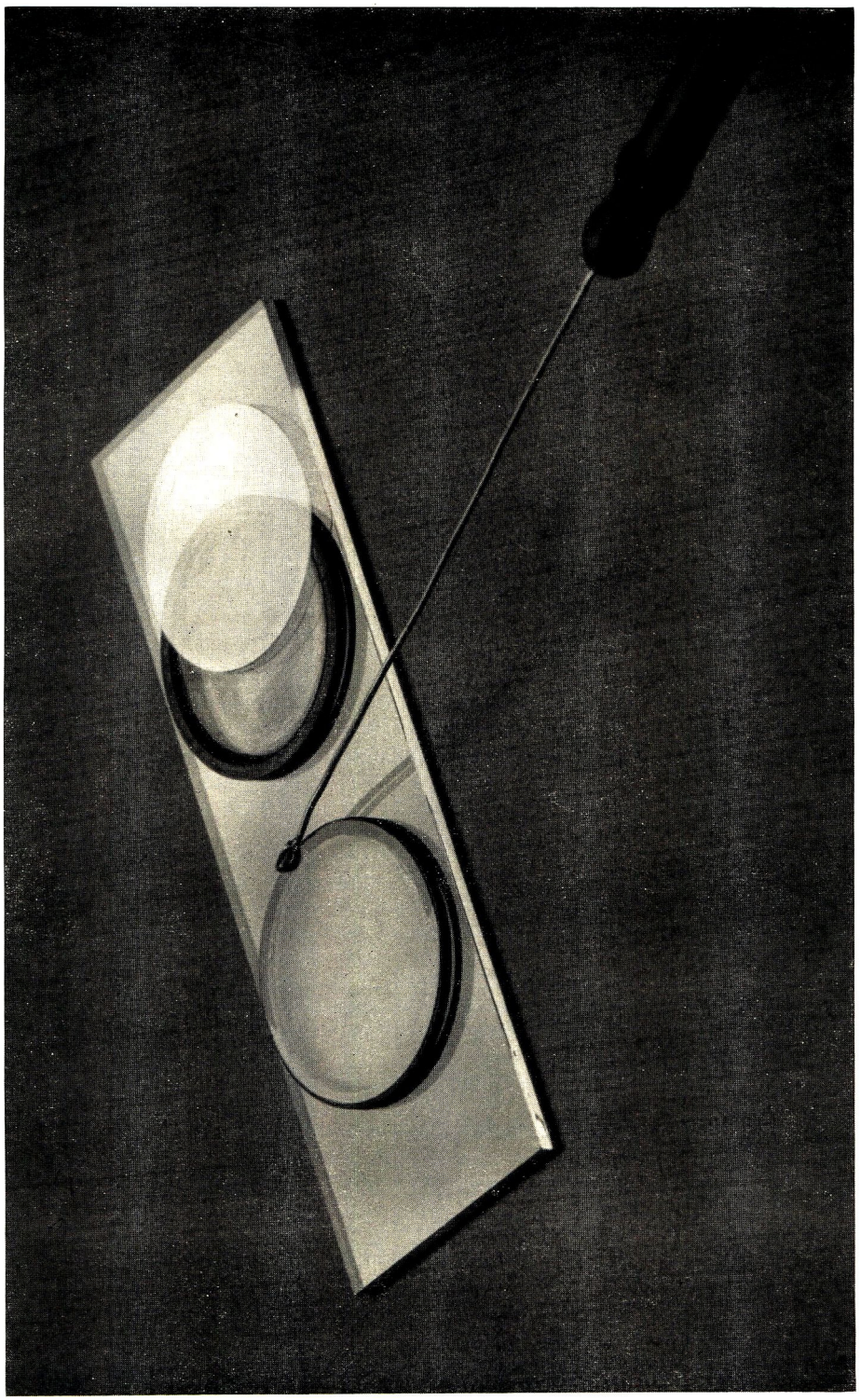
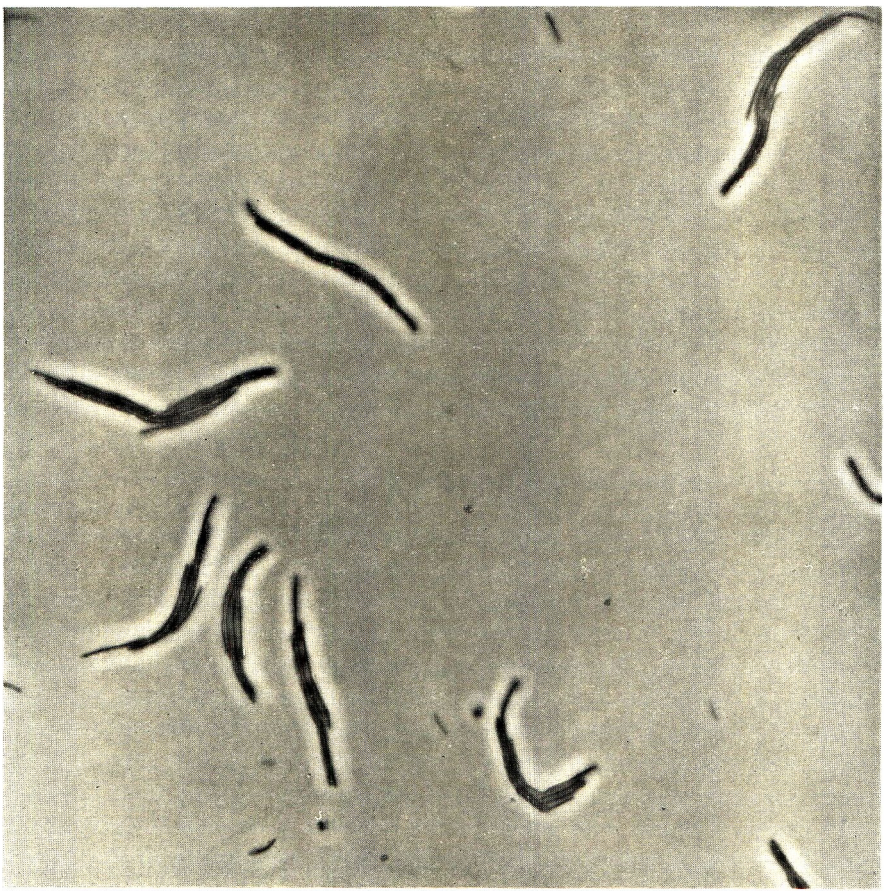


Plate 1



J. R. POSTGATE, J. E. CRUMPTON AND J. R. HUNTER

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The Effect of Asparagine on the Growth of a Gram-Positive Coccus

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SUMMARY

A *Pediococcus* sp., unusual in that it needed reducing conditions for growth, would not grow in a medium based on acid-hydrolysed casein but grew when asparagine was added. Neither glutamine nor aspartic acid + an ammonium salt satisfied this requirement. When the acid-hydrolysed casein was replaced by a mixture of amino acids, the omission of asparagine caused a decrease in growth in the presence of aspartic acid + an ammonium salt. No growth occurred in the absence of both aspartic acid and asparagine.

INTRODUCTION

Strains of Gram-positive cocci morphologically similar to *Pediococcus cerevisiae* but differing in other respects were described by Gunther (1958). Since a requirement for folinic acid may be a useful character for recognizing the pediococci (Felton & Niven, 1953), an attempt was made to determine this requirement for some of Gunther's strains. This was done in a casein hydrolysate medium based on that described by Sauberlich & Baumann (1948) for the assay of folinic acid with *P. cerevisiae* ATCC 8081. However, none of these strains grew in this medium even when folinic acid was present, although a normal response to this growth factor was given by the assay organism. It was found that growth occurred when Tween 80 and tryptic digest of casein were added to the medium (Garvie & Gregory, to be published). Therefore, a search to identify the active component in the tryptic digest was undertaken.

METHODS

Test organism and its maintenance. Strain NCDO 1250, sent by Dr H. L. I. Gunther under the reference number S344, was used. It is a catalase-negative Gram-positive coccus, growing in pairs, tetrads and clusters but not in chains, and is considered to belong to the genus *Pediococcus*. It differs from other known strains of this genus in being unable to grow aerobically in normal media. It was grown anaerobically in a McIntosh & Fildes jar filled with 90 % (v/v) H_2 + 10 % (v/v) CO_2 or alternatively in the presence of air by adding 0·5 ml. of a 1 % (w/v) Seitz-filtered solution of cysteine hydrochloride or ascorbic acid to each 10 ml. of medium immediately before inoculation.

The strain was maintained in litmus milk supplemented with 0·3 % (w/v) Yeastrel, 1 % (w/v) glucose, 0·1 % (v/v) Tween 80, 1 % (w/v) chalk; cysteine hydrochloride

was added aseptically. After incubation at 30° for 24 hr. the cultures were stored at 4°.

Preparation of inoculum. The strain was grown in tomato glucose broth medium (Briggs, 1953) modified by Dr M. E. Sharpe, and having the following composition: (%, w/v) 1·5, peptone (Oxoid); 2·0, glucose; 0·5, Yeastrel; 0·5, sodium acetate; 0·5, KH_2PO_4; 0·2, di-ammonium citrate; (%, v/v) 10·0, tomato juice (Oxoid); 0·1, Tween 80; 0·5, salts B (Snell & Strong, 1939); adjusted to pH 6·5 and autoclaved (15 min. at 10 lb./sq.in.). Cysteine hydrochloride was added aseptically as described above. Serial transfers with an inoculum of *c.* 0·01 ml. were made after incubation for 48 hr. at 30°. A 24 hr. culture of the 3rd–5th transfer was used as the inoculum for the growth tests; one drop of a 1/100 dilution in quarter strength Ringer solution was inoculated into each assay tube.

Growth tests. The basal media (Table 1), which were modifications of that of Sauberlich & Baumann (1948), were made up at double strength and added in 5 ml. quantities to optically matched test tubes containing 5 ml. of test solution. The tubes were then covered with aluminium caps and autoclaved (10 min. at 10 lb./sq. in.). They were inoculated with strain NCDO 1250 and incubated at 30° for 48 hr., either aerobically or anaerobically, as described above. Relative amounts of growth were estimated turbidimetrically with a Lumetron 400-A colorimeter (Photovolt Co., New York).

Amino acid requirement of strain NCDO 1250. The growth of the cultures was measured in the defined medium A from which the amino acids were omitted, each in turn. Since cystine was one of the amino acids under test cysteine could not be used as a reducing agent and ascorbic acid was used instead in the tests incubated in air. Cultures which grew in the first test were serially transferred in the same medium to eliminate carry over of nutrients.

Estimation of the amino acid content of acid-hydrolysed casein and Tryptone by paper chromatography. Aqueous solutions (1 %, w/v) were adjusted to pH 2 by adding N-HCl and desalted on Dowex 50 (H^+) resin columns. The desalted eluates were concentrated under reduced pressure at 50° and used to prepare two-dimensional chromatograms on no. 1 Whatman paper by the procedure of Levy & Chung (1953).

Chemical estimation of glutamine and asparagine in Tryptone. Five ml. of 1 % (w/v) aqueous Tryptone (Oxoid) was hydrolysed in N-HCl for the estimation of total amide-N, and in borate phosphate buffer at pH 6·5 for the estimation of the amide-N of glutamine (Vickery *et al.* 1935). The ammonia produced was distilled into boric acid containing a mixed indicator of bromcresol green and methyl red (Conway, 1957). Solutions of glutamine and asparagine were analysed as controls, and blank values were determined by using distilled water as the test solution.

Free amino acid content of strain NCDO 1250. The strain was grown in the defined medium A as described earlier but with (*a*) asparagine omitted, (*b*) aspartic acid omitted, (*c*) asparagine and aspartic acid omitted but with Tryptone (Oxoid 5 mg./ml.) added. Extracts of washed organisms were made in 10 % (v/v) acetic acid and chromatograms prepared from the extracts by the method of Mattick, Cheeseman, Berridge & Bottazzi (1956).

RESULTS

Growth of strain NCDO 1250 *in medium A and medium B.* Good growth was obtained in defined medium A which contained a mixture of 19 amino acids and an ammonium salt. In contrast, growth did not occur under aerobic or anaerobic conditions in the partially defined medium B in which the amino acid mixture was replaced by acid-hydrolysed casein + tryptophan, cysteine, tyrosine, phenylalanine and histidine (Table 1), even in the presence of 1 μg. folinic acid/ml. medium. However, growth did occur under both conditions of incubation when 0·5 % (w/v) Tryptone (Oxoid; a tryptic digest of casein) was added. These observations suggested that medium B was deficient in some nitrogenous component and accordingly the amino acid requirements of the strain were studied.

Table 1. *Composition of double strength media*

Medium A

Glucose	50 g.	DL-Alanine	400 mg.
Sodium acetate	40 g.	L-Arginine	400 mg.
NH_4Cl	6 g.	L-Aspartic acid	200 mg.
Salts A (Snell & Strong, 1939)	12 ml.	L-Asparagine	800 mg.
Salts B (Snell & Strong, 1939)	10 ml.	L-Cystine	100 mg.
Tween 80	2 ml.	L-Glutamic acid	600 mg.
Adenine, guanine, uracil, xanthine, each	20 mg.	Glycine	200 mg.
Thiamine hydrochloride	1000 μg.	L-Histidine	100 mg.
Pyridoxal ethyl acetal hydrochloride	2000 μg.	DL-Isoleucine	500 mg.
		DL-Leucine	500 mg.
Ca-D-pantothenate	1000 μg.	L-Lysine	400 mg.
Riboflavin	1000 μg.	DL-Methionine	200 mg.
Nicotinic acid	2000 μg.	DL-Phenylalanine	200 mg.
p-Aminobenzoic acid	200 μg.	L-Proline	200 mg.
Biotin	2 μg.	DL-Serine	100 mg.
Folic acid	20 μg.	DL-Threonine	400 mg.
		DL-Tryptophan	80 mg.
		L-Tyrosine	200 mg.
		DL-Valine	500 mg.

Volume to 1000 ml. pH 6·8.

Medium B

Medium B had the same composition as medium A except that 10 g. vitamin-free acid hydrolysed casein (Difco or Allen and Hanbury's), 80 mg. tryptophan, 200 mg. cystine and 400 mg. each tyrosine, phenylalanine and histidine were used in place of the amino acid mixture.

Amino acid requirement of strain NCDO 1250. Omission of the following amino acids (singly) from medium A prevented growth of strain NCDO 1250: arginine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine. In contrast, no effect on growth was observed when alanine, aspartic acid or lysine was omitted. The omission of asparagine, however, decreased growth by some 50 %.

The amino acid deficiency of medium B. Chromatograms prepared from the Allen and Hanbury's acid-hydrolysed casein indicated that, as compared with medium A, medium B was deficient only in methionine and asparagine. The addition of either or both of these amino acids showed that asparagine was the limiting growth factor. In contrast, chromatograms of Tryptone showed the presence

of asparagine. By chemical analysis 1 mg. Tryptone was found to contain 0·08 mg. asparagine and a negligible amount of glutamine. In medium B the growth responses to Tryptone and asparagine were similar (Fig. 1) and indicated that 1 mg. Tryptone was equivalent in activity to about 0·15 mg. asparagine.

Specificity of the requirement of strain NCDO 1250 *for asparagine.* The growth activity of asparagine in medium B was not replaced by aspartic acid at 25–200 μg./ml. or by glutamine (1–500 μg/ml.) or glutathione (1–500 μg./ml.). In the absence

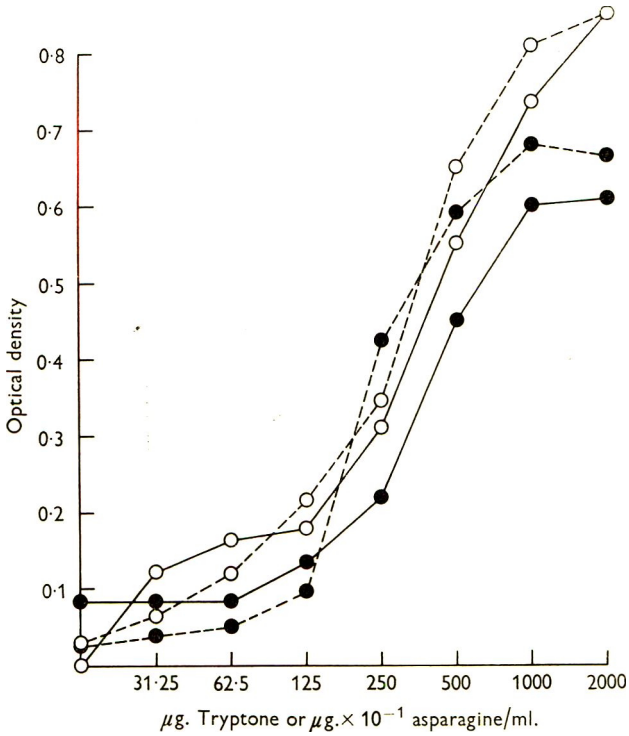

Fig. 1. Growth response of Pediococcus NCDO 1250 to Tryptone or asparagine after 48 hr. in medium B at 30°. ●—● = +asparagine aerobically with ascorbic acid; ●– – –● = +asparagine anaerobically; ○—○ = +Tryptone aerobically with ascorbic acid; ○– – –○ = + Tryptone anaerobically.

of both asparagine and aspartic acid from the defined medium A no growth occurred until either one or other of the acids was replaced. The growth response to asparagine (aspartic acid absent) was greater than that to aspartic acid (asparagine absent) at the same concentration by weight (0·1 or 0·4 mg./ml.). Increasing the glutamic acid up to 0·9 mg./ml. had no effect on growth.

Effect of asparagine on the amino acid pool. The amino acids found in the extracts of organisms grown in the defined medium A when either asparagine or aspartic acid was omitted, or with both omitted and tryptone added, were qualitatively and quantitatively similar. There were two outstanding features. First, no asparagine could be detected in the organisms even when it had been added to the medium, and, secondly, even when aspartic acid was omitted from the medium, it could still be found in the organisms in undiminished concentration.

DISCUSSION

The use of asparagine in bacteriological media is widespread. In media based on acid-hydrolysed casein it has been reported to stimulate the growth of lactobacilli (Evans & Niven, 1951) and, together with glutamine, of *Streptococcus lactis* (Niven, 1944). Asparagine also promoted the growth of butyric acid bacteria in corn mash but aspartic and glutamic acids were equally effective (Tatum, Peterson & Fred, 1935). Our finding that the Pediococcus strain NCDO 1250 in acid-hydrolysed casein medium had a requirement for asparagine which could not be replaced by glutamine or aspartic acid is similar to that of Niven (1944) for certain of his strains of *Streptococcus lactis*. However, when the casein hydrolysate was replaced by a mixture of amino acids the requirement of the Pediococcus for asparagine was satisfied by aspartic acid + an ammonium salt. It seemed possible that the requirement for asparagine in acid-hydrolysed casein medium was due to an imbalance of amino acids in the medium. For example, the large amount of glutamic acid in acid-hydrolysed casein might inhibit the transport of aspartic acid into the cell (Gale & van Halteren, 1951). However, no support for this hypothesis was obtained. Growth was not promoted when the concentration of aspartic acid was increased nor did high concentrations of glutamic acid inhibit growth in the defined medium.

Stokes, Koditschek, Rickes & Wood (1949) suggested a relationship between asparagine and strepogenin activity. In agreement with this, a major part of the stimulatory activity of Tryptone for the Pediococcus in the acid-hydrolysed casein medium could be attributed to its asparagine content. Asparagine peptides in the Tryptone would not be estimated by the procedure used and might account for the remaining activity (Miller & Waelsch, 1952). The precise role of asparagine in the metabolism of the Pediococcus strain examined remains to be established. Examination of the amino acid pool showed that asparagine was not concentrated there, suggesting that it was rapidly metabolized to provide as one of its functions the aspartic acid requirement of the cell. Whether under certain conditions it is more effective than aspartic acid because it is more easily transported into the cell remains to be determined.

The authors wish to thank Dr H. L. I. Gunther (Queen Elizabeth College, London University) for supplying the strain used in the investigation. They also thank Dr M. E. Sharpe (National Institute for Research in Dairying) for permission to publish the recipe of the tomato glucose broth.

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Comparative Carbohydrate Metabolism and a Proposal for a Phylogenetic Relationship of the Acetic Acid Bacteria

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SUMMARY

The oxidation of 20 carbohydrates and derivatives by 45 strains of acetic acid bacteria, representing most of the known species, was studied and correlated with the enzymic constitution of the organisms. The strains of the mesoxydans group of Frateur and the acetate-oxidizing Gluconobacters oxidized the greatest variety of substrates and contained the most complex enzyme system. The strains investigated could be arranged in two main lines, each one with a stepwise decreasing gradation of oxidative properties. In contrast to the expectations evoked by their names, the strains of the 'oxydans' group had only limited oxidative powers and the 'peroxydans' bacteria even less so. The enzymic mechanism of the catabolism of several carbohydrates was discussed. It is proposed to split the acetic acid bacteria into two biotypes: *Gluconobacter oxydans* and *Acetobacter aceti*, and to consider the existing species as varieties within the two main types. An approach to the phylogenetic interpretation of the intermediary carbohydrate catabolism of these bacteria is discussed.

INTRODUCTION

The present classification of bacteria is nearly always a system for grouping together apparently closely related strains and a useful key for the identification of unknown bacteria. The objections against the basis of this system, of which *Bergey's Manual of Determinative Bacteriology* (1957) is the standard compilation, have been voiced many times (van Niel, 1946, 1953; Skerman, 1949; Winogradsky, 1952; Sneath, 1957*a*) and need not be repeated here. The status of bacterial classification is still far removed from the situation in the taxonomy of higher plants and animals, for it is no reflexion of bacterial phylogeny. The considerations of van Niel (1946, 1953) on this problem and his views on a re-organization of our knowledge of the bacterial world in a natural relationship (adansonian relationship (Adanson, 1763) where morphological, physiological, biochemical, enzymic, ecological, immunological and other properties all have equal value) may open the way to an understanding of its phylogeny. To obtain this goal, a file-index with the complete properties of existing strains would be required, which is a task beyond practical possibilities for most investigators. Nevertheless, a first step in this direction has been made by the analysis with an electrical computing machine of many properties of Chromobacterium (Sneath, 1957*b*; Lysenko & Sneath, 1959) and of many bacteria from the (British) National Collection of Type Cultures (Sneath & Cowan, 1958).

A few years ago we began a study of the comparative carbohydrate biochemistry as a possible basis to show a natural relationship and phylogeny of the species within the group of 'acetic acid bacteria'. These bacteria are well suited for this kind of investigation because their species differentiation is mainly based on their carbohydrate metabolism. In Frateur's system of classification (Frateur, 1950) all the strains are arranged in four groups with the characters summarized in Table 1.

Table 1. *Summary of the properties of the four groups of acetic acid bacteria according to Frateur* (1950)

Group	Catalase	CO_2 production from acetic and lactic acids	Ketogenesis (glycerol to dihydroxyacetone; mannitol to fructose; glucose to 5-keto-gluconate)	Oxidation of glucose to gluconic acid
Peroxydans	−	+	− or very weak	−
Oxydans	+	+	− or very weak	+ (− for *A. ascendens*)
Mesoxydans	+	+	+	+
Suboxydans	+	−	++	++

Extensive investigations in our laboratory of the enzymic equipment of many strains have revealed that often only a few enzymes are responsible for the oxidative properties of intact organisms and that the interpretation of the Warburg experiments was often surprisingly simple and clear-cut in terms of the enzymic outfit of the organisms. Thus it was invariably found that strains which consumed 0·5 mole O_2/mole glucose, oxidized this substrate quantitatively to gluconate with an oxidosome-linked glucose oxidase or a soluble coenzyme-linked glucose dehydrogenase, or both. (Oxidosomes are oxidase-bearing, ultra-microscopic fragments of the 'ghost' or cytoplasmic membrane (De Ley, 1960).) The same applied to many other substrates (see below). Also, several substrates were oxidized by two pathways, one of them being the hexose monophosphate oxidative cycle (also called Horecker cycle, pentose phosphate cycle or shunt), the other usually a non-phosphorylative mechanism. In many cases the relative importance of these two mechanisms could easily be calculated (see, for example, De Ley & Schell, 1959*a*). The study of the oxidative capacities of acetic acid bacteria had thus a considerable advantage over most other bacteria, e.g. the Enterobacteriaceae and others, where the results of Warburg experiments do not allow conclusions about the pathways involved. Resting acetic acid bacteria have been used several times for qualitative studies (Bertho, 1932; Cozic, 1933; Kondo & Ameyama, 1958) or quantitative studies on oxygen uptake. We reported on *Acetobacter peroxydans* (De Ley, 1958*a*), the type species of the 'peroxydans' group, on *A. aceti* (De Ley & Schell, 1959*a*) from the 'mesoxydans' group, on *Gluconobacter suboxydans* (De Ley & Stouthamer, 1959) and on *G. liquefaciens* (De Ley & Dochy, 1960*a*, *b*). Several other species were studied by Stouthamer (1959, 1960). Yet we felt that the potentialities of this

method had not been exploited to their full extent. In the present paper we wish to clarify in part this situation by summarizing some of our results with the 45 strains of our collection, which represents most of the known species of acetic acid bacteria. We preferred to use authentic strains which have been described and named by other authors, instead of isolating fresh ones, since the former have often been the origin for the definition of new species, later used in the construction of several systems of classification (Henneberg, 1909; Lafar, 1914; Hermann & Neuschul, 1931; *Bergey's Manual*, 1957; Frateur, 1950). The recent papers by Shimwell (1959) and Shimwell & Carr (1959) rejecting the validity of most of the species of the acetic acid bacteria also induced us to submit this report. The fundamental differences between the interpretation of Shimwell and our own on the relationship amongst these bacteria are discussed below.

METHODS

Organisms. The origin of the strains used are mentioned in Table 2. Upon arrival all the strains were checked for purity by plating on a medium containing (w/v) 10% glucose, 3% $CaCO_3$, 1% yeast extract (Difco) and 2·5% agar, and were incubated for several days at 30°. Most of the cultures were pure. In some cases mixtures of colony types were seen. From those the strain, corresponding to the name on the label, was isolated. Pure strains were stored on agar slopes of the same medium at 4° and transferred bi-monthly. The purity of the stock cultures was checked several times a year by plating on several media. Occasionally, the stock cultures were also preserved on beer solidified with 12% (w/v) gelatine. These cultures have been used in our laboratory for about 3 years and in most cases no obvious change in their properties was noticed, which is at variance with the results of Shimwell (1957, 1959) and Shimwell & Carr (1959).

Mass cultures. The bacteria from a 2- or 3-day-old culture on a slope were suspended in sterile 0·01 M-KH_2PO_4-Na_2HPO_4 buffer (pH 6·0) and used to inoculate Roux flasks of the same glucose medium as above. In some experiments (see below) other sugars in 5% (w/v) concentration were used instead of glucose. The bacteria were grown for 2–3 days at 30°, harvested from the agar surface with 0·01 M of the same buffer (pH 6·0) with the aid of a magnetic stirrer, washed at least twice and finally suspended at a concentration of 100 mg. (wet wt.) living bacteria/ml. of the same buffer. These suspensions were used at once for the Warburg and other experiments. *Acetobacter ascendens*, strain A, was unable to grow on glucose; it was therefore grown on beer solidified with 12% (w/v) gelatine and incubated at 20° for 2–3 days.

Warburg experiments. These were carried out in the conventional apparatus at 30°. The main vessel contained 50–100 mg. wet wt. bacteria, suspended in 1·8 ml. 0·02 M-phosphate buffer (pH 6·0). The side arm contained 10 μmole of the substrate in 0·1 ml. and the central well 0·1 ml. of 20% (w/v) KOH. The oxygen uptake was followed until the reaction was over. When the oxidation was slow, it was followed up to 5 hr. The results were corrected for endogenous respiration, which was usually nearly negligible. With some strains the endogenous oxygen uptake remained nearly zero for 2–3 hr. and then increased logarithmically to 20 or more μmole O_2 after 5 hr. It appeared to be a spontaneous autolysis of some of the cells, providing oxidizable substrates for the living ones.

Fermentation experiments. A Kluyver type fermentometer (Kluyver, 1914) was filled with pure mercury. One ml. of a 0·033 M-phosphate buffer (pH 6·0) containing 100–500 mg. wet wt. living bacteria and 0·5 mmole glucose was introduced into the upper part of the closed arm and the apparatus incubated at 30° for at least

Table 2. *Origin and specification of the strains, mentioned in this paper*

Collection (name under which the organism was received)	Strain no. used in this paper	Generic name used in this paper
The National Collection of Industrial Bacteria (NCIB), Torry Research Station, Aberdeen		
Acetobacter aceti (Kützing) Beijerinck	8554	*Acetobacter*
A. acetosus (Henneberg) Bergey *et al.*	2224	*Acetobacter*
A. ascendens (Henneberg) Bergey *et al.*	4937	*Acetobacter*
A. ascendens (Henneberg) Bergey *et al.*	8163	*Acetobacter*
A. capsulatus Shimwell	4943	*Gluconobacter*
A. kuetzingianus (Hansen) Bergey *et al.*	3924	*Acetobacter*
A. melanogenus Beijerinck	8086	*Gluconobacter*
A. mesoxydans Frateur	8622	*Acetobacter*
A. mobilis Tosic & Walker	6428	*Acetobacter*
A. pasteurianus (Hansen) Beijerinck	8856	*Acetobacter*
A. peroxydans Visser 't Hooft	8618	*Acetobacter*
A. rancens Beijerinck	8619	*Acetobacter*
A. suboxydans Kluyver & de Leeuw	3734	*Gluconobacter*
A. turbidans Cosbie, Tosic & Walker	6424	*Acetobacter*
A. viscosus Shimwell	8131	*Gluconobacter*
A. xylinum (Brown) Holland	8747	*Acetobacter*
Bacterium vini acetati Henneberg	4939	*Acetobacter*
Bacterium xylinoides Henneberg	4940	*Acetobacter*
Laboratory for Microbiology, Technological University, Delft, Netherlands		
Acetobacter aceti var. *muciparus*	5	*Acetobacter*
A. ascendens (Henneberg) Bergey *et al.*	A	*Acetobacter*
A. lovaniensis Frateur	13	*Acetobacter*
A. melanogenus Beijerinck	16	*Gluconobacter*
A. mesoxydans var. *saccharovorans*	4	*Acetobacter*
A. paradoxus Frateur	30	*Acetobacter*
A. pasteurianus (Hansen) Bergey *et al.*	11	*Acetobacter*
A. rancens Beijerinck	15	*Acetobacter*
A. suboxydans Kluyver & de Leeuw	10	*Gluconobacter*
A. xylinum (Brown) Holland	12	*Acetobacter*
A. xylinum (Brown) Holland	25	*Acetobacter*
Dr I. Mehlman, Department of Food Technology, University of California, Davis, California, U.S.A.		
Acetobacter rancens Beijerinck (received as *A. peroxydans*)	'Davis'	*Acetobacter*
A. ketogenus	24	*Acetobacter*
Gluconobacter cerinus var. *rosiensis*	22	*Acetobacter*
Acetobacter suboxydans Kluyver & de Leeuw	26	*Gluconobacter*
A. rancens Beijerinck	23	*Acetobacter*
Prof. Asai, Institute for Applied Microbiology, Tokyo, Japan		
Gluconobacter liquefaciens Asai	20	*Gluconobacter*
G. cerinus Asai	21	*Gluconobacter*
Prof. J. Frateur, Laboratory for Microbiology, the University, Louvain, Belgium		
Acetobacter aceti (Kützing) Beijerinck	Ch 31	*Acetobacter*
Dr J. G. Carr, Department of Agriculture and Horticulture, the University, Bristol		
Acetobacter estunensis Carr	E	*Acetobacter*
Dr A. J. Stouthamer, Laboratory for Microbiology, the University, Utrecht, Netherlands		
Acetobacter suboxydans Kluyver & de Leeuw	SU	*Gluconobacter*

3 days. The amount of gas produced was read every day. Fermentations were also carried out in the Warburg apparatus in a CO_2 or N_2 atmosphere, with different amounts of glucose or fructose.

The detection of enzymes. As reported in 'Results', this was carried out by the methods described or referred to in previous papers (De Ley, 1954; De Ley & Verhofstede, 1957; De Ley, 1958*a*; De Ley & Schell, 1959*a*, *b*; De Ley & Dochy, 1960*a*, *b*; De Ley & Defloor, 1959).

RESULTS

Forty-five strains were studied. The properties of *Acetobacter peroxydans*, as previously reported (De Ley, 1958*a*) were included for comparison. The species names of the strains, as received from the different laboratories, were used unchanged. In accordance with Leifson (1954) we retained the generic name of *Acetobacter* for the peritrichous or non-flagellated strains of the mesoxydans, oxydans and peroxydans groups of Frateur (1950). For the polarly flagellated bacteria, most of which belong to the suboxydans group of Frateur, we adhered to the name of *Gluconobacter*, originally proposed by Asai (1934, 1935), for reasons to be mentioned below.

Because the bacteria were nearly always grown on glucose, the shape of the oxidation curves in the Warburg experiments might give an indication about the constitutive or inducible nature of the enzymes concerned in the metabolism of the other substrates.

Metabolism and oxidation of glucose

All the strains of our collection could be sharply divided into three groups a, b and c.

Group a. *Acetobacter ascendens*, strain A, *A. paradoxus* 30 and the strains of *A. peroxydans* did not attack glucose at all. We detected neither glucose oxidase nor glucose dehydrogenase. For *A. ascendens*, strain A, this explains the original description of Henneberg (1898), who noticed that this species was unable to make acid when growing on glucose media. Other so-called *A. ascendens* strains, e.g. NCIB 4937 and NCIB 8163, oxidized glucose with the uptake of 0·5 or more mole O_2/mole substrate. If we adhere to the definition of Henneberg and to the above results with strain A, we cannot include these strains NCIB 4937 and NCIB 8163 in the species *ascendens* but have to relegate them to another one.

Group b. Some strains oxidized glucose only with the uptake of 0·5 mole O_2/mole substrate. Here belong *Acetobacter rancens* 15, 23 and 'Davis', *A. pasteurianus* 11 and 8856, *A. kützingianus* 3924, *A. rancens* var. *turbidans* 8619 and *A. ascendens* 8163. The other oxidative capacities of these strains were also almost identical. Frateur (1950) redefined the species *A. rancens*; as an additional criterion to his definition we can now add that this species is able to oxidize glucose only to gluconic acid. On the enzymic level this means that *A. rancens* contains only a glucose oxidase and a glucose dehydrogenase or both, but no hexokinase or other mechanism to introduce glucose into the hexose monophosphate oxidative cycle. *A. lovaniensis* 13 carries out the same reaction with glucose, but is able to grow on ethanol and NH_4^+ as sole carbon and nitrogen source, which differentiates it from *A. rancens*. Both *A. rancens*, as redefined now, and *A. lovaniensis* are unable to use glucose as a carbon source; they grow chiefly at the expense of the yeast extract. The production of gluconate from glucose is an accidental event which has nothing or very little to

do with the physiological processes involved in the growth of the organisms. The production of gluconate in both species appears to be a useless reaction, which may possibly be regarded as an atavism, a remnant of a metabolic pathway from other acetic acid bacteria, such as the one we unravelled for *Gluconobacter suboxydans* (De Ley & Stouthamer, 1959). This interpretation would also explain the results of Hermann & Neuschul (1931) who found that many strains of acetic acid bacteria, while growing on glucose, converted this substrate nearly quantitatively into gluconate.

Group c. All the other strains of the collection oxidized glucose with an uptake of more than 0·5 mole O_2/mole substrate: from 1·2 for *Acetobacter estunensis* E to 4·9 O_2 for *A. mesoxydans* var. *saccharovorans* 4. On the whole most strains took up 1–3 mole O_2/mole substrate, which was far below the theoretically expected value of 6. The explanation for this phenomenon was given in a previous paper (De Ley & Schell, 1959*a*). Indeed, glucose was metabolized by two independent pathways, which competed for the substrate; one was the oxidation to gluconate and the other the hexokinase, followed by the hexose monophosphate oxidative cycle. If the former mechanism alone was active, an uptake of 0·5 mole O_2/mole glucose was expected; with the latter alone, 6 mole O_2/mole glucose. Intermediate O_2 uptake between these two extremes allows one to calculate how much of the glucose was oxidized by each pathway. We found (De Ley & Schell, 1959*a*) that *A. aceti* Ch 31 converted about 80 % of the glucose into gluconate, the remaining quantity being used as carbon and energy source. This calculation was corroborated by chemical analysis of the end products. By applying similar calculations to the strains of the present paper, which did not oxidize gluconate further, it was found that, for example, *A. vini acetati* 4939 converted 84 % of the glucose to gluconate, *A. estunensis* E about 87 %, etc. The remaining glucose was thus used by way of the hexose monophosphate oxidative cycle, which explains the irisation which occurs when these strains grow on the glucose–$CaCO_3$–yeast extract–agar medium. For most of the strains of the mesoxydans group and for Gluconobacter, these calculations could not be used, because ketohexonates were also formed.

Efficiency of glucose as a carbon and energy source for growing cells. For most of the strains the yield (wet weight) of organisms/Roux flask was determined. Figure 1 shows that there was a clear correlation between the yield of organisms and their ability in a resting state to oxidize glucose. The higher the O_2 uptake/mole substrate (that is the more glucose was consumed via the hexose monophosphate oxidative cycle), the higher the yield of the organisms. Organisms which oxidized glucose only to gluconate grew poorly as compared to the others, and all belonged to the species *rancens* as redefined above, which also strengthened the former conclusions. Again, this way of interpretating the results applied best to the bacteria of the oxydans group. For the strains of the mesoxydans group and for Gluconobacter, the excess O_2 uptake over 0·5 mole O_2 did not represent a true measure of the disappearance of glucose by way of the hexose monophosphate oxidative cycle. Yet, as a whole, the same general tendency was observed.

Glycolysis and the fermentation of glucose in the acetic acid bacteria. Neuberg & Simon (1928*a*, *b*) and Simon (1930) found that *Acetobacter ascendens*, *A. pasteurianus* and *Gluconobacter suboxydans* fermented glucose with the formation of about equal amounts of ethanol and CO_2. From a recalculation of their results it may be seen

that only part of the glucose fermented was accounted for by these end products. The remainder (usually 50–80 %) was apparently converted into unidentified end products. Simon (1930) withdrew the previous results and reported that indeed all of the glucose was accounted for as ethanol + CO_2. Why then was glucose not fermented to completion? The answer may be deduced from an experiment of Simon, in which he showed that ethanol appeared to inhibit the fermentation. These authors also described the presence of enzymes of glycolysis in these bacteria. Their conclusion, that acetic acid bacteria contained an active glycolytic mechanism, found its way in the literature (see, for example, Thimann, 1955). In fact, the results of

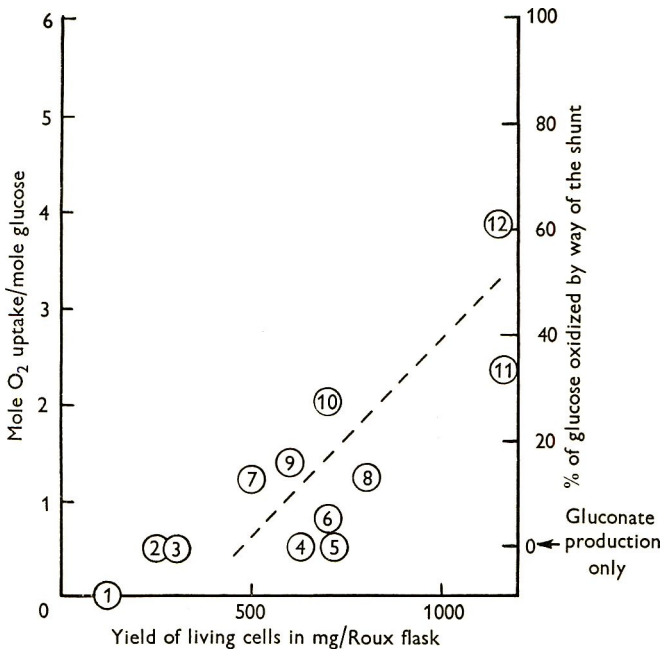

Fig. 1. The relationship between the yield of living cells (wet weight) from the oxydans group after 3 days of growth on the glucose medium in Roux flasks and the final O_2 uptake with glucose as a substrate in the Warburg with the same cells in a resting state. Strains used: 1, *Acetobacter ascendens* A; 2, *A. lovaniensis* 13; 3, *A. kützingianus* 3924; 4, *A. pasteurianus* 8856; 5, *A. pasteurianus* 11; 6, *A.* '*ascendens*' 8163; 7, *A. estunensis* E; 8, *A.* '*ascendens*' 4937; 9, *A. vini acetati* 4939; 10, *A. mobilis* 6428; 11, *A. turbidans* 6424; 12, *A. acetosus* 2224.

Neuberg & Simon are difficult to understand. Indeed, the typical *A. ascendens* cannot oxidize glucose at all; it contains neither glucose oxidase nor hexokinase. *A. pasteurianus* contains no hexokinase and oxidizes glucose only to gluconate. How then could these strains ferment glucose? A tentative explanation is that the strains used were neither ascendens nor pasteurianus. In the hope of detecting either the unknown end products or the mechanism of glucose fermentation by using labelled substrates, we repeated the experiments of Neuberg & Simon, but were unable to confirm their results. Most of the strains of our collection were used with the Kluyver fermentometer. In most cases only a trace of gas was formed. The gas production rarely exceeded 10 % of the theoretically expected amount of CO_2

(22·4 ml.) according to an alcoholic fermentation. At the end of the reaction 1 ml. of 20% (w/v) KOH was introduced and it appeared that only about three-quarters of the gas was CO_2. The nature of the residue was not assayed; only about 0·5 ml. or less was available. It could not have been due to an air leak, since the gas was always under a slight excess pressure, due to the level of the mercury in the open tube. In some cases more than 10% of the expected amount of CO_2 was formed, but it could invariably be shown that the suspension had become contaminated during storage in the fermentometer, generally with coliform organisms. Neither could gas formation be observed in the anaerobic Warburg experiments, even when they were continued for 5–7 hr. and with varying concentrations of sugar. Prof. J. Frateur kindly informed us (in 1959) that he had been unable to corroborate the results of Neuberg & Simon. Stouthamer (1960) also found no fermentation with *Gluconobacter liquefaciens*. Kitos *et al.* (1958) reported that in *G. suboxydans* the shunt accounted for the bulk of the oxidation. The general conclusion from all these results appears to be that only a few acetic acid bacteria may contain a very weak glycolytic system, that the claims of Neuberg & Simon have on the whole not been confirmed, and that it seems desirable to erase this opinion from the literature.

Oxidation of gluconate

Most of the strains of the oxydans group of Acetobacter were unable to oxidize gluconate. Nearly all the strains of the mesoxydans group and of *Gluconobacter* attacked this substrate vigorously. Only *Acetobacter aceti* 8554 and Ch 31, *A. aceti* var. *muciparus* 5 and *A. ketogenus* 24 attacked it very weakly.

Oxidation of 2- and 5-ketogluconate

None of the strains of the oxydans group were able to attack either 2- or 5-ketogluconate. In the mesoxydans groups of Acetobacter and in Gluconobacter, the oxidation occurred at random and could not be used for taxonomic purposes. The oxidative abilities for these substrates denoted merely the presence or absence of enzyme systems, described previously (De Ley, 1958*a*; De Ley & Defloor, 1959; De Ley & Stouthamer, 1959), which appeared to be arbitrarily distributed throughout the representatives of the latter groups. It should be stressed, however, that the Warburg method is unable to indicate the presence of weak enzymes. This explains the apparent discrepancy between our results and those of Frateur, Simonart & Coulon (1954). These authors found by paper chromatography that several strains of the oxydans group were able to form 2-ketogluconate when grown on glucose for up to 30 days. In the Warburg apparatus an uptake of 1 μmole O_2/5 hr./100 mg. wet wt. bacteria would surely be regarded as negligible. Yet it can be calculated that this low enzyme activity would be sufficient to allow the production of 2·9 mmole or 617 mg. calcium 2-ketogluconate from glucose/month/g. wet wt. bacteria.

The oxidation of mannitol and fructose

Most strains of the oxydans group of Acetobacter oxidized neither mannitol nor fructose; some strains oxidized both very slowly. Nearly all the strains of the mesoxydans group of Acetobacter and of Gluconobacter oxidized both substrates. Arcus & Edson (1956) showed a DPN-linked mannitol dehydrogenase in *G. suboxydans*

and we found a TPN-specific dehydrogenase in *A. aceti* Ch 31 (De Ley & Schell, 1959*a*). Fructose, which was formed or excreted outside the cell, became unavailable. We explained this by postulating a fructose permease. Mannitol, which was oxidized to fructose within the cells, was further metabolized. We have encountered similar situations in *G. viscosus* 8131 and *G. capsulatus* 4943. From all the strains investigated from the latter groups, only *A. mesoxydans* 8622, was unable to oxidize mannitol. It thus appears that the oxidation of mannitol by resting cells might be used as an additional criterion to decide whether a strain belongs in the oxydans or peroxydans groups of Acetobacter (mannitol negative), or belongs in the mesoxydans group of Acetobacter or in Gluconobacter. Fructose oxidation is less suitable for this purpose.

The oxidation of mannose, galactose and pentoses

Most of the strains investigated were able to oxidize mannose, galactose and pentoses to the corresponding sugar acids. Nearly all the strains except a few representatives of the oxydans group of Acetobacter oxidized mannose with the uptake of 0·5 mole O_2/mole substrate, presumably to mannonic acid. The same is also true for the oxidation of galactose and xylose, from which the corresponding galactonic and xylonic acids were formed. Only *Gluconobacter cerinus* 21 and *G. liquefaciens* 20 oxidized galactose with the uptake of 1·0 or more mole O_2/mole substrate, possibly to the 2-ketogalactonate stage or beyond. *G. cerinus* 21 oxidized xylose with the uptake of 0·95 mole O_2/mole substrate. About half of the strains from the oxydans group of Acetobacter did not attack these substrates. There was a remarkable correlation between the oxidation of both substances: strains which oxidized galactose also converted xylose into xylonic acid and strains which did not attack galactose did not oxidize xylose. This might suggest that the same enzyme was possibly responsible for the oxidation of both substrates.

Ribose and L-arabinose were never oxidized beyond the stage of the corresponding pentonates. We showed previously that all the above substrates are oxidized by oxidases, located on the cytoplasmic membrane (De Ley & Dochy, 1960*a*, *b*). D-Arabinose was not oxidized by any of our strains.

Two main conclusions can be drawn from these results. (i) Mannose, galactose, xylose, L-arabinose and ribose are unsuitable carbon sources for the growth of acetic acid bacteria. On the other hand, these bacteria show excellent promise for the eventual industrial production of these sugar acids. The corresponding enzymes effect an oxidation which has no or very little physiological meaning for the growth of the organisms, which, however, oxidize the substrate when it is present, because they happen to possess the oxidase. The situation is similar to the oxidation of glucose by *Acetobacter rancens* and *A. lovaniensis*. This possibility was supported by the following experiments. *A. turbidans* 6424 and *A. xylinum* 12 were grown on a medium containing either 5% galactose or 5% xylose, 3% $CaCO_3$, 1% yeast extract (Difco) and 2·5% Bacto-agar (Difco). These organisms were harvested and washed as usual. The yields of organisms, when grown on glucose, galactose, or xylose were nearly identical, showing that indeed they did not adapt by growing on the latter two substrates and that they were unable to use them as carbon or energy sources. Furthermore, organisms grown on either of the three substrates oxidized them in the Warburg at the same rate and to the final stage of galactonate and xylonate. (ii) The ubiquitous oxidative capacity for mannose, galactose and

the pentoses in the mesoxydans group of Acetobacter and in Gluconobacter, and the distribution in the oxydans group of Acetobacter made this a poor taxonomic character.

The oxidation of maltose and saccharose

The ability to oxidize maltose and sucrose was distributed at random over the strains examined. Again the oxydans group of Acetobacter was the poorest. About half of the strains of the mesoxydans group of Acetobacter and of Gluconobacter was able to oxidize either both or one of these substrates. Therefore it seemed appropriate to abolish the varieties 'maltovorans' and 'saccharovorans', as used in the system of Frateur. Lactose and raffinose were not oxidized by any of our strains.

Oxidation of glycerol

Glycerol was oxidized nearly to completion by all strains tested, except by *Acetobacter peroxydans*, *A. ascendens* 4937 and hardly by *A. vini acetati* 4939. It is probably an excellent carbon and energy source. An O_2 uptake of at least 2 mole O_2/mole glycerol was quite common. In some cases an induction period was noted, possibly pointing to the formation of an inducible enzyme (glycerokinase) in the enzyme chain.

Oxidation of i-erythritol

i-Erythritol seemed to be very promising for use in classification. None of the strains of the oxydans group of Acetobacter were able to attack it, while all the strains of the mesoxydans group of Acetobacter and of Gluconobacter attacked it. A strain which we had received as *G. cerinus* var. *rosiensis* 22 appeared to be the only exception. Closer inspection of its properties showed it to be closely related to either *A. turbidans* 6424 or *A. acetosum* 2224. Very often the oxidation proceeded beyond the erythrulose stage (see Table 3). i-Erythritol was oxidized by an oxidase located on the cytoplasmic membrane (De Ley & Dochy, 1960*a*, *b*).

The oxidation of D-*lactate, ethanol and acetate*

All the strains of the oxydans, mesoxydans and peroxydans groups of Acetobacter oxidized D-lactate, ethanol and acetate nearly to completion. We described previously the enzymic mechanism of D-lactate and ethanol oxidation in *A. peroxydans* (De Ley & Schell, 1959*a*). Unpublished experiments carried out with several other species, such as *A. aceti* Ch 31, *A. ascendens* A, showed that this type of mechanism is a general one in all species investigated. The ability to use D-lactate and ethanol as carbon and energy sources seemed to be the really essential physiological activity for Acetobacter. In several cases an induction period was noticed in the oxidation of acetate. In the polarly flagellate Gluconobacter there appeared to be two distinct groups: (i) *G. liquefaciens* 20, which was able to oxidize ethanol, D-lactate and acetate as already pointed out (Asai & Shoda, 1958; Stouthamer, 1959, 1960; De Ley & Dochy, 1960); (ii) bacteria such as *Gluconobacter suboxydans* 26, 10 and SU, *G. viscosus* 8131, *G. capsulatus* 4943 and *G. melanogenus* 8086 and 16, which were unable to oxidize acetate and oxidized D-lactate and ethanol not beyond acetate. The latter group corresponded to the 'suboxydans' bacteria of Frateur. It may be noted that *G. cerinus* 21, although it did not oxidize acetate, nevertheless oxidized D-lactate and ethanol nearly to completion.

Table 3. *The oxidation of some carbohydrate derivatives by some representative strains of acetic acid bacteria*

Content of the Warburg vessels: see text. The results are expressed as mole O_2 uptake per mole substrate at the end of the oxidations. In some cases the reaction was not yet over after 5 hr. and is marked by the sign >

Strain	Glucose	Sodium gluconate	Sodium 2-keto-gluconate	Sodium 5-keto-gluconate	Mannitol	Fructose	Mannose	Galactose	Xylose	Glycerol	i-Erythritol	Sodium D-lactate	Ethanol	Sodium acetate
Peroxydans group														
A. peroxydans (8618)	0	0	0	0	0	0	0	0	0	0	0	2·7	2·6	1·4
Oxydans group (22 strains investigated)														
A. ascendens (A)	0	0	0	0	0	0	0	0	0	2·3	0	2·5	2·3	1·8
A. rancens (15)	0·5	0	0	0	0	0	0	0·1	0·2	2·2	0	2·5	> 1·6	> 0·65
A. lovaniensis (13)	0·5	0	0	0	0	0	0·46	0·5	0·5	2	0	> 1·96	> 1·4	> 0·5
A. ascendens (4937)	1·0	0	0	0	0	0	0	0	0	0	0	> 1·7	2·4	> 1·2
A. vini acetati (4939)	1·4	0	0	0	0	0	> 0·2	> 0·3	> 0·3	> 0·3	0	> 2·2	2·4	1·8
A. estunensis (E)	1·2	> 0·3	0	0	0	0	> 0·3	0·4	0·5	2·2	0	2·3	2·35	1·85
A. turbidans (6424)	2·3	> 1·6	0	0	0	0	0	0·4	0·3	2·3	0	2·5	2·8	2·0
A. mobilis (6428)	2·0	> 0·8	0	0	> 0·5	0·2	0·2	0·6	0·4	2·5	0	2·5	2·4	> 1·8
Mesoxydaus group (14 strains investigated)														
A. aceti (8554)	1·8	0·2	0	0	> 1·4	> 0·3	0·5	0·5	0·5	2·5	> 0·7	2·7	2·2	1·9
A. mesoxydans (8622)	> 3·0	> 2·1	0	0	0·2	0	0·4	0·5	0·4	2·5	0·3	2·1	2·4	> 1·7
A. mesoxydans (4)	4·9	4·4	> 3·1	> 3·4	> 1·3	> 1·1	0·4	0·6	0·5	2·7	> 1·2	2·9	> 1·7	> 0·7
A. xylinum (8747)	> 3·5	> 3·3	0·6	> 0·4	> 3·3	> 1·7	0·4	0·4	0·5	> 2·1	> 0·8	> 2·1	> 2·0	> 1·1
Suboxydans group (*Gluconobacter*) (9 strains investigated)														
G. capsulatus (4943)	1·4	0	0	0	> 1·9	0	0·5	0·5	0·5	0·9	0·5	0·23	0·9	0
G. suboxydans (26)	3·1	2·5	0	0·1	4·3	> 1·7	0·6	0·5	0·5	1·7	> 1·1	1·0	0·8	0
G. melanogenus (8086)	2·2	1·3	0·5	0	4·8	4·4	0·5	0·5	0·5	> 1·6	0·6	0·7	0·8	0
G. cerinus (21)	4·5	4·0	0·3	> 1·5	3·5	1·0	0·3	0·9	0·9	2·9	2·2	3·0	2·6	0
G. liquefaciens (20)	2·2	1·1	0·5	2·6	> 2·4	1·8	0·6	> 1·0	0·7	2·35	0·5	1·9	1·9	1·4

DISCUSSION

Use of the generic name Gluconobacter. There is convincing evidence in the literature that the acetic acid bacteria can be divided into two distinct groups: (i) the peritrichously flagellate or non-flagellate bacteria of similar physiology, named Acetobacter, as redefined by Leifson (1954); (ii) the polarly flagellate or non-flagellate strains of similar physiology for which either the name Gluconobacter Asai or Acetomonas Leifson amended Shimwell are used. Asai (1934, 1935) realized for the first time the sharp distinction within the acetic acid bacteria and gave generic rank to two groups, *Acetobacter* and *Gluconobacter*, based on several physiological properties. These results were considerably extended by Asai & Shoda (1958). Twenty years after Asai's first papers Leifson (1954) rediscovered the duality of these bacteria from his studies of the type of flagellation and proposed a new genus *Acetomonas* for the polarly flagellate strains which oxidized neither lactate nor acetate. This genus was studied and reviewed by Shimwell & Carr (1959). These genera, *Gluconobacter* and *Acetomonas*, are almost identical and a slight redefinition of the former would suffice to make the latter superfluous. Asai (1934, 1935) and Asai & Shoda (1958) included several strains into *Gluconobacter* which did not oxidize ethanol to acetic acid and were possibly pseudomonads. The exact position of the acetate-oxidizing *Gluconobacter* species has not yet been settled. According to Asai & Shoda (1958) both *G. liquefaciens* and *G. melanogenus* are polarly flagellate. According to Shimwell & Carr (1959) *G. melanogenus* had sparse peritrichous flagella, and Stouthamer (1960) published a not very convincing picture of a supposedly peritrichous *G. liquefaciens*. Pending further investigations, we accept Asai's view. On the evidence of our results about the gradual change in the enzymic make-up within the cluster of strains (see below), it would not seem very probable that an abrupt distinction between polarly and peritrichously flagellate strains would exist. One can expect a gradual transition from one type into the other, where it might often be hard to make an impartial decision. For historical reasons we prefer to use the name Gluconobacter with the redefinition as given by Asai & Shoda (1958), but excluding the strains which are unable to oxidize ethanol to acetic acid.

The oxidative properties of the groups according to Frateur's classification. Frateur's system and nomenclature was built up on the physiological properties of intact organisms. Unfortunately it is a general belief that acetic acid bacteria and in particular those of the oxydans group, possess strongly oxidative properties whereby they oxidize their substrates nearly to completion. This was supposed to be the reason why ketogluconates, fructose, sorbose, dihydroxyacetone, etc. did not accumulate as intermediates from their respective starting compounds. We may quote, for example, Bernauer (1940): 'So sehen wir z.B. bei den Essigbakterien, dasz manche derselben (wie *A. rancens*) nur in geringe Masse primäre Abbauprodukte anhaüfen. Diese Bakteriënart hat ein sehr starkes Oxydationsvermögen und baut in der Regel gleich bis zu CO_2 ab'. Also Frateur (1950, p. 307) says: 'Le groupe oxydans est caractérisé par un pouvoir oxydatif très brutal. Ses représentants sont capables d'oxyder complètement, jusqu'au stade CO_2 et H_2O, une grande quantité de substrats organiques'. Table 3 shows that, quite opposite to expectation, the bacteria from the oxydans group had very poor oxidizing capacities which were nearly comparable to the representatives from the peroxydans group. On the

other hand, the mesoxydans group, which, as the name indicates, was supposed to occupy a position between the oxydans and the suboxydans group, exhibited the greatest diversity of oxidative abilities. The bacteria from the suboxydans group occupied a separate position, since most of them lacked the Krebs cycle. Neverthe-

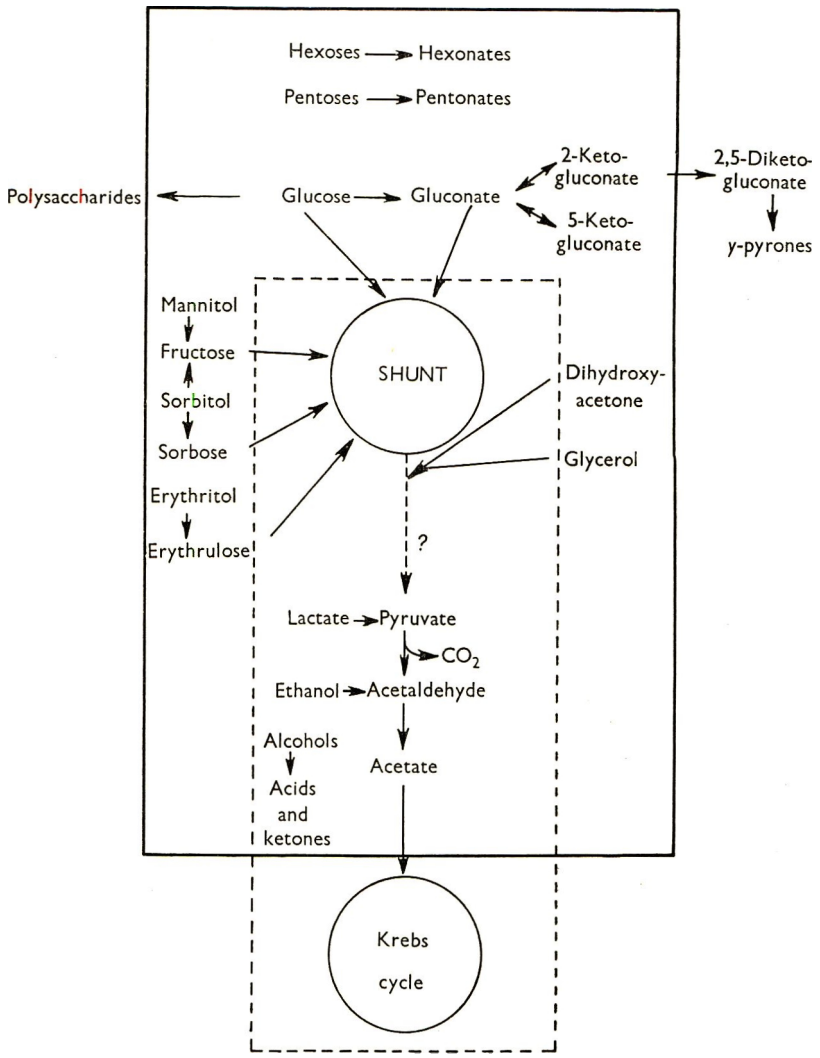

Fig. 2. Simplified metabolic map of the acetic acid bacteria. The overall picture represents the main part of the metabolism of *Gluconobacter liquefaciens*. Although the latter strain is not known to make polysaccharides, this property was included because it is common to many strains. Two typical extreme divergencies are *G. suboxydans* (metabolic map within the full lines) and *Acetobacter peroxydans* (broken lines).

less, they oxidized nearly the same range of substrates as the mesoxydans group, and they did it to the same extent. These results agree on the whole with the reports of Stouthamer (1959, 1960). Previously it was shown (De Ley, 1958) that strains of the peroxydans group can oxidize only a few substrates. It can thus be

seen that the nomenclature of the four groups according to Frateur (1950) becomes misleading in view of the better knowledge of the enzymic constitution of the different organisms.

Comparative carbohydrate biochemistry of the acetic acid bacteria. The oxidative behaviour of resting bacteria represents only the overall activity of the enzyme systems which have been studied in previous papers. The core of their intermediary carbon catabolism appears to consist of three essential parts: (i) Hauge, King & Cheldelin (1955), Kitos *et al.* (1958) and Gromet, Schramm & Hestrin (1957) showed the presence of the hexose monophosphate oxidative cycle in *Gluconobacter suboxydans* and *Acetobacter xylinum*. Unpublished work in our laboratory with many other strains has confirmed these results. Stouthamer (1960) detected the same mechanism in *G. liquefaciens*. (ii) The presence of the Krebs cycle enzymes was detected in *A. pasteurianus* (King, Kawasake & Cheldelin, 1956), in *A. aceti* (Rao, 1955) and in *G. liquefaciens* (Stouthamer, 1960). It seems likely that this mechanism is present in all the acetic acid bacteria, except for the strains of *Gluconobacter* which are unable to oxidize acetate (see, however, *G. cerinus*, above). Rao (1955) found indeed that *G. suboxydans* showed only a few steps of this cycle. (iii) Lactate and ethanol are oxidized to acetate by two pathways; one is effected by a set of oxidosome-linked enzymes (De Ley, 1960) and the other by soluble enzymes. This enzymic mechanism was extensively studied in *A. peroxydans* (De Ley & Schell, 1959*a*) and in many other strains (Schell, 1960). Species differentiation is mainly based on the carbohydrate metabolism of the strains (e.g. polysaccharide formation, ketogenic properties, glucose oxidation, etc.). The enzymic explanation of these differences shows that it is very often only a matter of one or at most a few enzymes, located in the outer regions of the metabolic map (Fig. 2).

The natural relationship of the acetic acid bacteria and a proposal for their phylogeny. As pointed out in the introduction, the ideal approach to the rational classification of bacteria consists in giving equal weight to all the properties exhibited by the strains. Many of these properties are an outward reflexion of the inner enzymic anatomy of the organism and in several cases they represent the truth as little as the shadow of an object tells about the object itself. Within one group of closely related bacteria one can considerably limit the number of properties to be studied, analyse their diverse physiological and biochemical behaviour in terms of their enzymic constitution and compare afterwards their metabolic maps. The comparative biochemical approach might apparently give better results in constructing a natural relationship of bacteria and lead to a model of their phylogeny. The former objective is within reach, because it is a description of an existing situation; the latter objective (phylogeny) can be little more than an hypothesis, because it suggests changes in the course of time, deduced from the present-day state. It may be impossible to construct a complete phylogenetic tree for bacteria from the beginning, because of the great time period involved in their evolution, their short generation time, the enormous possibilities for mutation and because of the lack of evidence of comparative morphology and anatomy, embryology and palaeontology, which have been imperative for our knowledge of the evolution of higher organisms. We can at best hope that the knowledge of the comparative biochemistry of existing bacteria might help to elucidate the latest stages in their evolution. Modern trends in biology allow speculation through the concept of 'regressive

evolution'. It is now a generally accepted belief that the most recent stages in the evolution of many higher organisms consist mostly in gradual loss of properties. On the biochemical level there are several examples of consecutive losses of enzymes, accompanying further stages in evolution (Florkin, 1944). The same trend exists in microbiology. For example, Braun (1953) mentioned several possibilities of a regressive physiological evolution in micro-organisms, the most clear-cut being the commonly occurring mutation from prototrophy to auxotrophy and the transition coliform organisms→typhoid bacilli→dysentery bacilli, involving successive losses

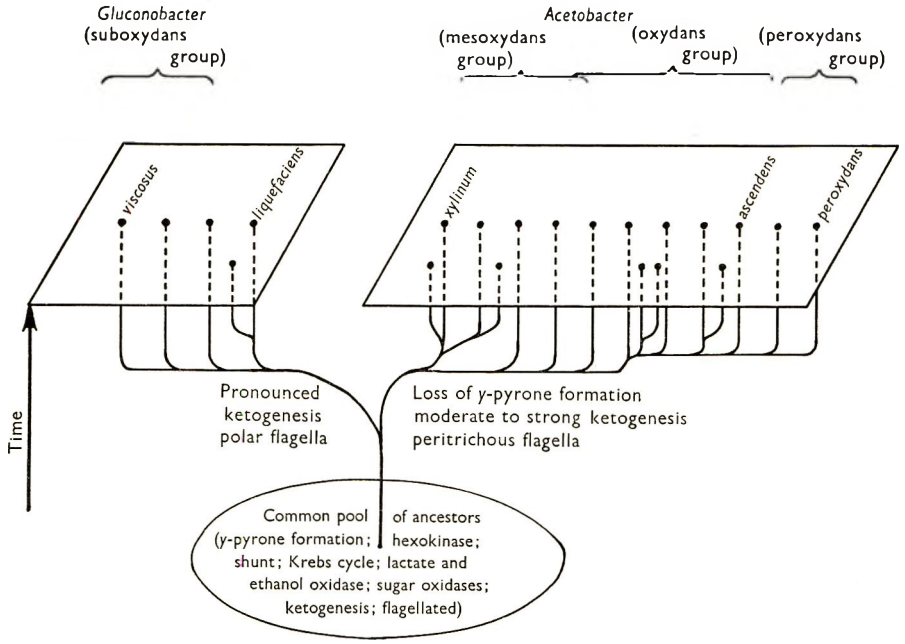

Fig. 3. Possible origin of the acetic acid bacteria from our collection, based mainly on comparative carbohydrate metabolism. For details see Fig. 4 and 5.

of fermentative and biosynthetic steps. A bacterial classification constructed in accordance with this concept, in which the strains of a genus are arranged in a sequence of decreasing capacities, might come closest to representing a phylogenetic relationship. This principle is used in Table 3. From there it is a small step to the hypothesis of phylogenetic lines represented in Figs. 3–5. The genera *Acetobacter* and *Gluconobacter* have many features in common, such as the enzymic mechanism of the oxidation of D-lactate and ethanol to acetate, the enzymes of the hexose monophosphate oxidative cycle, but in particular the oxidosome-linked nature of hexose and pentose oxidation, the formation of 2-ketogluconate, the ketogenic characteristics (glycerol to dihydroxyacetone, mannitol to fructose, sorbitol to sorbose, gluconate to 5-ketogluconate, etc.), their acid resistance, etc., that little doubt can remain about their close phylogenetic relationship. In fact many of the quoted properties are so typical for the acetic acid bacteria that they are not encountered in other bacteria, except sometimes in Pseudomonas. One can imagine that both genera evolved from the same pool of ancestors as shown in Fig. 3. In

spite of the present uncertainty on the exact position of the acetate-oxidizing *Gluconobacter* species it is clear, according to the above concept, that these strains would be the closest representatives of the ancestral pool of the acetic acid bacteria. From those, or closely related strains, might have evolved in the course of time the presently existing acetic acid bacteria. The relationship between the strains which we studied is represented in Figs. 4 and 5. Both figures represent only some of the 45 strains actually investigated. If a much larger collection had been used, or all the known strains had been included, a much more ramified picture might have emerged.

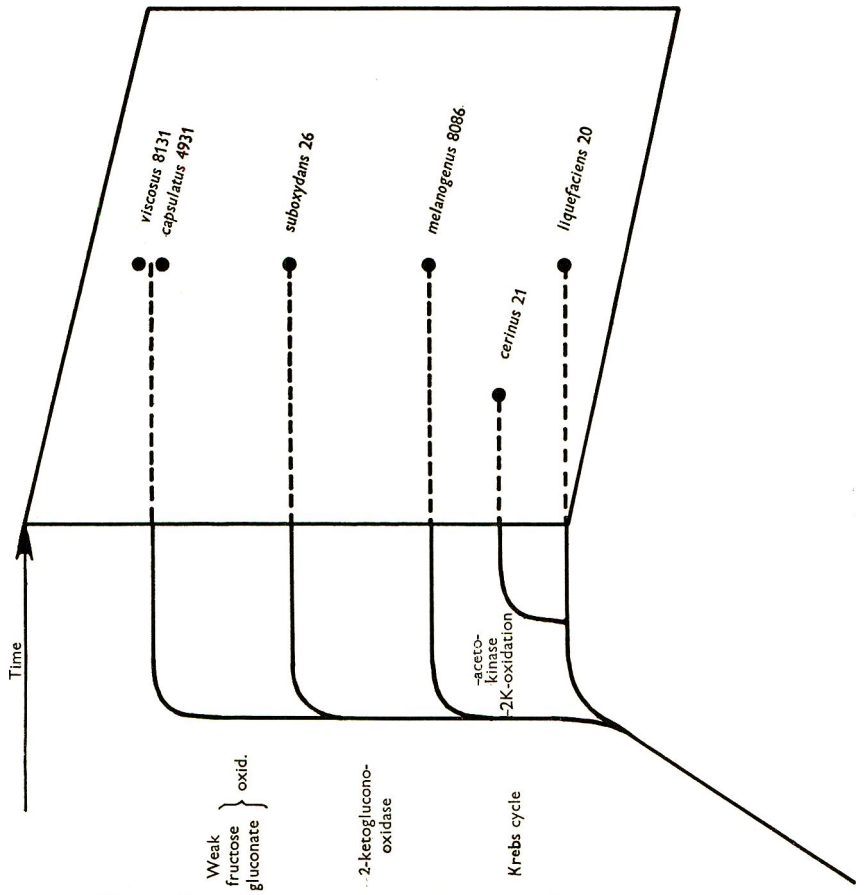

Fig. 4. Details of the scheme in Fig. 3. The *Gluconobacter* biotype.

The value of the 'species' concept in the acetic acid bacteria. A considerable number of 'species' of acetic acid bacteria have been described. Frateur simplified the situation by relegating closely related varieties to ten main species. Shimwell (1959) and Shimwell & Carr (1959) followed this attitude to an extreme and established only one species, *Acetomonas oxydans*, for the suboxydans group of Frateur and no type species at all in the genus *Acetobacter*. The use of species names implies that each 'species' is characterized by a number of typical and specific morphological and physiological differences, which set them apart from all the others. Our experience with these bacteria (see Figs. 4 and 5) shows that there are no such

abrupt changes in the sequence of strains and that, in each phylogenetic line, they can be arranged in a smooth gradation from the most complex one to the one with the poorest enzymic equipment (from *Gluconobacter liquefaciens* to *G. viscosus* and from *Acetobacter xylinum* to *A. peroxydans*). The differences between a strain and its two immediate neighbours consists at most in one or a few enzymes. With this in

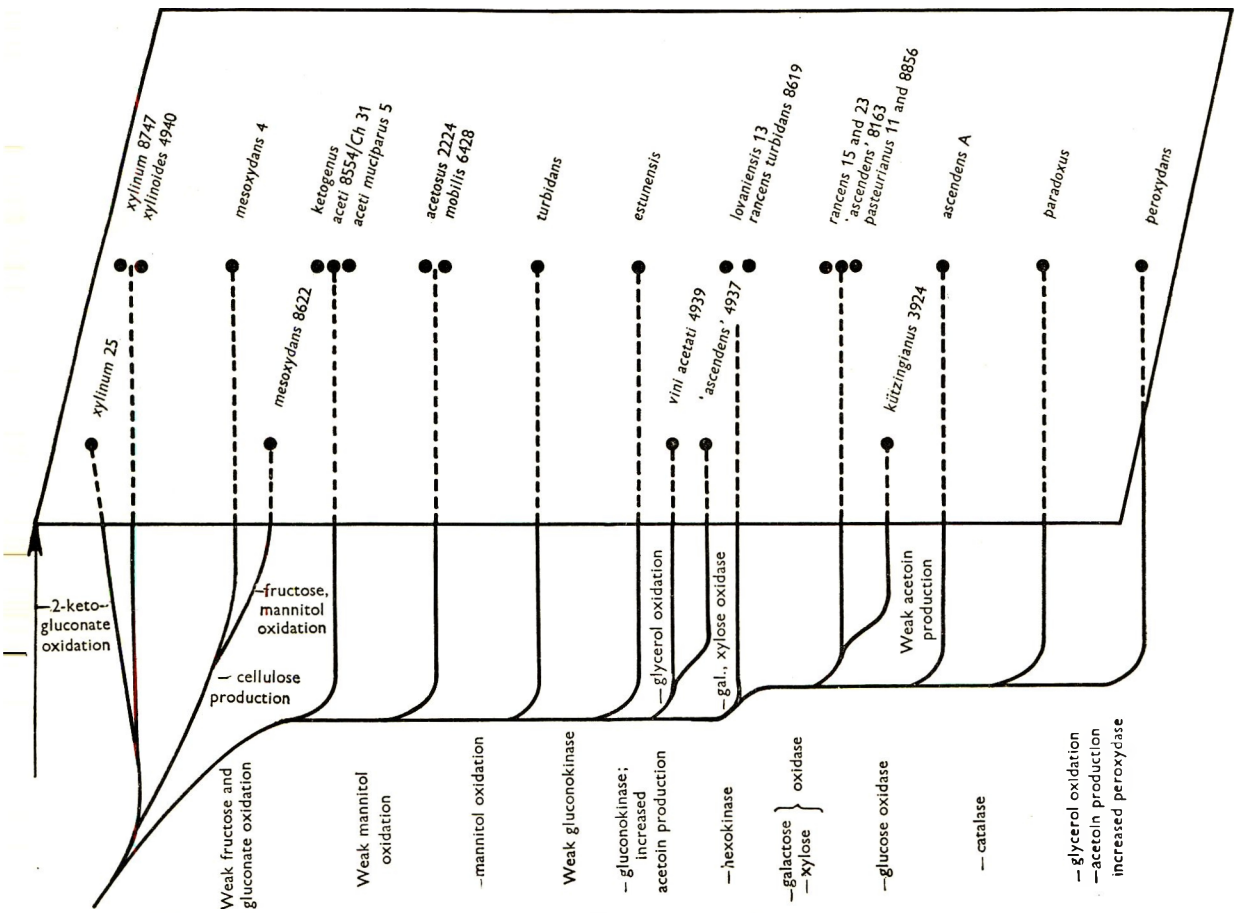

Fig. 5. Detail of the scheme in Fig. 3. The *Acetobacter* biotype. The property to make polysaccharides was not taken into account, since it is a characteristic easily lost and gained by adaptation and mutation.

mind, it is hard to see where to draw the line in this gradation of properties for defining the limits of a 'species'. The most logical attitude would be to regard the entire cluster of strains (a 'biotype' according to Winogradsky, 1952) from each phylogenetic line as variations of one species and to drop their species names altogether. For sake of clarity and also for historical reasons we shall call the two biotypes *Acetobacter aceti* (Beijerinck, 1898) Beijerinck, 1900, and *Gluconobacter oxydans* (Henneberg) comb.nov. There is apparently one practical disadvantage to this new proposal. This is that within each biotype a considerable variety is encountered: compare, for example, *A. xylinum* and *A. peroxydans*, each being at a different end of the line and yet making part of the same biotype. Yet each strain

requires a label for recognition, whether it be a name or a number. For the sake of clarity and for historical reasons the strains could be labelled as, for example, *A. aceti* (peroxydans), *A. aceti* (xylinum), *Gluconobacter oxydans* (melanogenum), etc., much as our strains now bear a collection number. The previous species names would then be degraded merely to catalogue numbers and indicate a set of enzymic properties. The characteristics for new and unknown strains could be determined according to the criteria of Frateur, flagella staining (possibly electron microscopy) and the Warburg apparatus (O_2 uptake) criteria as in the present paper. The creation of new species names should be discouraged in the future, at least when the new strains are closely related to those already existing.

It might appear that our proposal to abolish species names for the acetic acid bacteria and to substitute two names of biotypes, is merely a confirmation of the conclusions of Shimwell (1959) and Shimwell & Carr (1959). In fact, the proposals are fundamentally different and the conclusions were reached on entirely different grounds. According to Shimwell, the acetic acid bacteria are 'overwhelmingly' unstable, each species mutating spontaneously into another in the laboratory. With such changing properties classification would be impossible; stable strains would not exist. This is contrary to our experience with these bacteria; most of our strains showed unchanged sets of properties over a period of three years. We can thus not accept Shimwell's view of the abnormally high mutability of acetic acid bacteria. Frateur (1950 and personal communication) also emphasized the stability of his strains. Undoubtedly, several authors, including ourselves, have observed spontaneous mutations in this group (but probably not more frequently than with other bacteria). A direct enzymic analysis of the spontaneous change of *Gluconobacter melanogenus* into *G. suboxydans* (De Ley & Stouthamer, 1959) showed that at most two enzymes only were affected. The enzymic interpretation of the 'polysaccharide-less' mutants (*Acetobacter xylinum* to *A. mesoxydans* and *A. pasteurianus* to *A. rancens*) as well as of the other results of Shimwell leads to the same conclusion. The change in the enzymic constitution of the mutants is really never so dramatic as might appear from the papers from Shimwell. A further study of the eventual genetic homogeneity of both biotypes by means of mutations, transformations and recombinations is in progress in our laboratory.

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The Effect of Monovalent Cations on the Inhibition of Yeast Metabolism by Nystatin

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SUMMARY

Glycolysis of yeast is inhibited by the polyene antibiotic nystatin. This inhibition was prevented or reversed at or above pH 5·8 (the approximate intracellular pH of yeast) by adding NH_4^+, K^+ or Rb^+ (in order of decreasing effectiveness). These ions do not interfere with the absorption of nystatin by the yeast cell. At pH 4 both glycolysis and respiration are highly sensitive to nystatin but are not protected by the monovalent cations. At pH 7 respiration of intact cells was insensitive; only with yeast protoplasts could respiration be inhibited by nystatin. Temporary protection was obtained with NH_4^+ or K^+.

K^+ and NH_4^+ did not prevent the fungicidal or fungistatic actions of nystatin even under conditions where the protection of glycolysis by these ions was essentially complete. The original yeast cells were not dependent upon added K^+. After a brief treatment with nystatin at pH 7·0, the cells showed an absolute requirement for K^+ or NH_4^+; it was shown that K^+ was rapidly lost by cells following contact with nystatin. It is concluded that nystatin directly damages the cell membrane and thus produces a rapid increase in permeability to small ions. The resulting depletion of cellular K^+ halts glycolysis. The addition of K^+ or NH_4^+ restores glycolysis but does not reverse the membrane alterations.

INTRODUCTION

Nystatin, a polyene antifungal antibiotic produced by *Streptomyces noursei* (Brown & Hazen, 1957), inhibits growth and utilization of various substrates by fungi, especially yeast (Lampen, Morgan & Slocum, 1957; Harman & Masterson, 1957; Meyer-Rohm, Hopff & Lange-Brock, 1957; Peynaud, Lafourcade & Lafon, 1957; Bradley, 1958*a*, *b*). Lampen *et al.* (1957) reported that nystatin was more effective at acid than at neutral pH values in inhibiting glycolysis and respiration of *Saccharomyces cerevisiae*. The increased binding of nystatin by yeast at acid pH was suggested as a possible cause for the greater sensitivity of metabolism at this pH. Evidence is now presented that this differential sensitivity is a function both of pH and of the monovalent cations in the test system.

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METHODS

Organisms. *Saccharomyces cerevisiae* strain A-B was commercial bakers' yeast obtained from Anheuser Busch, Old Bridge, New Jersey, in the form of a pressed filter cake. The cells were washed four times in water before use.

Saccharomyces cerevisiae strain LK2G12 (obtained from Dr H. Halvorson, University of Wisconsin, Madison) was grown for 16 hr. on a slope of Penassay agar (Difco Laboratories, Inc., 920 Henry St, Detroit, Mich.) containing 1% (w/v) glucose. The cells from one slope were transferred into 100 ml. of Wickerham's medium (Wickerham, 1951) and incubated for 16 hr. at 28° on a rotary shaker. These cultures had an optical density of *c.* 1·0 at 530 mμ when measured with the uninoculated medium as a blank. After washing twice with water, the cells (herein termed stationary-phase cells) were suspended in water at a concentration of 1×10^8/ml., as determined by counts in a haemacytometer (American Optical Co., Buffalo, N.Y.). By using the method described by Cavett (1931), the nitrogen content of 1×10^8 cells (3 mg. dry weight) was found to be *c.* 0·22 mg.

To prepare log-phase cells, 10 ml. of a stationary-phase culture was added to 100 ml. of Wickerham's medium and incubated as above for 4 hr. At this time an optical density of approximately 0·4 had been reached. The cells were washed twice with water and resuspended at a concentration of 1×10^8/ml. Buds were counted as individual cells. It was found that 1×10^8 cells (2 mg. dry wt.) contained *c.* 0·05 mg. N.

Protoplasts were obtained from log-phase cells by following essentially the technique described by Bachmann & Bonner (1959) for *Neurospora crassa*. Before adding the snail enzyme to the cells, 0·1 ml. of a 1% (w/v) aqueous cysteine-HCl solution was added to each ml. of enzyme to overcome the inhibitory effect of thiomersalate, present as a preservative in the commercial enzyme solution. The protoplasts were washed twice with sodium citrate + phosphate buffer (pH 5·8) plus 0·82 M-mannitol, and resuspended in buffered stabilizer solution. The nitrogen content was *c.* 0·075 mg./1×10^8 protoplasts.

Sensitivity tests. Defined medium N of Vogel (1956) was used with the following additions, per l., 10 g. glucose, 2·0 mg. *i*-inositol, 0·2 mg. calcium pantothenate 0·2 mg. pyridoxine.HCl and 0·2 mg. thiamine.HCl. A 50 ml. quantity of medium was inoculated with 0·5 ml. of a stationary-phase culture of strain LK2G12. Nystatin was sterilized by the method of Donovick *et al.* (1955) and added aseptically to the autoclaved medium. The cultures were incubated for 16 hr. at 28° on a rotary shaker. The criterion of growth was the optical density value at 660 mμ.

Manometry. The conventional Warburg respirometer was used at 30°. The contents of the flasks will be described below. Since 0·82 M-mannitol was present as an osmotic stabilizer in the experiments with protoplasts, it was generally added in tests with log-phase cells to permit direct comparison between the two cell types. The sensitivity of the log-phase cells to nystatin was increased somewhat by the addition of mannitol, but the characteristics of the inhibition did not appear to be altered. The sensitivity of stationary-phase cells was not affected by mannitol.

Preparation of materials. A highly purified preparation of nystatin (Lot HV942, obtained from the Squibb Institute for Medical Research, New Brunswick, N.J.) was dissolved in dimethyl sulphoxide and diluted in water. The maximum concen-

tration of dimethyl sulphoxide in any reaction mixture (0·07 %) had no detectable effect on metabolism. Buffers were prepared as described by Gomori (1955). Na^+ was replaced in certain experiments by K^+ or NH_4^+.

RESULTS

Protection of glycolysis by potassium ions

In the experiments previously reported (Lampen *et al.* 1957), the buffers used at neutral pH value contained considerable quantities of both sodium and potassium ions. In subsequent tests with only a single cation present (Table 1), it became evident that glycolysis was highly sensitive to nystatin at pH 7·0 in Na^+-buffer, but was unaffected in K^+-buffer. At pH 4·0 there was no significant protection by K^+. The amount of nystatin absorbed by the cells was the same whether Na^+ or K^+ was present (Table 1); thus K^+ did not protect by preventing the access of nystatin to the yeast cells. More nystatin was absorbed at acid than at neutral pH, but, as can be seen from Fig. 1, the sensitivity of glycolysis to nystatin was not greatly affected. The inhibition did occur more rapidly at pH 4·0 than at pH 7·0; however, the rate in the absence of nystatin was also higher at pH 4·0. Thus, the total CO_2 production in the presence of differing nystatin levels was similar at both pH values.

Table 1. *The effect of* Na^+ *and* K^+ *ions on the inhibition of yeast glycolysis by nystatin at different pH values*

Glycolytic activity is expressed as μl. CO_2 evolved in 60 min./1 $\times 10^8$ cells. Uptake of nystatin is given as μg./mg. (dry weight) of yeast in 30 min. (Lampen *et al.* 1959). Each vessel contained 9×10^7 stationary-phase cells of *S. cerevisiae* LK2G12 and 2·8 ml. citrate + phosphate buffer, with use of the sodium or potassium salts indicated below. The nystatin and 60 μmole of glucose were added at 0 min. Final volume: 3·0 ml. Gas phase: N_2. Temperature: 30°.

	Glycolysis		Uptake of nystatin
μg. nystatin/ml.	0	5	5
pH 4·0 (Na^+)	572	69	3·0
pH 4·0 (K^+)	595	68	3·0
pH 7·0 (Na^+)	384	89	0·7
pH 7·0 (K^+)	421	359	0·7

Protection by various cations

With the A-B cells or stationary-phase cells of strain LK2G12, essentially complete protection of glycolysis was obtained when either K^+ or NH_4^+ was added to sodium citrate + phosphate buffer (pH7) system. This is illustrated in Fig. 2. At a concentration of 4 mM-KCl or NH_4Cl, good glycolytic activity was usually maintained; in subsequent experiments both were generally added at 9 mM to insure adequate protection. The addition of K^+ or NH_4^+ or both to the control vessels caused a slight stimulation of the rate of glycolysis or respiration at pH 7·0. The rate of increase, however, was a relatively minor effect when compared with the reversal rate of completely inhibited cells after the addition of K^+ or NH_4^+.

NH_4^+ was more effective than K^+ in protecting log-phase cells or the protoplasts derived from them. Thus, with log-phase cells in sodium citrate + phosphate buffer, a rapid inhibition of glycolysis was produced by 0·25 μg. nystatin/ml. The effect of

1 μg./ml. was prevented by NH_4^+ but only partially by K^+. At 10 μg. nystatin/ml., the addition of either ion (or both) yielded only *c.* 40 % of the uninhibited rate of CO_2 production. The superiority of NH_4^+ was equally evident in tests with protoplasts. Glycolysis was rapidly eliminated by 0·25 μg. nystatin/ml.; as shown in Fig. 3, the action of 1 μg./ml. was prevented for at least 60 min. by NH_4^+, and only delayed by K^+.

At pH 7·0 respiration of protoplasts was inhibited only when a nystatin concen-

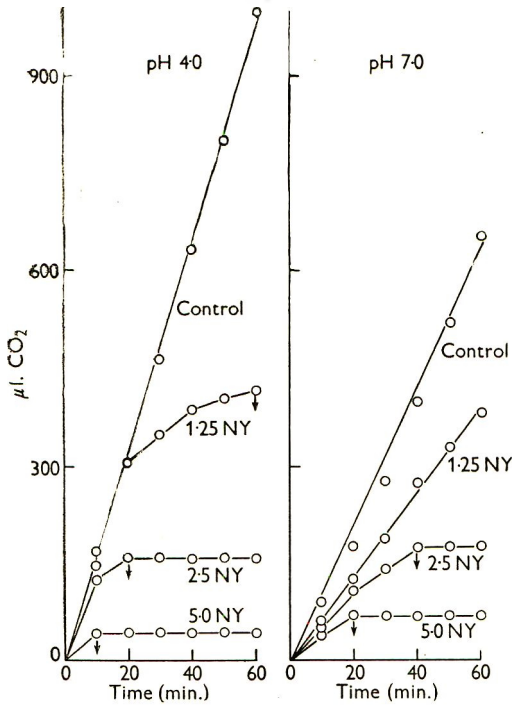

Fig. 1

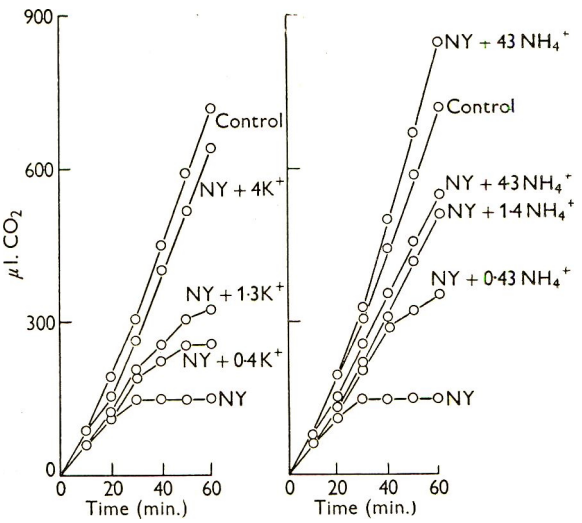

Fig. 2

Fig. 1. Sensitivity of glycolysis to nystatin at neutral and at acid pH. Each vessel contained 2·5 mg. (dry weight) of *S. cerevisiae* strain A-B cells, 1·5 ml. pH 4·0 or 7·0 sodium citrate + phosphate buffer, and water to bring the final volume to 3·0 ml. Nystatin (NY) concentrations are in μg./ml. The nystatin and 60 μ mole glucose were added from the side-arm at 0 min. Gas phase: N_2. Temperature: 30°. The (↓) indicates the point at which metabolism ceased.

Fig. 2. The protection of yeast glycolysis by various concentrations of K^+ or NH_4^+ against inhibition by nystatin. Each vessel contained 2·5 mg. (dry weight) of *S. cerevisiae* strain A-B cells, 1·5 ml. sodium citrate + phosphate buffer (pH 7), K^+ or NH_4^+ in the indicated final concentrations (expressed in m-equiv./l.), and water to 3·0 ml. At 0 min., 120 μmole glucose and, where indicated, 15 μg. nystatin were added from the side-arm. Gas phase: N_2. Temperature: 30°.

tration of 20 μg./ml. was attained. Addition of NH_4^+ or K^+ resulted in temporary protection, but after 40 min. respiration slowed abruptly and it was observed that the protoplasts had lysed.

Respiration by intact cells, either from log- or stationary-phase cultures, was highly resistant to nystatin at neutral pH. No inhibition was observed in sodium

citrate+phosphate buffer upon the addition of 40 μg. nystatin/ml.; at pH 5·8, log-phase cells were inhibited by 20 μg./ml. but stationary-phase cells were only partially inhibited by 40 μg./ml. No protection was achieved by adding a mixture of K^+ and NH_4^+. At pH 4·0 respiration was relatively sensitive to nystatin: A-B cells were inhibited by 2 μg./ml. and log-phase LK2G12 cells by 0·25 μg./ml. No significant protection was afforded by 0·01–0·1 M-KCl and/or 0·01 M-NH_4Cl.

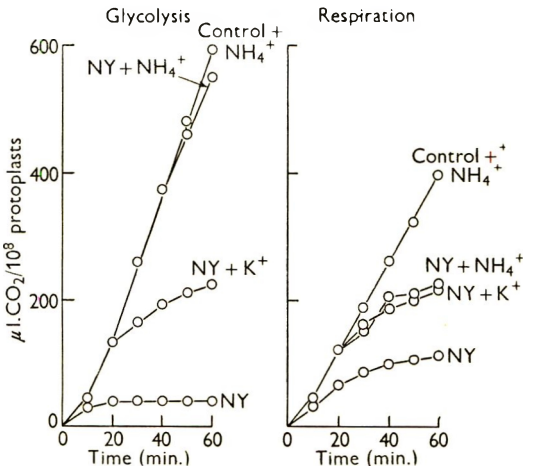

Fig. 3. Protection of glycolysis and respiration in yeast protoplasts. For measurements of glycolysis each vessel contained 8×10^7 protoplasts prepared from *S. cerevisiae* LK2G12, 9 mM-KCl or NH_4Cl (where indicated), 1 μg. nystatin/ml. (where indicated), and a gas phase of N_2; for respiration, $1{\cdot}6 \times 10^8$ protoplasts, 18 mM-KCl or NH_4Cl, 20 μg. nystatin/ml., and an atmosphere of air were used. All vessels received 2·7 ml. sodium citrate + phosphate buffer (pH 7·0) containing 0·82 M-mannitol and water to a total volume of 3·0 ml. Temperature: 30°. The nystatin and 120 μmole glucose were added at 0 min.

Reversal of the inhibition of glycolysis

In cells inhibited by nystatin at neutral pH, glycolysis could be initiated by adding K^+ or NH_4^+ as long as 1 hr. after total inhibition had occurred. From the experiment presented in Fig. 4A, it may be noted that CO_2 production resumed within 10 min. after addition of the supplement and attained a rate approximately that in control vessels.

Reversal of inhibition could also be obtained (Fig. 4B) by the addition of rubidium salts at relatively high levels (*c.* 50 mM). Lithium chloride and magnesium sulphate were without effect at 9 or 50 mM, and were somewhat toxic at 50 mM in the absence of nystatin. Calcium, ferrous or manganous salts were inactive at 9 mM. At this value none of these ions prevented complete reactivation when a $K^+ + NH_4^+$ mixture was subsequently added.

Since 0·1 or 0·2 M sodium salts were present in most experiments, it was conceivable that the inhibition by nystatin was essentially an induced sodium toxicity. When 0·1 M-imidazole-HCl buffer (pH 7·0) was utilized, the sensitivity of glycolysis to nystatin did not differ significantly from that observed in sodium citrate + phosphate buffer or in unbuffered medium. Thus a high concentration of Na^+ was not essential for the inhibition.

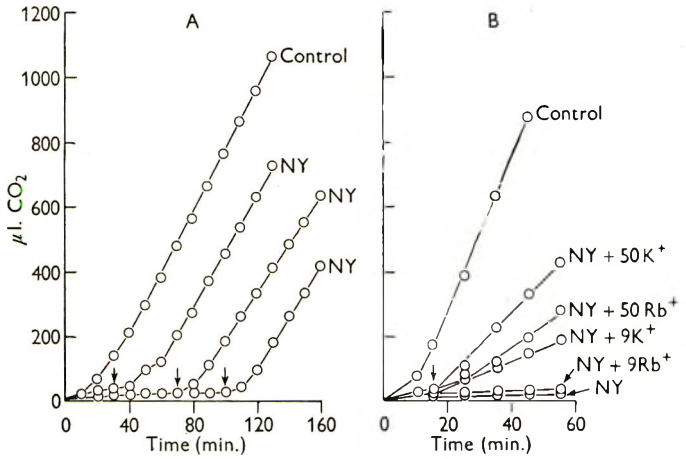

Fig. 4. Reversal by monovalent cations of the inhibition of glycolysis by nystatin. Each vessel contained 1×10^8 cells of *S. cerevisiae* LK2G12 from a log-phase culture and 2·5 ml. sodium citrate + phosphate buffer (pH 7·0), containing 0·82 M-mannitol. The nystatin (3 μg.) and 120 μmole glucose were added from the side-arm at 0 min. Final volume: 3·0 ml. Gas phase: N_2. Temperature: 30°.

A. At 30, 70 or 100 min. as indicated by (↓), KCl and NH_4Cl were tipped into individual vessels to yield final concentrations of 9 mM.

B. At 15 min. (↓) various concentrations of RbCl or KCl were tipped into individual vessels. Concentrations are expressed as m-equiv./l.

The specific anions present do not appear to be critical. Reversal occurred in test mixtures containing either phosphate, citrate or chloride ions, and the addition of phosphate did not affect the rate at which reversal was attained. The concentration of K^+ or NH^+ required for protection was similar whether chloride, nitrate or sulphate salts were used.

Protection or reversal of the inhibition was obtained with a $K^+ + NH_4^+$ mixture only at pH 5·8 and above (Table 2). Conway & Downey (1950) have reported that the intracellular pH of yeast is 5·8–6·2. At a pH below 5·8 an increase in cellular permeability (induced by nystatin) may produce a rapid acidification of the intracellular space and initiate irreversible damage to the glycolytic system. Consistent

Table 2. *Effect of* pH *value on the reversal by* K^+ *and* NH_4^+ *of the inhibition of glycolysis by nystatin*

Each vessel received either 1×10^8 log-phase cells of *S. cerevisiae* LK2G12 or 8×10^7 protoplasts and 2·6 ml. sodium citrate-phosphate buffer containing 0·82 M-mannitol. The nystatin and 120 μmole glucose were added from the side arm at 0 min. Final volume: 3·0 ml. Gas phase: N_2. Temperature: 30°. After 20 min. incubation, glycolysis had ceased in those vessels containing nystatin. KCl and HN_4Cl were then added to all vessels in final concentrations of 9 mM, and gas evolution was measured for an additional 40 min.

		Cells								Protoplasts	
		4·0		5·0		5·8		7·0		7·0	
pH											
μg. nystatin/ml.		0	1	0	1	0	1	0	1	0	1
μl. CO_2 produced/10^8 cells	0–20 min.	203	11	146	12	104	13	104	31	125	40
	20–60	712	2	514	7	284	122	280	229	335	90

with this suggestion is the observation that cells treated for 15 min. at pH 4·0 with 5 μg. nystatin/ml. and then washed, did not resume glycolysis even when tested in the absence of nystatin in potassium citrate + phosphate buffer (pH 7·0) containing 10 mM-NH_4Cl.

The differential effect of pH on protection by K^+ is not a function of the conditions under which the original contact between nystatin and the cells occurs. Cells incubated with nystatin (5 μg./ml.) at pH 7·0 in potassium citrate + phosphate buffer and then washed free of unabsorbed nystatin were inactive when tested at pH 4·0 in K^+-buffer; good glycolytic activity was retained at pH 7·0 provided K^+-buffer was used.

With protoplasts, reversal by K^+ and NH_4^+ of an established inhibition was incomplete even at pH 7·0 (Table 2), although excellent protection by NH_4^+ was obtained (Fig. 3). The changes produced by nystatin may result in more severe damage to the protoplast membrane than to the membrane of intact cells which have a protective rigid cell wall. In fact, with protoplasts lack of reversal by K^+-NH_4^+ appeared to correlate with lysis of the preparations.

Effects of nystatin not prevented by K^+ *or* NH_4^+

Despite the fact that K^+ and NH_4^+ protect glycolysis against concentrations of nystatin rapidly inhibitory in sodium citrate + phosphate buffers, it is clear from several lines of evidence that these ions do not protect the cells completely from the action of nystatin. High concentrations of nystatin are inhibitory to glycolysis even in the presence of excess K^+ and NH_4^+. The mechanism by which this inhibition is produced will be considered in a subsequent publication.

With low concentrations of nystatin (5 μg. or less/ml.), the addition of K^+ and NH_4^+ afforded complete protection of glycolysis against the agent but did not prevent its fungicidal action. As is illustrated in Table 3, a decrease of *c.* 99·99 % in colony-forming units occurred regardless of the presence or absence of the ions.

Table 3. *Lack of effect of* NH_4^+ *and* K^+ *on the fungicidal action of nystatin*

The manometric measurements are expressed as μl. CO_2 evolved in 40 min. Each vessel contained $2{\cdot}8 \times 10^8$ cells of *S. cerevisiae* LK2G12, harvested from a stationary-phase culture, and 1·5 ml. sodium citrate + phosphate buffer (pH 7·0). KCl and NH_4Cl were added where indicated to yield final concentrations of 9 mM. The nystatin and 120 μmole glucose were added from the side-arm at 0 min. Final volume: 3·0 ml. Gas phase: N_2. Temperature: 30°. After 40 min. the cells were diluted in potassium citrate + phosphate buffer (pH 7·0) and plated on Wickerham's medium adjusted to pH 7·2 and containing 1·5 % agar. Colonies were counted after incubation of the plates for 48 hr. at 28°.

μg. nystatin/ml.	Addition	CO_2	Colony count/ml.
None	None	338	$9{\cdot}2 \times 10^7$
5	None	80	$7{\cdot}5 \times 10^3$
None	$NH_4Cl + KCl$	346	$6{\cdot}6 \times 10^7$
5	$NH_4Cl + KCl$	348	$7{\cdot}5 \times 10^2$

Evidence that nystatin initiates a rapid alteration in cellular permeability is presented in Table 4. Glycolysis by the original cells was essentially independent of exogenous K^+ (Table 1). Even with cells grown on the defined medium modified to contain growth-limiting amounts of potassium salts there was only a slight

stimulation of glycolysis when 9 mM-KCl was added to the usual sodium citrate + phosphate buffer (pH 7·0). In contrast, when cells were incubated with 5 μg. nystatin/ml. in potassium citrate + phosphate buffer (pH 7·0) for 30 min., washed quickly with water, and their glycolytic activities again measured, these cells now had an absolute requirement for exogenous K^+ (Table 4). Respiration, insensitive to nystatin at pH 7·0 by direct test, was also totally dependent on added K^+. These nystatin-treated cells could also glycolyse or respire actively when resuspended in ammonium citrate-phosphate buffer (pH 7·0); in fact, the rates were higher in the presence of NH_4^+ than of K^+.

Table 4. *Requirement of* K^+ *or* NH_4^+ *for glycolysis and respiration after nystatin treatment*

Treatment (1) $1{\cdot}4 \times 10^8$ cells of *S. cerevisiae* LK2G12 from a stationary-phase culture were suspended in 3·0 ml. of the K^+ citrate + phosphate buffer (pH 7·0, 175 m-equiv. K^+/l.) in the presence of 60 μmole glucose and, where indicated, 5 μg. nystatin/ml. The test mixture shaken 40 min. at 30° in an atmosphere of N_2.

Treatment (2) The cell samples were washed twice with water and added to Warburg flasks containing 1·5 ml. sodium, potassium, or ammonium citrate-phosphate buffer (pH 7·0) in a final volume of 3·0 ml. At 0 min. 60 μmole glucose was added from the side-arm. Gas phase: N_2 or air. Temperature: 30°.

	μg. nystatin/ml. during treatment (1)			
Buffer	0	5	0	5
Treatment (2)	μl. CO_2/60 min.		μl. O_2/60 min.	
Na^+	440	12	342	11
K^+	850	525	330	230
NH_4^+	880	695	338	306

The growth of yeast is highly sensitive to nystatin at pH 7·0, even in the presence of high concentrations of K^+ and NH_4^+. For instance, the minimal inhibitory concentration for strain LK2G12 was *c.* 0·5 μg. nystatin/ml. in either the crude Wickerham medium (20 m-equiv. K^+/l.) or Vogel's defined medium (36 m-equiv. K^+/l.). If the potassium salts in the latter medium were replaced by sodium salts so that

Table 5. *Leakage of* K^+ *from yeast cells following treatment with nystatin*

The test mixtures contained 5×10^8 cells of *S. cerevisiae* LK2G12, 15 ml. sodium or ammonium citrate + phosphate buffer (pH 7·0), 1200 μmole glucose, nystatin where indicated, and water to 30 ml. After incubation for 30 min. at 30° in an atmosphere of N_2, the cells were collected by centrifugation, washed once with water, and resuspended in 5 ml. of water. The suspensions were placed in a boiling water bath for 3 min., the cellular debris was removed by centrifugation, and the supernatant fluid analysed for potassium. Conventional flame photometric techniques were employed in conjunction with a Beckman spectrophotometer, model B.

		μg. K^+/mg. cells (dry wt.)	
μg. nystatin/ml.	Buffer cation	Stationary-phase cells	Log-phase cells
0	(H_2O)	14	8
0	Na^+	9	10
5	Na^+	0·8	1·1
5	NH_4^+	1·0	—

only a trace of K^+ was present, the extent and rate of growth were decreased substantially. Nevertheless, under these conditions the concentration of nystatin required to inhibit growth was still one-half that observed with ample K^+. The sensitivity of growth was unchanged when, in K^+-deficient medium, NH_4^+ was replaced by 1% Casamino acids (Difco). Thus K^+ and NH_4^+ are not decisive in determining the sensitivity of growth to nystatin.

Leakage of K^+ *from cells*

Incubation of log- or stationary-phase yeast cells with nystatin produced a rapid decrease in the intracellular concentration of K^+. This is illustrated by the experiment (Table 5) in which bound K^+ (not readily removed by washing with water) was measured. The efflux of K^+ was not prevented by levels of NH_4^+ which completely protected glycolytic activity. Thus NH_4^+ did not prevent the damage to the cell membrane by nystatin.

DISCUSSION

Previous studies on the inhibition of yeast by nystatin were conducted primarily at acid pH. These experiments focused attention on an irreversible inhibition of glycolytic and respiratory activity as a critical factor in the fungicidal action of nystatin. Evidence is now presented that the metabolic changes are secondary to alterations of the cell membrane. These alterations are themselves apparently irreversible, but can, at appropriate pH values, produce either a reversible inhibition of glycolysis or the destruction of the enzymes.

The most attractive working hypothesis is that nystatin reacts primarily with the cell membrane. Nystatin can be bound by isolated cell walls (Lampen & Arnow, unpublished observations), but the critical importance of the membrane is clear since the sensitivity of protoplasts to nystatin is essentially identical with that of the log-phase cells from which they were derived (Sutton, Marini & Lampen, 1960). The binding of nystatin causes the membrane to become highly permeable to K^+ and perhaps to other monovalent cations, and glycolysis quickly ceases as a result of the deficiency of K^+. At neutral pH, and when the concentration of nystatin does not exceed 10–20 μg./ml., the addition of K^+ or NH_4^+ will permit glycolysis to resume. There is no evidence for destruction of the glycolytic enzymes under these conditions since essentially complete reactivation with the monovalent cations is possible. The essentiality of K^+ or NH_4^+ for glycolysis has long been recognized. These ions are required for yeast phosphofructokinase (Muntz, 1947), aldolase (Richards & Rutter, 1960) and pyruvate kinase (Washio, Mano & Shimazono, 1959). NH_4^+ is more effective than K^+ in reactivating phosphofructokinase; this difference may in part account for the superiority of NH_4^+ for nystatin-treated cells. The loss of cellular K^+ which occurs in the presence of nystatin does not halt respiration unless the cells are subsequently washed. Since complete reactivation by K^+ and NH_4^+ can be achieved, an increased membrane permeability appears to be an adequate explanation of the observed inhibition.

The initial interaction of nystatin and the cell membrane at pH 4 may well be identical with that obtained at pH 7, since the concentration of nystatin required to inhibit glycolysis or respiration at pH 4 approximates that needed to inhibit glycolysis at pH 7 (in the absence of K^+ or NH_4^+). At reactions more acid than the

usual intracellular pH value of yeast, sudden increase in the permeability of the cell membrane to monovalent cations would produce a rapid acidification of the cell interior. This may activate latent lytic enzymes, as occurs following acidification of animal cells (De Duve, 1957), and thus initiate the decrease in extractable glycolytic enzymes which characterizes the action of nystatin at pH 4·5 (Scholz, Schmitz, Bücher & Lampen, 1959). It should be noted that the inhibition of glycolysis and respiration is clearly a result of the prior increase in membrane permeability. This change may be the primary event in nystatin action, since an irreversible loss of ability to maintain elevated internal concentrations of critical ions and cofactors would certainly be lethal.

Evidence has been reported by several laboratories that nystatin produces an alteration of cellular permeability with subsequent leakage of internal constituents. A critical role for K^+ and NH_4^+ has not been previously proposed, however. Osteux, Tran-Van-Ky & Biquet (1958) noted that the rate of excretion by *Candida albicans* of the acids of the citric acid cycle and of inorganic phosphate was increased in the presence of nystatin. The leakage of phosphate was correlated with the concentration of nystatin and the time of contact between the cells and the antibiotic. Bradley (1958*a*) observed a decrease in cellular phosphorus during a 3 hr. period following the addition of nystatin, but at shorter intervals actual accumulation of phosphate esters was noted by Scholz *et al.* (1959), while pyruvate and malate were rapidly released from the cell. Under the conditions of the present experiments, low concentrations of nystatin appear first to cause a deficiency of K^+ and NH_4^+. With higher concentrations of nystatin, leakage of various low molecular weight substances probably occurs, since restoration of activity requires the addition of all the cofactors needed in the usual soluble glycolytic system (Lampen & Arnow, unpublished observations).

Bradley & Farber (1960) observed that nystatin inhibited fermentation by *Candida stellatoidea* in various buffers only at relatively acid pH (pH 2–5). These workers emphasized the potential role of the anions in determining sensitivity, but the cations employed were not reported. Ribéreau-Gayon, Peynaud, Lafourcade & Lafon (1958) also reported that nystatin more effectively inhibited glycolysis of *Saccharomyces cerevisiae* at acid than at alkaline pH, but again these authors did not indicate the cations used in their buffer systems.

Although major attention has centred on the disorganization of cell-wall synthesis by antibacterial antibiotics (Salton, 1960), several agents are believed to affect primarily the cell membrane and hence cellular permeability. Newton (1956) demonstrated this for polymyxin; Anand & Davis (1960) and Anand, Davis & Armitage (1960) reported similar results for streptomycin. Gale (1955) offered evidence that β-propiolactone acts on the cell membrane of yeast. Scharf (1960) has recently observed K^+ leakage following treatment of yeast with benzalkonium chloride. Nystatin appears to be an excellent example of this type of antibiotic.

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Morphological and Biological Properties of a Strain of Chromogenic Acid-fast Bacteria and of its Phage-immune Variant

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SUMMARY

Phage-immune variants were detected among chromogenic acid-fast mycobacteria by the action of *Mycobacterium phlei* bacteriophage. One of the strains thus obtained showed not only phage immunity, but also a marked difference in colonial appearance, pigmentation, antigenic composition and pathogenicity for laboratory animals as compared with the original strain.

INTRODUCTION

During a recent survey of the phage susceptibility of chromogenic acid-fast bacteria isolated from patients with tuberculous disease, it was found that twenty-two out of ninety-eight of these strains were affected by exposure to one or more of the six phages used. In nine instances phage-immune dissociates were isolated from hazy plaques or from isolated colonies within clear plaques. Several of the phage-immune bacteria showed some morphological differences from the parent strain. In one case, the differences were so marked that this strain became the subject of the present study.

METHODS

Source of the strain of Mycobacterium. The chromogenic strain of acid-fast bacterium 41308 was isolated in 1956 from the sputum of a patient who, 26 months before, presented radiological evidence of widespread infiltration with bilateral cavitation. Typical *Mycobacterium tuberculosis* was isolated from the sputum at that time and twice again during the first 2 months of treatment. The patient received streptomycin, isonicotinic acid hydrazide and *p*-aminosalicylic acid for 2 years. Because the tuberculous disease appeared to be 'arrested', chemotherapy was suspended. Two months later, pure cultures of chromogenic acid-fast bacteria were recovered from the sputum.

Source of bacteriophages. The phages used were derived from *Mycobacterium phlei*, *M. friburgensis*, *M. rabinowitsch*, *M. smegmatis* and were received from Dr E. Vandra, Budapest, Hungary, and also phages D28 and D29 received from Dr S. Froman, Olive View, California, U.S.A.

Characters of the chromogenic acid-fast bacterium and phage-immune variant studied: (*a*) cultural requirements and colonial appearance; (*b*) microscopic examination of the bacterial elements; (*c*) comparison of their antigenic composi-

tions by agar diffusion precipitation tests; (*d*) susceptibility to antituberculosis agents; (*e*) virulence tested by the neutral red cytochemical reaction and animal inoculation.

RESULTS

Strain 41308 produced smooth light-yellow colonies on egg-yolk media after incubation periods of 10–12 days at 37°, and of 16–18 days at 22° (Pl. 1, fig. 1). The pigment was not affected by light, and the strain was thought to belong to group III of Runyon's classification (Runyon, 1959). According to agar diffusion precipitation studies (Mankiewicz, 1958) this strain belonged to a group of about 6% of acid-fast bacteria which show a close antigenic relationship with virulent tubercle bacilli. When this strain was spot tested with *Mycobacterium phlei* phage according to the technique described by Froman & Bogen (1954) and modified by Takeya, Yoshimura, Tamamura & Toda (1959), a slightly hazy plaque of regular contour was obtained. The subcultures of the phage-immune bacteria showed smooth colonies of darker pigmentation than those of the parent strain. Both strains grew only on media designed for *M. tuberculosis*.

When 0·2 ml. of the *Mycobacterium phlei* phage filtrate (referred to as phage M) containing 10^8 phage particles/ml. were added to 4·8 ml. of a 48-hr. culture of strain 41308 in Tween albumin medium, subcultures made during the first 2 weeks on egg-yolk medium yielded either the original light-yellow colonies, or the more darkly pigmented colonies described above. After 2 weeks fewer colonies of a different aspect appeared on subculture. These colonies of a phage-immune variant were umbilicated or wart-like and at 37° their pigment was orange. They lowered the pH value of the medium containing malachite green (Pl. 1, fig. 2). These growth characteristics were maintained through fifteen subcultures on egg-yolk medium at 37°. By maintaining the above-described conditions of incubation with phage M, the phage-immune variant was isolated on several occasions from single colonies of strain 41308 which were selected after repeated plating and inoculated into liquid Tween albumin medium.

Besides the difference in the colonial aspect, the phage-immune variant grew on egg-yolk medium in 3 days at 37°, and in 5 days at 22°. It grew readily on nutrient agar and in brain-heart infusion. On agar medium, the colonies resembled those of Nocardia: they were lighter in colour than those grown on egg-yolk media, and were dry and granular. In brain-heart infusion, the variant produced a granular deposit at the bottom of the tube, and a veil at the surface of the liquid. Like certain *Nocardia* spp., it was able to use paraffin as sole carbon source. In contrast to the parent strain, the pigment of the variant was affected by light. At room temperature and in the light, only a light pigment was formed; the colonies were small, granular and closely resembled those of *Mycobacterium tuberculosis* (Pl. 1, fig. 3).

On microscopic examination, strain 41308 showed slender granulated rods. Approximately 80% of the bacilli of a 10-day culture in Tween albumin medium were acid-fast. Under the same conditions only 10% of the slender granulated phage-immune bacteria were acid-fast. There were no differences in the susceptibilities of the two strains to streptomycin or isonicotinic acid hydrazide (INH); both strains were susceptible to 1 μg. streptomycin/ml. and resistant to 10 μg. INH/ml. However, there was a significant difference in their susceptibility to ethionamide

(TH 1314), the original strain being sensitive to 0·5 μg./ml. while the phage-resistant strain was resistant to 10 μg./ml. of this antituberculosis drug.

The most remarkable difference between strain 41308 and its variant was that the latter was lysogenic. Culture filtrates of the phage-immune strain lysed the parent strain 41308. In order to eliminate the possibility that the lysis was due to adsorbed phage, single colonies of the variant were transferred five times on egg-yolk media. One colony of the last culture was selected as inoculum for Dubos broth basal medium containing Tween 80, which is known to interfere, in the absence of albumin, with the adsorption of phages to bacteria (White & Knight, 1958). Filtrates from the Dubos broth basal culture lysed the same mycobacteria as did phage M, namely, *Mycobacterium phlei*, *M. stercoides*, *M. friburgensis*, strain 41308 and five out of twelve test strains of unnamed chromogenic bacteria. The lytic spectrum suggested that the lysogenic variant carries a prophage similar to, or identical with phage M.

Froman *et al.* (1955) reported that BCG strains did not show as high and as consistent a susceptibility to mycobacteriophages as did strains H37Rv and H37Ra. Takeya *et al.* (1959) found that attenuated strains of human tubercle bacilli of Imamura were less susceptible to phage action than the original virulent strains. The attenuated strains, especially those of Imamura, showed changes in colonial appearance which were similar to those undergone by the variant of 41308. A study of the antigenicity and pathogenicity of this variant was attempted as follows.

(*a*) The agar diffusion precipitation technique (Mankiewicz, 1958) was used to study the antigenic composition of the variant. It was found that the precipitation pattern had changed from that of the original strain. The variant shared antigens with *Mycobacterium phlei*, with *Nocardia asteroides* and with other chromogenic acid-fast bacteria to a much larger extent than with virulent tubercle bacilli.

(*b*) The cytochemical reaction with neutral red (Dubos & Middlebrook, 1948) was used with the following results:

Strain 41308	+
Its phage-immune variant	−
Controls	
H37Rv	++
BCG	(±)

(*c*) Animal experiments. Five guinea-pigs (250 g.) were inoculated intramuscularly with 1 mg. wet weight of strain 41308, and five others with the same weight of the variant. After 4 and 8 weeks, the animals inoculated with 41308 gave strongly positive reactions to intradermal Old Tuberculin, whereas the guinea-pigs inoculated with the variant remained tuberculin-negative throughout the experiment. After 9 weeks, the latter did not show any sign of disease at autopsy. The animals inoculated with the original strain showed enlarged regional lymph nodes. However, liver and lungs were free from disease and no acid-fast bacteria were isolated from these tissues.

Forty Swiss mice (Rockefeller strain; 20 g.) were inoculated intravenously with 0·5 mg. each of strain 41308; and forty others with the same amount of the variant organism. The animals were killed after 6 weeks. Thirty-two of the mice inoculated with the original strain showed macroscopic lesions in the lungs. None was observed

in the animals inoculated with the variant strain. Homogenates of the right lungs and of the right kidneys of the eighty animals were inoculated on egg-yolk medium. Chromogenic, smooth-growing acid-fast bacteria were recovered from cultures of the kidneys (twelve instances) and from the lungs (twenty-six instances) from animals inoculated with strain 41308. No bacteria were recovered from the forty kidney and forty lung cultures of the mice inoculated with the variant (Table 1).

Table 1. *Comparison between the chromogenic acid-fast bacterium* 41308 *and its phage-immune variant*

	Strain 41308	Variant strain
Growth on egg-yolk medium		
At 37° in the dark:	Yellow, smooth,	Orange, umbilicated,
Matures in:	10–12 days	3 days
At 22° in the light:	Yellow, smooth,	Light yellow, granular,
Matures in:	16–18 days	3 days
Growth on nutrient agar:	No growth	Light yellow, granular,
Matures in:		5 days
Susceptibility to ethionamide:	Sensitive	Resistant
Neutral red reaction:	+	−
Sensitization of guinea-pigs to Old Tuberculin:	+	−
Virulence for mice:	+	−
Lysogenicity:	−	+
Susceptibility to Phage M:	+	−
Culture filtrate of variant:	+	−

DISCUSSION

The questions which arise concern the origin and nature of the variant. An attempt may be made to narrow down the possibilities which appear to be as follows:

(1) Culture 41308 when isolated from the patient was 'mixed', containing phage-immune and phage-susceptible bacteria of different morphology.

(2) The phage-immune variant is the result of a spontaneous mutation. In both instances, phage M would have acted as a selective agent for the dissociate.

(3) Strain 41308 is an undetected or defective lysogenic strain.

(4) The change was phage-induced by (*a*) the process of transduction, or (*b*) by lysogenization.

The possibilities that culture 41308 was mixed, or that the variant resulted from spontaneous mutation, can be disregarded because extensive plating of the original strain on egg-yolk medium never produced the faster growing umbilicated variant. Without previous exposure to phage M, no growth was produced by 41308 on nutrient agar, or in Youman's medium with serum containing 10 μg. ethionamide/ml. Strain 41308 did not produce any lysis of strains selected at random. No lysis occurred within this strain after exposure to ultra-violet irradiation. It did not show any immunity to the lytic action of culture filtrates of the variant.

The changes described as characteristic for the variant were induced by phage M only. To establish that the process at work was that of transduction, one would have to know the donor bacterium on which the phage had multiplied. The properties acquired by the variant of 41308 (umbilicated colonial morphology, the particular pigment, resistance to ethionamide, lysogenicity) do not permit tying the variant to

Mycobacterium phlei, *M. stercoides*, *M. friburgensis* or to any of the acid-fast bacteria which were found to be susceptible to phage M. Although the possibility of transduction cannot be completely excluded, lysogenization appears the most plausible mechanism for the production of the variant.

The ability to produce phage and immunity are the most important changes due to lysogenization. Others have been described which concern the morphology and antigenicity of lysogenic bacteria. Ionesco (1953) observed that, due to the presence of prophage, the normally smooth colonies of *Bacillus megaterium* became rough and wart-like. Antigenic changes were described by Uetake, Nakagawa & Akiba (1955), Uetake, Luria & Burrows (1958), Staub, Tinelli, Luderitz & Westphal (1959), Stocker (1958) and Zinder (1957) in Salmonellae after they became hosts to prophages. In Mycobacterium no naturally occurring lysogenic strains have been detected so far. Takeya *et al.* (1959) found that a phage-immune mutant of *Mycobacterium* sp. ATCC 607, resistant to phages A-2 and A-4, was lysogenic.

A connexion between the chromogenicity of colonies of mycobacteria and their immunity to phagolysis has been shown for *Mycobacterium smegmatis* and *M. phlei* (Bassermann, 1959); the phage-immune mutants showed an increase in pigmentation of their colonies.

While extensive studies have been devoted to the localization and chemical nature of the phage receptor groups of phage-susceptible *Escherichia coli* and various salmonellas, little is known about phage receptors in mycobacteria. Penso (1955) suggested that in mycobacteria there exists a close relationship between antigen-antibody reaction and phage susceptibility. Tuberculo-antigens and phage receptors might be of similar molecular structure. The variant of strain 41308 showed that the loss of phage-susceptibility was linked with changes in the antigenic composition as demonstrated by the precipitation studies and the animal experiments. It is hoped that future studies will lead to a definition of the phage-affected areas in mycobacteria.

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EXPLANATION OF PLATE

The phage susceptible strain of chromogenic-aeid fast bacteria and the phage-immune variant.

Fig. 1. Colonies of the phage-susceptible strain after 12 days of incubation at 37°.

Fig. 2. Colonies of the phage-immune variant after 5 days of incubation at 37° in the dark.

Fig. 3. Colonies of the phage-immune variant after 5 days of incubation at 22° in the light.

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(*Facing p.* 68)

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Effect of Biotin Deficiency on the Synthesis of Nucleic Acids and Protein by *Saccharomyces cerevisiae*

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SUMMARY

A strain of *Saccharomyces cerevisiae*, grown in a medium containing a suboptimal concentration ($0{\cdot}4 \times 10^{-10}$ M) of biotin, was shown to contain less deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein but, during the early stages of growth, increased concentrations of acid-soluble ultraviolet (u.v.)-absorbing substances, as compared with the same organism grown in the presence of an optimal concentration ($8{\cdot}0 \times 10^{-10}$ M) of biotin. The concentration of acid-soluble u.v.-absorbing substances in the biotin-deficient yeast was higher, irrespective of the nature of the extracting acid (0·2 N-perchloric acid, 5 % (w/v) and 10 % (w/v) trichloroacetic acid, or 5 % (v/v) *n*-butanol in M/15 KH_2PO_4). Raising the temperature of extraction from 3° to 21° or 30° had little or no effect on the amounts of these u.v.-absorbing substances extracted. Analyses of the nucleotides and nucleobases in the yeast RNA showed these to have a ratio of purine:pyrimidine bases of 1·00–1·15, with the exception of the RNA from 5-day cultures of biotin-deficient yeast which had a slightly but consistently higher ratio. The significance of these results is discussed in relation to the metabolic function of biotin.

INTRODUCTION

Biotin has for some time been recognized as a growth factor for micro-organisms, but, although the metabolic roles of many other vitamins and growth factors have been elucidated, no specific function for biotin in the metabolism of micro-organisms has as yet been unequivocally established. Two main lines of study have been pursued in an attempt to gain information on the role of biotin in microbial metabolism. Growth of certain biotin-requiring micro-organisms in media containing suboptimal concentrations of biotin has been shown to be accompanied by the appearance in the medium of biosynthetic intermediates, the further metabolism of which is inhibited under conditions of biotin deficiency. Accumulation of an aromatic amine, 5-amino-imidazole riboside (Chamberlain, Cutts & Rainbow, 1952; Chamberlain & Rainbow, 1954; Moat, Wilkins & Friedman, 1956) and of hypoxanthine (Chamberlain & Rainbow, 1954) by *Saccharomyces cerevisiae* is the result of an impairment in the ability of the biotin-deficient yeast to complete the synthesis of purines. The inability to synthesize adequate quantities of purine under these conditions is also thought to cause a derangement in the synthesis of pyridine nucleotides by *S. cerevisiae*, which is manifested in the accumulation of

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nicotinic acid and nicotinic acid adenine dinucleotide in the culture medium (Rose, 1960*a*, *b*), while the same metabolic disturbance is probably responsible for the subnormal contents of adenosine triphosphate (ATP) and coenzyme A in biotin-deficient *Piricularia oryzae* (Katsuki, 1959*a*) and *Bacillus macerans* (Katsuki, 1959*b*).

Several workers have searched for a more specific locus of biotin function and have been able to show that, in certain biotin-deficient micro-organisms, the activity of some enzymes is markedly depressed. For example, a deficiency of biotin affects adversely the decarboxylation of oxaloacetate in *Lactobacillus arabinosus* (Lardy, Potter & Elvehjem, 1947), oxalosuccinate carboxylation in *Escherichia coli* (Shive & Rogers, 1947) and succinate decarboxylation in *Propionibacterium pentosaceum* (Delwiche, 1950). Biotin has also been shown to be essential for the activity of certain enzymes involved in amino acid metabolism, including those responsible for the aspartate to α-ketoglutarate transformation (Rossi, Rossi & Rossi, 1957), and for the deamination of aspartic acid (Lichstein & Umbreit, 1947), of threonine (Lichstein & Christman, 1948) and of serine (Nadkarni & Sreenivasan, 1957). Hexokinase activity in *Saccharomyces cerevisiae* (Strauss & Moat, 1958) and the ability to convert ornithine and carbamyl phosphate into citrulline in *Streptococcus lactis* (Estes, Ravel & Shive, 1956) have also been reported to be decreased under conditions of biotin deficiency.

However, the function of biotin in these biotin-dependent enzyme reactions has not yet been established. The activity of some of the enzymes in cell-free preparations is known to be stimulated on adding biotin, but a coenzymic role for the vitamin has not been demonstrated directly. Indirect evidence, such as the demonstration of a correlation between the activity of enzyme preparations and the content of bound biotin, is taken by some workers (e.g. Lichstein, 1955) to indicate a possible coenzymic role. There is, however, no evidence to show that the bound forms of biotin which have been isolated (such as ϵ-N-biotinyl-L-lysine, 'biocytin') are coenzymic forms of the vitamin (Wright *et al.* 1952). With other biotin-dependent enzyme systems no stimulation of activity occurs on adding biotin to the cell-free preparations, and this has led some workers to ascribe an indirect role for biotin, probably in enzyme synthesis. Sund, Ravel & Shive (1958), for example, did not obtain any immediate increase in activity of a preparation of the ornithine→citrulline enzyme from biotin-deficient *Streptococcus lactis* on adding biotin, and concluded that biotin is probably concerned in synthesis of the enzyme. Further evidence to support the view that biotin is concerned only indirectly in the activity of this enzyme comes from more recent data (Ravel, Grona, Humphreys & Shive, 1959), which showed that purified preparations of the enzyme contained less biotin than did the original cell-free extract. Similarly, Chambers & Delwiche (1954), as a result of their studies on the function of biotin in *Propionibacterium pentosaceum*, suggested that the vitamin functions in the synthesis of the coenzyme or apoenzyme concerned in the carboxylation of succinate.

The results from both of these lines of study suggest, therefore, that biotin is concerned in protein synthesis, either via the synthesis of purines or in the formation of specific enzymes. The work reported in this paper was carried out in order to examine the effect of biotin deficiency on the synthesis of nucleic acids and total protein in *Saccharomyces cerevisiae*. The results show that, under these conditions, synthesis of both of these groups of substances was impaired.

METHODS

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

Experimental cultures. The chemically defined medium of Rose & Nickerson (1956) was used. Portions of the medium (100 ml.), containing either an optimal ($8{\cdot}0 \times 10^{-10}$ M) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) concentration of biotin, were dispensed into 350 ml. conical flasks, which were plugged and sterilized by autoclaving momentarily at 10 lb./sq.in. The medium was inoculated by the procedure described by Rose (1960*b*), and cultures were incubated statically at 25°. Growth was measured turbidimetrically by determining the optical density of a portion (6 ml.) of culture in the Hilger 'Spekker' absorptiometer (model H 760), using neutral green-grey H 508 filters and a water blank. Optical density measurements were related to dry weight of yeast by a calibration curve.

Nucleic acid estimations. Yeast grown in media containing either an optimal or a suboptimal concentration of biotin was washed three times with M/15 KH_2PO_4, (pH 4·5), and triplicate 3 mg. portions of the crop were taken for nucleic acid estimations. The pellet of yeast was extracted twice, in 15 ml. tapered centrifuge tubes, with 2·0 ml. portions of 0·2 N-perchloric acid at room temperature to remove acid-soluble ultraviolet (u.v.)-absorbing substances. The extracts were pooled, neutralized with N-NaOH, and made up to 5·0 ml. with M/15 KH_2PO_4; the optical density of this extract was measured at 260 mμ, with the Unicam S.P. 500 quartz spectrophotometer, and the reading taken as a measure of the acid-soluble u.v.-absorbing substances in the yeast. The yeast pellet was then extracted twice with 3 ml. of a boiling mixture of 95 % (v/v) ethanol in water (3 vol.) + ether (1 vol.) for 2 min. to extract lipids, and the extracts rejected. The ribonucleic acid (RNA) in the residue was hydrolysed to acid-soluble nucleotides by suspending the material in 2·0 ml. N-NaOH for 1 hr. at room temperature (Schmidt & Thannhauser, 1945; Bonar & Duggan, 1955), after which perchloric acid (N) was added to a concentration of 0·2 N. The supernatant fluid containing the soluble RNA nucleotides was separated from the precipitate of deoxyribonucleic acid (DNA) and protein, which was then washed twice with 1·0 ml. portions of 0·2 N perchloric acid, and the washings combined with the RNA extract. The combined volume was neutralized with N-NaOH, made to 10·0 ml. with M/15 KH_2PO_4, and the optical density at 260 mμ taken as a measure of the RNA content of the yeast.

The residue of DNA and protein was suspended in 2·0 ml. N-perchloric acid, and held at 90° for 15 min. This hydrolysed the DNA to acid-soluble nucleotides, which were removed in the supernatant fluid. Extracts were made up to 3·0 ml. with N-perchloric acid, and the optical density of the solution at 260 mμ taken as a measure of the DNA content of the yeast.

Protein estimations. Protein in the residue remaining after the nucleic acids had been extracted was determined by the conventional micro-Kjeldahl technique (Markham, 1942) with a mercuric oxide catalyst (Miller & Houghton, 1945). Protein contents are expressed as mg. Kjeldahl nitrogen/3 mg. dry weight yeast.

Analysis of ribonucleic acids. For the electrophoretic separation of ribomononucleotides, the RNA extract from 40 mg. dry weight of yeast was adjusted to pH 4·0 by careful addition of 10·0 N-KOH, and potassium perchlorate removed by centrifugation (Davidson & Smellie, 1952). Portions (4·0 mg.) of a commercial preparation of yeast RNA (L. Light and Co. Ltd. Colnbrook, Buckinghamshire), which was used as a control, were dissolved in 1·0 ml. of 0·3 N-KOH, incubated at 37° for 18 hr., and the supernatant fluid removed by centrifugation after neutralization with 9·2 N-perchloric acid. The supernatant liquid was then adjusted to pH 4·0 with 10·0 N-KOH, and potassium perchlorate removed by centrifugation. Samples of the solutions (300–500 μl.) were applied as a short band (1·5–2·0 cm.) about 15 cm. from the end of a strip of Whatman no. 3 MM paper (57 cm. × 10 cm.). The paper was soaked in 0·02 M-citrate buffer (pH 3·5), and a potential gradient of 21 V./cm. applied for 7 hr. After removal, the paper was dried with a hair dryer, examined under u.v. radiation (Hanovia 'Chromatolite'), and the positions of the nucleotide spots marked with a pencil. The areas of paper containing the spots were cut out, and the nucleotides eluted from the shredded paper by soaking in 0·01 N-HCl overnight at 37° in a stoppered test tube. The eluate was centrifuged to remove cellulose fibres. The optical density of the eluate was then measured at the appropriate wavelength (adenylic acid, 260 mμ; guanylic acid, 260 mμ (Volkin & Carter, 1951); cytidylic acid, 278 mμ; uridylic acid, 262 mμ (Ploeser & Loring, 1949)), and the molar concentrations of the nucleotides in the eluates calculated from the optical density measurements by using the appropriate millimolar extinction coefficients. Controls of adenylic acid, guanylic acid, cytidylic acid and uridylic acid were run with each electrophoretogram.

Ribonucleobases were obtained by perchloric acid oxidation of the nucleotides. The extract of RNA mononucleotides from 40 mg. dry weight yeast was acidified to pH 5 and evaporated to dryness on a boiling water bath. This was followed by the addition of 0·2 ml. of 12·0 N perchloric acid, after which the solution was heated on the boiling water bath for 70 min. On cooling, 0·2 ml. distilled water was added, and the perchloric acid neutralized by addition of 10·0 N-KOH. The supernatant liquid was then acidified with pure HCl to 2·0 N-HCl, heated in a boiling water bath for a further 5 min. and cooled, after which the precipitate of potassium perchlorate and carbon was removed by centrifugation. This precipitate was washed with 0·1 N-HCl and the washings combined with the original extract. This was then applied as a band (1·0–1·5 cm.) on Whatman no. 3 MM paper, and examined by descending chromatography, using a solvent of *iso*propanol + conc. HCl + water (68 + 16·4 + 15·6; Wyatt, 1951). The paper was irrigated for 40 hr. at room temperature, dried, and examined beneath u.v. radiation. The nucleobases appeared as discrete absorbing spots, the guanine spot being easily distinguished by its bluish tinge. The appropriate areas of paper were cut out, and the nucleobases eluted from the shredded paper by soaking overnight at 37° in 0·10 N-HCl. The optical densities of the eluates were measured at the appropriate wavelengths (adenine, 260 mμ; guanine, 250 mμ; cytosine, 275 mμ; uracil, 260 mμ; Wyatt, 1951), and the molar concentrations of nucleobases calculated by using the appropriate millimolar extinction coefficients. Controls of adenine, guanine, cytosine and uracil were run with each chromatogram.

RESULTS

Effect of biotin deficiency on the concentrations of nucleic acids, protein and acid-soluble u.v.-absorbing substances in yeast

Cultures of the yeast, grown in media containing either an optimal ($8{\cdot}0 \times 10^{-10}$ M) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) concentration of biotin, were removed at intervals and, after growth had been measured, the yeast was washed and analysed for DNA, RNA, protein and acid-soluble u.v.-absorbing substances. The results are shown in Figs. 1 and 2. Under conditions of biotin deficiency, growth of the yeast was restricted and, after *c.* 120 hr. of incubation, the biotin-deficient yeast was coloured pink instead of the usual creamy-white (Chamberlain *et al.* 1952). This restriction in growth and change in colour of the yeast was accompanied by marked changes in

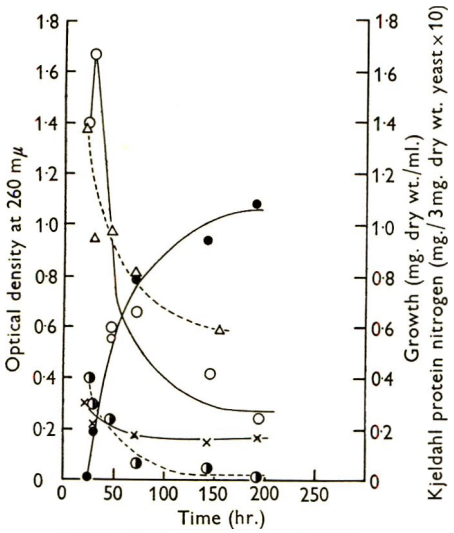

Fig. 1

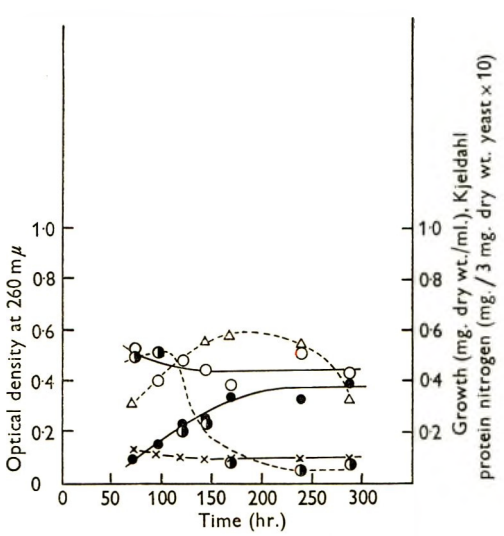

Fig. 2

Figs. 1, 2. Effect of incubation time on growth (●—●, mg. dry wt./ml.) and on the concentrations of DNA (× — ×), RNA (○—○), acid-soluble u.v.-absorbing substances (◑—◑) and Kjeldahl protein nitrogen (△—△, mg./3 mg. dry wt. yeast × 10) in yeast grown in media containing either an optimal ($8{\cdot}0 \times 10^{-10}$ M) (Fig. 1) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) (Fig. 2) concentration of biotin. Analyses were conducted on triplicate 3·0 mg. portions of yeast. Concentrations of DNA, RNA and acid-soluble u.v.-absorbing substances are expressed as the optical densities at 260 mμ of extracts from the yeast made up to 3·0, 10·0 and 5·0 ml. respectively with M/15 KH_2PO_4.

the concentrations of DNA, RNA, protein and acid-soluble u.v.-absorbing substances. After an initial slight decrease, the concentrations of DNA in both types of yeast remained constant throughout the period of growth, although under conditions of biotin deficiency, the concentration was significantly lower than in the yeast (biotin-optimal) grown in medium containing an optimal concentration of biotin. The sequence of changes observed in the RNA content of the biotin-optimal yeast was similar to that previously reported by other workers (Di Carlo & Schultz, 1948). In the biotin-deficient yeast, however, the concentration of RNA was, by comparison, low and remained so during the observed period of growth. The

concentration of Kjeldahl protein-nitrogen in the biotin-optimal yeast was highest during the very early stages of the exponential phase of growth, but declined steadily as the culture aged. In the biotin-deficient yeast, the protein nitrogen content increased up to 160 hr., when it was approximately half of that in exponential phase biotin-optimal yeast, but, thereafter, gradually declined. The biotin-optimal yeast contained an appreciable amount of acid-soluble u.v.-absorbing substances during the early stages of the exponential phase of growth but on further incubation the concentration declined rapidly and, at the end of the exponential phase, had become extremely small. The biotin-deficient yeast contained significantly higher concentrations of these substances during the early stages of growth although, after *c.* 200 hr., this concentration too had decreased to a low value.

Concentration of acid-soluble u.v.-absorbing substances and stability of RNA in biotin-deficient yeast

The comparatively high concentration of acid-soluble u.v.-absorbing substances in the biotin-deficient yeast during the early stages of growth was obviously of interest in relation to the inability of the yeast to synthesize normal amounts of RNA under conditions of biotin deficiency. It was possible that these u.v.-absorbing substances arose as a result of the breakdown of RNA in the biotin-deficient yeast during extraction with perchloric acid; alternatively, they may have represented purine- and pyrimidine-containing substances that had failed to be polymerized into DNA and RNA. A study was therefore made of the effect of using various acid solutions, including 0·2 N and 1·0 N-perchloric acid, 5 % (w/v) and 10 % (w/v) trichloroacetic acid, and 5 % (v/v) *n*-butanol in M/15 KH_2PO_4 (pH 4·5), to extract these u.v.-absorbing substances from 120 hr. biotin-deficient yeast and from exponential phase (40 hr.) and stationary phase (120 hr.) biotin-optimal yeast. Triplicate 3 mg. portions of washed yeast were taken, and were extracted five or, when necessary, more times with 4·0 ml. portions of the extracting solution at 3° for 5 min. After centrifugation, the supernatant liquid was decanted, adjusted to pH 4·5, and made up to 5·0 ml. with M/15 KH_2PO_4. The optical densities of these extracts were then measured at 260 mμ, with a blank of the appropriate reagent. Removal of the u.v.-absorbing substances was usually complete in five extractions, although complete removal from biotin-deficient yeast with the 5 % aqueous butanol required seven separate extractions. After all the u.v.-absorbing substances had been extracted, the residue was defatted and the RNA estimated in the usual way.

Perchloric acid (0·2 N) extracted all the u.v.-absorbing substances fairly rapidly from biotin-deficient yeast and from biotin-optimal yeast. Consistently larger amounts of u.v.-absorbing substances were extracted from biotin-deficient yeast than from either exponentially growing or stationary phase yeast grown in presence of optimal biotin (Fig. 3). Higher concentrations of perchloric acid (e.g. N) are known to hydrolyse RNA, and this is used as a means of extracting RNA from tissues (Ogur & Rosen, 1950). However, it would seem from the data shown in Fig. 4 that the RNA in biotin-deficient yeast was hydrolysed more quickly by N-perchloric acid as compared with RNA in biotin-optimal yeast.

Many workers have recommended the use of trichloroacetic acid for the extraction

of u.v.-absorbing substances before estimation of nucleic acids in tissues (Davidson, Frazer & Hutchinson, 1951; Schmidt & Thannhauser, 1945; Schneider, 1945). Trichloroacetic acid at concentrations of 5 % (w/v) or 10 % (w/v) rapidly extracted the bulk of the acid-soluble u.v.-absorbing substances from the biotin-deficient yeast and from the biotin-optimal yeast. The amounts extracted from biotin-deficient yeast again exceeded those from non-deficient yeast (Fig. 5). Little difference was

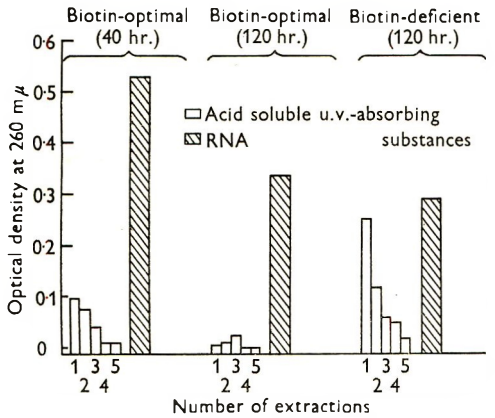

Fig. 3

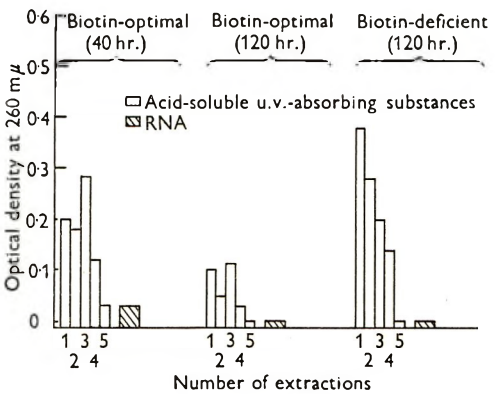

Fig. 4

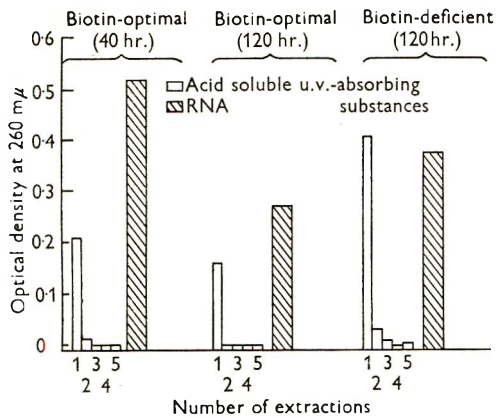

Fig. 5

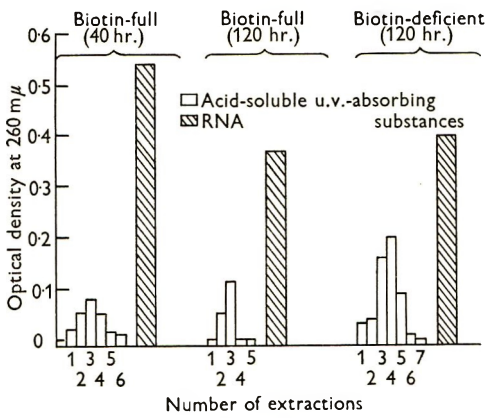

Fig. 6

Figs. 3–6. Extraction of acid-soluble u.v.-absorbing substances from 3·0 mg. portions of biotin-optimal (40 and 120 hr.) and biotin-deficient (120 hr.) yeast with separate 4·0 ml. portions of 0·2N-$HClO_4$ (Fig. 3), 1·0N$HClO_4$ (Fig. 4), 5 % (w/v) trichloroacetic acid (Fig. 5) or 5 % (v/v) *n*-butanol in M/15KH_2PO_4 (Fig. 6). Extracts were adjusted to pH 4·5, made up to 5·0 ml. with M/15 KH_2PO_4, and the optical densities measured at 260 mμ. RNA was extracted as described under Methods.

observed between the amounts extracted by 5 % and 10 % trichloroacetic acid, although a slightly decreased content of RNA in biotin-deficient yeast that had been extracted with 10 % trichloroacetic acid suggested that some of the nucleic acid may have been hydrolysed during extraction.

Extraction of u.v.-absorbing substances by aqueous *n*-butanol was used by Mitchell & Moyle (1951) in their studies on the chemical anatomy of *Staphylococcus*

aureus (*Micrococcus pyogenes*). This reagent is far milder than either perchloric acid or trichloroacetic acid, and was used in an attempt to minimize possible hydrolytic breakdown of RNA during extraction. Concentrations of *n*-butanol up to 4% (v/v) in M/15 KH_2PO_4 failed to extract detectable amounts of u.v.-absorbing substances from the yeast. But by using a concentration of 5% (v/v) butanol in M/15 KH_2PO_4, the u.v.-absorbing substances were extracted, although at least seven separate extractions were required for complete removal of these substances from biotin-deficient yeast (Fig. 6).

Effect of temperature. Further information about the stability of the RNA in biotin-deficient yeast during extraction of the acid-soluble u.v.-absorbing substances was obtained when these extractions were carried out at 21° or 30° instead of at 3°. The results obtained showed that elevation of the temperature had no significant effect on the amounts of u.v.-absorbing substances and of RNA extracted, as compared with the amounts extracted at the lower temperature. Extraction at these elevated temperatures did not affect the amounts of acid-soluble u.v.-absorbing substances extracted from biotin-optimal yeast. Spectrophotometric examination of the various extracts of acid-soluble u.v.-absorbing substances revealed that, in all instances, these showed maximum u.v. absorption at or very close to 260 mμ.

Effect of biotin deficiency on the nucleotide and nucleobase compositions of the yeast RNA

The nucleotide and nucleobase compositions were determined on the RNA extracted from 40 mg. dry wt. yeast. The yeast was extracted with 10 ml. portions of 5% (w/v) trichloroacetic acid at 3° until the acid-soluble u.v.-absorbing substances had been completely removed; this required five to eight separate extrac-

Table 1. *Molar concentrations of adenylic, guanylic, cytidylic and uridylic acids (based on adenylic acid = 10) in a commercial yeast RNA and in extracts of RNA from* Saccharomyces cerevisiae *grown in media containing an optimal* ($8{\cdot}0 \times 10^{-10}$ M) *or a suboptimal* ($0{\cdot}4 \times 10^{-10}$ M) *concentration of biotin*

Source	Age of culture (hr.)	Molar concentration				Ratio: purine/ pyrimidine
		Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	
Commercial yeast RNA	—	10·0	12·64	7·73	12·20	1·13
Biotin-optimal yeast	40	10·0	11·12	8·00	11·31	1·11
	96	10·0	10·6	8·20	11·60	1·03
Biotin-deficient yeast	120	10·0	11·90	7·40	9·76	1·28
	168	10·0	12·70	8·19	12·08	1·12

tions. The tissue was defatted by extracting twice with 10 ml. portions of a boiling mixture of 95% (v/v) ethanol in water (3 vol.)+ether (1 vol.), and the residue treated with 2·0 ml. 0·3 N-KOH for 18 hr. at 37° (Davidson & Smellie, 1952) to hydrolyse polyribonucleotides to soluble mononucleotides. Shorter periods of incubation were tried and, although these were sufficient to allow for the hydrolysis of RNA to acid-soluble nucleotides as detected spectrophotometrically, nevertheless it was shown electrophoretically that incubation for 18 hr. at 37° was necessary

to obtain complete hydrolysis to the mononucleotides. The nucleotide and nucleobase compositions of the RNA extract were then determined as described under Methods.

The data in Table 1 show the molar concentrations (with adenylic acid expressed as 10) of RNA nucleotides in a sample of commercial yeast RNA and in the RNA from *Saccharomyces cerevisiae* grown in media containing either an optimal or a suboptimal concentration of biotin. These results show the molar ratio of purine to pyrimidine nucleotides ranged from 1·00 to 1·15 in the commercial yeast RNA

Table 2. *Molar concentrations of adenine, guanine, cytosine and uracil (based on adenine = 10) in a commercial yeast RNA and in extracts of RNA from* Saccharomyces cerevisiae *grown in media containing an optimal* ($8{\cdot}0 \times 10^{-10}$ M) *or a suboptimal* ($0{\cdot}4 \times 10^{-10}$ M) *concentration of biotin*

Source	Age of culture (hr.)	Molar concentration				Ratio: purine/ pyrimidine
		Adenine	Guanine	Cytosine	Uracil	
Commercial yeast RNA	—	10·0	10·10	8·07	11·03	1·05
Biotin-optimal yeast	40	10·0	11·36	7·91	11·50	1·10
	96	10·0	11·14	8·50	11·40	1·04
Biotin-deficient yeast	120	10·0	11·60	7·56	10·10	1·23
	168	10·0	11·60	9·10	11·90	1·02

and in biotin-optimal yeast during the exponential and stationary phases of growth. In biotin-deficient yeast from 120 hr. cultures, the ratio was slightly but consistently higher, the average value obtained being *c.* 1·28; but in yeast from 7-day biotin-deficient cultures, the ratio had decreased to within the range 1·00–1·15. Closely similar results were obtained when the ratio of purine to pyrimidine bases in the yeast RNA was determined. As shown in Table 2, this ratio was in the range 1·00–1·10 in all of the samples of RNA studied, with the exception of that obtained from 120 hr. cultures of biotin-deficient yeast in which it averaged 1·23.

DISCUSSION

Since purine- and pyrimidine-containing nucleotides (e.g. ATP, DPN, coenzyme A) and polynucleotides (nucleic acids) are essential components of all living cells, it is to be expected that any metabolic stress which causes a derangement in the biosynthetic processes leading to the formation of purines or pyrimidines will result in the decreased synthesis of nucleotides and nucleic acids. Although the presence of diminished amounts of total purine in biotin-deficient micro-organisms has not been demonstrated directly, several workers have obtained evidence that biotin is concerned in the synthesis of these nitrogenous bases. The effects of this biotin-conditioned purine deficiency on the synthesis of certain nucleotides in biotin-requiring micro-organisms has already been reported. Thus, Katsuki (1959*a*, *b*) has shown that biotin-deficient *Piricularia oryzae* and *Bacillus macerans* contained diminished amounts of ATP, DNP and coenzyme A, and Rose (1960*b*) reported that the excretion of nicotinic acid and nicotinic acid adenine dinucleotide, two biosynthetic precursors of pyridine nucleotides which appear in the culture medium during growth of *Saccharomyces cerevisiae* under conditions of biotin deficiency, is suppressed on adding adenine to the medium. The results obtained in the present

investigation showed that biotin deficiency during growth of a strain of *S. cerevisiae* had a profound effect also on the synthesis of nucleic acids. It was somewhat surprising to discover that, during the early stages of growth, RNA in the biotin-deficient yeast contained an abnormally high content of purine, in view of the adverse effect of biotin deficiency on purine biosynthesis. There was further evidence of a difference between the RNA from 5-day biotin-deficient cultures and that from other cultures, in that the former was more readily hydrolysed by N-perchloric acid. This may indicate a certain instability in structure, a reflexion perhaps of the slightly abnormal base ratio. It is important to note, however, that the RNA extracted from the yeast was heterogeneous and consisted of a mixture of ribosomal, soluble and nuclear RNA. The slight difference in the overall base ratio of the mixture might then be caused by a more significant variation in the base ratio of one of these RNA fractions.

This diminution in the amounts of nucleic acids synthesized under conditions of biotin deficiency was accompanied, during the early stages of growth, by a significant increase in the concentration of intracellular acid-soluble u.v.-absorbing substances which, since they absorbed maximally at approximately 260 mμ, were taken to be purine- and pyrimidine-containing substances. The substances were detected initially in extracts made with the strong acids perchloric and trichloroacetic acids, and it was possible that they represented products from the acid degradation of RNA and DNA. When it was discovered, however, that these u.v.-absorbing substances were also extracted, albeit more slowly, with aqueous *n*-butanol at pH 4·5, a much less drastic reagent, then it was assumed that they were present in the yeast in the soluble state and that they did not represent artefacts of extraction. Some evidence to support this contention came from the discovery that the amounts of u.v.-absorbing substances extracted with acid did not increase significantly when the temperature of extraction was raised from 3° to 21° or 30°, as might have been expected had they arisen as the result of hydrolysis of RNA. No analyses of the composition of this acid-soluble fraction were made in the present study, so it is not known what type of purine- or pyrimidine-containing compounds were present. But at least two -u.v.-absorbing purine precursors, 5-amino-imidazole riboside (Moat *et al.* 1956; Lones, Rainbow & Woodward, 1958) and hypoxanthine (Chamberlain & Rainbow, 1954), are known to be excreted by *Saccharomyces cerevisiae* growing under conditions of biotin deficiency, so that it is likely that these also accumulate in the cells. It is possible too, that ribonucleotides are present in this fraction since these are known to accumulate intracellularly under conditions of decreased RNA synthesis. This has been demonstrated, for example, with a strain of *Escherichia coli* following addition to the culture of the purine analogue 6-azauracil (Skoda & Sorm, 1958).

The role of RNA in protein synthesis is well known, and there is also some reason for believing that protein synthesis is essential for the synthesis of RNA. The results obtained in the present study showed that, under conditions of biotin deficiency, synthesis of total protein by *Saccharomyces cerevisiae* was markedly diminished, and it is possible that this was a result, at least in part, of the decrease in the amount of RNA synthesized and of the formation of possibly abnormal RNA. But for protein synthesis to take place, it is also essential to have in the cell an adequate reservoir or pool of amino acids as well as sufficient energy, in the

form of ATP, to activate these amino acids. It is probable, however, that neither of these requirements is met in biotin-deficient yeast, for, not only does biotin-deficient *S. cerevisiae* contain reduced amounts of ATP (Dr M. H. Briggs, personal communication), but it has also been found to contain decreased concentrations of water-soluble ninhydrin-positive substances (Mr A. L. S. Munro, unpublished observations) as compared with biotin-optimal yeast. It would appear, therefore, that there are several metabolic deficiencies contributing towards this overall reduction in protein synthesis.

The diminution in the amount of protein synthesized in biotin-deficient yeast must clearly affect the enzymic activities of the yeast. It is possible that the production of only certain enzymes is affected under conditions of biotin deficiency, which would explain why only a limited number of enzymic activities have been reported to be impaired in yeast grown under this metabolic stress. On the other hand, biotin deficiency may have a non-specific effect on protein synthesis, with the result that production of all of the enzymes in the cell is decreased to about the same extent. Then the deficiency might only be observed in those metabolic reactions for which the enzymes are present in rate-limiting concentrations.

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The Cultivation of D-Galactosamine-metabolizing Lactic Acid Bacteria

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SUMMARY

Media have been devised containing tracheal hydrolyzates or D-galactose as an economical means for the cultivation of large quantities of galactosamine-metabolizing bacteria. With these media *Lactobacillus bulgaricus* and *L. casei* showed good growth; dried preparations were able to utilize galactosamine to produce ammonia and lactic acid.

INTRODUCTION

The wide distribution and importance of glucosamine and galactosamine have been amply reviewed (Kent, 1957; Roseman, 1959), and despite the considerable work done to elucidate the metabolism of glucosamine, little has been reported about the metabolism of galactosamine (Roseman, 1959). This communication describes cultural studies resulting in the production of large numbers of galactosamine-metabolizing bacteria. These included the study of bacteria grown in a medium containing bovine tracheal hydrolyzates as a source of D-galactosamine. Ammonia production and lactic acid formation by dried preparations of lactic acid bacteria, as well as the ability to support growth, were used as criteria of galactosamine utilization.

METHODS

Cultures and media. The following organisms were tested for their ability to grow on D-glucosamine or D-galactosamine: *Streptococcus faecalis* R. ATCC 8043, *S. liquifaciens*, *S. salivarius* ATCC 9756, *Lactobacillus bulgaricus* ATCC 521, *L. buchneri*, *L. casei* ATCC 7469, *L. plantarum* ATCC 8014, *Leuconostoc mesenteroides* P-60 (*Pediococcus*; Garvie, 1959), *Pediococcus cerevisiae*. These cultures were maintained in stabs of liver powder + yeast extract + glucose agar.

The inocula were prepared by seeding Micro Inoculum Broth (Difco) from stab cultures. The cultures after growth for *c.* 18 hr. were centrifuged and washed twice with sterile water. In some cases the inocula were carried over several transfers in medium B (see below), containing glucose, glucosamine or galactosamine at 5 mg./ml. medium, before testing.

For growth studies the inocula were diluted with sterile water to an optical density of 0·4 at 660 mμ in a Beckman Model B spectrophotometer with a light path of 18 mm. One drop of this suspension was then used to inoculate tubes containing 2–6 ml. of the test medium as described in the text.

The medium B was prepared as follows: Casamino acids (Difco), 5 g.; DL-tryptophan, 0·03 g.; L-cysteine.HCl, 0·2 g.; K_2HPO_4 and KH_2PO_4, 0·5 g. each; sodium acetate.$3H_2O$, 10 g.; NH_4Cl, 3 g.; $MgSO_4.4H_2O$, 0·01 g.; $FeCl_3$, 0·03 g.; yeast extract, 3 g.; adenine sulphate, guanine HCl, and uracil, 0·01 g. each; distilled water, 1000 ml.; adjusted to pH 6·8.

Freshly prepared neutralized hexosamine solutions were sterilized by filtration through UF sintered glass filters and added aseptically to previously autoclaved medium.

Preparation of bovine trachea hydrolyzates. Calf trachea, obtained frozen from a slaughterhouse, was cleaned by removing superficial tissues and fat and then passed twice through a meat grinder. The ground trachea was stored at $-12°$ until used. Eighty g. of ground trachea were suspended in 100 ml. of 2 or 4N-hydrochloric acid or 2 or 4N-sulphuric acid and heated in free-flowing steam (100°) for either 7 or 17 hr. The conditions of hydrolysis were arbitrarily chosen. The hydrolyzates were cooled to 5° and solid fat filtered off. The hydrochloric acid-digested material was adjusted to pH 6·8 with sodium hydroxide. The sulphuric acid-digested material was treated with 95% of the amount of barium hydroxide calculated to neutralize all the acid present. The precipitated barium sulphate was removed by filtration and the filtrate adjusted to pH 6·8 with sodium hydroxide.

Chemical determinations. Ammonia was determined by the microdiffusion technique of Conway & Byrne (1933) followed by nesslerization. Lactic acid was measured by the procedure of Barker & Summerson (1941).

D-Galactosamine.HCl and D-glucosamine.HCl were obtained from Mann Research Laboratories (136 Liberty Street, New York, New York, U.S.A.). Each yielded one reducing spot before and after ninhydrin treatment according to the method of Stoffyn & Jeanloz (1954).

Preparation of dried organisms. Dried *Lactobacillus bulgaricus or L. casei* were prepared from cultures grown in medium B which had been supplemented with glucose, glucosamine, galactose or galactosamine to 30 μmole/ml. medium. The flasks were inoculated as follows: for glucose or galactose-containing medium, organisms grown in inoculum broth were used; for flasks containing the amino sugars, organisms grown for several transfers in glucosamine or galactosamine were used. After 28 hr. of growth, the organisms were harvested by centrifugation, washed twice and dried by lyophilization. Lyophilized *L. bulgaricus* and *L. casei* were also prepared from cultures grown on trachea hydrolyzed in 4N-H_2SO_4 for 7 hr. and prepared with glucose supplements as described in the text.

RESULTS

The growth of the organisms

All of the organisms tested with glucose as substrate reached maximum growth at 24 hr.; however, the organisms which grew on the hexosamines required almost 35 hr. to achieve maximum growth. Except for *Lactobacillus buchneri* which showed no growth, all of the organisms grew well on glucosamine. With galactosamine only *L. mesenteroides*, *L. bulgaricus* and *L. casei* achieved more than minimal growth. *L. casei* and *L. bulgaricus* grew well on both amino sugars; these two organisms were studied further.

The growth-supporting activities of glucose, glucosamine or galactosamine were then investigated. Growth on each of these substrates was followed turbidimetrically during 36 hr. To decrease the lag-time for *Lactobacillus casei*, it was grown in a galactosamine-containing medium for several transfers before the test. For both lactobacilli, growth with glucose or glucosamine was superior to growth with galactosamine, at least up to 30 μmole substrate/ml.

Since results such as these raised the question of whether growth on galactosamine was due to mutation or adaptation, a series of plating experiments were designed in which organisms from one glucose supplemented tube were plated on each of three groups of ten agar plates supplemented with 30 μM/ml. of glucose, galactose or galactosamine. In the case of *Lactobacillus casei*, analysis of the data by Fisher's 't' test showed no significant differences between any of the substrates, indicating that the processes were adaptive. For *L. bulgaricus*, however, while glucose and galactose showed no significant differences by the 't' test, on plates containing galactosamine the mean counts were approximately one-half those found in the other two cases.

Some authors (Levene, 1916; Meyer & Rapport 1951) have reported that cartilage is rich in galactosamine. This suggested the use of tracheal hydrolyzates as an economical source for culture media containing this amino sugar. Preparations of hydrochloric acid- or sulphuric acid-hydrolyzed bovine trachea were studied to determine their efficacy as a galactosamine source for the growth of large quantities of organisms. Different conditions of hydrolysis were studied; the results obtained with sulphuric acid-hydrolyzed trachea with *Lactobacillus casei* are shown in Table 1. Hydrolyzates

Table 1. *Growth of* Lactobacillus casei *in media containing bovine trachea hydrolyzate*

The sulphuric acid-hydrolyzates contained 1 % (w/v) yeast extract in a final volume of 6 ml. After 64 hr. incubation, the organisms were centrifuged, washed once, resuspended in water to 6 ml. and the optical density measured. The hydrolyzates contained solids ranging from 0·082 to 0·088 g./ml.

	Strength of sulphuric acid (N) and period of hydrolysis (hr.)			
Hydrolyzate (ml.)	2N; 7	4N; 7	2N; 17	4N; 17
	Optical density			
1	0·39	0·37	0·35	0·33
2	0·64	0·59	0·55	0·50
3	0·77	0·76	0·72	0·62
4	0·94	0·86	0·80	0·73
5	1·00	1·02	0·85	0·83

were supplemented with 1 % (w/v) yeast extract. It is evident that with 2N- and 4N-sulphuric acid, the 7-hr. hydrolyzates were better than the corresponding 17-hr. hydrolyzates. For *L. bulgaricus* the growth response on the four hydrolyzate media was equivalent to that obtained for *L. casei* grown in the medium containing the 17-hr. hydrolyzates. The hydrolyzates from hydrochloric acid-hydrolyzed trachea were considerably inferior to those treated with sulphuric acid. When the different hydrolyzates containing 1 % (w/v) yeast extract were supplemented with various substances, they showed no significant increase in growth over unsupplemented media. The following substances singly, or in mixtures, were used: adenine,

guanine, uracil, xanthine, various B vitamins, sodium acetate, ammonium chloride, potassium phosphate, magnesium chloride, ferrous sulphate, tryptophan, cysteine, Casitone, Casamino acids. Although glucose supplementation of the medium increased the growth of both lactobacilli significantly, organisms grown in this manner lost much of their ability to produce ammonia and lactate in the presence of glucosamine or galactosamine.

Experiment with dried organisms

The activities of dried *Lactobacillus casei* and *L. bulgaricus* previously grown on glucose, galactose, glucosamine or galactosamine were tested by determination of their ability to produce ammonia and lactic acid from glucosamine or galactosamine. Ammonia production was taken as an indication of deamination of these amino sugars and lactate production as an indication of the metabolism of their carbon skeletons. Table 2 shows the results of such a study. It can be seen that while

Table 2. *Ammonia and lactic acid formation from glucosamine and galactosamine by dried organisms grown in medium B*

The reaction mixture contained: 0·05 ml. M-potassium phosphate (pH 7·0); 2 mg. dried organisms; 5 μmole glucosamine or galactosamine in total volume 1 ml. Tubes were incubated at 37° for 90 min. The reaction was stopped by adding 1 ml. 5% (w/v) trichloroacetic acid.

	Amino sugar substrate			
	Glucosamine		Galactosamine	
	Product (μmole/ml.)			
Organism	Ammonia	Lactic acid	Ammonia	Lactic acid
L. bulgaricus, glucose grown	2·58	3·04	0·10	0·00
L. bulgaricus, galactose grown	2·00	3·28	2·00	3·82
L. bulgarius, glucosamine grown	4·10	8·10	0·20	0·00
L. bulgaricus, galactosamine grown	4·10	7·30	5·49	10·60
L. casei, glucose grown	2·50	2·82	0·25	0·00
L. casei, galactose grown	2·15	3·36	3·85	7·70
L. casei, glucosamine grown	4·98	9·08	0·55	0·00
L. casei, galactosamine grown	1·54	1·10	5·54	9·28

glucose- and glucosamine-grown *L. bulgaricus* and *L. casei* had little activity toward galactosamine, they were active toward glucosamine. Galactose and galactosamine-grown *L. bulgaricus* and *L. casei* showed activity for both substrates, although *L. casei* metabolized glucosamine much less actively. With dried organisms the addition of adenosinetriphosphate to any of these assay systems did not increase ammonia or lactic acid production. When dried *L. casei* or *L. bulgaricus* grown in trachea hydrolysates were tested with glucosamine or galactosamine results like those shown in Table 3 were obtained. Supplementation of the media with glucose markedly inhibited the ability of dried preparations to metabolize galactosamine.

DISCUSSION

Glucosamine metabolism has been extensively studied and several modes for its microbial breakdown have been reported (Roseman, 1959). On the other hand, galactosamine, aside from its phosphorylation (Cardini & Leloir, 1953; Davidson,

Table 3. *Ammonia and lactic acid formation from glucosamine or galactosamine by dried organisms grown in bovine trachea hydrolyzates supplemented with glucose at different concentrations*

Assay conditions as in Table 2.

Organism	Glucose (%, w/v)	Amino sugar substrate: Glucosamine Product (μmole/ml.) Ammonia	Glucosamine Product (μmole/ml.) Lactic acid	Galactosamine Product (μmole/ml.) Ammonia	Galactosamine Product (μmole/ml.) Lactic acid
L. bulgaricus	0·0	3·38	6·72	2·67	4·26
L. bulgaricus	0·2	4·12	7·08	1·09	1·44
L. bulgaricus	1·0	4·12	7·88	0·40	0·00
L. casei	0·0	4·22	8·46	3·05	4·70
L. casei	0·2	2·11	3·90	0·35	0·00
L. casei	1·0	2·22	3·31	0·50	0·15

1960), has been studied mostly in terms of its uridine derivatives and their subsequent conversion to glucosamine (Glaser, 1959). In the present work we were concerned with the cultural conditions for obtaining a bacterial system that would metabolize D-galactosamine. The *Lactobacillus casei* and *L. bulgaricus* strains examined have such a system, which appears to be inducible. Although preparations of dried organisms made in various ways metabolized glucosamine, only galactose or galactosamine grown-organisms attacked galactosamine. Further investigations with cell-free systems are now in progress in order to ascertain the pathway of galactosamine metabolism.

One of the authors, T. Shiota, was a Research Associate, American Dental Association at the National Institutes of Health.

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Reversible Bicarbonate-Induced Enzyme Activity and the Point of no Return during Morphogenesis in Blastocladiella

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SUMMARY

With synchronous single-generation cultures of the non-filamentous water fungus *Blastocladiella emersonii* the activities of isocitric dehydrogenase, α-ketoglutaric dehydrogenase, and cytochrome oxidase were studied during morphogenesis. The total isocitric dehydrogenase activity per plant increased rapidly following germination of the spore and reached its peak at the point of no return in ontogeny; this increase was about seven times greater than the increase in total α-ketoglutaric dehydrogenase activity. During growth from the spore stage to the stage of irreversibility, the specific activity of isocitric dehydrogenase increased 350% while that of α-ketoglutaric dehydrogenase decreased 50%. By removing the external bicarbonate before the point of no return was reached, and thus reversing the morphogenetic pathway, the specific activity of isocitric dehydrogenase, which was in the process of increasing, decreased immediately, while the α-ketoglutaric dehydrogenase activity, which had decreased sharply, increased quickly once again. Similarly, the total activity of isocitric dehydrogenase per plant decreased precipitously while, without reversal, it continued to rise. Conversely, reversal of the morphogenetic path caused an immediate rise in the total α-ketoglutaric dehydrogenase activity per plant while, in the absence of reversal, enzyme activity remained on a level plateau. Thus, removal of bicarbonate induced a new morphogenetic path and, simultaneously, a 250% increase in the activity of one key enzyme and a 35% decrease in the activity of a second; on the other hand, the total protein and total activity of two other 'control' enzymes (glucose-6-phosphate dehydrogenase and cytochrome oxidase) remained essentially constant. In contrast to the above, removal of bicarbonate immediately after the point of no return in morphogenesis had no significant effect on these same enzymes. The data provide substantial evidence that a reversible bicarbonate-induced enzyme synthesis is involved in morphogenesis; i.e. in the mechanism of the bicarbonate-induced formation of resistant-sporangial plants in Blastocladiella.

INTRODUCTION

Of the two major morphogenetic pathways that can be taken by a spore of *Blastocladiella emersonii*, the one which leads to a two-celled thick-walled brown-

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pitted resistant sporangial (RS) plant is induced by bicarbonate. In the absence of bicarbonate, a thin-walled papillate ordinary-colourless (OC) plant is formed. Up to a certain point of no return in ontogeny, the young developing RS plant can be made to revert to an OC plant by removing the bicarbonate from its immediate environment. Similarly, before a critical stage in ontogeny, a young OC plant can be induced to form an RS plant by providing it with bicarbonate. But beyond this point of no return, these two diverse morphological pathways become fixed and irreversible. Previous studies have led to the belief that bicarbonate exerts its effect by interfering with decarboxylation sites in the tricarboxylic acid cycle and that, while some enzyme activities of the cycle are consequently lost, a triphosphopyridine nucleotide (TPN)-specific isocitric dehydrogenase remains active and mediates CO_2 fixation via reductive carboxylation of α-ketoglutarate. This bicarbonate trigger mechanism has been discussed in recent reviews (Cantino & Turian, 1959; Cantino, 1960, 1961).

The purpose of the work to be described here was threefold. (i) To look for precise and direct evidence, by using single-generation synchronous-culture techniques that (*a*) the α-ketoglutaric dehydrogenase system (which was expected to interfere with the bicarbonate trigger mechanism in that it would tend to compete with isocitric dehydrogenase by removing α-ketoglutarate from the carboxylating site) was either lost or severely diminished before the point of no return was reached during the bicarbonate-induced development of an RS plant, while (*b*) the isocitric dehydrogenase (essential for the reductive carboxylation of α-ketoglutarate) either increased in activity or, at least, remained fully functional. (ii) To look for direct evidence that reversal of the morphogenetic pathway (by removal of bicarbonate before the point of no return) caused a corresponding reversal in the activity of these enzymes, believed to be directly implicated in the bicarbonate trigger mechanism, but not in other enzymes more distantly removed from the locus of the bicarbonate effect. (iii) To look for direct evidence that, conversely, removal of bicarbonate after the point of no return in development did not induce significant changes in these same enzymic activities.

METHODS

Procedures for maintenance of stock cultures, and for the growth, sampling and harvesting of large-scale synchronous cultures of *Blastocladiella emersonii* were described previously (Lovett & Cantino, 1960*a*, *b*). Cultures were grown with aeration in a water bath at $24° \pm 0{\cdot}02°$. Zoospores were harvested and washed for enzyme studies according to McCurdy & Cantino (1960).

With the exception of zoospores, plants were homogenized in a Serval Omnimixer using 1·0 g. wet weight/10 ml. phosphate buffer (0·05 M; pH 6·8) and 10 g. washed glass beads (200 μ diam., Minnesota Mining and Manufacturing Co.). For zoospores we used 0·2 ml. packed organism (equiv. 25·0 mg. dry wt.)/10 ml. buffer + 10 g. beads. Dry weights were obtained for all samples in order to calculate the per plant data (Lovett & Cantino, 1960*b*). Homogenates were centrifuged for 5 min. at 14,500 *g* and the supernatant fluids used as the enzyme source. The entire procedure was carried out between 0 and 5°. Soluble protein in these preparations was determined by the turbidimetric method of Stadtman Novelli & Lipmann (1951), with slight modification.

In earlier studies (Cantino & Hyatt, 1953), the diphosphopyrimidine nucleotide (DPN)-dependent α-ketoglutaric dehydrogenase in Blastocladiella was assayed spectrophotometrically by following the reduction of nucleotide at 340 mμ or reduction of cytochrome *c* (via carrier nucleotide) at 550 mμ with a cyanide block for the terminal oxidase. In the present experiments, we followed the disappearance of α-ketoglutarate directly by chromatographic isolation and analysis of its 2, 4-dinitrophenylhydrazone (modified from Cavallini & Frontali, 1953; Cavallini & Mondovi, 1957). The specific activity of α-ketoglutaric dehydrogenase was defined as follows: μmole α-ketoglutarate disappearing/30 min./mg. protein in a standard assay containing *c.* 2–8 mg. protein, 100 μmole phosphate buffer (pH 6·8), 0·15 μmole coenzyme A, 0·25 μmole DPN, 50 μmole potassium α-ketoglutarate, in a final volume of 3·0 ml., at 30°. The reaction was stopped by acidification in a boiling water bath.

The activities of isocitric dehydrogenase, cytochrome oxidase, and the glucose-6-phosphate/16-phosphogluconic dehydrogenases (the latter two being equally active in OC plants (Cantino & Horenstein, 1959), and here assayed together as a unit, for simplicity and referred to as glucose-6-phosphate dehydrogenase) were determined spectrophotometrically under standardized assay conditions. In each case the specific activity has been expressed as units/mg. protein. For isocitric dehydrogenase and cytochrome oxidase, one unit was defined as an optical density change of 0·001/min. in the standard assay. For glucose-6-phosphate dehydrogenase, one unit was defined as an optical density change of 0·1/min. in the standard assay. All rates were determined as the average optical density change/min. (corrected for endogenous controls) for the first 3 min. following the addition of enzyme.

The assay for isocitric dehydrogenase contained *c.* 0·45 mg. protein, 100 μmole phosphate buffer (pH 6·8), 0·5 μmole TPN, 0·3 μmole KCN, 10 μmole $MgCl_2$, with and without 20 μmole sodium isocitrate, in a final volume of 3·0 ml., and the optical density change followed at 340 mμ. The assay for cytochrome oxidase contained *c.* 0·7 mg. protein, 100 μmole phosphate buffer (pH 6·8), 0·068 μmole cytochrome *c* (reduced with $Na_2S_2O_3$ followed by aeration to remove SO_2), with and without 0·3 μmole KCN, in a final volume of 3·0 ml., and the optical density change followed at 550 mμ. The glucose-6-phosphate dehydrogenase assay contained *c.* 0·06 mg. protein, 50 μmole phosphate buffer (pH 7·0), 10 μmole $MgCl_2$, 0·5 μmole TPN, with and without 30 μmole glucose-6-phosphate, in a final volume of 3·0 ml., and the optical density change followed at 340 mμ.

The values per plant were calculated from the known dry weights used in the experiments and the dry weight of individual plants at various stages in ontogeny as previously described (Lovett & Cantino, 1960*b*).

RESULTS

The activity of isocitric dehydrogenase, α-ketoglutaric dehydrogenase, and cytochrome oxidase during the growth of an RS plant

The total activity per plant of the isocitric dehydrogenase increased quickly during the growth of an RS plant up to its point of no return and reached a value almost 7000 times that of the spore, while net synthesis of the α-ketoglutaric dehydrogenase activity increased much less rapidly and reached a final value which was only about one-tenth of this amount (Fig. 1). During this same developmental

period, the specific activity of the isocitric dehydrogenase increased *c.* 350 % while, on the other hand, the specific activity of α-ketoglutaric dehydrogenase increased slightly and then actually decreased to about 50 % of its original value in the spore (Fig. 2). The activity (not plotted) of the terminal cytochrome oxidase of *Blastocladiella emersonii* followed a course essentially identical with that of α-ketoglutaric dehydrogenase. The final activity of both α-ketoglutaric dehydrogenase and cytochrome oxidase in fully mature RS plants is essentially zero (Cantino & Horenstein, 1955).

Changes in the reversibility of enzymic activity before and after the point of no return during morphogenesis

Plants of *Blastocladiella emersonii* were grown up to a stage (32 hr. at 24°) just preceding the point of no return, removed from the medium, freed from bicarbonate

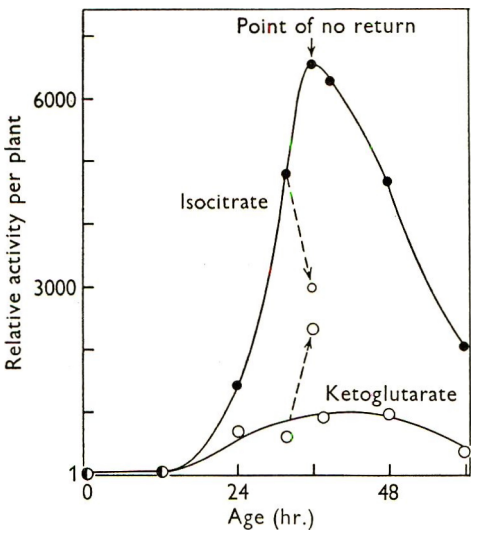

Fig. 1

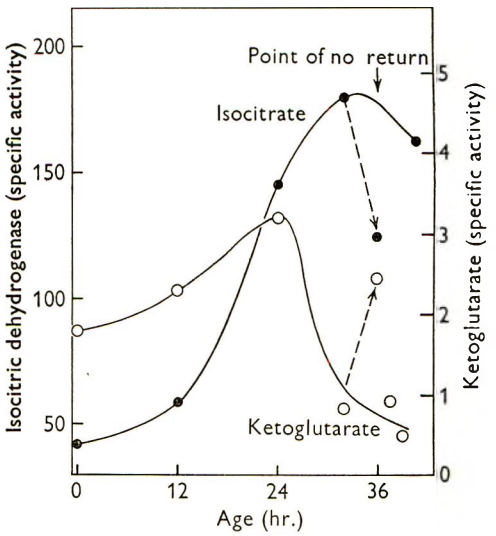

Fig. 2

Fig. 1. The total activities of isocitric dehydrogenase and α-ketoglutaric dehydrogenase per RS plant at different stages of development. The total activities per plant at the spore stage (α-ketoglutaric dehydrogenase, $1{\cdot}934 \times 10^{-7}$ unit; isocitric dehydrogenase, $4{\cdot}52 \times 10^{-6}$ unit) were assigned values of 1·0, and all other total activities at different stages of development were related to the spore level (see Methods for details). The dotted lines represent the changes in activity induced by the removal of bicarbonate.

Fig. 2. The specific activities of isocitric dehydrogenase and α-ketoglutaric dehydrogenase at different stages of development of an RS plant (see Methods for details). The dotted lines represent the changes induced by the removal of bicarbonate.

by washing, and then resuspended and agitated in water for 4 hr. at 24°. Under these conditions, such plants reverted to the alternative morphogenetic pathway and, by 36 hr., were ready to form papillae; i.e. they were fully mature thin-walled OC plants. Assays for enzymic activity in 32 hr. RS plants and the 36 hr. OC and RS plants derived therefrom (i.e. reversed and non-reversed, respectively) revealed that the morphological shift to an OC plant was associated with a pronounced and rapid enzymic shift; the specific activity of the α-ketoglutaric dehydrogenase,

which reached a low value at 32 hr., rose quickly once again; the specific activity of the isocitric dehydrogenase, which was in the process of ascending rapidly toward its peak, dropped sharply (Fig. 2). Even more pertinent from an interpretative point of view (see Discussion) were the per plant data, which revealed similarly striking reversals in total enzymic activity (Fig. 1). Under these identical conditions, however, the cytochrome oxidase and the glucose-6-phosphate dehydrogenase activities did not change significantly (Table 1). The significance of these observations is emphasized by analyses of RS plants subjected to the same treatment (i.e. removal of bicarbonate) after the point of no return in morphogenesis had been reached. At this later stage in ontogeny none of these enzymic activities changed appreciably following incubation for 4 hr. in the absence of bicarbonate.

Table 1. *Enzymic activities before and after a four-hour incubation of young RS plants of* Blastocladiella emersonii *in water*

	Cytochrome oxidase	Glucose-6-phosphate dehydrogenase
	Specific activity	
32 hr. RS plants before incubation	16·9	6·9
32 hr. RS plants after incubation	16·5	8·1

See Methods for details.

DISCUSSION

The bicarbonate trigger mechanism for morphogenesis, as originally visualized from the results of experiments with multiple-generation cultures of Blastocladiella involves the gradual cessation of a weakly functional tricarboxylic acid cycle and, concomitantly, increased carboxylation of α-ketoglutarate via a TPN-specific isocitric dehydrogenase as the developing plant becomes committed to the formation of a resistant sporangial (RS) plant (Cantino, 1961). More recently, data derived from experiments with synchronous, single-generation cultures have corroborated and amplified these notions (Cantino & Lovett, 1960; Lovett & Cantino, 1960*a*,*b*; McCurdy & Cantino, 1960). In this report we have now provided more precise and direct evidence that the isocitric dehydrogenase activity required for carboxylation does indeed, increase quickly and many fold, while the enzyme system which would compete with it for α-ketoglutarate by oxidatively decarboxylating this substrate (α-ketoglutaric dehydrogenase and an associated terminal cytochrome oxidase) decreases sharply. It is particularly noteworthy that during the development of an RS plant, bicarbonate also induces, simultaneously, an immediate exponential synthesis of isocitritase, a crucial enzyme required for removal of the isocitrate following its formation from α-ketoglutarate via isocitric dehydrogenase. This does not occur in the absence of bicarbonate during the early stages of development in an ordinary colourless (OC) plant (McCurdy & Cantino, 1960; Cantino, 1961).

An appreciable accumulation of evidence (Cantino & Turian, 1959; Cantino, 1960, 1961) suggests that these bicarbonate-induced changes in enzyme activity are intimately related in cause and effect fashion to the differentiation of an RS plant. On the basis of previous arguments (Cantino, 1961) and the data here reported, we are now strongly tempted to conclude that these alterations in measurable

enzymic activity actually reflect bicarbonate-induced reversible changes in enzyme synthesis and enzyme degradation.

Before the point of no return in ontogeny, the bicarbonate-triggered morphogenetic mechanism leading to an RS plant remains plastic and reversible; the activities of two of the three key enzymes (α-ketoglutarate dehydrogenase, isocitric dehydrogenase) presumed to be most directly involved in this mechanism, seem to be equally plastic and reversible, responding quickly to the addition or removal of bicarbonate; the third, isocitritase, has not yet been tested for reversibility. Beyond the point of no return, when morphogenesis becomes irreversible and continues to completion, the same two enzyme systems lose their plasticity. These observations seem particularly significant in the light of the obvious lack of pliability in the activities of other enzyme systems, namely the TPN-specific glucose-6-phosphate dehydrogenase and the terminal cytochrome oxidase, neither of which is directly involved in the bicarbonate trigger mechanism, nor is altered by the removal of bicarbonate, either before or after the irreversible point in morphogenesis.

The per plant data, however, provide the greatest support for our argument. While the morphogenetic shift is associated with a several-fold increase in the total α-ketoglutaric dehydrogenase activity per plant and a sharp decrease in the total isocitric dehydrogenase activity per plant, neither the total cytochrome oxidase per plant, the total glucose-6-phosphate dehydrogenase activity per plant, nor the total protein per plant changes significantly. Thus the changes in enzyme activity cannot be artifacts resulting from an alteration of the total protein content of the fungus which would result in an apparent unidirectional change in specific activity. While it is always possible that some kind of ill-defined 'masking effect' has been induced by the removal of bicarbonate, this alternative explanation seems improbable.

There is little if any published evidence to show that induced enzymic synthesis is related in a cause-and-effect fashion to morphogenesis (Markert, 1958). In the light of these and earlier (Cantino, 1961) experiments, it seems very likely that, in *Blastocladiella emersonii*, bicarbonate does induce both synthesis and degradation of adaptive enzymes whose activities, in turn help to initiate and direct morphological differentiation.

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The Endogenous Metabolism of *Euglena gracilis*

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SUMMARY

The endogenous respiratory rate of non-photosynthetic *Euglena gracilis* var. *bacillaris* is remarkably constant under varied conditions of growth and incubation. After 60–90 min. of slightly more rapid respiration, the endogenous oxygen uptake averages about 6·5 μl. O_2/million cells/ hr., and this rate can be maintained for at least 26 hr. The respiratory quotient of the endogenous metabolism is 1·0. Fluoroacetate inhibits endogenous oxygen consumption about 70 %. A variety of evidence suggests that the polysaccharide paramylum is the major endogenous substrate.

Tracer experiments indicate that the endogenous metabolism continues during the oxidation of exogenous substrates, but that the assimilated substrate mixes with, and dilutes the endogenous reserves. The time-course of this dilution process indicates that the most recently assimilated reserves are the first to be oxidized.

INTRODUCTION

In the absence of exogenous substrate, cells of *Euglena gracilis* var. *bacillaris* (non-photosynthetic strain) have a respiratory rate roughly one-quarter that on a substrate such as acetate or ethanol. Previous manometric experiments indicated that this endogenous respiration continues unabated during the metabolism of exogenous acetate (Wilson & Danforth, 1958). The experiments described here were designed to provide further information concerning this endogenous metabolism and its relation to the metabolism of exogenous substrates.

METHODS

Experiments were done on the same non-photosynthetic strain of *Euglena gracilis* var. *bacillaris* used in previous studies (Danforth, 1953; Danforth & Wilson, 1957; Wilson & Danforth, 1958). Cells were grown at *c.* 20° in a medium composed of (w/v) Bacto-Tryptone (Difco), 0·25 %; with Na acetate $3H_2O$, 0·3 % (w/v) at pH 7·3; or ethanol, 0·3 % (v/v) at pH 3·5 as carbon sources. Several experiments on the rate of endogenous oxygen consumption were performed on cells grown at 24–25° in the chemically defined medium of Cramer & Myers (1952), with acetate, 0·5 % (w/v); or succinate, 0·5 % (w/v) at pH 7·0 or fumarate, 0·5 % (w/v); malate, 0·5 % (w/v); or α-ketoglutarate, 0·5 % (w/v) at pH 5·0 as sole carbon sources. Cells were harvested and washed by centrifugation before experimental use. These

procedures have been described in more detail in the previous publications cited above.

'Partially-labelled' Euglena was prepared by incubating the cells for 10–20 min. in 0·01 M-phosphate buffer (pH 7·0) containing 5·0–6·4 μmole of Na acetate-2-^{14}C. 'Fully-labelled' cells were prepared by inoculating 1 ml. of Euglena culture into 300 ml. growth medium containing acetate-2-^{14}C. Cells were harvested after 8 days of growth in this medium. The labelled cells were dispersed into experimental media in Warburg flasks or 50 ml. Erlenmeyer flasks equipped with centre wells. Each centre well received 0·5 ml. (Warburg) or 0·1 ml. (Erlenmeyer) of 0·1 M-NaOH. Flasks were stoppered and shaken at 120 cyc./min. Experiments were performed at 21–26°. At approximately hourly intervals, the NaOH was withdrawn from the centre wells and the wells rinsed and refilled with the same volume of fresh NaOH. The entire withdrawal, rinsing, and refilling took less than a minute, so loss of CO_2 during this process was probably less than 2 % of the measured values. Samples of the NaOH withdrawn were spread uniformly on planchets with the addition of ethanol and were dried under an infra-red lamp. Radioactivity was counted with a thin-window Geiger tube for a period of time sufficient to give a total count of 1000 or more. Background was counted to the same degree of accuracy, and was subtracted from all counts. No correction was made for self-absorption, since preliminary experiments had indicated that differences in self-absorption between samples were negligible.

In some experiments samples of labelled Euglena were killed in known volumes of alkaline ethanol (0·01 M-NaOH in 95 % (v/v) ethanol), at the beginning and the end of the experiment. Known volumes of these samples were dried on planchets, and counted to determine the radioactivity of the cells.

Under the experimental conditions described above, there was a certain amount of retention of CO_2 in the incubation medium. To determine whether the incomplete recovery of CO_2 caused any serious error in the results, a number of experiments were performed by a method designed to eliminate the effect of CO_2 retention. In these experiments, samples were removed from the incubation flasks at intervals and centrifuged. The cells were washed and resuspended in fresh medium, and these new suspensions transferred to Warburg flasks whose centre wells contained 0·4 ml. of 0·1 M-NaOH and whose sidearms contained 0·5 ml. of 0·1 M-H_2SO_4. After incubation for the desired period, the sidearms were tipped, killing the Euglena and acidifying the medium. The flasks were shaken for another hour to complete the diffusion of CO_2 to the centre well, and the centre well contents were sampled and counted as before. The experiments shown in Figs. 3 and 6 were done by this method. In every case the results of such experiments were similar to those using the simpler method described above.

Respiration was measured by standard Warburg techniques, at 26°, in a gas phase of air. The respiratory quotient was determined by the acid end-point method described in a previous report (Wilson & Danforth, 1958). Respiration of cells grown in Cramer-Myers medium was measured in the same medium minus carbon source. Tris (2-amino-2'' hydroxymethylpropane – 1:3-diol) buffer + phosphate (tris + phosphate) or phosphate alone were used as buffers for other respiratory experiments.

To determine the volume of Euglena cells, measured portions of a heavy suspen-

sion of Euglena were centrifuged in Hopkins vaccine tubes until the cells were packed in the narrow graduated portions of the tubes. Centrifugation was continued until a pellet of constant volume was achieved. Because the plasticity of the Euglena pellicle permits tight packing, the volume of extracellular water in such pellets was probably small. The number of cells was determined by haemocytometer counts on samples of the same suspension.

Na acetate-2-^{14}C was obtained from the Volk Radiochemical Company, Chicago, Illinois; Na fluoroacetate (Compound 1080, 10% inert material) was the gift of Mr Tull C. Allen, Tull Chemical Company, Inc., Oxford, Alabama, U.S.A.

RESULTS

General characteristics of the endogenous metabolism. As was noted by Von Dach (1942) in the closely related Astasia, Euglena cells often have a slightly higher rate of endogenous metabolism during the first hour or two after harvesting than at later periods. When cells grown in Cramer–Myers medium with acetate, succinate, fumarate, malate, or α-ketoglutarate were incubated at pH 5·0, 5·5 or 7·0, eighteen experiments yielded an average initial endogenous respiration rate of $8{\cdot}2 \pm 1{\cdot}2$ (av. dev.) μl. O_2/million cells/hr. After this initial period of rapid respiration, the rate decreases to a constant level, $6{\cdot}5 \pm 1{\cdot}0$ μl. O_2/million cells/hr. in the same series of experiments. The same change has been noted in cells grown on Tryptone media with acetate or ethanol as growth substrates, and incubated in tris-phosphate buffer. The endogenous respiratory rate was independent of the growth substrate, the presence or absence of a nitrogen source in the incubation medium, the pH of incubation, and the other differences in incubation media described under 'Methods'.

The later, steady rate of endogenous respiration is maintained for long periods of time. In the longest experiment performed, the rate of endogenous metabolism during the 24th to 26th hour was 93% of that during the 3rd and 4th hour. Assuming that the rate of O_2 consumption in this experiment equalled the average rate of 6·5 μl./million cells/hr. (unfortunately the cell count sample in this experiment was lost), the total oxygen consumption during the entire experiment was 7·3 μmole/million cells, equivalent to the complete oxidation of 3·6 μmole of acetate or 1·2 μmole hexose/million cells. Since the respiratory rate was still appreciable at the end of the experiment, it is clear that these are minimum estimates of the endogenous reserves of Euglena cells.

Effect of fluoroacetate on endogenous metabolism. It would be of some interest to determine the pathways and intermediates involved in the endogenous metabolism of Euglena, particularly in view of the metabolic scheme presented in the discussion, which assumes a minimum of interaction between the endogenous metabolism and that of exogenous substrates. Some few data bearing on this problem have been obtained.

One source of such data are experiments involving the inhibitor, fluoroacetate. In higher animals, this substance has been shown to be incorporated, by way of the 'condensing enzyme', into fluorocitrate, which inhibits the enzyme aconitase and thus blocks citrate metabolism (Peters, 1952, 1957). Danforth (1952) found that fluoroacetate inhibited acetate oxidation by Euglena, but was unable to demonstrate the expected accumulation of citrate. Holz (1954), however, was able to show

accumulation of large amounts of citrate in fluoroacetate-poisoned Euglena of the same strain. Thus, it seems that the mechanism of fluoroacetate inhibition in Euglena is the same as that in the tissues of higher animals.

Figure 1 shows that 0·001 M-fluoroacetate inhibited endogenous oxygen consumption of Euglena by about 68 %. Further increases in concentration did not result in appreciably greater inhibition. It seems likely, therefore, that the endogenous metabolism occurs, in large part at least, through pathways in which citrate is an intermediate.

The respiratory quotient of the endogenous metabolism. Determination of the respiratory quotient of the endogenous metabolism also yielded some information concerning the nature of the substrates involved, though such data cannot provide positive identification of the substrates. Six experiments, using cells grown on acetate + Tryptone at pH 7·3 and ethanol + Tryptone at pH 3·5, resulted in an average respiratory quotient of $1{\cdot}05 \pm 0{\cdot}19$. The respiratory quotient of the endogenous, like its rate, was independent of the pH value and substrate of growth, and of the composition and pH value of the experimental media.

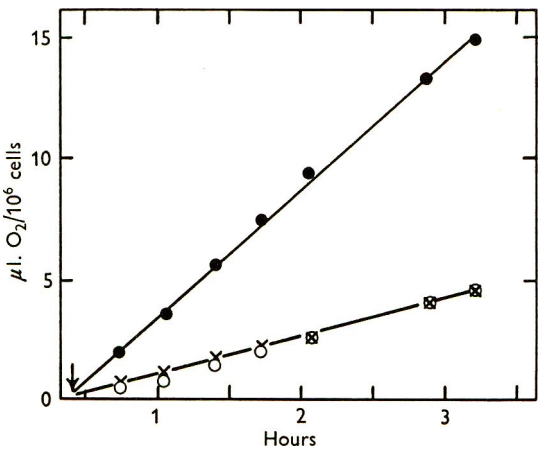

Fig. 1. Effect of fluoroacetate on the endogenous oxygen consumption by Euglena. Warburg flasks contained 17·4 million Euglenas in 3·0 ml. 0·01 M-phosphate buffer (pH 7·0). Temperature was 26°. Time is measured from the time of addition of fluoroacetate; the arrow indicates the time manometer readings were begun. ●, No fluoroacetate; ×, 0·001 M-fluoroacetate: ○, 0·04 M-fluoroacetate.

The respiratory quotient of approximately 1·0 suggests that the endogenous store is carbohydrate in nature.

Interaction of endogenous and exogenous metabolism. Wilson & Danforth (1958) concluded, on the basis of respirometric data, that the endogenous metabolism of Euglena continues unchanged during the oxidation of exogenous acetate. This conclusion was based on indirect evidence; it was assumed that the ratio of acetate oxidized to acetate assimilated in short-term experiments should be independent of the total amount of acetate utilized. It was found that the data fitted this assumption only when corrected for a continuing endogenous metabolism. In the case of ethanol, this criterion did not permit a decision as to whether or not the endogenous metabolism continued.

Blumenthal, Koffler & Heath (1957) showed that the manometric method described above and measurements based on release of radioactive carbon dioxide from ^{14}C-labelled cells may lead to apparently contradictory conclusions concerning the effect of exogenous substrates on endogenous metabolism. It seemed desirable, therefore, to compare results obtained by the tracer method with our earlier respirometric studies. Figures 2 and 3 show the results of two such experiments, in which Euglena which had assimilated small amounts of radioactive acetate were then incubated in the presence and in the absence of exogenous substrate.

In the absence of substrate, the rate of $^{14}CO_2$ production decreases rapidly with time; the decrease follows an approximately logarithmic time-course. This decrease cannot be attributed solely to the decreasing radioactivity of the cells, since at the

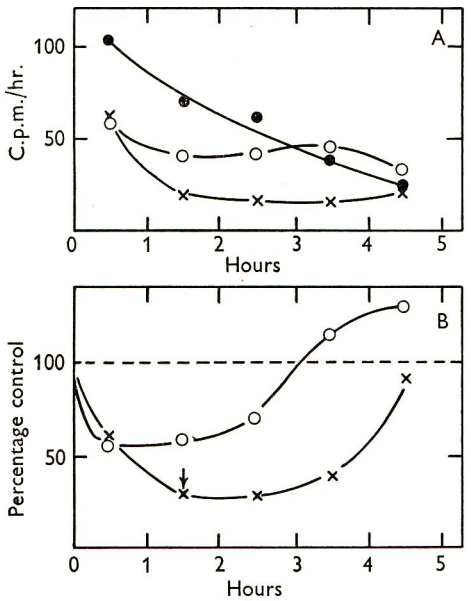

Fig. 2

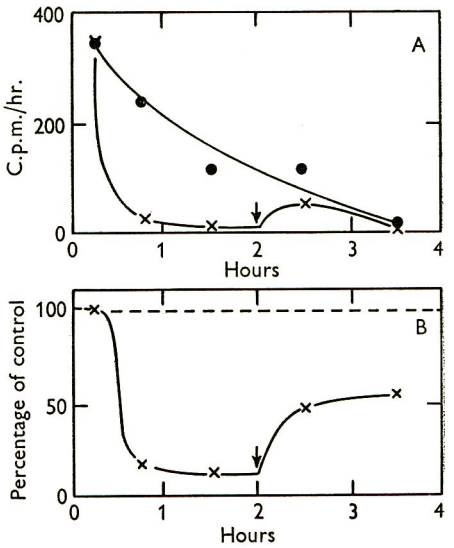

Fig. 3

Fig. 2. Effect of nonradioactive substrate on release of $^{14}CO_2$ by 'partially labelled' Euglena. CO_2 trapped by first method (see 'Methods'). Incubation mixtures contained per flask, 15·3 million Euglenas, 1450 c.p.m., in 1·0 ml. 0·03 M-phosphate buffer (pH 7·0). Temperature was 25–27°. A, Rates of $^{14}CO_2$ release. The average rate of release is plotted in the mid-point of the measuring interval. B, Rates of $^{14}CO_2$ release as % rate in the absence of substrate. ●, No substrate; ×, 20 μmole acetate; ○, 20 μmole ethanol. Arrow indicates the most probable time of acetate exhaustion.

Fig. 3. Effect of non-radioactive acetate on release of $^{14}CO_2$ by 'partially labelled' Euglena. CO_2 trapped by second method. Incubation mixtures contained per flask, 2·0 million Euglenas in 1·5 ml. 0·008 M-phosphate buffer, pH 7·0. Temperature was 26°. A and B, Data plotted as in Figs. 2A and 2B, respectively. Arrow indicates time of removal from acetate. ●, No substrate; ×, 20 μmole acetate/ml.

end of the experiment shown in Fig. 2, the cells retained more than 90 % of their original radioactivity, while the rate of release of the $^{14}CO_2$ had dropped to about 25 % of the initial rate.

It is apparent that the presence of exogenous acetate or ethanol markedly reduces the rate of $^{14}CO_2$ production by labelled cells. The course of this 'inhibition' is

clearest when the data are plotted as in Figs. 2B and 3B, expressing the rates of $^{14}CO_2$ production in the presence of substrate as percentage of the endogenous rates over the same time intervals. The inhibition develops gradually after addition of the substrate; in the experiment shown in Fig. 3, there was no inhibition during the first half hour after addition of acetate. With time, the rate of $^{14}CO_2$ release recovers toward or beyond the endogenous rate. It seemed probable that the recovery began at the time when the exogenous substrate was exhausted. In the experiment shown in Fig. 2, the probable time of exhaustion of acetate was estimated from the amount of acetate added and the average rate of acetate utilization by Euglena cells in other experiments performed under the same conditions. The vertical arrow in the figure indicates the most probable time of acetate exhaustion. In the experiment shown in Fig. 3, the known time of transfer from acetate-containing to endogenous medium is shown by a similar arrow.

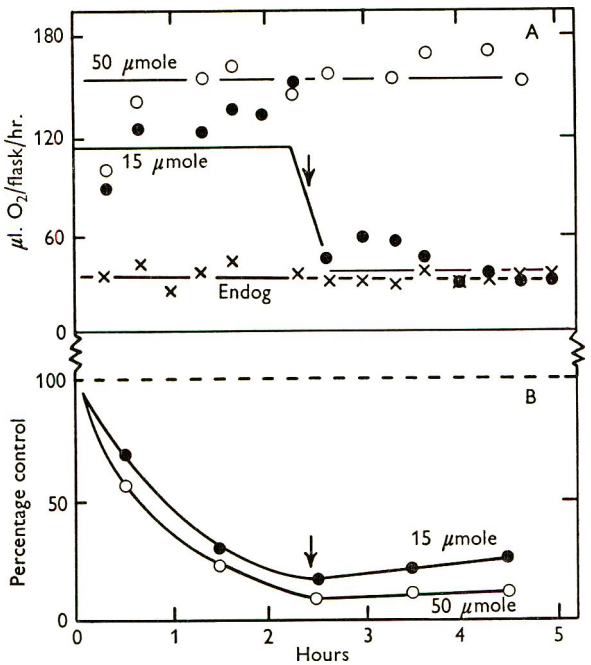

Fig. 4. Effect of nonradioactive acetate on respiration and rate of release of $^{14}CO_2$ by 'partially labelled' Euglenas. CO_2 trapped by first method. Mixtures contained per flask, 6·0 million Euglenas, 4750 c.p.m., in 1·0 ml. 0·008 M-phosphate buffer, pH 7·0. Temperature was 24°. A, Rates of oxygen consumption. B, Rates of $^{14}CO_2$ release as % of rate with no substrate. Figures beside the curves indicate the amounts of acetate added.

It is clear that the decreased rate of $^{14}CO_2$ production persists for as long as several hours after depletion of exogenous substrate. This fact is apparent in Fig. 4, which records an experiment in which rates of oxygen consumption and $^{14}CO_2$ production were measured simultaneously in duplicate flasks. Since the respiratory quotient of both endogenous and acetate (Wilson & Danforth, 1958) is 1·0, the total (labelled plus unlabelled) CO_2 production may be taken as equal to the oxygen consumption. Two initial values of acetate, 15 and 50 μmole, were used. The former was exhausted during the course of the experiment, while the latter was not. As before, the inhibi-

tion of radioactive CO_2 production developed gradually and persisted for several hours after the sharp drop in respiratory rate to the endogenous value, which indicates exhaustion of the acetate.

Blumenthal *et al.* (1957) also showed that the apparent effect of exogenous substrates on endogenous metabolism might vary, depending on whether the cells were labelled by brief exposure to radioactive substrate or were grown from a small inoculum entirely on uniformly labelled substrate. In the first case, various endogenous substrates may be labelled to different degrees, while in the second all cellular constituents are uniformly radioactive. For this reason, experiments were performed on Euglena grown through several-hundredfold multiplication on radioactive acetate. Such cells were not strictly 'uniformly-labelled', since the acetate

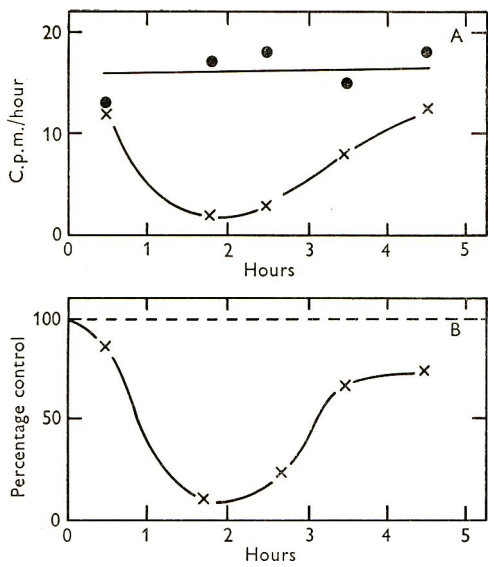

Fig. 5

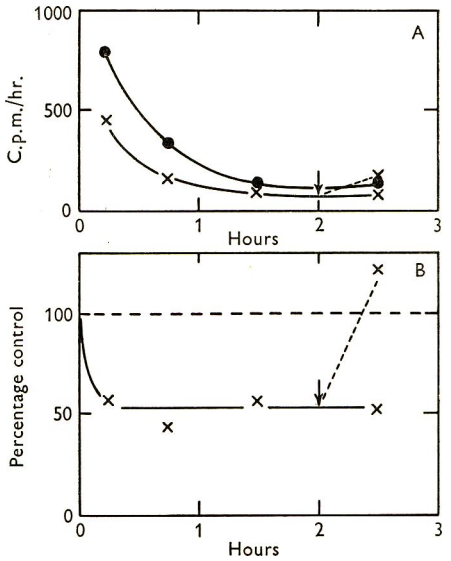

Fig. 6

Fig. 5. Effect of non-radioactive acetate on release of $^{14}CO_2$ by 'fully labelled' Euglena. CO_2 trapped by first method. Incubation mixtures contained per flask, 6·8 million Euglenas, 4380 c.p.m., in 1·0 ml. 0·008 M-phosphate buffer, pH 7·0. Temperature was 21°. A and B, Data plotted as in Fig. 2A and 2B respectively. ●, No substrate; ×, 10 μmole acetate.

Fig. 6. Effect of non-radioactive acetate on release of $^{14}CO_2$ by 'partially labelled' Et-grown Euglena. CO_2 trapped by second method. Incubation mixtures contained per flask, 18·5 million Euglenas in 1·5 ml. 0·008 M-phosphate buffer (pH 7·0). Temperature was 26°. At the time indicated by the arrow, one sample of cells was transferred from acetate medium to endogenous medium (dotted curve). A and B, Data plotted as in Fig. 2A and 2B, respectively. ●, No substrate; ×, 20 μmole acetate/ml.

used for growth was labelled only in the methyl carbon, and since the nonradioactive Bacto-Tryptone in the medium probably supplied some carbon to the cells. But such cells certainly more closely approach uniformity of labelling than do those used in the preceding experiments.

Figure 5 shows the results of an experiment with such 'fully-labelled' cells. A comparison with Figs. 2 and 5 shows a number of interesting differences. Although the initial radioactivity of the 'fully-labelled' cells was about twice that of the

partially labelled, the rate of release of $^{14}CO_2$ was much lower in the fully labelled. This would be expected if newly assimilated acetate is converted mainly into the cellular constituents most readily available as endogenous substrates. This explanation is supported by the fact that the rate of production of radioactive CO_2 by partially labelled cells decreases rapidly with time, while that of the fully labelled cells remains constant over the same period of time.

The effect of exogenous acetate on the release of radioactive CO_2 is, however, the same in fully-labelled cells as in partially labelled. There is the same gradual decrease in rate to about 10–30% of the endogenous rate, and the same slow recovery following disappearance of the exogenous acetate.

All of the preceding experiments were done on Euglena which had been grown on acetate at pH 7·3, the 'Ac-grown' cells described in previous publications (Danforth & Wilson, 1957; Wilson & Danforth, 1958). Cells grown on ethanol at pH 3·5 ('Et-grown') have much lower rates of acetate metabolism. We were interested, therefore, in comparing the effect of acetate on endogenous metabolism in Et-grown cells with that in Ac-grown cells. Figure 6 shows the results of an experiment similar to that of Fig. 3, but performed with Et-grown Euglena. Two differences are apparent; with Et-grown cells the inhibition develops more rapidly, but the final level of inhibition is less than with Ac-grown cells.

The volume of Euglena cells. The volume of Euglena cells was estimated from a series of twenty-three determinations on four different cell suspensions. The averages for the four suspensions ranged from 1·15 to 1·80 μl./million cells, with a grand average of 1·58 μl. $\pm$ 0·12 μl./million cells. This volume is about half that found by Neff (1960) for a green strain of *Euglena gracilis* var. *bacillaris*. Strain differences or differences in culture methods may account for this discrepancy.

DISCUSSION

Effect of substrates on the endogenous metabolism. The utilization of exogenous acetate or ethanol greatly depresses the rate of release of radioactive CO_2 by Euglena previously labelled with radioactive carbon. At first glance, these results seem to indicate that, contrary to our earlier conclusions, the presence of exogenous substrate depresses the rate of endogenous CO_2 production. Two facts, however, suggest that the situation is not quite so simple. First, the rate of release of radioactive CO_2 remains depressed for more than an hour after the substrate has been exhausted. But numerous manometric experiments have shown that, after acetate exhaustion, the respiratory rate returns promptly to the endogenous level, as illustrated in Fig. 4. Secondly, it would be difficult to explain the gradual onset of the inhibition of $^{14}CO_2$ production by any mechanism involving a direct competition between the endogenous and acetate metabolism, since acetate metabolism begins at maximum rate almost immediately upon the addition of acetate. On the other hand both the gradual onset of the inhibition and the persistence of the inhibition after acetate exhaustion can be explained if it is assumed that the rate of total CO_2 production from endogenous sources remains unchanged in the presence of acetate, but that the specific radioactivity of the endogenous reserves is lowered by the assimilation of non-radioactive substrate, thus decreasing the radioactivity of the CO_2 produced from this source.

A metabolic scheme in which such dilution might occur is shown in Fig. 7. In this model, it is assumed that the endogenous reserves are composed of two (or more) components, a 'labile reserve' which is the immediate source of substrate for endogenous metabolism, and a larger pool of 'stable reserve(s)' from which the labile reserve is replenished. It is further assumed that endogenous acetate or ethanol is assimilated directly into the labile reserve, and enters the stable reserve only indirectly. Such a model explains the fact, noted previously, that the most recently-assimilated reserves are most readily available for endogenous metabolism. The decrease with time of the rate of endogenous $^{14}CO_2$ production by partially labelled cells would occur not only because of rapid re-oxidation of the new, radioactive reserves, but also because of the exchange of material between highly radioactive labile reserve and the less active stable reserve.

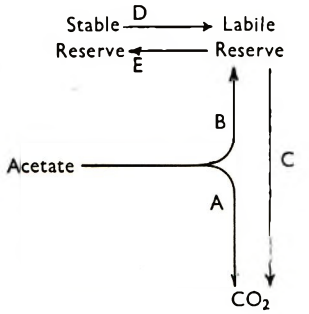

Fig. 7. Hypothetical model of acetate and endogenous metabolism in Euglena.

In this model, then, reaction C is the continuing endogenous metabolism of Wilson & Danforth (1958), reaction A the oxidation of exogenous acetate, and reaction B the assimilation of acetate. When unlabelled acetate is present, the assimilation of non-radioactive carbon directly into the labile reserve would dilute these reserves, lowering the specific activity of the CO_2 produced via reaction C, even though the total rate of CO_2 production from this pathway remained unchanged. The extent of dilution would increase with time, and the dilution would persist for a time after the exhaustion of substrate, in agreement with the experimental findings.

This model also accounts for the fact that 'inhibition' of $^{14}CO_2$ production by acetate is less in Et-grown than in Ac-grown cells, since both reaction A and reaction B (oxidation and assimilation of acetate) are slower in Et-grown cells. The labile reserves would therefore be less diluted by acetate in the case of Et-grown cells. The precise mechanism of the more rapid onset of inhibition in Et-grown cells is not known, but the phenomenon is not inconsistent with the model presented here.

The only essential feature of the model presented is the assumption that the newly-assimilated material 'pools' more rapidly with the immediate substrate of endogenous metabolism than with the remainder of the endogenous reserves. In other respects, the model is probably oversimplified. In particular, it seems doubtful that only two compartments are involved, and that the distinction between labile and stable reserves is a sharp one. A series of many compartments with varying degrees of 'lability' would probably be closer to reality.

Nature of the endogenous reserves. While the present experiments do not permit conclusive identification of the nature of the endogenous reserves, they provide a number of important clues. For example, from the rate and duration of endogenous respiration, it was calculated that the reserves contain at least the equivalent of about 1 μmole of hexose per million cells. From the measured volume of the cells, this would amount to the equivalent of an approximately 0·6 M-hexose solution, a concentration larger than any reasonable estimate of the total osmolar concentration of Euglena cytoplasm. Thus, the bulk of the reserves must be in the form of high molecular weight compounds, or of insoluble materials.

The respiratory quotient of 1·0 suggests that the endogenous reserves are carbohydrate in nature. Paramylum, a polymer of D-glucose, is a likely candidate for the stable reserve substance. It is present in Euglena as granules which are frequently so numerous as to be the most conspicuous cellular inclusions. These granules have been observed to decrease in size and number during prolonged starvation (Gojdics, 1953; Padilla & Buetow, 1959).

Pathway of endogenous metabolism. The effectiveness of fluoroacetate as an inhibitor of endogenous respiration suggests that the endogenous metabolism occurs via the Krebs tricarboxylic acid cycle. Since acetate is also probably oxidized through the Krebs cycle (Danforth, 1953), the apparent lack of competition between acetate metabolism and endogenous metabolism is puzzling. The present experiments provide no information concerning the mechanism which permits the two processes to go on side by side without interference. Approximately 33 % of the endogenous oxygen uptake is insensitive to fluoroacetate inhibition. This is roughly the amount of oxygen which would be required for the partial oxidation of carbohydrate to the level of acetate, the point at which the fluoroacetate block occurs.

Role of the endogenous metabolism. The constancy of the endogenous metabolism in the face of varying conditions, and its persistence during oxidation of exogenous nutrients, suggest that the endogenous pathways play a special role in Euglena metabolism which goes beyond merely providing a reserve of carbon and energy. It is worth noting, in this connexion, that *Euglena gracilis* will grow at maximum rates in the presence of substances which do not stimulate respiration appreciably above the endogenous level (Wilson, Buetow, Jahn & Levedahl, 1959). Thus, the rate of endogenous metabolism is sufficient to supply all the energy needed by growing cells. It is possible, therefore, that the endogenous metabolism is the direct source of energy for growth, movement, osmotic regulation, and other 'life processes', and that exogenous substrates are used only to replenish or increase the supply of endogenous reserves.

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The Fixation of Tetanus Toxin by Ganglioside

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SUMMARY

The fixation of tetanus toxin by brain ganglioside has been confirmed. A method has been developed for assaying the toxin-fixing capacity of ganglioside in the analytical ultracentrifuge. Under appropriate conditions ganglioside will fix many times its own weight of toxin. Ganglioside preparations from brain contain at least three gangliosides; at least two of these differ in their sialic acid contents and toxin-fixing capacities. The sialic acid residues (and their free carboxyl groups) are essential for toxin-fixation. Tetanus toxin does not appear (so far) to bring about any change in the ganglioside molecule.

INTRODUCTION

In a previous paper (Van Heyningen, 1959*b*) the tetanus toxin receptor in nervous tissue was tentatively identified as ganglioside. Brain ganglioside contains fatty acid (stearic acid), sphingosine, hexose (glucose and galactose), hexosamine (*N*-acetylgalactosamine) and sialic acid (*N*-acetylneuraminic acid) residues. The fatty acid, sphingosine and one of the hexose residues comprise a cerebroside moiety (Klenk & Lauenstein, 1953; Bogoch, 1958; Rosenberg & Chargaff, 1958). The presence of hydrophobic and hydrophilic groups can lead to micelle formation in aqueous solution, and the values for a molecular weight of about 250,000 reported by Folch, Arsove & Meath (1951), Bogoch (1958) and Rosenberg & Chargaff (1958) may be a reflexion of such micelle formation. Klenk & Gielen (1960) have recently reported that the ultracentrifugal behaviour of ganglioside in solution in dimethylformamide is consistent with a molecular weight of *c.* 1500. Their preparation of ganglioside appears to be a mixture of two gangliosides, for which they have suggested detailed structures. Each contains one moiety of cerebroside, one hexose residue and one sialic acid residue, and while one contains a hexosamine residue the other has an additional hexose residue instead. There is some uncertainty, however, about the sialic acid and hexosamine contents of brain ganglioside, and the reported values vary (Table 1).

Because of the formation of micelles in aqueous solution, conclusions about the homogeneity of preparations subjected to ultracentrifugation and electrophoresis are of questionable value. In the present work a ganglioside preparation, although electrophoretically and ultracentrifugally homogeneous, was found on investigation by chromatographic methods to contain a number of constituents. Some of these were eliminated, to leave a mixture of at least three gangliosides which were partially separated. In addition, a more refined method of assay of toxin-fixing

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capacity has been evolved. A study has been made of the effect of modifications in ganglioside on its capacity to fix toxin, and the important question of whether ganglioside is changed as a result of its interaction with toxin has been investigated.

Table 1. *Sialic acid and hexosamine contents of ganglioside preparations as variously reported*

Reference	Residues per cerebroside residue: Sialic acid	Hexosamine
Svennerholm (1956*a*, 1957*a*)	1·0	1·0
Klenk & Gielen (1960)	1·0 1·0	1·0 0
Folch, Meath & Bogoch (1956); see also Meltzer (1958)	1·5	0·67
Bogoch (1958)	1·5	1
Rosenberg & Chargaff (1958)	1·5	0·36

METHODS

Toxin. The tetanus toxin preparation, TD 464 D (Van Heyningen, 1959*a*), was kindly supplied by Dr C. G. Pope and Dr R. O. Thompson of the Wellcome Research Laboratories. It contains 40 % (w/w) protein (the rest is salt); 75 % of this protein is toxin.

Ganglioside. Fresh beef brain (8 kg.) was freed from blood and meninges under running tap water, minced in a Waring blendor with an equal volume of acetone, centrifuged, and the acetone extract discarded. The extraction was repeated twice to yield 1·3 kg. acetone-dried powder. This material was extracted for 5 hr. in a Soxhlet extractor with chloroform + methanol (1 + 2 vol.), to yield 380 g. extract (see Svennerholm, 1956*a*). Four 90 g. batches of extract were each dissolved in 3·5 l. chloroform + methanol (2 + 1 vol.) and the solutions dialysed for two days against running tapwater. During this time two phases formed within the dialysis tubes. The material in the upper aqueous phases from the four batches was concentrated in a rotating evaporator and freeze-dried to yield 14 g. crude ganglioside containing 0·3 μmole sialic acid/mg. (see Folch *et al.* 1951). Of this crude ganglioside 10 g. were dissolved in 400 ml. chloroform + methanol (2 + 1 vol.) containing 10 % (w/v) $CaCl_2.H_2O$. After standing for some hours at room temperature the deposit was filtered off (see Rosenberg & Chargaff, 1958), the filtrate dialysed against running tap water for two days, and the ganglioside recovered from the upper phase as before; the yield was 4·1 g. crude ganglioside containing 0·6 μmole sialic acid/mg. This material was again dissolved in chloroform + methanol, treated with calcium chloride, and recovered by partition-dialysis to yield 3·0 g. ganglioside containing 0·82 μmole sialic acid/mg. This was the calcium salt of ganglioside. To convert it to the free acid 3 g. were dissolved in 80 ml. water and the solution brought to pH 1·4 with 12 N-HCl. The acidic solution was dialysed in the cold with agitation against several changes of 1 l. of distilled water until the pH value was constant at 3·0 (Folch *et al.* 1951). The final yield was 2·7 g. of partially purified ganglioside containing 0·73 μmole sialic acid/mg., 0·31 μmole hexosamine/mg. This material was ninhydrin-negative, and contained less than 0·4 % protein according to the method

of Lowry *et al.* (1951). It showed a single peak on electrophoresis (Fig. 1 (ii)) and ultracentrifugation (Fig. 2 (i)), but on paper chromatography (Fig. 6*c*) several substances were detected. Spots 4 and 5 were much fainter than spots 1, 2 and 3. Spots 3, 4 and 5 were non-metachromatic and stained blue-mauve, spots 1 and 2 were metachromatic and stained pink-mauve.

Column chromatography. Mallinckrodt's Analytical Reagent Silicic Acid 100 mesh (Savory and Moore, London) was ground to 300 mesh and treated according to Hirsch & Ahrens (1958). Silicic acid (10 g.) was suspended in 35 ml. chloroform, poured into tubes, 1·5 cm. diameter, 25 cm. long, and washed with a further 50 ml. chloroform. Ganglioside was dissolved in 6 % (v/v) methanol in chloroform and loaded on the column, at not more than 25 mg./g. sialic acid. Elution was performed with methanol + chloroform mixtures of stepwise increasing methanol concentration as indicated below. The chloroform used was washed with water, dried with $CaCl_2$ and distilled before use; methanol was distilled before use.

Paper chromatography. Schleicher & Schull 2045B smooth paper (Gallenkamp-Towers, London) was used, in strips 20 cm. long and 11 cm. wide, after washing overnight with 5 % (v/v) acetic acid by descending chromatography, followed by washing with water and air drying. Samples (200 μg.) dissolved in 10 μl. water were placed on the papers in 1 cm. streaks and the chromatograms developed by the ascending technique with freshly prepared mixtures of di-iso-butylketone, acetic acid, H_2O (40:30:7, v/v; Marinetti & Stotz, 1956), in tightly closed cylindrical jars, 21 cm. high, 12·5 cm. diameter. After overnight running the papers were air dried, stained by immersion in 0·02 % (w/v) cresyl violet in 1 % (v/v) acetic acid in water and heating at 60° for 10 min., and washed with 1 % (v/v) acetic acid. This dye stains lipids, acid lipids showing metachromasy, particularly in wet papers.

Analyses. Sialic acid was determined by the resorcinol method of Svennerholm (1957*b*), with *N*-acetylneuraminic acid (kindly supplied by Professor G. Blix and Dr L. Svennerholm) as a standard. Hexosamine was determined by the Procedure A of Svennerholm (1956*b*), with glucosamine hydrochloride as a standard.

Ultracentrifugal studies. The Spinco Model E Analytical Ultracentrifuge, with the RTIC unit and a phase plate in the schlieren-optical system, was used. Toxin and ganglioside were dissolved in 0·1 M-phosphate buffer (pH 7·0). The temperature of the rotor varied in different runs from 18° to 21°. Runs were carried out at 59,780 rev./min. for 23 min. in single sector cells and at 42,040 rev./min. for 60 min. in double sector cells. Areas under the peaks of the schlieren diagrams (phase plate angle 60°) on the photographic plates were measured by projecting the diagrams in a photographic enlarger with an enlargement of 10 diameters, tracing the diagrams on paper, drawing a midline through the thickness of the phase-plate lines, and measuring the areas so enclosed by means of a polar planimeter with an accuracy of $\pm 0{\cdot}5$ %. The schlieren diagrams shown in this paper are derived from these tracings.

Electrophoresis. The Antweiler microelectrophoresis apparatus, with schlieren optical attachment (Boskamp; Shandon, London), was used. Samples were dissolved in 0·05 M-phosphate buffer (pH 7·0) and dialysed against the same buffer and subjected to electrophoresis at 75 v. for 10 min.

RESULTS

Studies on the combination of toxin and ganglioside

Electrophoretic studies. In previous studies (Van Heyningen, 1959*b*) the reaction between the water-soluble tetanus toxin and water-soluble ganglioside was demonstrated in the preparative (Spinco Model L) ultracentrifuge by determining the

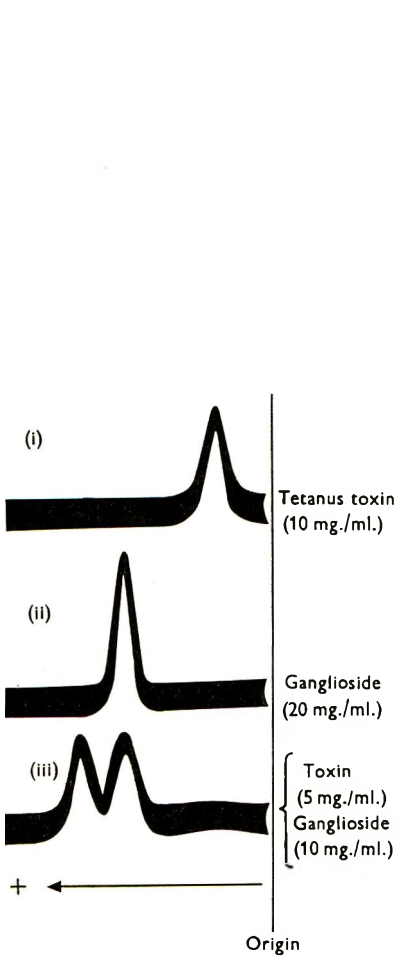

Fig. 1

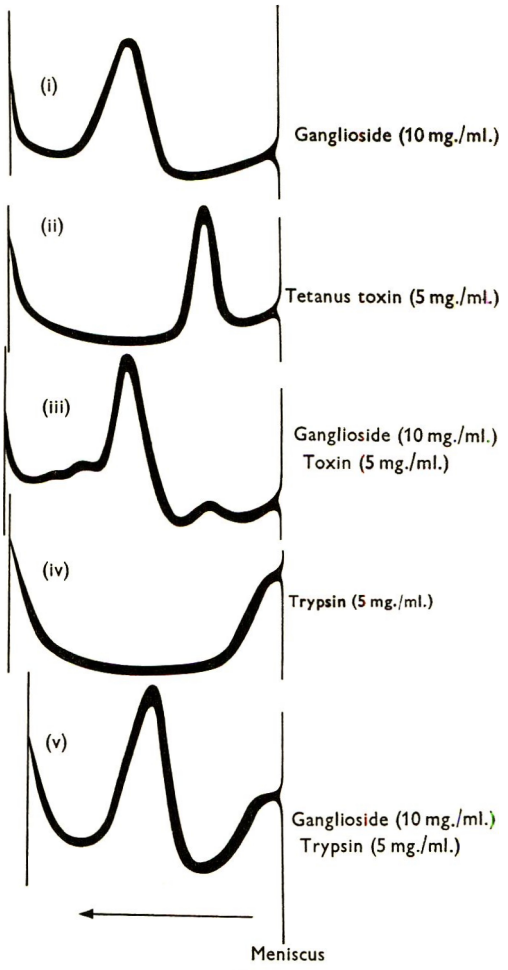

Fig. 2

Fig. 1. Fixation of tetanus toxin by ganglioside demonstrated by boundary electrophoresis.

Fig. 2. Fixation of tetanus toxin, and non-fixation of trypsin, demonstrated in the analytical ultracentrifuge.

effect of the more rapidly sedimenting ganglioside on the rate of migration of the toxin from the upper to the lower half of the centrifuge tube. This was done by measuring the protein contents of the upper and lower halves of centrifuge tubes after centrifuging the toxin at 40,000 rev./min. for 1 hr. in presence and absence of ganglioside. The combination of toxin and ganglioside can also be demonstrated by

electrophoresis. Fig. 1 (i) shows that toxin alone migrated towards the positive pole much more slowly than ganglioside (Fig. 1 (ii)). Fig. 1 (iii) shows that when toxin and ganglioside were mixed the more slowly moving toxin peak was absent and instead a complex of peaks appeared, moving at about the same rate as ganglioside.

Ultracentrifugal studies; qualitative. The combination of toxin and ganglioside can also be demonstrated in the analytical ultracentrifuge. Fig. 2 (i) shows that ganglioside alone sedimented faster than toxin alone (Fig. 2 (ii)). Fig. 2 (iii) shows the sedimentation diagram of a mixture of toxin and ganglioside. The slowly moving toxin peak has almost disappeared (see section *quantitative* below), and we now have a complex of peaks sedimenting as fast as, and faster than the ganglioside peak, since complexes of greater molecular weight than ganglioside alone are formed. Fig. 2 (iv) shows that under the same conditions of centrifugation the peak of trypsin, a protein which does not combine with ganglioside at the buffer concentration used (see van Heyningen 1959*a*), did not move completely away from the meniscus. Fig. 2 (v) shows that the sedimentation of trypsin, unlike that of tetanus toxin, was unaffected by the presence of ganglioside.

The following substances were found not to fix tetanus toxin. (1) Sialic acid-free ganglioside, prepared by acid hydrolysis of brain ganglioside and kindly supplied by Professor E. Klenk. (2) Brain ganglioside in which the carboxyl groups of the sialic acid residues had been methylated with diazomethane. When this substance was saponified (in 0·1 N-NaOH for 2 hr. at 37°) it fixed toxin again. (3) Sialic acid alone (the preparative ultracentrifugal technique was used for this demonstration; although 40 % of the toxin in the upper half of a centrifuge tube migrated to the lower half during spinning for 2 hr. at 40,000 rev./min., no measurable amount of sialic acid migrated in the presence or absence of toxin under the same conditions). (4) Ovine mucoid substance with a sialic acid content of *c.* 0·5 μmole/mg. and a molecular weight of *c.* 40,000, kindly supplied by Professor Gunnar Blix. (5) Hexosamine-free ganglioside from horse red blood cells (Klenk & Lauenstein, 1953), kindly supplied by Professor E. Klenk.

Ultracentrifugal studies; quantitative. The qualitative demonstration of the fixation of toxin by ganglioside in the analytical ultracentrifuge led to the development of a quantitative assay of toxin-fixing capacity. This involved centrifuging a mixture of toxin and ganglioside and determining the concentration of toxin not fixed, by measuring the area of the toxin peak in the schlieren diagram. This area is a linear function of the concentration of toxin. These measurements require that the base-lines of the peaks should be accurately delineated, and for this purpose double-sector cells were used, with buffer in one sector (to give the base lines) and the solution of toxin and ganglioside in buffer in the other (to give the peaks). Double sector cells cannot be spun at speeds greater than 42,040 rev./min. Fig. 3*a* shows the series of schlieren diagrams obtained when constant concentrations of 5 mg. toxin protein/ml. were mixed with concentrations of ganglioside increasing from 0–10 mg./ml. With increasing ganglioside concentration the area of toxin peak progressively decreased as more toxin was sedimented with the ganglioside. At concentrations of 2 mg. ganglioside/ml. and above there was no further decrease in the area of the toxin peak and a peak of constant size remained, corresponding in area to 25 % of the original peak. This residual peak is due to the 25 % of non-toxic protein in the toxin preparation (75 % pure, see Methods) which is not fixed by the ganglioside.

It is interesting to see in these diagrams that the rapidly sedimenting ganglioside-toxin peaks are complex, representing several ganglioside-toxin combinations, and that the smaller the concentration of ganglioside the greater the speed with which the complexes sedimented. (Control experiments showed that the rate of sedimentation of ganglioside alone was independent of concentration.) Presumably at high ratios of toxin to ganglioside more toxin is fixed per unit weight of ganglioside and consequently the weight of the resultant particle is greater.

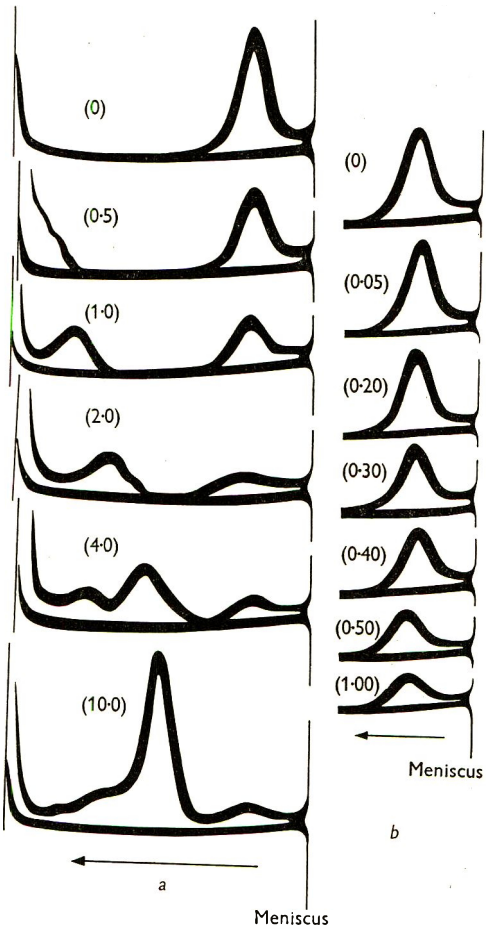

Fig. 3. Quantitative assay of toxin fixation by ganglioside in the analytical ultracentrifuge. Toxin protein concentration 5 mg./ml. in all cases, ganglioside concentration (mg./ml.) shown in brackets. *a*, Wide range of ganglioside concentrations; *b*, toxin protein peaks after treatment with smaller range of ganglioside concentrations.

To provide a quantitative assay of toxin-fixing capacity, a smaller range of ganglioside concentrations was mixed with constant concentrations of 5 mg. toxin protein/ml. Fig. 3*b* shows the peaks of the unfixed protein during centrifugation. From the areas of these peaks the amounts of unfixed protein, and thus of fixed protein, were calculated. (In 10 separate experiments the values obtained for the area due to 5 mg. toxin protein, including pipetting errors, were 31·6, 34·5, 32·4, 29·8, 33·8, 32·8, 32·7,

32·3, 31·2, 31·4; mean 32·3 mm.^{2}). The points in Fig. 4 show the relation between amount of ganglioside and amount of toxin fixed. Evidently the curve through these points is very steep at low concentrations of ganglioside. Where the data are replotted to show weight of toxin fixed per unit weight of ganglioside (on the ordinate) as a function of weight of toxin fixed per unit volume, the intercept with the ordinate (i.e. infinite ratio of unfixed toxin to ganglioside) corresponds to a value of 23·5 mg. toxin fixed/mg. ganglioside (or 1 mole toxin/2 mole ganglioside). The (unbroken) curve in Fig. 4 is the Standard Curve obtained by replotting the smooth (broken) curve drawn through the points in this figure, with mg. toxin fixed on the ordinate and Receptor Units on the abscissa. The Receptor Unit (RU) is defined as the amount of ganglioside that fixes 20 μg. tetanus toxin (= 10L+ units toxin at 2 μg. toxin/L+ unit; see van Heyningen, 1959*a*) at an infinite ratio of toxin to

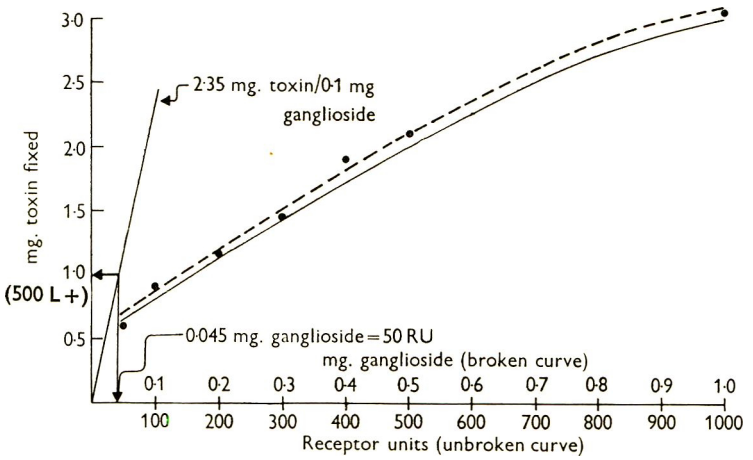

Fig. 4. Relation between weight of toxin fixed and weight of ganglioside used (broken curve), and the Standard Curve (unbroken) derived from these data.

ganglioside. (The RU now defined differs to an unknown degree from the RU previously defined under different conditions for the insoluble ganglioside + cerebroside receptor complex; van Heyningen, 1959*a*). The steps in determining the RU content of a ganglioside preparation are now as follows. To one 0·25 ml. volume containing 25 mg. tetanus toxin preparation TD 464 D (i.e. 10 mg. toxin protein)/ml. 0·1 M-phosphate buffer (pH 7) is added 0·25 ml. buffer, and to another 0·25 ml. volume of toxin solution is added 0·25 ml. of a ganglioside solution of appropriate concentration in buffer. A portion (0·4 ml.) of the solution containing toxin only is placed in one sector of the double sector wedge cell, and 0·4 ml. of the ganglioside + toxin solution is placed in one sector of the double sector standard cell. Buffer (0·4 ml.) is placed in the remaining sectors of each cell. The cells are then spun at 42,040 rev./min. and after 1 hr. the schlieren diagrams are photographed, and the areas of the protein peaks measured. From the decrease in area due to the presence of ganglioside the mg. protein fixed is calculated and from this value the RU content of the ganglioside preparation is derived from the Standard Curve (Fig. 4).

Purification of ganglioside

The partially purified sample of ganglioside (0·73 μmole sialic acid, 0·31 μmole hexosamine, 1100 RU/mg.; see Methods) was further purified by chromatography on a column of silicic acid by a procedure recommended by Dr L. Svennerholm. Ganglioside (200 mg.) was dissolved in a minimum volume of 6 % (v/v) methanol in chloroform, and the column successively eluted with 50 ml. 10 %, 50 ml. 25 % and 150 ml. 50 % (v/v) methanol in chloroform. The eluate was collected in 10 ml. volumes and the sialic acid content determined. The ganglioside all emerged in the 50 % (v/v) methanol eluate (Fig. 5). Evidently it was still inhomogeneous with respect to sialic acid content and toxin-fixing capacity, although the hexosamine

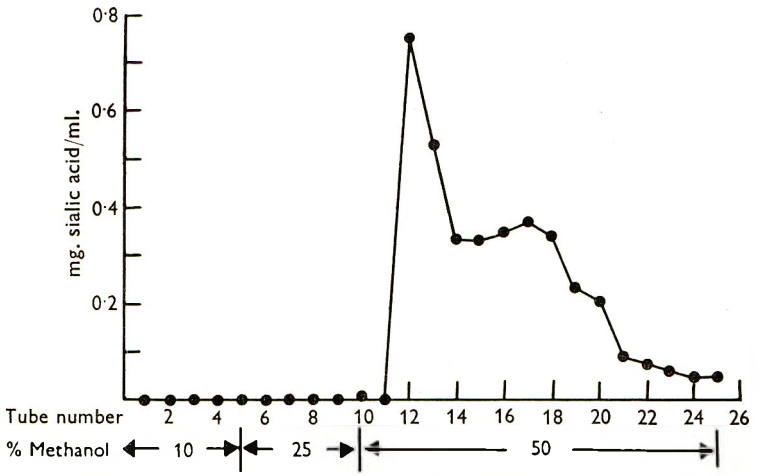

Fig. 5. Inhomogeneity of ganglioside emerging in 50 % methanol in chloroform eluate from silicic acid column.

content of the material in all the tubes appeared to be constant (Table 2). On paper chromatography the pooled material from the 50 % methanol eluate showed spots in positions 1, 2 and 3, and spots 4 and 5 were absent (compare Fig. 6*c*). This material was placed on a fresh column of silicic acid, and after elution with 50 ml. 10 % (v/v) methanol in chloroform and 50 ml. 25 % methanol it was eluted with 30 % methanol likewise in chloroform. Ganglioside first emerged in the 30 % methanol eluate and the column was eluted with this solvent (*c.* 200 ml.) until only negligible quantities of sialic acid-containing material emerged. Seventy-five mg. of material, which we shall call 'fast' ganglioside, was recovered in the 30 % methanol eluate. The column

Table 2. *Data on ganglioside preparations eluted from a silicic acid column with* 50 % *(v/v) methanol in chloroform (see Fig.* 5)

Pooled tubes	Hexosamine (μmole/mg.)	Sialic acid (μmole/mg.)	RU/mg.	RU/μmole sialic acid
12, 13	0·27	0·77	950	1234
14, 15	0·26	0·83	1090	1314
16–25	0·28	0·88	1170	1330

was then eluted with 200 ml. 50 % methanol, and 90 mg. 'slow' ganglioside was recovered. Diagrams of the paper chromatograms of the 'fast' and 'slow' preparations are shown in Fig. 6, *a* and *b*. The 'fast' preparations consist mainly of the non-metachromatic material (spot 3), and the 'slow' mainly of metachromatic material (spot 1). Both preparations contained a proportion of the metachromatic component 2. The metachromatic and non-metachromatic materials were eluted from

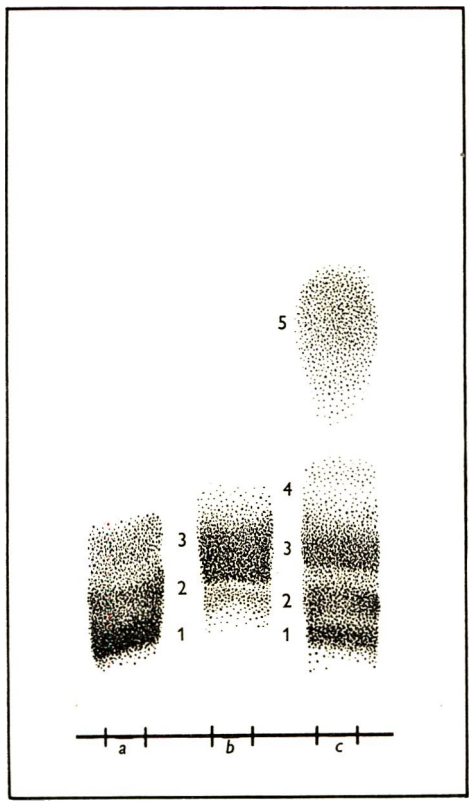

Fig. 6. Paper chromatograms of 'slow' (*a*), 'fast' (*b*), and partially purified (*c*), preparations of ganglioside. Developed by ascending technique in di-*iso*butyl ketone, acetic acid, water and stained with cresyl violet. Spots 1 and 2 metachromatic (pink-mauve) 3, 4 and 5 non-metachromatic (blue-mauve).

the paper and rechromatographed, separately and together, and no interconversion was observed. Both 'slow' and 'fast' preparations have the same hexosamine content (0·31 μmole/mg. in 'slow', 0·30 μmole/mg. in 'fast'), but the 'slow' ganglioside contains more sialic acid (0·93 μmole/mg.) and has a greater toxin-fixing capacity (1310 RU/mg.) than the 'fast' (0·75 μmole sialic acid/mg., 950 RU/mg.). We have observed similar differences in chromatographic properties, sialic acid content and toxin-fixing capacities between ganglioside preparations of other workers. Thus a mucolipid preparation provided by Dr Erwin Chargaff was mainly 'slow' and metachromatic on paper chromatography and contained 0·89 μmole sialic/mg and 1190 RU/mg., whereas a ganglioside preparation provided by Professor E. Klenk was mainly 'fast' and non-metachromatic and contained 0·66 μmole sialic acid/mg.

and 540 RU/mg. Since sialic acid is split off when ganglioside is heated at 100° at pH 3, and possibly on prolonged standing in solution at room temperature (e.g. see Bogoch, 1958), we wondered whether the 'fast' ganglioside is a degradation product derived from the 'slow' by loss of sialic acid during preparation, or whether they both exist in nervous tissue. We were not able to change the relative proportions of 'fast' and 'slow' in ganglioside preparations by the following variations in procedure: (*a*) plunging chopped-up beef brain in dry ice-cooled acetone immediately after killing, as compared with standing brain at room temperature overnight before acetone-dehydration; (*b*) extracting dehydrated brain with chloroform-methanol at room temperature as compared with extraction in a Soxhlet apparatus; (*c*) deionizing by treatment with Amberlite CG 50 as compared with dialysis at pH 1·4–3.

Is ganglioside changed by interaction with tetanus toxin?

We examined the question whether ganglioside is changed as a result of its interaction with tetanus toxin. Ganglioside (5 mg./ml.) and tetanus toxin (10 μg., or 75,000 LD 50/ml.) were incubated together in 0·1 M-phosphate buffer (pH 7) for 5 hr. at 37° and the solution then dialysed overnight at 2° with constant agitation against an equal volume of water. The diffusate was retained and dialysis of the residue continued for 5 hr. against running tap water. Tests for free amino groups, sialic acid and hexosamine were then carried out on the residue and the diffusate. The concentration of ganglioside was such that a 10 % liberation of amino groups, or of hexosamine or sialic acid in a diffusible form, would have been detectable. The only positive reactions observed were those for sialic acid and hexosamine on the non-diffusible residue, which suggests that the amino groups of the hexosamine, sialic acid and sphingosine residues were not liberated, and that there was no liberation of diffusible material containing hexosamine or sialic acid. Bangham & Dawson (1959) showed that lecithin was not attacked by lecithinase unless the positive charge was changed to a negative charge by the addition of various negatively charged substances at threshold concentration. Positively charged cations or amphiphatic molecules reversed the effect. With the thought that similar considerations of charge (either negative or positive) might apply to the possible enzymic cleavage of ganglioside, the experiments above were repeated in the presence of 0·02 M-$CaCl_2$ (in which case collidine acetate buffer, pH 6·8, was used), and cetyltrimethylammonium bromide, sodium dodecyl sulphate, dicetylphosphoric acid, and saponin, all at 0·25 mg./ml. None of these substances brought about any positive results. Since ganglioside occurs in extracts of nervous tissue in the form of a complex with cerebroside (van Heyningen, 1959*b*) the same tests were carried out with ganglioside cerebroside complex, again with negative results.

Ganglioside (10 mg./ml. 0·05 M-phosphate buffer, pH 7) was incubated overnight at 37° in the presence and absence of tetanus toxin (10 μg./ml., 75,000 LD 50/ml.); the two preparations behaved identically in the electrophoresis apparatus and in the analytical ultracentrifuge.

If tetanus toxin were an enzyme with ganglioside as its substrate, one would not expect the ganglioside to fix the toxin after the toxin had catalysed a change in it. This point was tested in the following way. An insoluble ganglioside + calcium + cerebroside complex was made (Van Heyningen, 1959*b*) and suspended in water to

give a concentration of 1 mg. ganglioside/ml. To samples of this suspension were added equal volumes of 0·133 M-phosphate buffer (pH 7) or a solution containing 1 mg. (= 7 million LD50) toxin/ml. 0·133 M-phosphate buffer (pH 7). These two mixtures were incubated for 16 hr. at 37°, spun at 5000 rev./min., the pellets washed with water, and the adsorbed protein removed with 0·1 N-NaOH. The ganglioside + calcium + cerebroside complexes which had thus been treated with toxin overnight and deproteinized with alkali, and the control preparation that had not been treated with toxin, were resuspended to the same concentration as before and again added to equal volumes of toxin solution and the amount of adsorbed protein measured in the manner previously described (van Heyningen, 1959*a*). The receptor complex which had been treated with toxin adsorbed 88 μg. protein/mg. on the second suspension in toxin solution, and the control preparation 81 μg. protein/mg. It appeared therefore that the toxin-fixing capacity of the ganglioside + cerebroside complex was unaffected by treatment overnight with toxin at a concentration of 3·5 million LD50/ml.

We also tested the possibility that complement might be fixed during the toxin + ganglioside interaction, but found that guinea pig complement (2MHD) still haemolysed sensitized sheep red cells after incubation with toxin + ganglioside mixtures. The toxin concentration ranged from 0·11 mg./ml. (800,000 LD50/ml.) to 0·000,000,4 mg./ml. (3 LD50/ml.) and the ganglioside concentration from 0·4 mg./ml. to 0·05 mg./ml. The reagents were added in the order complement, toxin, ganglioside, 1 hr. at 37°, haemolytic system, 20 min. at 37°.

DISCUSSION

In our most purified preparations of ganglioside at least three components have been observed on paper chromatograms, but on silicic acid columns only two were distinguished, and the absence of the third component was probably due to inadequate resolution. The three components have not been sufficiently separated from each other to merit a complete chemical analysis. The presence of adsorbed proteins might account for inhomogeneity on columns, but our preparations contain no proteins, and we believe we are dealing with at least three different gangliosides, and that irrelevant impurities have been removed. The 'fast' and 'slow' fractions separating on the silicic acid columns have the same hexosamine content, but differ in rate of movement and staining with cresyl violet on paper chromatography, in sialic acid content, and in toxin-fixing capacity. They contain the third, metachromatic, component in common.

The reported sialic acid contents of ganglioside vary (Table 1). Comparison of our preparations with those of others suggests that the preparation of Klenk & Gielen (1960), which is a mixture of two gangliosides each containing 0·66 μmole sialic acid/mg., is 'fast' and non-metachromatic, whereas the preparation of Rosenberg & Chargaff (1958), which contains more sialic acid (0·89 μmole/mg.) is 'slow' and metachromatic. We are not in a position to say whether the 'fast' and 'slow' gangliosides exist as such in nervous tissue, or whether the 'fast' is derived from the 'slow' by loss of some sialic acid during extraction and purification.

The fixation of tetanus toxin is dependent on the presence of the sialic acid residue in ganglioside, and, at least within the range provided by the 'fast' and 'slow'

preparations, roughly proportional to the sialic acid content. The free carboxyl group of the sialic acid residue is essential for toxin-fixation.

The hexosamine-free ganglioside from horse red blood did not fix toxin, but this may be due to factors other than the absence of hexosamine, e.g. *N*-glycolyl rather than *N*-acetyl substituents in the sialic acid residues, differences in fatty acid residues, etc.

If tetanus toxin brings about a change in ganglioside it is by no means obvious. All the tests that we were able to devise to detect any change were negative and therefore inconclusive.

We are grateful to Dr A. Woodin for helpfully critical discussions; to Forskare L. Svennerholm for receiving one of us (W. E. van H.) in his laboratory and giving him invaluable advice on column and paper chromatography of gangliosides, as well as a sample of pure sialic acid; to Dr G. G. Pope and Dr R. O. Thompson for the tetanus toxin; to Professor G. Blix for samples of sialic acid and mucoid substances; to Professor E. Klenk for various ganglioside preparations; to Dr E. Chargaff for a sample of mucolipid; to the Oxford and District-Co-operative Society Ltd. for the supply of beef brain; to Mr J. Otway for technical assistance and to Mrs Galatea Collins for non-technical assistance. Very special thanks are due to the Wellcome Trustees for the most generous grant to W. E. van H. of the Spinco Analytical Ultracentrifuge and the Antweiler Microelectrophoresis apparatus. W. E. van H. also acknowledges with gratitude that this work was assisted in part by a grant from the Office of Naval Research of the United States Department of the Navy (Project No. 103–474). P. A. M. was the John Simon Guggenheim Memorial Fellow.

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The Relation between the Tetanus Toxin-Fixing and Influenza Virus-Inhibiting Properties of Ganglioside

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SUMMARY

The capacity of a number of gangliosides to fix tetanus toxin and to inhibit haemagglutination by influenza virus was investigated. Toxin fixation increased with total sialic acid content but not in strict proportion. Whether or not the sialic acid is bound by neuraminidase-labile linkage appears to be irrelevant in determining whether toxin is fixed. Viral inhibition by ganglioside also is related to the total content of sialic acid; but there exists at least one ganglioside which has very little capacity to react with virus and whose sialic acid is virtually all insensitive to neuraminidase. Tetanus toxin does not prevent neuraminidase from attacking ganglioside.

INTRODUCTION

Ganglioside from nervous tissue has been shown to act as a specific receptor for tetanus toxin (Van Heyningen, 1959) and the sialic acid residue(s) of this compound has been shown to be essential for toxin-fixation (Van Heyningen & Miller, 1961). It is well known that sialic acid-containing substances also act as influenza virus receptors. Rosenberg, Howe & Chargaff (1956) found that their preparation of mucolipid, which contained a polypeptide moiety in addition to ganglioside, inhibited influenza virus, and was susceptible to viral and cholera neuraminidase, whereas samples of ganglioside which did not contain amino acid residues were inactive. They considered that the polypeptide moiety, in addition to the sialic acid residue, was essential for viral inhibitory activity. It has been observed that amino acids are present in ganglioside preparations extracted from wet brain, but not in preparations extracted from acetone-dehydrated brain (Folch, Arsove & Meath, 1951; Svennerholm, 1956). Bogoch (1957) showed that a preparation of brain ganglioside from wet brain inhibited viral haemagglutination. Kuhn (1958) separated two fractions of brain ganglioside from dehydrated brain on a cellulose column eluted with methanol + pyridine + water and found that the more rapidly moving fraction (G 2), containing 21 % sialic acid, did not inhibit viral haemagglutination and was not susceptible to *Vibrio cholerae* neuraminidase; the more slowly moving fraction (G 0·5) containing 31 % sialic acid, inhibited viral haemagglutination and was susceptible to neuraminidase. Van Heyningen & Miller (1961) separated brain ganglioside (prepared from dehydrated brain and containing no amino acid

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residues) into 'fast' and 'slow' fractions from silicic acid columns eluted with methanol + chloroform. The 'fast' preparation contained 0·75 μmole sialic acid/mg. and the 'slow' 0·93 μmole/mg. The tetanus toxin-fixing capacities of these fractions were approximately proportional to their sialic acid contents. They also showed that the mucolipid preparation of Rosenberg *et al.* (1956) was 'slow' and contained 0·89 μmole sialic acid/mg., whereas an amino acid-free preparation supplied by Professor E. Klenk was 'fast' and contained 0·66 μmole sialic acid/mg. The tetanus toxin-fixing capacities of these preparations were also roughly proportional to their sialic acid contents. We have now investigated the influenza virus-inhibiting properties of these various ganglioside preparations in relation to their toxin-fixing capacities.

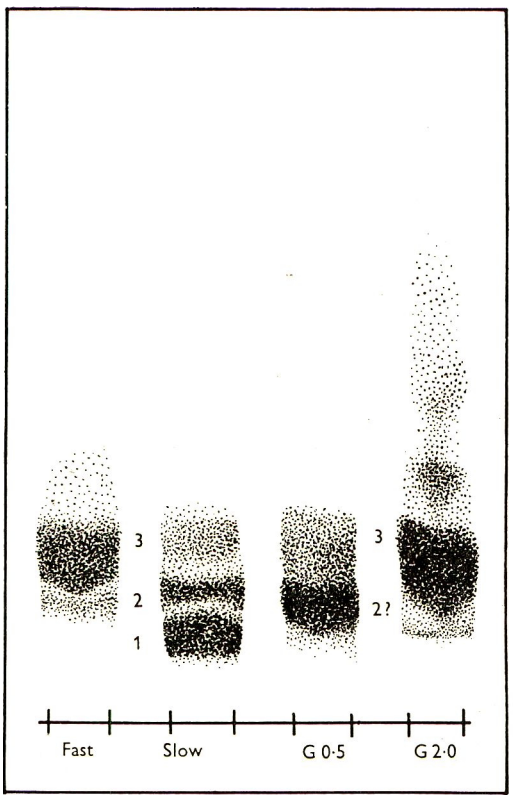

Fig. 1. Paper chromatograms of various ganglioside preparations. Components 1 and 2 metachromatic, 3 non-metachromatic.

METHODS

Ganglioside preparations. The preparation (or source) and properties of the 'partially purified', 'fast', 'slow', 'Chargaff' and 'Klenk' gangliosides are described in the preceding paper (Van Heyningen & Miller, 1961). The G 2·0 and G 0·5 preparations were kindly supplied by Professor Richard Kuhn. Their paper chromatographic behaviour in comparison with 'fast' and 'slow' ganglioside preparations is shown in Fig. 1.

Paper chromatography. As described by Van Heyningen & Miller (1961), except that for Fig. 3 the ratio of di-*iso*butylketone, acetic acid, water was 40:25:5, rather than 40:30:7.

Assays. Total sialic acid was estimated by the resorcinol method of Svennerholm (1957); free sialic acid by the thiobarbituric acid assay of Warren (1959); tetanus-toxin fixing capacity (RU/mg.) by the method of Van Heyningen & Miller (1961); influenza virus-inhibiting capacity essentially by the method of Howe (1951). The PR 8 strain of influenza virus was harvested from eggs, the combined allantoic fluid centrifuged and the sedimented virus resuspended in 0·1 vol. 0·15 M-NaCl. Virus prepared in this way (haemagglutinin titre 64,000) was kindly supplied by Dr A. Isaacs. Indicator virus was prepared by heating this preparation for 30 min. at 55–56°. The titrations were carried out in MRC pattern Perspex plates. Serial two-fold dilutions of ganglioside contained in 0·2 ml. 0·15 M-NaCl, buffered to pH 7·2 with 0·01 M-phosphate, were mixed with 1–2 haemagglutinin units of indicator virus and allowed to stand in the refrigerator for 30 min.; 0·1 ml. of a 2 % suspension of washed chicken erythrocytes was then added, and after standing 1–2 hr. in the cold, the patterns of the sedimented erythrocytes were observed. The end-point used was the smallest amount of ganglioside giving just short of complete inhibition of haemagglutination.

Neuraminidase. Two preparations were used: (*a*) crude freeze-dried *Vibrio cholerae* filtrate (N. V. Philips-Roxane, Amsterdam), (*b*) crystalline *V. cholerae* neuraminidase kindly supplied by Dr G. L. Ada. Neuraminidase (RDE) activity was estimated by the method of Burnet & Stone (1947). The Ca + acetate + saline used in experiments with neuraminidase contained per l. distilled water: 12·38 g. sodium acetate, 5·0 g. NaCl, 1·0 g. $CaCl_2.H_2O$; adjusted to pH 6·2 with 2 N-acetic acid.

RESULTS

Inhibition of haemagglutination

The viral haemagglutination-inhibiting property of the various ganglioside preparations is shown in Table 1.

Action of neuraminidase on ganglioside

The thiobarbituric acid assay (Warren, 1959) is useful for the measurement of enzymic liberation of sialic acid from ganglioside since it estimates only free sialic acid. Figure 2*a* shows the time course of the liberation of sialic acid from 'slow' ganglioside by crude *Vibrio cholerae* neuraminidase (75 units/ml.). Maximal splitting was reached in about 8 hr. Figure 2*b* shows the liberation of sialic acid from 'slow' ganglioside by increasing concentrations of crystalline neuraminidase in 16 hr. Maximal splitting was obtained with 75–100 enzyme units/ml.

Comparative susceptibility of various ganglioside preparations to neuraminidase. Neuraminidase-labile sialic acid of the several ganglioside preparations was estimated by incubating 160 μg. ganglioside with 4000 units of crystalline neuraminidase in a volume of 0·4 ml., pH 6·2, at 36°. After 16 hr. free sialic acid was measured (Warren, 1959) and the values obtained corrected for small amounts of colour and turbidity given by untreated ganglioside. The results are shown in Table 1.

Table 1. *Chromatographic behaviour, neuraminidase-sensitivity, viral-inhibition and toxin-fixation of ganglioside preparations*

Ganglioside preparation	Chromatographic properties of main component(s)		Total sialic acid (μmole/mg.)	Fraction of total sialic acid liberated by neuraminidase (%)	Amount required to inhibit 1–2 haemagglutinin units of influenza virus (μg.)	Toxin-fixing capacity	
	Movement	Staining with cresyl violet				RU/mg.	RU/μmole sialic acid
Partially purified	Fast and slow	N and M*	0·73	44	8	1100	1505
Fast	Fast	N	0·75	35	8	950	1280
Slow	Slow	M	0·93	57	1	1310	1400
Kuhn G0·5	Slow	M	0·82	33	0·5	820	1000
Kuhn G2·0	Fast	N	0·58	2	20	580	1000
Chargaff mucolipid	Slow	M	0·89	47	0·5	1190	1340
Klenk ganglioside	Fast	N	0·66	21	10	540	820

*M = metachromatie, i.e. stains pink-mauve; N = non-metachromatic, i.e. stains blue-mauve.

A preparation of influenza virus, 50 times as concentrated (the greatest practicable) as the allantoic fluid from which it was derived, released 48 % of the total sialic acid of the 'slow' ganglioside in 16 hr. at 36° (compare 57 % released by *Vibrio cholerae* neuraminidase).

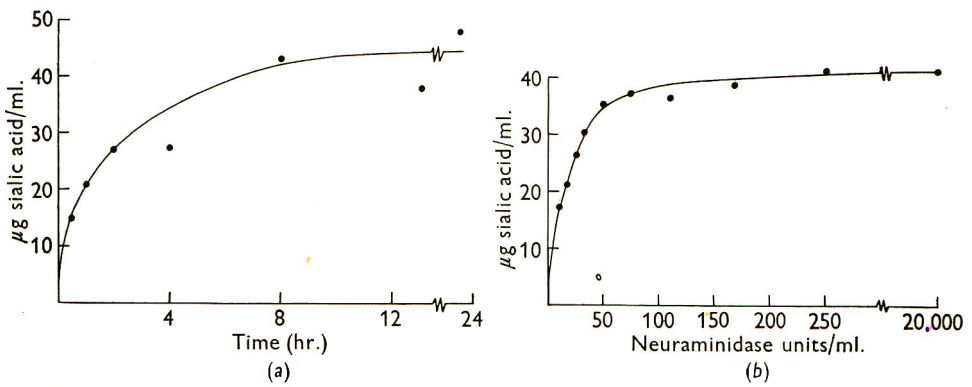

Fig. 2*a*. Course of appearance of sialic acid in a mixture of ganglioside and *Vibrio cholerae* filtrate. One ml. crude *V. cholerae* filtrate reconstituted to twice its original volume with calcium + acetate + saline (pH 6·2) was added to one ml. of the same diluent containing 0·8 ml. 'slow' ganglioside. During incubation at 36°, 0·2 ml. samples were removed at the times indicated and delivered into tubes containing 0·1 ml. sodium periodate in phosphoric acid, and free sialic acid subsequently estimated. The values are corrected for colour given by the *V. cholerae* filtrate.

Fig. 2*b*. Liberation of sialic acid as a function of enzyme concentration. Each point represents the free sialic acid found after incubating for 16 hr. at 36° 80 μg. 'slow' ganglioside in 0·1 ml. calcium + acetate + saline (containing 0·1 % gelatin) + 0·1 ml. crystalline *V. cholerae* neuraminidase in same diluent. The values are corrected for the small amount of colour given by the ganglioside alone.

Fixation of tetanus toxin

The tetanus toxin-fixing capacities of the various ganglioside preparations are shown in Table 1. It is evident that even when practically none of the sialic acid was neuraminidase-sensitive (as in preparation G 2·0) ganglioside still fixed toxin. The data suggest that neuraminidase-insensitive as well as neuraminidase-sensitive sialic acid is concerned in toxin-fixation. In order further to test this point a solution

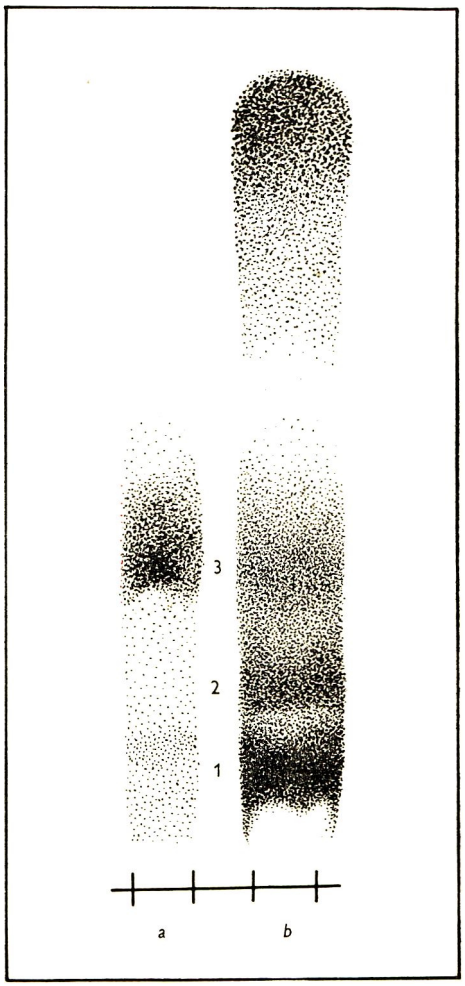

Fig. 3. Paper chromatogram of 'slow' ganglioside after treatment with neuraminidase (*a*) compared with partially purified ganglioside (*b*). Components 1 and 2 metachromatic, 3 non-metachromatic.

containing 1·2 mg. 'slow' ganglioside and 6000 units neuraminidase in 3 ml. Ca + acetate + saline was incubated for 16 hr., with liberation of 48 % of the total sialic acid. The mixture was dialysed overnight against running tap water and freeze-dried. The product was found to contain 580 RU/mg., or 1140 RU/μmole sialic acid.

Figure 3 shows a paper chromatogram of the neuraminidase-treated preparation,

compared with unfractionated ganglioside. It can be seen that the neuraminidase had converted 'slow' ganglioside into 'fast' ganglioside.

The possibility was tested that tetanus toxin might competitively inhibit the action of neuraminidase on ganglioside, but as much as 2·6 mg. toxin/ml. did not significantly diminish the rate of liberation of sialic acid from ganglioside as compared to that of neuraminidase acting on ganglioside in the absence of toxin. The concentration of toxin was sufficient to combine with all the ganglioside present; the concentration of neuraminidase was *c.* 0·01 μg./ml. We were unable to test the reverse concentrations of toxin and neuraminidase because of the limited supply of pure neuraminidase at our disposal.

DISCUSSION

Differences in the ability of various gangliosides to inhibit viral haemagglutination appear to reside in differences in sialic acid content rather than in the presence or absence of amino acid residues as suggested by Rosenberg *et al.* (1956). Not all of the sialic acid which is responsible for viral inhibition is necessarily neuraminidase-sensitive, for comparison of the G0·5 and 'slow' preparations shows the proportion of neuraminidase-sensitive sialic acid to be much lower in G0·5 than in 'slow', while the latter, if anything, had slightly less viral inhibitory capacity.

The gangliosides having a greater sialic content are 'slow' and metachromatic on paper chromatography, and Figs. 1 and 3 (as well as Fig. 6 in Van Heyningen & Miller, 1961) show that there are apparently two such gangliosides. There is a suggestion in Fig. 3 that the second of these gangliosides is the same as G0·5. Since the G0·5 has 33 % neuraminidase-sensitive sialic acid while the mixture of the first and second components (i.e. 'slow') has 57 % neuraminidase-sensitive sialic acid, the proportion of neuraminidase-sensitive sialic acid in the first component is probably greater than 57 %. It is unlikely that the two metachromatic gangliosides are identical with the two gangliosides of Klenk & Gielen (1960) since these latter are 'fast' and non-metachromatic (Van Heyningen & Miller, 1961).

The work reported in this paper confirms the observation of Van Heyningen & Miller (1961) that tetanus toxin-fixation by ganglioside is dependent upon sialic acid content. Although not all the sialic acid of ganglioside is concerned in viral inhibition, it does all appear to be concerned in toxin-fixation. Thus, for example, G2·0, which has practically no viral inhibitory capacity and no neuraminidase-sensitive sialic acid, nevertheless fixes tetanus toxin in proportion to its sialic acid content. Similarly, when 'slow' ganglioside is treated with neuraminidase it retains its toxin fixing capacity in proportion to its residual sialic acid.

It is notable that there are sialic acid-containing substances having biological properties that are the reverse of those of the G2 type of ganglioside, for example, ovomucoid which is an effective inhibitor of viral haemagglutination but does not fix tetanus toxin (Van Heyningen & Miller, 1961).

The figures for the neuraminidase-sensitive sialic acid are subject to some variation and may not in every case represent maximal values, for the tendency of ganglioside to form aggregates may render them inaccessible to the enzyme bonds which would otherwise be attacked. There seem to be at least two possible explanations for differences in neuraminidase sensitivity of sialic acid in ganglioside: there may

be more than one kind of sialic acid linkage in ganglioside, or, the gangliosides themselves may form different types of micelles in which the availability of the bonds to the enzyme differs.

In addition to the acknowledgements made in the preceding paper by van Heyningen & Miller (1961) we wish to thank Professor R. Kuhn for samples of gangliosides, Dr G. L. Ada for crystalline neuraminidase, and Dr A. Isaacs for influenza virus and helpful advice. This work was in part supported by a grant (to W. E. van H.) from the Office of Naval Research of the United States Department of the Navy (Project No. 103-474). Grateful acknowledgement is made to the Commonwealth Fund for a Fellowship granted to A. W. B.

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Further Studies on the Repression of Methionine Synthesis in *Escherichia coli*

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SUMMARY

The observation that growth in the presence of methionine results in organisms with a greatly decreased ability to form methionine from homocysteine has been extended to other strains of *Escherichia coli*, in particular strain PA15, an organism for which there is a considerable background of knowledge concerning the enzymic mechanism of this stage of methionine synthesis. When organisms grown in the absence of methionine ('active' organisms) are transferred to a medium containing methionine the activity is not lost, but simply diluted by new organisms having much less activity. Both organisms and ultrasonic extracts derived from them show the same comparative loss of activity when the organisms are incubated with methionine.

Organisms grown in the presence of methionine ('inactive' organisms) regain activity when incubated in a growth medium without methionine. The major part of such regeneration of activity occurs before growth is significant. Enzyme formation occurs linearly and without lag and is stopped by the addition of methionine at any stage. A number of analogues of methionine also repress enzyme formation, but none is as active as methionine.

Regeneration of enzyme is inhibited by chloramphenicol and is greatly diminished when the supply of individual amino acids is restricted either by the use of an inhibitory analogue (*p*-fluorophenylalanine) or by the use of amino acid-requiring auxotrophs. Formation of enzyme therefore requires *de novo* synthesis of protein. Similar techniques gave no evidence that concurrent synthesis of ribonucleic acid was also required.

Compounds which act as substrates or cofactor for methionine synthesis (serine, homocysteine, cobalamin) also repressed enzyme formation to some extent and, after a lag, full repression was obtained with a mixture of all three. These effects are probably due to methionine itself whose synthesis by the enzyme initially present is stimulated by the test substances.

INTRODUCTION

In earlier work in this laboratory it was shown that growth of a number of strains of *Escherichia coli* in a medium containing methionine resulted in an almost total loss by the organisms of their ability to form methionine from homocysteine and serine. Furthermore, strong evidence was obtained that the change was a purely phenotypic one (Wijesundera & Woods, 1953, 1960). The phenomenon thus appeared to be a typical case of enzyme repression (Vogel, 1957) by the ultimate

product of the activity of the enzyme in question. All the earlier work, and also that of Cohn, Cohen & Monod (1953), had been carried out with intact organisms and it remained theoretically possible that the failure to metabolize homocysteine after growth on methionine was due to a loss of the ability of homocysteine to permeate the organism. Part of the object of the present work was to determine by experiments with cell-free enzyme preparations whether enzymic activity for the conversion of homocysteine to methionine was also lacking or reduced after culture on methionine.

It was also shown (Wijesundera & Woods, 1960) that a single subculture in the absence of methionine completely restored the ability of the organisms to synthesize this amino acid. A second object of the present work was to determine under more controlled conditions the factors necessary for the production of the enzyme, and particularly whether *de novo* synthesis of protein occurred. A similar study of the synthesis of the enzyme ornithine transcarbamylase was made by Maas & Gorini (1957); the formation of this enzyme, which is concerned in the synthesis of arginine, is repressed by growth of the organism (*Escherichia coli*) in the presence of arginine.

Following the terminology of Wijesundera & Woods (1960) 'inactive' organisms will be used in reference to organisms harvested after growth in the presence of methionine and having severely restricted ability to convert homocysteine to methionine; 'active' organisms are those which have not been exposed to methionine.

The strain (PA15) of *Escherichia coli* used for most of the work was chosen because there was a considerable background of knowledge, both with intact organisms and cell-free enzyme preparations, concerning the mechanism of the reaction between homocysteine and serine which yields methionine (Gibson & Woods, 1960; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960).

METHODS

Organisms. Escherichia coli PA15 is an auxotroph requiring serine or glycine for growth; it was obtained originally from Dr Barbara Wright. Other strains used for certain experiments required for growth arginine and uracil (C21), thymine (15T$^-$) and homocysteine or methionine (26/18). All strains were maintained on tryptic digest of meat agar slopes, subcultured monthly and stored at 4° after incubation for 18 hr. at 37°.

Preparation of suspensions of organisms. The basal medium (GL) used throughout was the glucose + lactate medium of Guest *et al.* (1960); it was normally supplemented with glycine (10mM) for strain PA15, though DL-serine (10 mM) was used in some experiments. Other supplements were: uracil (mM) + L-arginine (10 mM) for strain C21; thymine (mM) for strain 15T$^-$; DL-homocysteine (mM) for strain 26/18).

The inoculum for the main cultures was developed as follows. Medium GL (6 ml.) containing the appropriate growth factor was dispensed in 150 × 150 × 16 mm. ⊥-shaped tubes (Monod, Cohen-Bazire & Cohen, 1951) and autoclaved at 115° for 7 min. After seeding from an 18 hr. culture on tryptic digest of meat agar the tubes were rocked for 8 hr. at 37° at 36 oscillations/min. of excursion 10 cm.

The main cultures were grown in 1 l. conical flasks containing 200–500 ml. of medium GL supplemented with the appropriate growth factors and autoclaved at 115° for 7 min. After inoculation the flasks were shaken (circular motion, 220 rotations/min. of radius 2 cm.) in air at 37° for 16–18 hr. The size of the inoculum was so adjusted (0·05–0·5 ml. of the liquid culture described above) that the culture density with the various strains reached the equivalent of 0·2–0·7 mg. dry wt. organism/ml. in the time stated. These precautions were taken in order to obtain young cultures of approximately similar physiological age with all strains. The organisms were harvested by centrifuging (15 min. at 2000 *g*) and washed once with the culture volume of water. For direct tests of ability to synthesize methionine they were resuspended at a concentration equiv. 0·3–0·4 mg. dry wt./ml. in 0·066 M-potassium phosphate buffer pH 7·4.

Preparation of extracts of organisms. Enzyme-containing extracts were prepared by resuspending washed organisms in water (20–30 mg. dry wt./ml.) and subjecting to ultrasonic vibration and further treatment as described by Guest *et al.* (1960). The protein content of the final preparation was determined spectrophotometrically according to Layne (1957). Extract of heated *Escherichia coli* (strain PA15) was also prepared according to Guest *et al.* (1960).

Study of enzyme formation. Organisms were harvested from medium GL (supplemented with appropriate growth factors) but containing also 10 mM-DL-methionine. After washing as above they were resuspended at about 0·3 mg. dry wt./ml. in fresh medium, but with methionine omitted. The suspension (100 ml. in 1 l. conical flasks) was shaken at 37° as described for the growth of cultures. Samples were removed at intervals (usually hourly) to determine the extent of growth and the ability to synthesize methionine. In the latter case the organisms were centrifuged out, washed with the sample volume of water, resuspended in 0·066 M-potassium phosphate buffer (pH 7·4) and assayed as described below. In some experiments cell-free enzyme activity was also measured: in this case it was necessary to have larger samples and to start with a larger (250 ml.) volume of suspension. Ultrasonic treatment was as described above.

Measurement of the synthesis of methionine. In the case of intact organisms the procedure was similar to that of Gibson & Woods (1960). The washed organisms were suspended at a concentration equiv. *c.* 0·17 mg. dry wt./ml. in solution MSA which contained: DL-homocysteine (6 mM) DL-serine (10 mM) (or, in some experiments, glycine (10 mM)), glucose (20 mM), cobalamin (18·4 μmM) and potassium phosphate buffer (pH 7·4; 33 mM). Reactions were carried out in a final volume of 2 ml. After incubation for 3 hr. in air at 37° the suspensions were heated at 100° for 10 min., centrifuged and the supernatant fluids assayed for methionine. A control in which the complete reaction mixture had been heated initially at 100° for 5 min. was included in each experiment.

The test for methionine synthesis by ultrasonic extracts of organisms was based on the procedure of Guest *et al.* (1960). The enzyme at a final concentration of 2·5–5 mg. protein/ml. was added to solution MSB which contained in potassium phosphate buffer (0·125 M; pH 7·8): L-serine (5 mM), DL-homocysteine (5 mM), glucose (10 mM), $MgCl_2$ (5 mM), adenosine triphosphate (5 mM), diphosphopyridine nucleotide (0·5 mM), pyridoxal phosphate (0·25 mM) and either extract of heated *Escherichia coli* (0·1 ml./ml.) or tetrahydropteroylglutamic acid (0·5 mM) + cobalamin

(0·37 μM). In some experiments serine was replaced as donor of the one carbon atom unit by glycine (5 mM) or by the formaldehyde derivative of tetrahydropteroylglutamic acid (1·65 mM); in the latter case formaldehyde was mixed with tetrahydropteroylglutamic acid (Kisliuk & Woods, 1960) before adding to the reaction mixture and the concentration of homocysteine was decreased to a half. The tests were carried out in a final volume of 2 ml. and incubation was at 37° for 3 hr. under an atmosphere of H_2. After heating (100°, 3 min.) the reaction mixture was centrifuged to remove precipitated protein and the supernatant fluid used for the assay of methionine. In each experiment there was a control in which the complete reaction mixture was heated initially to 100° for 5 min.

Methionine was determined microbiologically with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 as described by Gibson & Woods (1960). It was found incidentally that this organism responds as well to DL-methionine sulphoxide as to DL-methionine.

Measurement of mass of organisms. Extent of growth and the dry weight of organisms was assessed as described by Wijesundera & Woods (1960) using an EEL photoelectric colorimeter (Evans Electroselenium, Halstead, Essex). For strain PA15 a reading of 15 corresponded to a dry weight of 0·5 mg./ml.

Expression of results. Since the interest was in enzyme activity per unit of organism rather than in total enzyme present in a culture or suspension, results have been expressed as μmole L-methionine formed/mg. dry wt. organism. In the case of cell-free extracts the basis used is mg. protein of the extract.

Chemicals. Most of the special chemicals used were as described by Guest *et al.* (1960). DL-Methionine sulphoxide was a laboratory specimen (m.p. 237° uncorrected) whose origin could not be traced. The sources of other analogues of methionine were: DL-α-methylmethionine (Sigma Chemical Co., St Louis, Mo., U.S.A.); L-S-methyl-cysteine (California Corporation for Biochemical Research, Los Angeles, U.S.A.); DL-methionine sulphone (Nutritional Biochemicals Inc., Cleveland, Ohio, U.S.A.); DL-ethionine (Roche Products Ltd., Welwyn Garden City, Herts).

The analogues and other amino acids were tested for possible contamination with methionine by assay with *Leuconostoc mesenteroides* P60. They were also tested by chromatography on paper; descending chromatograms were developed on Whatman no. 1 paper with a saturated solution of water in collidine and with ninhydrin (2% in *n*-butanol saturated with water) as detecting agent. Methionine and possible impurities were used as markers.

RESULTS

Since the strains of *Escherichia coli* used in the present work differed from those studied by Wijesundera & Woods (1960) it was first necessary to establish that growth in the presence of methionine also decreased the ability of the present strains to synthesize methionine from homocysteine. This proved to be the case (Table 1), 90–100% repression being obtained with strain PA15 and two other strains; the repression was, however, only about 60% with the strain requiring either homocysteine or methionine for growth.

With the one strain tested (PA15) the ability of organisms grown without methionine to synthesize this amino acid varied somewhat with the age of the culture

from which the organisms were harvested. It was maximal at a culture density of 0·5 mg. dry wt./ml., but was only 30 % at 1·5 mg. dry wt./ml. Young cultures were therefore used for the remaining work.

Table 1. *Effect of growth with methionine on the synthesis of methionine by various strains of* Escherichia coli

Organisms grown on medium GL (plus stated growth factor) with and without DL-methionine (10 mM). Organisms were harvested at a similar stage of growth and tested for methionine synthesis in solution MSA; incubated 3 hr.

Strain	Growth factor(s) added	L-Methionine formed (μmole/mg. dry wt.)		% decrease when methionine present during growth
		Methionine absent during growth	Methionine present during growth	
PA15	Glycine	2·40	0·10	96
C21	Uracil + arginine	0·90	0·07	92
15T⁻	Thymine	1·05	0·11	90
26/18	Homocysteine	0·84	0·31	63

Table 2. *Effect of growth on methionine on the activity of ultrasonic extracts of* Escherichia coli *PA*15

Organisms were grown on medium GL + glycine with the stated concentration of methionine. Ultrasonic extracts prepared from the harvested organisms and assayed for methionine synthesis in solution MSB. Cobalamin (0·37 μM) was present in all tests in which tetrahydropteroylglutamate or its formaldehyde derivative was used. Incubated 3 hr. EHC = extract of heated *E. coli*; PtH_4G = tetrahydropterolyglutamic acid and PtH_4G-CH_2O = its formaldehyde derivative.

C_1 donor ...	Serine	Serine	PtH_4G	Glycine	Glycine
Folic acid cofactor ...	EHC	PtH_4G	$-CH_2O$	EHC	PtH_4G
DL-Methionine (M) in growth medium	L-Methionine formed (μm mole/mg. protein)				
0	170	180	61	40	40
10^{-6}	158	173	57	39	—
10^{-5}	141	151	51	39	—
10^{-4}	122	117	35	31	—
10^{-3}	34	34	20	18	—
10^{-2}	22	25	17	15	10

Comparable figures (μm mole L-methionine/mg. dry wt.) for whole organisms tested with serine as C_1 donor were (M concentration of DL-methionine in growth medium in parenthesis) 1700 (0), 1500 (10^{-6}), 1300 (10^{-5}), 780 (10^{-4}), 240 (10^{-3}) and 140 (10^{-2}).

Enzymic extracts of organisms grown in the presence of methionine

Guest *et al.* (1960) established three conditions for the synthesis of methionine from homocysteine by ultrasonic extracts of *Escherichia coli* PA15 according to the nature of the donor of the C_1 unit and of the source of folic acid acting as the cofactor for the C_1 transfer reaction. These were: (1) serine as donor and extract of heated *E. coli* as cofactor; (2) serine as donor and tetrahydropteroylglutamate (in the presence of cobalamin) as cofactor; (3) the formaldehyde derivative of

tetrahydropteroylglutamate (again in the presence of cobalamin) both as donor and cofactor. Extracts of organisms grown in the presence of a range of concentrations of methionine were tested under all three conditions; in addition glycine was used as C_1 donor in place of serine under conditions (1) and (2). In all cases growth in the presence of methionine at 10 μM and above led to significant decrease in enzyme activity; at 10 mM the decrease reached 80–90 % (Table 2). The sharpest increase in repression occurred between 0·1 mM and mM methionine. The maximum degree of repression was not, however, quite so great as that observed with intact organisms (about 95 %). Mixtures of ultrasonic extracts prepared from 'active' and 'inactive' organisms had an activity equal to the sum of the separate activities; the 'inactive' extracts therefore did not contain an inhibitor.

Synthesis of methionine with glycine as C_1 donor is less active than with serine, but marked repression was again obtained (Table 2). Growth of strain 26/18 on homocysteine instead of methionine increased the activity of ultrasonic extracts by two- to threefold.

Loss of ability to synthesize methionine

'Active' organisms were resuspended in fresh medium GL+methionine and incubated with shaking. Samples were removed, the organisms re-harvested and again tested for their ability to synthesize methionine. The specific activity of the organisms decreased as would be expected if new enzyme were formed only in the restricted amount anticipated for organisms growing with methionine. The total activity present in the culture rose only slowly and the activity per unit organism decreased theoretically with the increasing amount of new growth (Fig. 1). The theoretical curve in Fig. 1 was computed on the assumption (from previous experiments) that the specific activity of organisms grown in the presence of methionine is 0·12 μmole L-methionine/mg. dry wt./3 hr. Then if the original activity of the organisms grown without methionine is a μmole L-methionine/mg. dry wt./3 hr., then the specific activity after n generations is

$$a/2^n + 0{\cdot}12(2^n - 1)/2^n.$$

A control in which the organisms were resuspended in medium GL without methionine showed no significant changes in specific activity (Fig. 1).

When the harvested organisms were re-incubated with methionine in conditions in which no growth occurred (mM-2:4-dinitrophenol present or source of nitrogen omitted from medium GL) the specific activity of the organisms fell only very slowly (15 % in 5 hr., the time equivalent to three generations when growth occurred.

Release from repression; formation of the enzyme by growing cultures

'Inactive' organisms were harvested after growth with methionine (10 mM) and resuspended in fresh medium without methionine at a density about equal to that of the original culture at the time of harvesting. The organisms rapidly regained ability to synthesize methionine from homocysteine (Fig. 2) and even before detectable multiplication began there was usually a six- to tenfold increase in activity. A control batch of organisms resuspended in medium containing methionine maintained its low order of activity, though growth was similar to that in the

culture without methionine (Fig. 2). Ultrasonic extracts prepared from samples taken during the period of increasing activity of the whole organisms also showed a large increase in specific activity (Fig. 3).

Effect of methionine. Addition of methionine at any stage during the recovery of enzyme activity caused the rapid cessation of new enzyme formation and the specific activity ultimately decreased as expected for the dilution of existing organisms by new organisms containing only limited enzyme activity (Fig. 4).

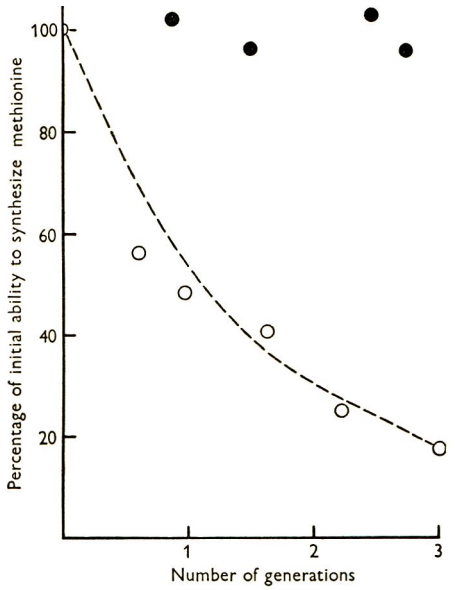

Fig. 1

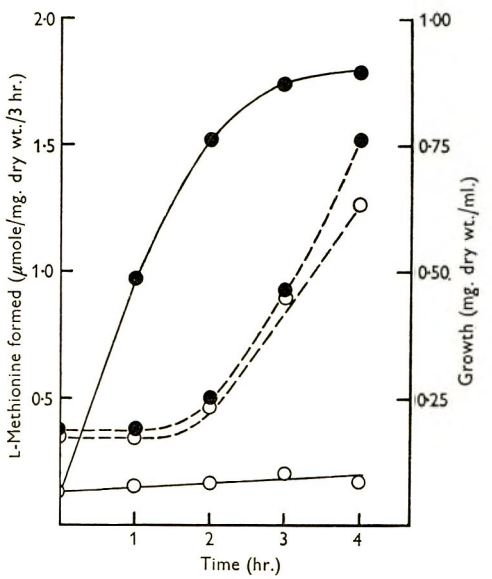

Fig. 2

Fig. 1. Effect of the transfer of organisms grown without methionine to fresh medium with or without methionine. Organisms were harvested from medium GL and resuspended (0·2 mg. dry wt./ml.) in medium GL with (○) or without (●) 10 mM-DL-methionine and incubated with shaking at 37°. Ability of organisms harvested from samples to synthesize methionine was tested in solution MSA. Theoretical curve for ○ (see text) indicated by – – –.

Fig. 2. Effect of the transfer of organisms grown with methionine to fresh medium with or without methionine. Organisms were harvested from medium GL and resuspended (0·2 mg. dry wt./ml.) in medium GL with (○) or without (●) 10 mM-DL-methionine and incubated with shaking at 37°. ———, ability to synthesize methionine (tested in solution MSA). – – –, growth.

The initial decrease was not so great as expected, indicating that there was a lag period before methionine exerted its full effect. This lag was more apparent when methionine was added at 1 hr. (Fig. 4) since new growth was negligible between 1 and 1·5 hr. Graded concentrations of methionine were also added initially before incubation; the extent of recovery of enzyme activity decreased with increasing methionine concentration, the sharpest change occurring between μM and 10 μM (Table 3).

Effect of chloramphenicol. Recovery by 'inactive' organisms of their ability to synthesize methionine when transferred to a medium without methionine was

Table 3. *Effect of the initial addition of methionine on the recovery of enzyme activity*

'Inactive' organisms (strain PA15) harvested from growth in the presence of DL-methionine (10 mM). Resuspended (0·3 mg. dry wt./ml.) in medium GL + glycine and the stated concentrations of DL-methionine. Organisms re-harvested from samples and tested for methionine synthesis in solution MSA.

DL-Methionine added to suspension (M)	L-Methionine formed (μmole/mg. dry wt./3hr.) by organisms after incubation of suspensions for (hr.)		
	0	1	3
0	0·13	0·85	1·85
10^{-6}	0·13	0·7	1·82
10^{-5}	0·13	0·34	0·39
10^{-4}	0·13	0·20	0·28
10^{-3}	0·13	0·18	0·24
10^{-2}	0·13	0·15	0·16

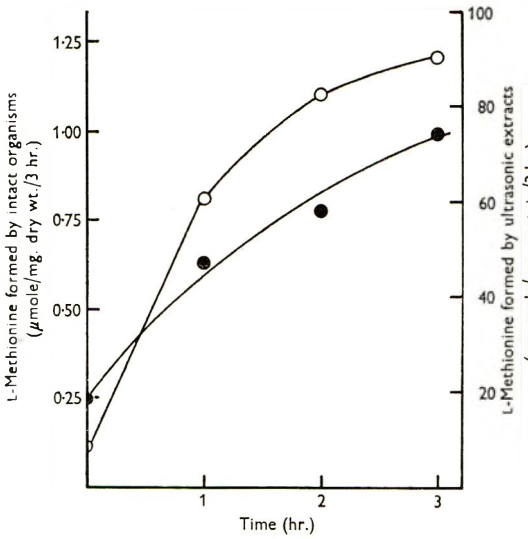

Fig. 3

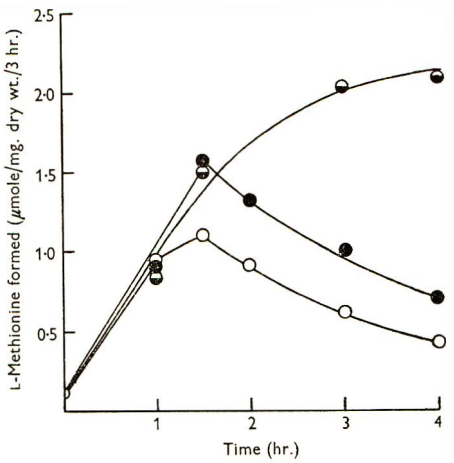

Fig. 4

Fig. 3. Effect of the transfer of organisms grown with methionine to fresh medium without methionine on the activity of ultrasonic extracts. Procedure as for Fig. 2 except that ultrasonic extracts were also prepared from the samples and tested for methionine synthesis in solution MSB. Activities: ○, intact organisms; ●, ultrasonic extract with DL-serine as C_1 donor and extract of heated *Escherichia coli* as cofactor.

Fig. 4. Effect of the addition of methionine at intermediate stages in the recovery of enzyme activity. 'Inactive' organisms (strain PA15) were harvested from growth with DL-methionine (10 mM), resuspended (0·25 mg. dry wt./ml.) in fresh medium without methionine and incubated. DL-methionine (10 mM) was added after 1 hr. (○), 1·5 hr. (●) or not at all (control) (◒).

completely prevented by chloramphenicol; furthermore, the antibiotic also stopped enzyme production when added at intermediate stages of the recovery (Fig. 5). Since chloramphenicol at the concentration used also almost completely inhibited

growth, the specific activity of the enzyme in this case remained constant or decreased slowly rather than showing the sharp decline which occurs when new organisms dilute the existing activity. Chloramphenicol is known to inhibit protein synthesis and these results suggest that there is *de novo* synthesis of enzyme protein when the organism recovers its ability to convert homocysteine to methionine.

Effect of amino acids. At the concentration used in most experiments (mM), *p*-fluorophenylalanine inhibited growth of the suspensions completely when added initially. There was very slight growth (10 % of the control) when the addition was delayed for 1 hr. Inhibition of growth was not however material from the present point of view since, as noted above, considerable enzyme formation occurred initially under the experimental conditions without detectable growth. Recovery of activity in synthesizing methionine was decreased by 80 % when *p*-fluorophenylalanine (mM) was added initially, while it was stopped completely when the addition of analogue was delayed for 1 hr., i.e. until recovery was already in progress (Table 4). A lower concentration of *p*-fluorophenylalanine (0·3 mM) inhibited enzyme production by about 60 %. The inhibitory effect of *p*-fluorophenylalanine was overcome completely by an equimolar concentration of phenylalanine, indicating that the inhibition specifically concerned the utilization of the latter amino acid. This is again in accord with the hypothesis that recovery is dependent on *de novo* synthesis of protein.

Table 4. *Effect of* p-*fluorophenylalanine on the recovery of enzyme activity*

'Inactive' organisms (strain PA15) were harvested from growth in the presence of DL-methionine (10 mM), resuspended (0·4 mg. dry wt./ml.) in medium GL + glycine and incubated with the supplements stated (added initially unless otherwise indicated). Organisms were re-harvested from samples and tested for methionine synthesis in solution MSA.

Expt.	Additions to suspension	L-Methionine formed (μmole/mg. dry wt./3 hr.) by organisms after incubation of suspension for (hr.) 0	1	3
1	None (control	0·13	0·82	1·99
	p-Fluorophenylalanine (mM)	0·13	0·40	0·50
	p-Fluorophenylalanine (0·3 mM)	0·13	0·56	0·73
	p-Fluorophenylalanine (mM) + phenylalanine (mM)	0·13	0·86	1·98
2	None (control)	0·12	0·42	1·34
	p-Fluorophenylalanine (mM)	0·12	0·29	0·41
	p-Fluorophenylalanine (mM)*	0·12	0·58	0·48

* Added after incubation in progress for 1 hr.

Another method by which the amount of a given amino acid present in a culture may be strictly controlled is by the use of auxotrophic strains which cannot synthesize the amino acid in question. Strains PA15 and C21 required for growth glycine (or serine) and arginine, respectively; 'inactive' organisms of both strains showed greatly diminished recovery of ability to synthesize methionine when the required amino acid was omitted from the suspension (Table 5). In the one case tested (strain PA15) the extent of recovery was proportional to the concentration

of amino acid (glycine) added. The fact that limited recovery occurred in the absence of added amino acid may have been due to some being present within the organism at the beginning of the experiment; the organism had, of course, been grown originally on a medium containing the required amino acid.

Table 5. *Effect of the absence of certain amino acids on the recovery of enzyme activity*

'Inactive' organisms were harvested after growth on medium GL + DL-methionine (10 mM) and glycine (strain PA 15) or arginine + uracil (strain C21). Resuspended (0·4 mg. dry wt./ml.) in medium GL containing the supplements stated and, in the case of strain C21 only, uracil (mM). Organisms re-harvested from samples and tested for methionine synthesis in solution MSA.

Strain	Addition to suspension	L-Methionine formed (μmole/mg. dry wt./3 hr.) by the organisms after incubation of suspension for (hr.) 0	1	3
PA 15	None	0·13	0·33	0·49
	Glycine (0·05 mM)	0·13	0·71	0·64
	Glycine (0·2 mM)	0·13	0·96	0·93
	Glycine (mM)	0·13	1·20	1·45
C21	None	0·07	0·18	0·13
	Arginine (10 mM)	0·07	0·30	0·42

Table 6. *Effect of the absence of certain pyrimidines on the recovery of enzyme activity*

'Inactive' organisms harvested after growth on medium GL + DL-methionine (10 mM) and thymine (strain 15T⁻) or arginine + uracil (strain C21). Resuspended (0·5 mg. dry wt./ml.) in medium GL containing the supplements stated and, in the case of strain C21 only, L-arginine (10 mM). Organisms re-harvested from samples and tested for methionine synthesis in solution MSA.

Strain	Additions to suspension	L-Methionine formed (μmole/mg. dry wt./3 hr.) by organisms after incubation of suspensions for (hr.) 0	1	3
C21	None	0·07	0·25	0·54
	Uracil (mM)	0·07	0·30	0·50
15T⁻	None	0·10	0·49	0·96
	Thymine (mM)	0·10	0·57	1·02

Effect of pyrimidines and purines. Auxotrophic strains requiring uracil and thymine respectively for growth were used to examine whether the presence of pyrimidines was necessary for the recovery of enzyme activity by 'inactive' organisms. Recovery occurred without addition of uracil (Table 6). Thymine was also not necessary and its presence did not significantly affect the rate of formation of new enzyme (Table 6).

Possible requirements of purines and pyrimidines for regeneration of the enzyme were also explored with strain PA15 by the use of potentially inhibitory analogues of these substances. 8-Azaguanine (2 mM) did not prevent 'inactive' organisms regaining activity under the usual conditions nor did it affect the growth rate of the suspensions under test; it did, however, increase markedly the lag phase in growth

tests with small inocula. 8-Azaxanthine (0·2 mM) was also without effect on regeneration of enzyme, as were also three pyrimidine analogues (2-thiouracil, 2-thiocytosine and 2-thiothymine, each tested at 0·2 mM). The last four compounds at the concentration tested also did not significantly affect the growth rate of the suspensions.

Source of energy. 'Inactive' organisms failed to regenerate the enzyme when glucose and lactate were omitted from the medium in which the organisms were suspended or when an uncoupling agent (mM-2:4-dinitrophenol) was added to the medium. There was also no growth under either condition.

Effect of analogues of methionine on the production of the enzyme

These tests were also carried out in the test system in which harvested 'inactive' organisms regain ability to synthesize methionine when transferred to a medium not containing the amino acid. Norvaline and norleucine were included among the compounds tested since they inhibit the growth of strain PA15, such inhibition being overcome specifically by methionine. All the analogues tested were free from methionine as judged by chromatography on paper and (except in the case of methionine sulphoxide which could not be tested in this way) assay with *Leuconostoc mesenteroides* P60.

Table 7. *Effect of analogues of methionine on enzyme formation*

'Inactive' organisms (strain PA15) were harvested from growth on medium GL + DL-methionine (10 mM) + glycine. Resuspended (0·35 mg. dry wt./ml.) in medium GL + glycine and the supplements indicated. Organisms re-harvested after incubation for 3 hr. and tested for methionine synthesis in solution MSA.

Compound added to suspension	L-Methionine formed (μmole/mg. dry wt./3 hr.)	Repression of activity (%)
None	2·20	—
DL-Methionine (mM)	0·22	90
DL-Methionine sulphoxide (mM)	0·32	86
DL-Methionine sulphoxide (0·1 mM)	0·53	76
DL-Methionine sulphoxide (0·01 mM)	1·96	11
DL-Methionine sulphoxide (μM)	2·20	0
DL-Methionine sulphone (mM)	0·95	57
DL-Ethionine (mM)	1·38	37
L-S-Methylcysteine (mM)	1·20	45
DL-α-Methylmethionine (mM)	0·92	58
DL-Norvaline (mM)	1·15	48
DL-Norleucine (mM)	0·66	70

Methionine sulphoxide (mM) repressed enzyme formation almost as much as methionine itself at the same concentration (Table 7), but a titration showed that it had at most only a quarter of the activity of methionine (compare Tables 3 and 7). It is probable that the activity of the sulphoxide is due to its conversion to methionine. Sourkes & Trano (1953) showed that methionine sulphoxide was reduced to methionine by suspensions of *Escherichia coli* in the presence of H_2, and auxotrophs of the same organism which require methionine for growth also respond to the sulphoxide (Lockingen, Humphrey & Wyss, 1958; unpublished observations by the

present authors). In qualitative tests by the paper chromatographic technique, both intact organisms and ultrasonic extracts of the present test organism (*E. coli* strain PA15) converted methionine sulphoxide partially to methionine.

Methionine sulphone (mM) caused some repression (60 %); it is unlikely that this compound is converted to methionine since it does not support the growth of auxotrophs of *Escherichia coli* which require methionine. The result with ethionine (about 40 % repression) with strain PA15 conflicts with that obtained by Wijesundera & Woods (1960) who found no activity with another strain (Y44).

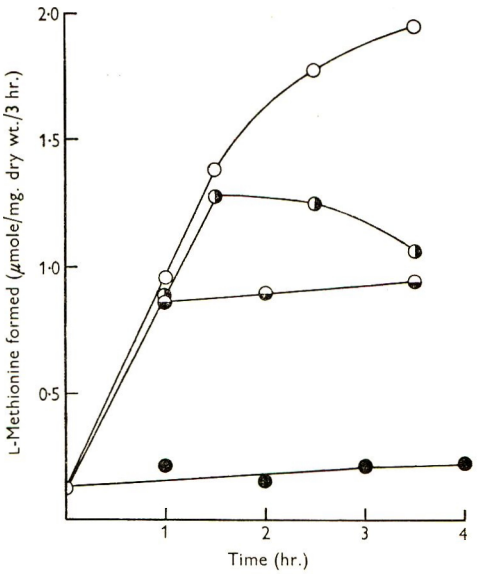

Fig. 5

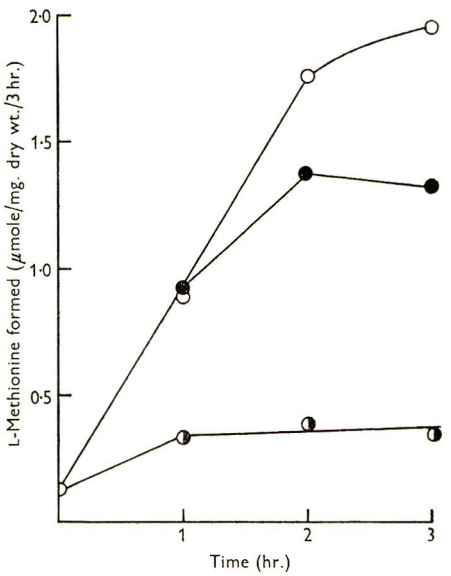

Fig. 6

Fig. 5. Effect of chloramphenicol on the recovery of enzyme activity. Experimental conditions as for Fig. 4. Chloramphenicol (0·125 mM) was added initially (●) or after 1 hr. (◒) or after 1·5 hr. (◑). Control without chloramphenicol (○).

Fig. 6. Effect of serine and other substances required for methionine synthesis on the recovery of enzyme activity. 'Inactive' organisms were harvested from growth on DL-methionine (10 mM) and resuspended in medium GL + the substances indicated. Organisms were re-harvested from samples and tested for methionine synthesis in solution MSA. Substances added to the suspension: 10 mM-glycine (○); 10 mM-DL-serine (●); 10 mM-DL-serine + 5 mM-DL-homocysteine + 15 μmM-cobalamin (◑).

Norvaline and norleucine (both mM) gave 50–70 % repression (Table 7). In growth tests from small inocula both amino acids inhibited growth by about 75 % at 0·1 mM. Under these conditions, however, organisms grown with norvaline had normal ability to convert homocysteine to methionine, while with those grown with norleucine the activity was only about 40 % of that of the controls grown without norleucine.

Effect of substrates for methionine synthesis on the formation of the enzyme

The main substrates required for methionine synthesis (serine and homocysteine) might affect recovery of ability to synthesize methionine in either of two ways. First, they might have intrinsic activity in repressing the formation of the enzyme. Secondly, as the enzyme was formed, their presence might lead to methionine

production by the suspension, the methionine in turn repressing the formation of more enzyme; in this case a lag would be expected before the effect became maximal. Cobalamin was included in some of the experiments to be described because it markedly increases synthesis of methionine by suspensions of strain PA15 (Gibson & Woods, 1960).

Strain PA15 will grow when provided with either serine or glycine, though glycine was the supplement used in medium GL for all experiments so far described. The effect on the recovery of enzyme activity of substituting serine for glycine in the medium to which 'inactive' organisms were transferred is shown in Fig. 6. During the first hour of incubation there was no significant difference but later the rate of increase of specific activity decreased in the presence of serine more rapidly than with glycine. When both serine and glycine were present the results were the same as those for serine alone. When the suspension fluid was supplemented with a mixture of serine, homocysteine and cobalamin there was initially only a small increase in enzyme activity and this did not increase further (Fig. 6). The suspending fluid was found to contain 0·05 mM-L-methionine at the end of the experiment. The individual effect of homocysteine was comparable with that of serine though that of cobalamin, though significant, was smaller (Table 8).

Table 8. *Effect of homocysteine and cobalamin on enzyme formation*

'Inactive' organisms (strain PA15) were harvested from growth on medium GL + DL-methionine (10 mM) + glycine. Resuspended (0·3 mg. dry wt./ml.) in medium GL + glycine and the supplements indicated. Organisms were re-harvested from samples and tested for methionine synthesis in solution MSA.

	L-Methionine formed (μmole/mg. dry wt./3 hr.) by organisms after incubation of suspension for (hr.)		
Addition to suspension	0	1·5	4
None	0·10	0·82	1·73
Cobalamin (3 μmM)	0·10	0·78	1·54
DL-Homocysteine (mM)	0·10	0·59	1·28

Similar, though more marked, repression of enzyme activity is found when the test substances are added initially to cultures developed from small inocula. Thus Gibson & Woods (1960) found the activity of organisms (strain PA15) grown on serine to be about half that of organisms grown on glycine. In the present work growth with cobalamin (3 μmM) or DL-homocysteine (mM) gave organisms with only 30 and 25 %, respectively, of the activity of control organisms grown without these substances.

Taken as a whole the results presented in this section suggest that the effects of serine, homocysteine and cobalamin on the development of the enzyme are due to the fact that they cause increased synthesis of methionine which in turn represses the formation of the enzyme system concerned in such synthesis.

DISCUSSION

The major part of the regeneration of enzyme activity takes place before multiplication of organisms has become significant and it occurs without lag and at a constant rate (Fig. 2). It is likely therefore that the new activity appears in the majority of organisms in the population and the conclusion of Wijesundera & Woods (1960) that the change is a phenotypic one is supported. When methionine is added to a suspension of active organisms either initially (Fig. 1) or during the development of activity (Fig. 4) there is no actual loss of enzyme, that present being simply diluted out by new organisms which contain much less enzyme.

Evidence has been obtained (Vogel, 1960) that growth of *Escherichia coli* on arginine represses a system which permits its precursor, acetylornithine, to enter the organism. In the present case repression of the ability of whole organisms to synthesize methionine from homocysteine was accompanied by almost as great a comparative decrease in the activity of cell-free enzyme preparations (Table 2). It is clear therefore that a change to decreased permeability to homocysteine could account at most for a minor part of the present effect.

Limitation of the supply of amino acids, either by the use of auxotrophs unable to synthesize a given amino acid or by the use of an amino acid analogue (*p*-fluorophenylalanine), leads to severe restriction of new enzyme formation. In addition, chloramphenicol inhibits the process either when added initially or during the course of the development of the enzyme. A source of energy is also required. It is likely therefore that *de novo* synthesis of protein from amino acids is required for the regeneration of ability to synthesize methionine rather than it being a case of the reactivation of an inactive or masked protein already present.

There is evidence that the formation by *Staphylococcus aureus* of the inducible enzyme, β-galactosidase, requires the concurrent synthesis of ribonucleic acid (Gale & Folkes, 1955); furthermore, such enzyme formation is inhibited by the purine analogue 8-azaguanine (Creaser, 1956). 8-Azaguanine did not however inhibit β-galactosidase formation in a strain of *Escherichia coli* (unpublished observations of Dr J. Lascelles in this laboratory). Furthermore, ribonucleic acid synthesis does not appear to go on when repressed enzymes are regenerated in *Aerobacter aerogenes* (Magasanik, Magasanik & Neidhart, 1959). In the present case restriction of uracil (by the use of an uracil auxotroph) or of purine (by the use of 8-azaguanine) did not affect regeneration of the ability to form methionine from homocysteine; it is provisionally concluded that concurrent synthesis of ribonucleic acid is not required. Similarly, lack of any effect when thymine was restricted by the use of a thymine-requiring mutant suggested that synthesis of deoxyribonucleic acid was not essential, though its presence is no doubt required.

A number of compounds structurally related to methionine also repressed formation of the enzyme though none approached the activity of methionine itself. The inhibition of growth by norleucine may be partly due to repression of formation of the enzyme which synthesises methionine, since when growth was limited by norleucine the organisms showed 60 % repression of the enzyme. With norvaline, however, higher concentrations were required for repression than for inhibiting growth. Cohen & Jacob (1959) found that repression by methionine was not shown by norleucine-resistant mutants of *Escherichia coli*.

The addition to suspensions capable of regenerating the enzyme of substrates (serine, homocysteine) or a cofactor (cobalamin) for methionine synthesis led to some decrease in enzyme formation. This was more marked when the compounds were added to cultures growing from small inocula and was probably due to stimulation of methionine synthesis by the enzyme initially present followed by repression of new enzyme formation by the methionine when formed. When a mixture of serine, homocysteine and cobalamin was added to a suspension of organisms, enzyme formation ceased almost completely after a short initial lag (Fig. 6) and methionine was detected in the reaction mixture at a concentration known to give repression of enzyme formation. This experiment demonstrates also the practical reality of control of methionine synthesis exercised through enzymic repression.

It is often considered (see, for example, Magasanik, 1959) that enzymic repression would be too slow in action to control the biosynthesis of metabolites and that its physiological function may be rather to enable the organism to attain the most economic enzyme composition for growth in a particular environment. It is also considered that control of metabolite synthesis is probably exercised by the ultimate product through inhibition of enzyme activity (rather than formation) by a feed-back mechanism (see Pardee, 1959). In the case of methionine synthesis no control mechanism of the feed-back type has so far been demonstrated. The concentration of methionine required to achieve marked repression of enzyme synthesis is small (10 μM) and may possibly be reached locally by the organism even when growing in a medium containing no preformed precursors of methionine. It seems possible that in the present case, and during the overall growth of a culture, enzymic repression may well control the amount of methionine formed, as well as adjusting the enzymic constitution of the organism when methionine is already present in the external environment. There is evidence that enzymes are repressed in other biosynthetic pathways, presumably by the endogenously synthesized metabolite, when the organism is growing in a simple medium (Ames & Garry, 1959; Maas & Gorini, 1957).

The present case of enzymic repression differs from many others (see Pardee, 1959) in that methionine inhibits the formation of the enzyme system concerned in the last step in its synthesis rather than one required at an earlier stage. Evidence has recently been obtained (unpublished experiments of R. J. Rowbury) that methionine also represses formation of cystathionase, an enzyme required for the preceding step in methionine synthesis.

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Observations on the Specificities of Extracellular Antigens of the Genera *Aeromonas* and *Serratia*

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SUMMARY

Members of the genus *Aeromonas* produced extracellular toxic antigens which were specific for this group and antisera produced against these antigens could be used to identify these organisms. Strains of *A. liquefaciens*, *A. punctata*, *A. hydrophila* and *A. formicans* were apparently very closely related; differences which existed between them were not greater than differences found among the strains of *A. liquefaciens*. Fresh isolates tended to be strongly haemolytic, produced extracellular toxins which were lethal to mice and elicited haemorrhagic lesions upon injection into the skin of rabbit. Old laboratory cultures tended to be much less haemolytic and less toxigenic. *A. salmonicida* appeared to possess extracellular antigens specific for this species but some strains of *A. liquefaciens* were found to possess this antigen.

Members of the genus *Serratia* were included in this study since Stevenson (1959) suggested that all Aeromonas strains are non-pigmented forms of Serratia. All strains of Serratia examined, regardless of their classification into species, were homogeneous and reacted with antiserum produced with one strain. Moreover, no cross-reaction was found between the antigens of Aeromonas and Serratia and it appeared unlikely that Aeromonas could be regarded as a non-pigmented member of Serratia. No cross-reaction was found between the extracellular antigens of these two genera and the other members of the families Enterobacteriaceae and Pseudomonadaceae and antisera produced with these antigens appeared useful in the identification of Aeromonas and Serratia.

INTRODUCTION

The genus *Aeromonas* is a group of Gram-negative bacilli in the family Pseudomonadaceae; they are usually motile by means of polar flagella. Unlike organisms in the genus *Pseudomonas*, those belonging to the genus *Aeromonas* ferment carbohydrate in a manner similar to that of the coliform bacteria and, therefore, the chief differences between the species of the genus *Aeromonas* and those in the genus *Paracolobactrum* are found in the arrangement of their flagella, in the less active fermentation of carbohydrates by the former, and in their pathogenicity. Although the characteristics of Aeromonas are fairly well established, no definite criterion is available at present to establish species within the genus. Snieszko (1957) used the source of isolation to differentiate three species, *A. liquefaciens*, *A. punctata* and *A. hydrophila*; and *A. salmonicida* is recognized by its pathogenicity to fish,

particularly Salmonidae. These criteria are not adequate because similar organisms may be isolated from different sources and the virulence of an organism may be lost after prolonged cultivation on artificial media. Due to the absence of any criterion for establishing a species, several new species have been proposed without adequate comparative study with standard strains of well-established species (Pivnick & Sabina, 1957; Stevenson, 1959).

The present study was initiated to find dependable criteria to establish the species within the genus *Aeromonas*. In the usual bacteriological procedures for identification of a Gram-negative bacillus of the coliform type, biochemical pattern and analyses of cellular antigens by agglutination tests are used. However, a few differences in biochemical reactions are always found even among strains of a well-known species of Aeromonas and it is difficult to determine which reactions should be taken as the differential criteria. The serological tests which use cellular antigens are frustrated by the fact that many strains of the same Aeromonas species possessed different cellular antigens. Finally, the possibility of the production of an extracellular toxin was examined because many Aeromonas strains are known to be pathogenic to cold-blooded animals, and the work of Russell (1898) suggested that the production of a toxin other than the endotoxin was possible. It was found that many fresh isolates of *A. liquefaciens* or *A. hydrophila* produced extracellular toxins, but many old laboratory cultures were relatively non-toxigenic; toxigenicity tests did not identify old laboratory strains. However, even old laboratory cultures produced non-toxic extracellular antigens which were also possessed by fresh isolates and these extracellular antigens appeared to be the most dependable criteria for identification of these Aeromonas strains. The present communication describes these findings.

METHODS

The strains of Aeromonas and Serratia used in this study are listed in Tables 1 and 2, respectively. Most of the strains of Aeromonas were obtained from Dr S. F. Snieszko (U.S. Fish and Wildlife Service, Eastern Fish Disease Laboratory, Leetown, W. Va.) and Dr R. Hugh (George Washington University, Washington, D.C.). Two strains of *A. hydrophila* isolated from sick frogs in this laboratory were also included. The strains of *A. margarita* (Stevenson, 1958) isolated from diseased desert locusts (*Schistocerca gregaria* Förs k) and one strain of *Pseudomonas noctuarum* (Lysenko, 1958) were obtained from Dr J. P. Stevenson (Department of Zoology, University College, London). Dr Snieszko stated in a personal communication that he considered that *A. liquefaciens*, *A. punctata* and *A. hydrophila* were physiological variants of one species. *A. margarita* and *P. noctuarum* were considered by Stevenson (1959) to be identical with *S. marcescens*.

All strains of Serratia were obtained from Dr L. S. McClung (Indiana University, Bloomington, Indiana). Many of these strains were from the collection of the late Dr R. S. Breed (New York State Experiment Station, Geneva, N.Y.) and were received after his death. These strains were reclassified by Hamilton (1957) according to his criteria and, as can be seen in Table 2, the designation of the species of *Serratia* differed considerably according to the criteria selected for their classification.

Technique for the production of extracellular antigens. The term 'extracellular antigen' indicates all antigenic substances produced by the organisms which

Table 1. *The list of Aeromonas strains used*

Strain no.	Genus	Species	Obtained from	Original source or source of isolation
U-14	*Aeromonas*	*liquefaciens*	Dr Snieszko*	Guppy
U-15	*Aeromonas*	*liquefaciens*	Dr Snieszko	Guppy
U-21	*Pseudomonas*	*punctata*	Dr Snieszko	Dr Rucker (Seattle)
U-23	*Pseudomonas*	*punctata*	Dr Snieszko	Dr Lassleben (Germany)
U-40	*Aeromonas*	*liquefaciens*	Dr Snieszko	Dr Kluyver
U-41	*Aeromonas*	*liquefaciens*	Dr Snieszko	Dr Kluyver
Canada-1958	*Aeromonas*	*salmonicida*	Dr Snieszko	Salmon
Leetown-1959	*Aeromonas*	*salmonicida*	Dr Snieszko	Salmon
A-H-1	*Aeromonas*	*hydrophila*	This laboratory	Frog
A-H-2	*Aeromonas*	*hydrophila*	This laboratory	Frog
251	*Aeromonas*	*hydrophila*	Dr Hugh†	ATCC 7965§
513	*Aeromonas*	*formicans*	Dr Hugh	ATCC 13136. Pivnick's strain 258
514	*Aeromonas*	*formicans*	Dr Hugh	ATCC 13137. Crawford's Pacific Grove strain
618	*Aeromonas*	sp.	Dr Hugh	Water of Potomac River
944	*Aeromonas*	*hydrophila*	Dr Hugh	Murray's 6, 1:A-1 from trout
829–5	*Aeromonas*	sp.	Dr Hugh	Water from Rock Creek
832-5	*Aeromonas*	sp.	Dr Hugh	Minced beef
SL 10	*Aeromonas*	*margarita*	Dr Stevenson‡	Desert locust
SL 11	*Aeromonas*	*margarita*	Dr Stevenson	Desert locust
SM 006	*Pseudomonas*	*noctuarum*	Dr Stevenson	Dr Lysenko (Czechoslovakia)

* U.S. Fish and Wildlife Service, Eastern Fish Disease Laboratory, Leetown, W. Va.
† George Washington University, Washington D.C.
‡ Department of Zoology, University College, London
§ American Type Culture Collection.

Table 2. *The list of Serratia strains used*

Strain no. assigned by Hamilton*	Name assigned by Hamilton	Name as received by McClung*	Strain no. as received by McClung	Original source
8	*S. indica*	*S. marcescens*	53A	U. of Wisconsin
11	*S. indica*	*S. marcescens*	53B	U. of Wisconsin
16*a*	*S. marcescens*	*S. rubra*	—	Ohio State Univ.
16*b*	*S. marcescens*	*S. rubra*	—	Ohio State Univ.
59*a*	*S. plymuthica*	*Serratia* sp.	2579	CDC†
60	*S. marinorubra*	*Serratia* sp.	2704	CDC
61	*S. marcescens*	*Serratia* sp.	1155	CDC
62	*S. marinorubra*	*Serratia* sp.	1176	CDC
63	*S. marcescens*	*Serratia* sp.	2268	CDC
113	*S. kiliensis*	*Erythrobacillus* sp.	PI4	Breed collection
139	*S. plymuthica*	*S. plymuthica*	HB	Breed collection
146	*S. plymuthica*	*S. plymuthica*	MW	Breed collection
151	*S. kiliensis*	*S. marcescens*	SMH	Breed collection
230*a*	*S. marcescens*	*Serratia* sp.	SM	Beth Israel Hosp. N.Y.

* Indiana University, Bloomington, Indiana.
† Communicable Disease Center, U.S. Public Health Service.
One strain of *Serratia marcescens* from the stock cultures of this department was included in this study.

could be separated readily from the cells. This antigen was produced with the cellophan plate technique already described (Liu, 1957). It consisted essentially in cutting a sheet of cellophan to fit the bottom of a Petri dish, sterilizing it by steam under pressure and placing it in the dish after the appropriate medium was poured and solidified. After the cellophan sheet was moistened, the wrinkles were carefully stretched out. About two drops of broth culture of the organism were placed on the cellophan and spread over the whole plate with a sterile cotton swab. The plate was incubated aerobically at the optimal temperature of each organism, which was 24° for *Aeromonas salmonicida* and 28° for all other Aeromonas and all Serratia species. The medium used for this purpose was the Trypticase-soy agar (Baltimore Biological Laboratories) enriched with 0·5 % glycerol. At the end of the incubation period, which was usually about 48 hr., 3 ml. sterile saline was used to wash off the growth which was centrifuged for 60 min. at 2000 *g* to remove the bacterial cells. The supernatant fluid containing the extracellular antigen was Seitz-filtered and preserved by the addition of enough thiomersal (Merthiolate, Lilly) to make the final concentration of 1/5000.

Production of antisera to the extracellular antigen. Aeromonas strains U-15, U-40, Canada-1958 and five strains of Serratia representing five species (8, 16*a*, 59*a*, 60 and 113) were used for the production of antisera. Strain U-15 was a relatively recent isolate of *A. liquefaciens* and it produced strong haemolysis on blood agar. U-40 on the contrary was an old laboratory culture which was used by the late Dr A. J. Kluyver as a typical strain of *A. liquefaciens*. It was almost rough in its colonial morphology and the extracellular antigens of this strain were found to be the least toxic among those produced by various strains of *A. liquefaciens*. It was used, therefore, as a representative of *A. liquefaciens* which became rough and non-toxigenic after a prolonged culturing on artificial media. The two strains of *A. salmonicida* did not vary much in their toxigenicity and the strain Canada-1958 was used because it was much more chromogenic and appeared to be more typical of this species.

After sterility tests of the extracellular antigen, it was mixed with an equal amount of Freund's incomplete adjuvant (a mixture of Arlacel and Bayol in the ratio of 1·5/8·5) and injected subcutaneously into rabbits at weekly intervals; 0·2 ml. was the dose for all antigens in the first week and 0·5 ml. in the second week. In the cases of U-40, Canada-1958 and all Serratia strains, 0·5 and 1·0 ml. of antigen without the adjuvant were injected intravenously in the 3rd and 4th weeks. The antigens of U-15 were so toxic that 0·2 ml. injected intravenously killed a rabbit within 8 hr. The preparation was diluted, therefore, ten times for intravenous injection. Beginning in the 3rd week, 0·2, 0·5, 1·0 and 2·0 ml. of the diluted antigen were injected intravenously at 3-day intervals. The sera were obtained 1 week after the last injection and preserved with thiomersal.

Identification tests. The technique used for the identification of these organisms with antisera was essentially that devised by Elek (1948) for the *in vitro* toxigenicity test of *Corynebacterium diphtheriae*. The medium used for this purpose contained Tryptose (Difco) 0·1 %, Casamino acids (Difco) 0·3 %, glycerol 0·5 %, Na_2HPO_4 0·3 %, NaCl 0·2 % and Bacto-agar 1·2 % (pH 7·4). The paper strip contained 0·15 ml. undiluted antiserum. Before inoculation of the organisms the plate was incubated overnight at 37° to ensure a dry surface. The inocula were always taken from fresh

cultures on blood agar. The plates which contained *Aeromonas salmonicida* were incubated at 24° because these organisms did not grow well at a higher temperature. All other plates were incubated at 28°.

RESULTS

Intradermal lesions produced by the extracellular antigens of Aeromonas and Serratia

The lesions produced by the extracellular antigens of various species of *Aeromonas* in the skin of rabbit are shown in Pl. 1, fig. 1. The extracellular antigens of U-15, A-H-1 and 514 contained toxins which produced haemorrhagic lesions. This was characteristic of most of the fresh isolates of motile Aeromonas strains, regardless of species. The haemorrhagic lesions appeared within 5 min. of the intracutaneous injection of 0·1 ml. of the crude preparation and were similar to the lesions produced by the toxin of *Pseudomonas pseudomallei* described by Nigg, Heckley & Colling (1955). The lesions reached maximum development within 3 hr. There were a few strains of the motile Aeromonas (such as U-40 and U-21 and mostly old laboratory cultures) which did not produce the haemorrhagic toxin (Pl. 1, fig. 1). It is possible that these strains produced toxin when they were first isolated but had lost this ability after prolonged cultivation on artificial media. The two strains of *A. salmonicida* did not produce the haemorrhagic toxin; since both were relatively recent isolates, the lack of haemorrhagic toxin in their extracellular antigens probably indicates a characteristic of the species.

The lesions produced by the extracellular antigens of Serratia are shown in Pl. 1, fig. 2. Fifteen strains of Serratia representing five species were tested. Preparations from some strains produced only abscesses and redness similar to the lesions produced by the endotoxin of Gram-negative bacteria, but preparations from other strains contained toxin which produced haemorrhagic and necrotic lesions. There was no correlation between pigment production and the production of these toxins, or between the type of lesion produced by a strain and its classification into species.

Neutralization of the toxic effects by antiserum

The haemorrhagic toxin of *Aeromonas hydrophila*, *A. punctata* and *A. formicans* could be neutralized by the antiserum to one strain of *A. liquefaciens* (U-15) as shown in Pl. 1, fig. 3. Quantities of serial two-fold dilutions of antiserum in saline were added to an equal amount of the toxin of *A. hydrophila* and *A. formicans* and the mixtures incubated at 37 for 1 hr.; 0·1 ml. each of the mixtures was injected intracutaneously into the rabbit. It can be seen in Pl. 1, fig. 3, that the toxins of both *A. hydrophila* and *A. formicans* were neutralized by the antiserum of *A. liquefaciens*.

Attempts to demonstrate neutralization by protection tests in mice were not successful since the lethal effect of U-15 toxin was not completely neutralized by its homologous antiserum.

Identification tests with Aeromonas species

In Pl. 2, figs. 4 and 5, are shown some of the results obtained in the agar diffusion identification test. Positive results could be noted readily by the formation near the paper strip of the so-called 'arrow head figure' which was due to precipita-

tion of antigens with their homologous antibodies. Most of the motile strains of Aeromonas used in this study were found to react readily with U-15 antiserum. There were a few strains of *A. liquefaciens* (including U-40) which did not react with U-15 antiserum and instead, they were found to react with Canada-1958 (*A. salmonicida*) antiserum, as shown in Fig. 5.

Identification tests with Serratia species

In their extracellular antigens the members of the genus *Serratia* appeared to be homogeneous. All strains of Serratia used in this study, regardless of their pigmentation and designation into species, gave a reaction with antiserum produced with one strain of Serratia. None of the Aeromonas strains except the two of *Aeromonas margarita* and the strain of *Pseudomonas noctuarum* showed reaction with antiserum of the Serratia group.

Table 3. *Summary of the reactions of various strains of Aeromonas and Serratia to three antisera*

Organisms	No. strains used	No. strains reacting to antisera		
		A. liquefaciens (U-15)	*A. salmonicida* (Canada-1958)	*S. marcescens* (16*a*)
Aeromonas liquefaciens	4	3	1	0
A. hydrophila	4	4	0	0
A. punctata	2	1	1	0
A. formicans	2	2	0	0
A. salmonicida	2	0	2	0
Aeromonas sp.	3	2	1	0
A. margarita	2	0	0	2
Pseudomonas noctuarum	1	0	0	1
S. marcescens	6	0	0	6
S. indica	2	0	0	2
S. plymuthica	3	0	0	3
S. kiliensis	2	0	0	2
S. marinorubra	2	0	0	2

The results of these identification tests are summarized in Table 3. It should be noted that most of the motile strains of Aeromonas belonging to the species *A. hydrophila*, *A. punctata* and *A. formicans* reacted readily with antiserum of *A. liquefaciens* and a few of them reacted with antiserum of *A. salmonicida*. The two strains of *A. margarita* and one strain of *Pseudomonas noctuarum* reacted readily with antiserum of *Serratia marcescens* and therefore these organisms appeared to be merely non-pigmented forms of Serratia, as suggested by Stevenson (1959). However, the generalization that all Aeromonas strains are non-pigmented forms of Serratia could not be substantiated because none of the other strains of Aeromonas showed any cross-reaction with antisera of the Serratia group.

No cross-reaction was observed between *Aeromonas*, *Serratia* and other genera of the families Enterobacteriaceae and Pseudomonadaceae; therefore antisera prepared with extracellular antigens of strains of Aeromonas and Serratia appeared to be useful in the identification of these genera.

DISCUSSION

The evidence presented in this paper indicates that the organisms belonging to the genera *Aeromonas* and *Serratia* produce extracellular antigens specific for each genus and that antisera produced with these antigens can be used to identify organisms belonging to these genera. Members of the genus *Aeromonas* are known to be pathogenic to various cold-blooded animals, such as frogs and fish (Emerson & Norris, 1905), and infection in man has been reported (Caselitz & Buck, 1958). Members of the genus *Serratia* are not generally considered to be animal pathogens but infections with members of this genus have been reported (Wheat, Zuckerman & Rantz, 1951; Vernon & Hepler, 1954). The production of toxic extracellular antigens by these organisms may play some role in the pathogenesis of these organisms.

Strains of Aeromonas which have been determined as *A. liquefaciens*, *A. hydrophila*, *A. punctata* and *A. formicans* are apparently very closely related organisms separated into different species on the basis of the source of isolation and a few biochemical reactions. The differences between these species were not greater than the differences found between the various strains of *A. liquefaciens*. The fresh isolates of this group tended to be strongly haemolytic, produced extracellular toxins and various extracellular enzymes, and most of their extracellular antigens reacted with antiserum against toxigenic strains of *A. liquefaciens*, such as U-15. *A. salmonicida* is a different organism as can be seen by its lower optimal temperature of growth, production of pigment and non-motility. However, the cross-reactions between this species and some strains of motile Aeromonas indicate that all strains of Aeromonas are closely related, and the absence of cross-reaction with other Gram-negative bacilli indicates that organisms belonging to the genus *Aeromonas* are a distinct group.

Members of the genus *Serratia* were included in this study at a later stage because Stevenson (1959) suggested that Aeromonas species are non-pigmented members of the genus *Serratia*. Complete absence of cross-reaction between strains of these two genera would seem to exclude the possibility that Aeromonas can be regarded as non-pigmented forms of Serratia. Stevenson (1959) drew his conclusion because of the similarity of *A. margarita* and *S. marcescens* and did not use typical strains of Aeromonas for comparative study. Since both strains of the so-called *A. margarita* used in this study reacted readily with antisera of the Serratia group and not with those of the Aeromonas group it was obvious that these two strains were non-pigmented members of Serratia and not Aeromonas. The results of the present study indicated that members of the genus *Serratia* were quite closely related in spite of the differences in pigment production and a few biochemical reactions which were used to differentiate them into species. Antisera produced against extracellular antigens of one strain of Serratia will identify practically all strains of Serratia regardless of their classification into species, which, according to Breed (1957), depends on minor differences such as solubility of the pigments and pellicle formation, characters that are variable. Inspection of Table 2 will show how different and confusing the classification of this group can be, depending on the criteria preferred by different workers. In view of the cross-reactions between the antigens of various strains of Serratia, classified into different species, it is doubtful whether any good can be

achieved by such a classification. A comparison between the classification of *Corynebacterium diphtheriae* and that of the Serratia species will serve to elucidate this point. The species *C. diphtheriae* has been partially established on the basis of its production of an exotoxin. The species has a certain biochemical pattern by which organisms belonging to it can be identified. Strains of the species ferment glucose, maltose and dextrin but not sucrose or lactose. The species *C. diphtheriae* is further divided into gravis, mitis and intermedius varieties by their colonial morphology and a few other biological characteristics. For example, the gravis variety tends to form a pellicle on the surface of broth and to ferment starch. Neither mitis nor intermedius varieties ferment starch and they do not usually form a pellicle. Mitis is usually haemolytic but intermedius is not. In the case of Serratia the characteristic of pellicle formation was used by Breed (1957) to separate *S. indica* from *S. marcescens*. If this criterion were to be taken for the classification of corynebacteria, a pellicle-forming strain of *C. diphtheriae* of the gravis variety would be assigned the rank of an independent species. Since some gravis strains do not form a pellicle but do ferment starch, another species would have to be created for this kind of strain. Solubility of pigments was used by Breed (1957) as a criterion in the separation of Serratia species, but pigment production may be lost by some strains. The so-called *Aeromonas margarita* is an excellent example of a non-pigmented form which is difficult to identify if pigment production is used as a distinguishing criterion.

The differences that existed between the various strains of Serratia tested, although classified into different species, did not appear to be greater than the differences that can be found among strains of other bacteria placed in a single species. The group as a whole could be placed in a single species and different subspecies might be recognized by the characteristics, such as pigment formation and the production of gas or acetylmethylcarbinol, which are not common to all strains.

The extracellular antigens produced by organisms belonging to the family Pseudomonadaceae have received very little, if any, attention in the past. This is due undoubtedly to the fact that the procedures and techniques used at present for the identification of Gram-negative bacilli are those developed primarily for the identification and classification of members of the family Enterobacteriaceae. The enteric bacteria are not active producers of extracellular enzymes or toxins and, therefore, the classification and identification of these organisms depend primarily on their biochemical reactions and cellular antigens. The members of the family Pseudomonadaceae and the genus *Serratia* (although it is included by Breed & Murray (1957) in the family Enterobacteriaceae) are much more active producers of extracellular antigens and some of these antigens, in being heat labile, have some characters of exotoxins. Both from the viewpoint of identification of the organisms and the understanding of their role in pathogenesis, these antigens deserve more attention than they have been accorded hitherto.

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EXPLANATION OF PLATES

Plate 1

All photographs in these three figures were taken 18 hr. after the injections.

Fig. 1. The lesions produced by the extracellular antigens of various species of Aeromonas in the skin of rabbit. They are (from left to right, upper row); 514 (Crawford's Pacific Grove strain of *A. formicans*), A-H-1 (*A. hydrophila*), U-15 (*A. liquefaciens*), (left to right, lower row); Canada-1958 (*A. salmonicida*), U-21 (*A. punctata*) and U-40 (*A. liquefaciens*).

Fig. 2. The lesions produced by the extracellular antigens of fifteen strains of Serratia representing five species. The antigens of some strains contained haemorrhagic and necrotic toxins while those of the other strains did not. There was no correlation between pigment production, species designation of a strain and its toxin production.

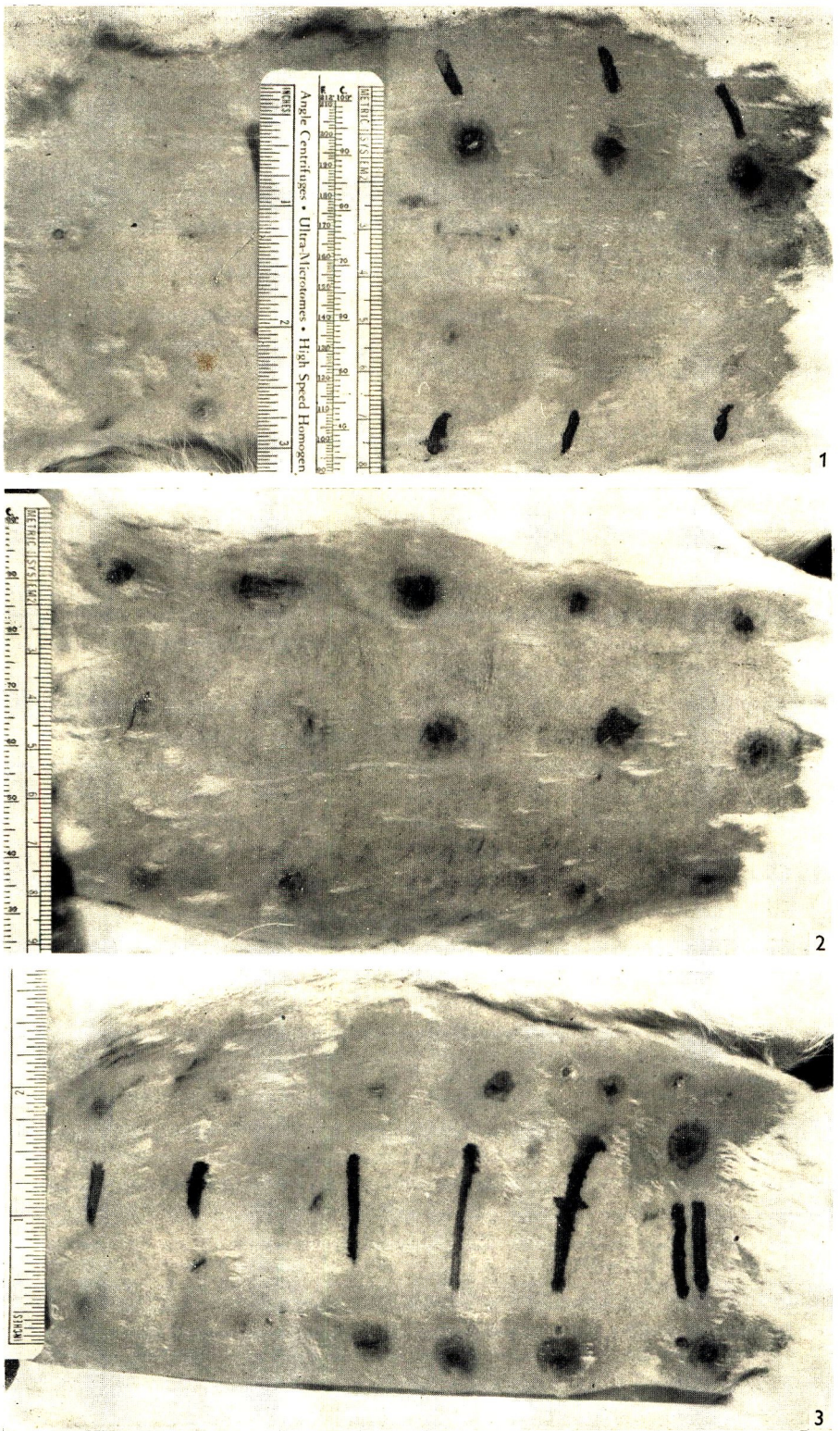
Fig. 3. Neutralization of the haemorrhagic toxins of *A. formicans* and *A. hydrophila* by *A. liquefaciens* antiserum. The upper row are the toxin of 514 (*A. formicans*) added to an equal amount of twofold dilutions of an antiserum of U-15 (*A. liquefaciens*), starting from $\frac{1}{4}$ from left side. 0·1 ml. of the mixtures was injected intracutaneously. The lower row are the toxin of A-H-1 (*A. hydrophila*) added to the same dilution of the same antiserum. The corresponding dilutions of the antiserum are connected by a black line. The control toxins of both species are connected by double lines.

PLATE 2

Fig. 4. Five strains of Aeromonas tested with antiserum of U-15 (*A. liquefaciens*). They are (left side of the paper strip from top to bottom): U-15, U-21 (*A. punctata*), U-40 (*A. liquefaciens*); (right side of paper strip from top to bottom), Canada-1958 and Leetown-1959 (both *A. salmonicida*). Note that only the strain U-15 is reacting to this serum (the so-called arrow head figure).

Fig. 5. The same five strains in the same arrangement as shown in Fig. 4 tested with an antiserum of Canada-1958. Note that both strains of *A. salmonicida* are showing strong reactions. The two strains of motile *Aeromonas* (U-21 and U-40) are also showing some reactions, although they are not as strong as those of *A. salmonicida*.

The two photographs in Figs. 4 and 5 were taken after 5 days incubation at 24°.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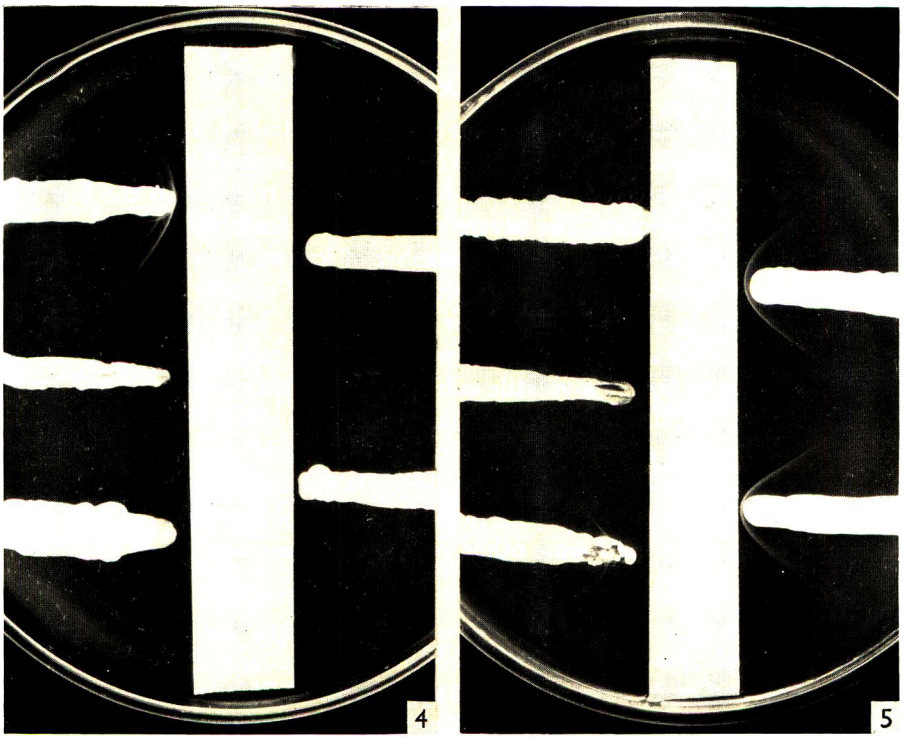
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A Volatile Substance Controlling Early Stages of Zygospore Formation in *Rhizopus sexualis*

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SUMMARY

The inhibitory effect of low temperature (*c.* 10°) on initiation and early development of zygospores of *Rhizopus sexualis* is less severe in mature cultures (in which numerous zygospores have already been formed before transfer to the low temperature) than in young ones. The severe effect of low temperature on immature cultures is not counteracted by any of a number of known growth substances, by extracts of mature mycelium and zygospores, or by used culture media. The effect of mature cultures in counteracting low temperature inhibition of zygospore production in young ones is able to pass across a gap of 5 mm. between the cultures and must therefore be due to a volatile substance (or substances) produced by the mature mycelium. By the use of small chambers which allow young test cultures to be kept at 10° while a stream of air from mature ones growing at 20° passes over them, it is confirmed that the active factor is volatile. This substance is not carbon dioxide or ammonia and is basic in nature. Its probable identity is discussed and comparison is made with some other volatile substances reported to influence growth and development of fungi.

INTRODUCTION

The initiation and early development of zygospores of *Rhizopus sexualis* (Smith) Callen and of some other members of the Mucorales are prevented by temperatures of 10° or lower (Hawker, Hepden & Perkins, 1957). Zygospores which have reached a later stage of development before being subjected to the low temperature continue to develop and eventually reach maturity. Growth of mycelium and sporangia also continues slowly at these low temperatures. Hawker *et al.* (1957) concluded that the inhibition of the early development of zygospores is due to 'a temperature-induced block in the synthesis of some substance or substances essential to further development, or to a slowing up of translocation of such substances into the gametangia from the supporting hyphae'. Occasionally young spores continue to develop in a colony exposed to the critical low temperature when older zygospores are also present. This effect might be due to the presence in relatively old cultures of a reserve of the hypothetical stimulatory substance, the synthesis of which is inhibited by low temperature.

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METHODS

The experimental methods were generally similar to those described in the previous paper (Hawker *et al.* 1957). Special methods will be described in the appropriate places in the text.

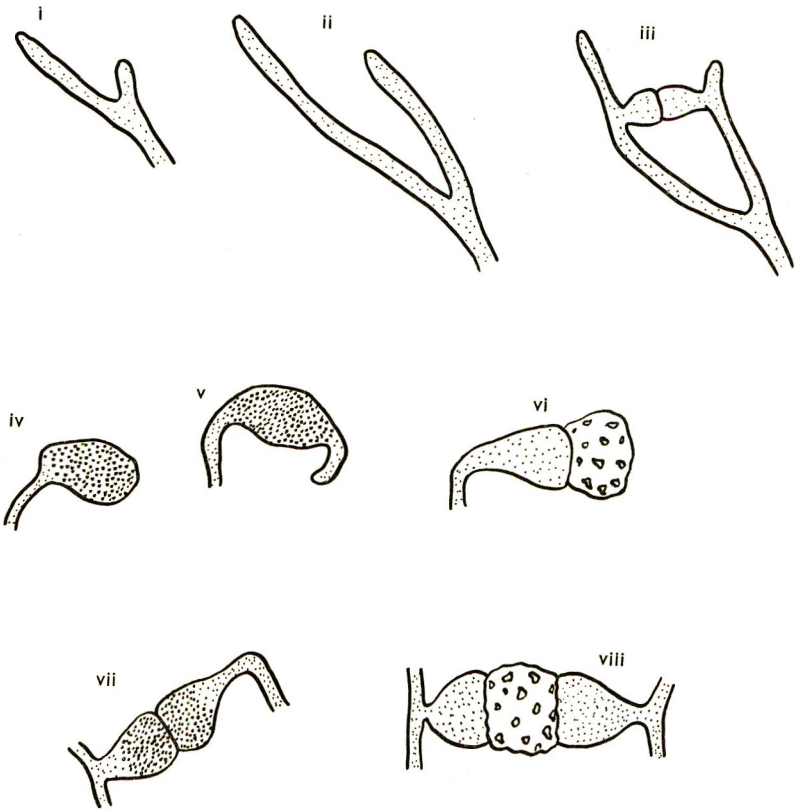

Fig. 1. Normal and abnormal development of zygospores of *R. sexualis*. i, ii, stages in formation of zygophores; iii, production of normal conjugating progametangia from zygophores; iv and v, pseudophores (or abnormal progametangia); vi, azygospore developing from a pseudophore; vii and viii, further development of normal zygospore (for comparison with pseudophore and azygospore). Diagrammatic.

In the earlier paper intensity of reproduction was expressed as the zygospore index (ZI) which was calculated from the numbers of zygospores at various defined stages of development in arbitrarily chosen microscopic fields. In the present investigation the differences were small and the zygospores were not uniformly distributed over the colony so that the use of the zygospore index was not always appropriate. Alternatively, results were expressed as total numbers of zygospores or average number in particular microscopic fields. In many experiments, however, zygospores were not formed, but the zygospores developed swellings (termed pseudophores by Callen (1940)) which have been interpreted as potential progametangia (Fig. 1) or which occasionally develop into azygospores. No method was devised to measure the frequency of these.

The culture used in early experiments was that used throughout the work described in the previous paper. During the present investigations, however, this isolate became variable and showed a general tendency to be less fertile and to produce an increased proportion of azygospores. Through the kindness of Dr Kiyosi Kominami of Tokyo, a new isolate was obtained which was used in all later experiments. The behaviour of this Japanese isolate was similar to that of the original one before the latter deteriorated.

RESULTS

Effect of natural extracts and known growth-promoting substances

Used media and extracts of mycelium. Culture media in which *Rhizopus sexualis* or other fungi had been grown were strained to remove mycelial debris, sterilized by autoclaving or by filtration and solidified with agar. When these used media were re-inoculated with *R. sexualis* growth was sometimes poor and in no example was there any counteraction of the inhibitory effect of low temperature.

Zygospore-bearing mycelium of *Rhizopus sexualis* and of some other members of the Mucorales was homogenized in a small volume of sterile distilled water. Extracts of the homogenate or of the used culture medium were concentrated (i) by evaporation under reduced pressure at 25°, (ii) by rapid evacuation and storage *in vacuo* at low temperature, or (iii) by freezing and removal of ice crystals. These extracts were added to malt agar plates, either by incorporation in the medium or by placing known volumes in holes cut from the agar after the plates had been poured. Again no counteraction of the inhibitory effect occurred.

Extracts of other natural materials. Rhizopus sexualis was grown on extracts of malt (from 1·0 to 3·0 %), potatoes, carrots, raisins, prunes and yeast, either alone or supplemented with nutrient solutions, in liquid form or in media solidified with agar. Only on carrot extract agar was the inhibition at low temperature modified; a few new zygospores were initiated on this medium after transfer to low temperature. In a typical experiment the zygospore index (calculated from twelve arbitrarily chosen microscopic fields in each of three plates) was 46 on 1 % malt extract agar and 157 on carrot agar after 50 hr. at 20°. After a further 72 hr. at 7° these figures rose to 48 and 200, an increase of approximately 4 and 22 %, respectively.

Since carrot contains a number of vitamins it was possible that one or more of these was responsible for the stimulation.

Pure vitamins. A number of vitamins (thiamine, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, cobalamin (B_{12}) and biotin), which are known to influence growth and sporulation of certain micro-organisms, were added to cooling malt extract agar or glucose asparagine agar (glucose, 10 g.; asparagine, 2 g.; KH_2PO_4 1·75 g.; $MgSO_4$, 0·175 g.; distilled water, 1 l.). In other experiments known volumes of solutions of the vitamins were placed in holes cut in plates of agar media in advance of the margin of a growing colony of *Rhizopus sexualis*. None of the vitamins tested, with the exception of B_{12}, decreased the inhibitory effects of low temperature. When a number of zygospores were already present prior to chilling, zygospore initiation continued for a limited time at low temperature when B_{12} was supplied, i.e. the effect of the presence of mature zygospores was enhanced, but not replaced, by this vitamin.

Purines. Robbins & Kavanagh (1942) showed that, for the production of mature zygospores, *Phycomyces blakesleeanus* requires an external supply of hypoxanthine in addition to the thiamin needed for mycelial growth. This result was confirmed.

Accordingly the effects of purines on *Rhizopus sexualis* were investigated. In preliminary experiments (Hawker, 1957) there was some evidence that adenine, hypoxanthine and guanine, when added to malt agar or to glucose asparagine agar, increased the rate of growth and of zygospore formation in *R. sexualis* at 20°. Furthermore, these purines showed a partial counteraction of the inhibitory effect of low temperature. In later experiments no such effect could be consistently demonstrated. The experiments were repeated many times by a variety of methods and with a wide range of concentrations of the purines, but the results with *R. sexualis* were usually negative. In a few experiments, in which the source of nitrogen was an inorganic salt, some stimulatory effect of low concentrations of purines occurred. This might be attributed to the use of these as a source of organic nitrogen and not necessarily to a specific stimulatory effect on zygospore initiation.

The effect of the presence of mature living mycelia and zygospores

The stimulatory effect on young colonies of old ones in close contact with them. Since used culture media, mycelial extracts and known growth-promoting substances did not replace mature living zygospores as a means of counteracting the low temperature effect, the influence of old cultures in close contact with young ones was studied. Segments were cut from agar plate cultures in advance of a young colony and were replaced by segments from older colonies of the same or different species. The plates were then placed at 7–10°. Zygospores continued to develop in such colonies. In a typical experiment the numbers of mature zygospores per microscopic field were 6·7 in the sector nearest the inserted segment of the mature colony and 2·6 in the rest of the young colony. No zygospores developed in comparable young colonies in the absence of the mature segment.

In other experiments an old colony of *Rhizopus sexualis*, or of a zygospore-producing species of the homothallic genus *Zygorhynchus*, growing on malt agar, was covered with boiled cellophane over which cool fresh medium was poured. The fresh medium was then inoculated with *R. sexualis*. This method also resulted in the initiation and development of a few zygospores at temperatures below that permitting spore formation in young colonies in the absence of older ones.

These results are consistent with the supply by the mature cultures of small amounts of an essential metabolite, the synthesis of which is prevented by low temperatures. Since this hypothetical substance was lacking in used culture media and mycelial extracts, it must be unstable, or volatile, or both. Attempts were made to detect the presence of such volatile substances.

The stimulatory effect on young colonies of old ones not in contact with them. The segment transfer experiments were repeated but with gaps left between the agar media on which the young (test) colony and the old (source) colony were growing. When this gap was not more than 5 mm. there was some counteraction of the effect of low temperature on zygospore initiation in the young test culture. Since the stimulus could pass over a gap of 5 mm. it is likely to be due to a volatile substance formed by the old colony.

Zygospore formation by *Phycomyces blakesleeanus* is not inhibited at 7° (Hawker *et al.* 1957). Segments of this fungus used as a source colony, without contact, induced initiation and maturation of a number of zygospores in young colonies of *Rhizopus sexualis* at that temperature.

Slide cultures of *Rhizopus sexualis* supported on glass rods over older agar plate cultures of the same or other species also produced a few zygospores at low temperature.

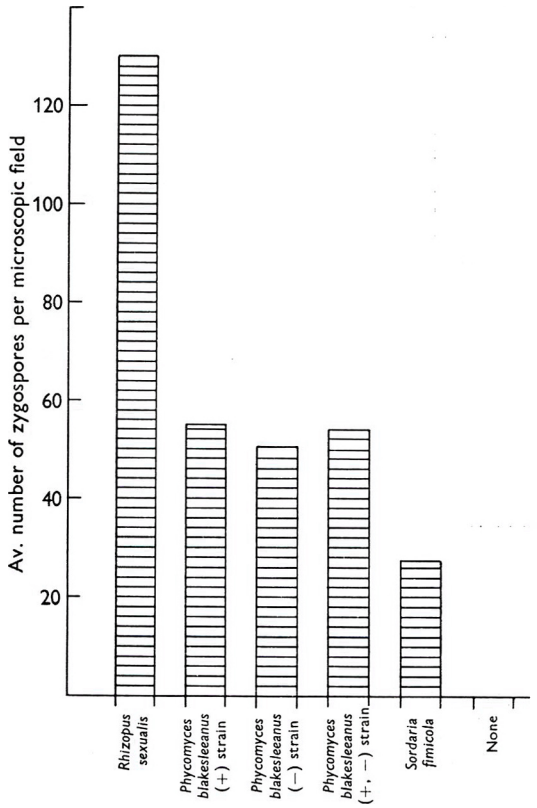

Fig. 2. The effect of volatile products of living fungi on zygospore production by *R. sexualis*. Each block represents the number of zygospores produced by *R. sexualis* in the presence of volatile substances from living cultures of the fungi indicated at base of diagram.

Stimulatory effects also occurred at a temperature of 20° which is near the optimum for *Rhizpous sexualis*. Figure 2 shows the number of zygospores in slide cultures supported over living cultures of *R. sexualis*, *Phycomyces blakesleeanus* and a Pyrenomycete (*Sordaria fimicola*) and in control slide cultures (supported over sterile plates of agar). In control cultures a number of aerial hyphae (stolons) developed before any zygophores were produced, but in treated cultures the first-formed aerial hyphae were zygophores. Zygospore production was stimulated equally by single or by paired compatible strains of the heterothallic *P. blakesleeanus*. In later experiments other sporing species of the Mucorales were tested and gave similar results. None was as effective as *R. sexualis* itself, but all were more effective than the unrelated Sordaria.

Rhizoctonia solani, a non-sporing fungus, was also tested as a source. Slide cultures of *Rhizopus sexualis* grown in the presence of *Rhizoctonia solani* showed no increase in zygospore production as compared with control cultures which had no source culture present.

Effects of a volatile stimulatory substance produced by mature colonies. Glass cylinders, 2 cm. deep and 3 cm. in diameter, with two glass tubes 3 mm. in diameter, fused into opposite sides as near the top as possible, were sealed by glass plates stuck on to the lower and upper surfaces with silicone grease (Fig. 3). The tubes were plugged with cotton wool and the units could then be autoclaved. The upper glass plate was temporarily removed and 10 ml. of molten sterile malt agar medium

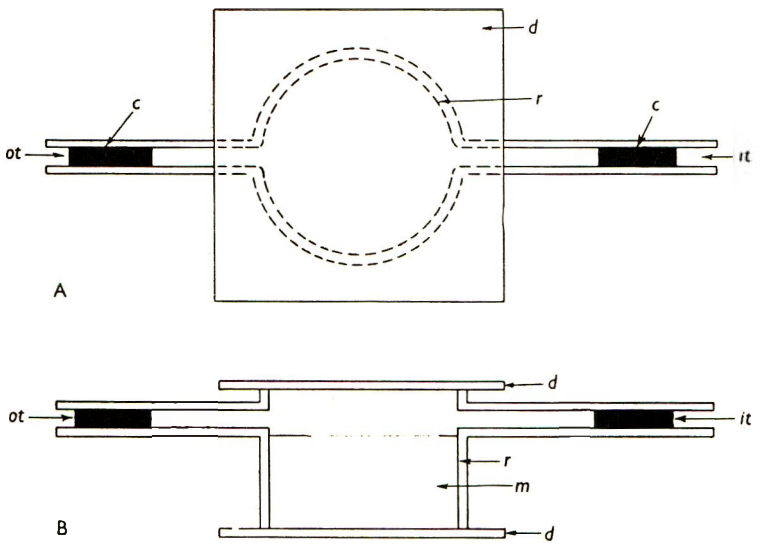

Fig. 3. Vessel designed to allow flow of gas over culture. A, plan and B, elevation of culture vessels used in experiments to test effect of volatile substances from mature cultures on zygospore production of *R. sexualis*. *r* = glass ring, *it* = inlet tube, *ot* = outlet tube, *c*, cotton-wool plug, *d* = glass disk sealed to ring (see text), *m* = agar culture medium.

were allowed to solidify on the bottom of the chamber. A suspension of sporangiospores was then poured over the agar medium and the chamber was rocked until the suspension was evenly distributed over the surface of the agar, after which the glass lid was finally replaced. The inoculated units could be connected together and an air stream passed through the series. The series could be arranged so that the air stream passed over mature cultures (usually growing in large flasks) as *source cultures*, before passing over young ones (*test cultures*); the cultures could be maintained at different temperatures so that the air passed from cultures at optimum temperatures over others at low temperatures; the effluent from mature cultures could be scrubbed by passage through various solvents to remove metabolic products likely to be present, or known gases could be passed through the system. The test cultures could be conveniently examined *in situ*.

In all experiments the source cultures were grown for 48 hr. at 20°, by which time zygospores were numerous, before being used. Test cultures were grown at

20° for 24 hr., by which time a few young zygospores but no mature ones had developed, or for 48 hr. before being treated. Air was then passed from source to test cultures. In the first few experiments the air was passed in continuous flow, but in later ones the total gas space of the system was determined and the air supply was controlled manually in such a way that the air over the source cultures was moved on over the test ones every 4 hr., thus allowing a longer period for any volatile product to accumulate over the source colonies and later for it to influence the test colonies. Moreover, this method avoided differences due to slight irregularities in flow rate. The results were more consistent than those with continuous flow but otherwise confirmed them. Control cultures received air which had passed over uninoculated culture medium.

The results of three typical experiments are shown in Table 1. In the first and second of these both source and test cultures were maintained at 20° and treatment was commenced 24 hr. after inoculation of the test culture. The rate of zygospore

Table 1. *The effect on zygospore production at* 20° *and at* 7° *of a stream of air which has previously passed over a mature culture growing at* 20°

Age of test culture at commencement of treatment	Temperature at which test culture was treated	Treatment of test cultures*	No. of replicates (10 fields counted in each)	Av. no. of zygospores per microscopic field
24 hr.	20°	Treated	9	75
		Control	6	51
		Treated	8	17†
		Control	12	6†
48 hr.	7°	Treated	6	10
		Control	6	7

* Treated and control cultures received air which had previously passed over mature source cultures and over uninoculated culture media, respectively.

† Only mature zygospores counted in this experiment.

production and maturation was greater in the treated than in the control cultures. In the third experiment the test cultures were transferred to 7° and treatment was begun 48 hr. after inoculation. The number of new zygospores initiated and the number of young ones maturing was greater in the treated than in the control cultures. In contrast the number of sporangia was greater in the controls. In a further experiment in which both source and test cultures were transferred to 7° 48 hr. after inoculation there was no stimulatory effect of the source upon the test one. Under these conditions it is likely that the rate of metabolism of the cultures would be so slow that the amount of volatile stimulatory substance produced by the source culture would be negligible, or that the substances would pass over too quickly for the test colonies to use it.

It is thus clear that some volatile product of metabolism stimulated zygospore development and that an adequate supply of it from external sources counteracted the inhibitory effect of low temperature. One must conclude that this inhibitory effect was at least partially due to failure of the fungus to synthesize the unknown volatile product at the low temperature.

Possible nature of the volatile stimulatory substance. The observation that cultures of *Rhizoctonia solani* do not produce volatile substances capable of stimulating zygospore production by Rhizopus indicates that the active factor is unlikely to be a common metabolic product, such as carbon dioxide, ammonia or ethanol. Moreover, no increase in zygospore production resulted when carbon dioxide or ammonia, at concentrations permitting vegetative growth, were passed over a young culture in a chamber of the type described in the previous section. The removal of carbon dioxide or ammonia, by passage through traps placed between source and test cultures and containing 20 % aqueous potassium hydroxide or Nessler's reagent, respectively, did not alter the activity of the effluent. Ethanol (70 % aqueous) placed between source and test cultures also failed to influence zygospore formation. Thus it is clear that none of these substances is the cause of the stimulatory effect.

Passage of the effluent through dilute sulphuric acid removed the activity, even when the humidity was restored by subsequent passage through water. This indicates that the substance is probably basic. Titration of the acid against 0·01 N-NaOH, however, did not show any change in the titration value; any substance removed by the acid must have been present in very small quantities.

Hepden & Folkes (1960), in considering the possible relationship between nucleic acid metabolism and the initiation of zygospores, suggest that the volatile substance is likely to be a methyl donor. They discuss the possibility that this might be methylamine and outline a probable mechanism for its action. The removal of the stimulatory effect of gaseous effluent from mature cultures of Rhizopus by passage through acid is consistent with methylamine being at least partially effective. Accordingly a number of methyl donors and other methylated compounds were tested for their effects on *Rhizopus sexualis*.

In some experiments these substances were incorporated in glucose-asparagine agar, glucose-ammonium salts agar or 1 % malt agar, to which they were added when they were cooling. In others compounds were placed on disks of filter paper which were laid directly on the agar plate or were placed on sterile glass coverslips which were then laid on the medium. In another series of experiments, *Rhizopus sexualis* was grown on glass microscope slides in a film of liquid culture medium. The non-volatile substances to be tested were added to this liquid; the volatile ones were placed in Petri dishes and the test slide cultures were supported above them. Both plate and slide cultures were tested at 20° and at 7°, to which they were transferred after 24 hr. at 20°. The results of all these experiments are collected in Table 2.

All the methyl donors tested were either ineffective or were more or less toxic even at low concentrations. None of them induced an increase in zygospore initiation and maturation comparable to that shown in Table 1 as resulting from the volatile substances produced by mature cultures. Choline, methylamine, DL-serine, formaldehyde, formic acid and methanol showed some counteraction of the effect of low temperature on cultures which had already begun to form zygospores before transfer to 7°. None of these substances, however, significantly increased the number of zygospores developing in cultures which, at the time of transfer to low temperature, bore no mature zygospores. In all cultures there was an increase in the number of pseudophores which, as already pointed out, have been interpreted

as potential progametangia and which, under suitable conditions, sometimes develop into azygospores. Callen (1940) reported an increase in the number of pseudophores produced by *Rhizopus sexualis* in the presence of the related *R. nigricans*. It is thus possible that the production of pseudophores indicates activity comparable to that of the naturally produced volatile substance, but that the beneficial effects are counteracted by the toxic nature of the pure compounds. Concentrations of these pure substances too low to be toxic had no effect on the production of pseudophores or of normal zygospores. These results do not exclude the possibility that the active substance is methylamine or some other methyl-donor.

Table 2. *Effects of various methyl donors and methylated compounds on growth and zygospore formation at* 20° *and at* 7–10° *after* 24 *hr. at* 20°

			Effects at 20°		Effects at 7–10°	
Substance	Percentage concentration	Method of supply	On growth	On zygospore formation	On growth	On zygospore formation
Methionine	0·1–0·2	A	None[1]	None	+	None
Methylamine	7·0–70·0	B, C D	−, p	None	±	p
Methylamine. HCl	0·1	A	−	None	±	p
Methanol	10–100	C, D	−	p	None	p
Thymine	0·1	A	None	None	None	None
Choline chloride	0·001–1·0	A	±	None	±	+ +
Formaldehyde	0·08–0·64	A, D	−	−[2]	−	p
Formic acid	0·09–0·46	B, D	−	p	−	p
Glycine	0·01–0·1	A	None	None	None	None
DL-Serine	0·01–0·1	A	+	+	None	+ +
Betaine. HCl	0·01–0·1	A	None	None	None	None

Key to Table 2

A, incorporated in culture medium.
B, on paper disk laid on agar medium.
C, on glass coverslip laid on agar medium.
D, on dish with slide culture supported above.
+, slight increase.
+ +, increase.
−, decrease.
±, slight increase or decrease according to concentration.
p, increase in number of pseudophores or of irregular swellings resembling pseudophores.
[1], slight increase in absence of other source of nitrogen.
[2], pseudophores developed in slide cultures only.

DISCUSSION

The experiments show that a volatile substance or substances produced by mature cultures of *Rhizopus sexualis* stimulates zygospore production by young cultures of the same fungus at optimal temperatures (*c.* 20°) and partially counteracts the inhibitory effects of low temperatures (7–10°). It is established that the active factor in the effluent gases is not carbon dioxide, ammonia or ethanol, that it is basic and probably produced in extremely small quantities. The identity of the active substance has not been established but the experimental results are not inconsistent with its being methylamine. It is clear, however, that the effects of the naturally produced substance cannot be exactly reproduced by low concentrations of pure methylamine.

The effects of the presence of cultures of related species were less than those of mature cultures of *Rhizopus sexualis* itself, and the unrelated *Sordaria* was even less effective. It is possible, therefore, that the activity of the effluent from mature cultures is not due to a single volatile substance but to a mixture of several such substances. The exact composition and proportions of such a mixture might well vary with different species, thus accounting for the high degree of specificity observed and the decreasing effect on *R. sexualis* with decrease in the relationship between test and source cultures. It is thus unlikely that volatile substances stimulating other fungi are exactly identical with that produced by *R. sexualis*. Of such substances, an unidentified one described by Banbury (1954) as stimulating zygotropism in *Mucor hiemalis* might conceivably be of similar nature; one described by McTeague, Hutchinson & Reed (1959) as stimulating growth of germ tubes of *Agaricus campestris* and identified as 2-3-dimethylpentene is unlikely to resemble closely that produced by *R. sexualis*. It is hoped that further work now in progress may establish the nature of the latter and elucidate the part that it plays in zygospore initiation.

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