J. gen. Microbiol. (1961), 24, 313–325
Printed in Great Britain

Virolysin, a Virus-Induced Lysin: Its Appearance and Function in Phage-Infected Staphylococci

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(*Received* 14 *September* 1960)

SUMMARY

The formation and role of enzyme, virolysin, in *Staphylococcus aureus* K_1 infected with phages P_1 and P_{14} are described. Virolysin is a by-product of the metabolism of the cell which is actively producing phage, not of the normal cell. Virolysin is first detected within 10–15 min. in a 40–50 min. latent period and increases linearly until lysis. Normal cell autolysin remains constant during infection. Observations on lysis and phage release show that (1) certain inhibitors which prevent lysis of the cocci by external virolysin also prevent lysis and phage release when added at the end of the latent period; (2) the rate of premature lysis of, and phage release from, cocci chilled during the latent period depends upon their virolysin content. Both observations suggest that virolysin functions in phage release.

INTRODUCTION

Lysins obtained from phage-infected bacteria were reported by Bronfenbrenner & Muckenfuss (1927) for a staphylococcal system, by Sertić (1929) for a coliphage system, and by Humphries (1948) for a klebsiella system. More recent publications describing phage-induced cell-wall or capsule-dissolving agents are those of Panijel & Huppert (1954) for an *Escherichia coli* system, Ralston and collaborators (Ralston, Baer & Krueger, 1955*a*; Ralston, Baer, Lieberman & Krueger, 1955*b*, 1957*a*; Ralston, Lieberman, Baer & Krueger, 1957*b*) for a *Staphylococcus aureus* system; and Adams & Park (1956) for klebsiella, Murphy (1957) for *Bacillus megaterium*, Maxted (1957) for streptococci, Koch & Dreyer (1958) for *E. coli* bacterium + bacteriophage systems. In addition to virus-induced lysins, other instances of virus-induced enzymes include DNA-synthesizing enzymes in *E. coli* B-T phage systems (Kornberg, Zimmerman, Kornberg & Josse, 1959; Flaks & Cohen, 1959; Flaks, Lichtenstein & Cohen, 1959), adenosine triphosphatase associated with avian myeloblastosis virus (Mommaerts *et al.* 1952) and the amidase of influenza virus (Gottschalk, 1952) in animal host + virus systems.

Virolysin is a lysin found in lysates of *Staphylococcus aureus* K_1 infected with phage P_1. In previous publications (Ralston *et al.*, 1955*a*, *b*; 1957*a*, *b*) we showed the following features: virolysin has the properties of an enzyme; it cannot be found in uninfected cocci but a similar enzyme, autolysin, is present. The two enzymes differ in their substrate specificity, antigenicity and pH optimum. For virolysin to act externally the cocci must be pretreated with phage, heat, acetone, urea or a

variety of other damaging agents. We suggested that: (1) virolysin acts on a substrate forming the framework of the bacterial wall; (2) in order for the substrate to become available to the enzyme, a preliminary alteration (presumably of the wall) must occur.

Preliminary studies (Ralston *et al.*, 1955*b*) suggested that virolysin appeared intracellularly during phage infection and that it acted in the final lysis to release virus. The present work was undertaken to obtain further information about the relationship between phage infection, virolysin appearance and the role of virolysin in lysis. More specifically, it would be of interest to determine (1) when and how the enzyme appears in the infected coccus; (2) what effect phage infection has on the quantity of normal cell autolysin; (3) whether virolysin can be induced in the absence of phage or whether these syntheses are keyed one to the other; (4) whether phage release is dependent upon the presence and operation of the enzyme at the end of the latent period.

METHODS

Media. Tryptose phosphate broth or agar (Difco Labs., Detroit, Mich., U.S.A.) was used for growth of bacteria and studies on phage-infected cells. In this paper, the media are referred to as TP 1× or 2× broth (i.e. single or double strength broth) and TP agar.

Bacterium + bacteriophage systems and phage assay. The phages P_1 and P_{14} and their bacterial hosts *Staphylococcus aureus*, strains K_1 and 145, were described previously (Ralston & Krueger, 1952, 1954). The method of assaying phage by a rapid plaque technique on the surface of microscope slides was previously reported (Jones & Krueger, 1951; Ralston & Baer, 1960). The following abbreviations are used throughout: P = phage; C = cocci; $P_{14}(K_1)$ = phage 14 produced on strain K_1 cocci and $P_1(145)$ = phage 1 produced on strain 145 cocci, and so forth.

Preparation of lysins. To prepare autolysin, 1×10^8 resting uninfected K_1 C/ml. were shaken in TP 2× broth for 6 hr. at 37° (final concentration = 3×10^9 C/ml.) and stored at 4° until autolysis occurred (24–72 hr.). Preparation of virolysin $P_1(K_1)$ involved shaking 1×10^8 resting K_1 C/ml. with 1×10^6 P_1 phage/ml. in TP 2× broth at 37°. Lysis of the culture occurred in 3 hr. The autolysates and lysates were spun at 20,000*g* for 1 hr. at 6°. The K_1 autolysin and $P_1(K_1)$ virolysin were present in the supernatant fluids.

Determination of lysin activity. Virolysin and autolysin assays were performed by turbidimetric measurement of the lysis of heat-killed K_1 cocci under standardized conditions. The enzyme concentration in any particular sample of autolysin or virolysin was determined by mixing a suitable dilution of lysin with heat-killed K_1 cocci at 37° (sometimes, however, at 4°). Generally a 1/2 to 1/100 dilution of lysin in TP 2× broth at pH 7·5 for virolysin, and pH 6·5 for autolysin, was mixed with cocci so that the final volume in the tube was 5 ml. and the final concentration of cocci was 1×10^9 C/ml. The decrease in turbidity was followed in a Klett-Summerson photoelectric colorimeter by taking readings at suitable intervals. From the data, the course of lysis can be represented by plotting the logarithm of the number of unlysed cocci against time at any given temperature. Only half of the heated cocci lysed and a correction was made for the resistant cocci in the population. A straight line was generally obtained (Ralston *et al.*, 1957*b*). Estimates of

lysin activity were based upon determinations of the initial velocity constants, K/min., calculated from the equation:

$$K = \frac{2{\cdot}3}{t} \log \frac{C_0}{C_0 - C_t},$$

in which C_0 equals the initial concentration of cocci/ml., C_t equals cocci/ml. lysed in any given time interval, t. These relative velocity constants are reported only for purposes of comparison within an individual experiment. Less accurate estimates of lysin activity were made by determining the % cocci lysed in a short time interval, and comparing the % lysis instead of the velocity constants, K, of individual samples.

Preparation of virolysin antiserum. A phage $P_1(K_1)$ lysate was spun at 20,000g, for 1 hr. at 6° in a Spinco Model L centrifuge. The pellet containing the phage was discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid at 0° to 40 % of saturation. The precipitated material was centrifuged at 4000 rev./min. for 15 min. and resuspended in 0·85 % (w/v) NaCl. At least a tenfold concentration of the original activity was obtained. A rabbit was inoculated subcutaneously with progressively higher amounts (0·5–2·0 ml.) of the concentrated material in a series of sixteen injections over 3–4 weeks. Serum was collected 2 weeks after the last injection. A second rabbit was inoculated with autolysed cell contents, which exhibited no lysin action, to determine whether anything in the cell contents contained antigens similar to virolysin. A number of rabbit sera tested, including several phage antisera, contained a lysin for heated K_1 staphylococci. This lytic activity was not removed by heating the sera at 56° for several hours, but was radically diminished by filtering the sera through Supercel filter-aid.

Antiserum inhibition tests. Antiserum to autolysin or virolysin was used at a dilution of 1/25 and mixed with enzyme for 20 min. at 20° before adding heat-killed bacteria. The enzyme control contained enzyme mixed with a similar dilution of normal rabbit serum.

Specificity of virolysin antiserum. From Tables 1 and 2 it is seen that: (1) antiserum to either P_1 or P_{14} virolysins (produced on strain K_1) did not affect the normal K_1 cell autolysin, indicating that the virolysin was antigenically unrelated to autolysin; (2) the antibody to $P_1(K_1)$ virolysin inactivated virolysin formed in either strain K_1 or strain 145 cocci, indicating that the antigenic specificity of the virus-induced enzyme depended on the phage rather than the host of production; (3) antiserum to the virolysin induced by the closely related phage P_{14} also inactivated P_1 virolysin, regardless of the host, showing that the differences between the phages did not in-

Table 1. *Staphylococcal virolysin. Effect of antiserum to $P_1(K_1)$ virolysin on $P_1(K_1)$ virolysin and staphylococcus strain K_1 autolysin*

	K/min. at 37°
Virolysin + inactive autolysin	0·026
Autolysin + inactive virolysin	0·010
Autolysin + inactive virolysin + virolysin antiserum	0·008
Virolysin + autolysin	0·039
Virolysin + autolysin + virolysin antiserum	0·010

volve genes concerned with virolysin induction. K_1 autolysin antiserum is specific for autolysin and does not affect virolysin (Lieberman, 1956; Ralston *et al.*, 1957*b*). The following controls gave no lysis of heated cocci: inactive virolysin + inactive autolysin; these two + virolysin antiserum; virolysin + inactive autolysin + virolysin antiserum. Inactive lysin was obtained by boiling a lysin sample for 5 min.

Table 2. *Staphylococcal virolysin. Effect of antiserum to $P_{14}(K_1)$ virolysin on $P_{14}(K_1)$, $P_1(K_1)$, and $P_{14}(145)$ virolysins*

Test mixture	Virolysin from P_{14} infection of strain K_1	Virolysin from P_1 infection of strain K_1	Virolysin from P_{14} infection of strain 145
	K/min. at 37°		
Enzyme	0·0204	0·0530	0·0154
Enzyme + antiserum to $P_{14}(K_1)$ virolysin	0·0000	0·0000	0·0000

Analysis of total lysin, autolysin and virolysin content of phage-infected and uninfected cocci. Total lysin (virolysin + autolysin) in phage-infected cocci can be assayed on heated K_1 cocci. The relative velocity constants, K/min., of a mixture of the two has been shown to be equal to the sum of the activities of the individual lysins (Ralston *et al.*, 1957*b*). Autolysin in a mixture of the two lysins can be determined in three ways: (1) K autolysin = $K_{\text{total lysin}} - K_{\text{residual lysin}}$ in presence of autolysin antibody; or (2) $K_{\text{residual lysin}}$ in presence of virolysin antibody; (3) by assay on acetone-treated *Micrococcus lysodeikticus*, which is lysed by autolysin but not by virolysin. Virolysin is determined by: (*a*) K virolysin = $K_{\text{total lysin}} - K_{\text{residual lysin}}$ in presence of virolysin antibody; (*b*) $K_{\text{residual lysin}}$ in presence of autolysin antibody.

RESULTS

Virolysin production in strain K_1 staphylococci during multiplication of phage P_1

The following two experiments were designed to determine the appearance and increase of virolysin in the phage-infected cocci, to ascertain changes, if any, in the autolysin content of the cocci after infection, and to compare these with the appearance and increase of mature phage. The experiments differed from each other in the phage used and in the method used to obtain and assay the intracellular contents; the results of the two experiments corroborate each other.

Experiment 1. Staphylococci (strain K_1), grown for 4 hr. at 37°, were suspended at $2{\cdot}5 \times 10^8$ C/ml. in TP broth, and infected with P_1 phage at a phage:coccus ratio of 4:1. The phage + cocci mixture was allowed to remain at room temperature for 3 min. and then shaken at 37°. Samples were chilled at intervals and tyrothricin was added to 0·001 mg./ml., to cause lysis (Fong & Krueger, 1950); the concentration of tyrothricin used did not inactivate free phage or lysin. Lysin activities were determined by using virolysin antiserum and by assays on *Micrococcus lysodeikticus*.

Experiment 2. Staphylococci (strain K_1) were grown on TP agar at 37° for 4 hr., suspended in TP $2\times$ broth to $2{\cdot}5 \times 10^8$ C/ml., chilled to 4°, and infected with

phage 14(K_1) at a phage:coccus ratio of 4:1. After 20 min. adsorption at 4°, the infected cocci were shaken at 37° until lysed. Samples were removed at intervals, chilled to stop phage and enzyme development, and the cocci centrifuged down at low speed. To collect the intracellular materials, the chilled infected cocci were exposed to a lysing medium, composed of non-plaque-forming phage 14(145) (inactivated by ultraviolet irradiation) at about 10–20 particles/coccus and a 1/50 dilution of 14(145) phage-free virolysin. The lysates were spun down at 4000 rev./min. and the supernatant fluids were analysed for phage and lysin content. Autolysin was distinguished from virolysin by the use of autolysin antiserum.

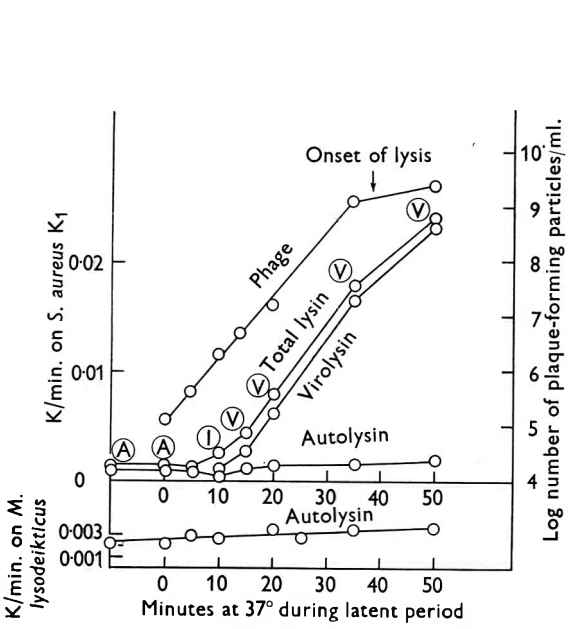

Fig. 1

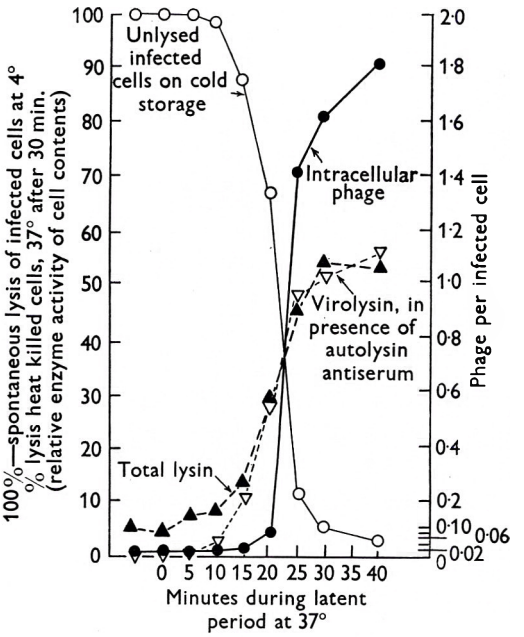

Fig. 2

Fig. 1. Formation of virolysin and phage during infection of staphylococcus strain K_1 organisms by phage P_1. The intracellular autolysin was determined from: (1) the total activity of the lysed coccal contents for *Micrococcus lysodeikticus*; (2) the residual activity of the lysates for strain K_1 cocci after exposure to virolysin antiserum. The circled letters, A, I and V, above the values for the total lysin indicate that the pH optimum of the sample was characteristic of autolysin (A), virolysin (V), or had an intermediate value (I). The phage was added at 0 min.

Fig. 2. Formation of virolysin and phage during infection of strain K_1 cocci with phage P_{14}; spontaneous lysis of infected cocci on storage in the cold. The virolysin content was determined by the residual activity of the lysates for strain K_1 cocci after treatment with autolysin antiserum. The phage was added at 0 min.

Lysin formation. Figures 1 (Expt. 1) and 2 (Expt. 2) show that a low value of lysin was present in uninfected cocci, and at 0–10 min. in a 40–55 min. latent period at 37°. This lysin was identifiable as autolysin. The autolysin content of uninfected cocci remained essentially constant (in a few experiments it increased slightly, but never as much as twofold). Material identifiable as virolysin first appeared at 10–15 min., and then it increased essentially linearly until the end of the latent period. Figure 1 shows that during the latent period a change in the pH optimum

of the total lysin occurred, from that for autolysin (pH 6·5) to that for virolysin (pH 7·5).

Tests were made for possible intracellular inhibitors or activators of the enzymes. The lysed cell contents of samples of infected cocci removed at various times in the latent period were mixed with each other at final dilutions of 1/5 in TP broth. Their activity indicated that under these conditions there was nothing present at any time which interfered with any lysin present, or which activated any unknown enzymes (Table 3, Expt. 1).

Table 3. *Lack of enzyme inhibition or enhancment by mixtures of coccal contents during latent period of phage multiplication on lysin activity of individual samples*

Individual samples (time during latent period)	Enzyme activity*	
Uninfected cocci	1	
0 min. infected cocci	1	
10 min. infected cocci	3	
20 min. infected cocci	8	
35 min. infected cocci	11	
Phage lysate	17	
	Enzyme activity	
Mixture of samples	Theoretical†	Observed
Uninfected cocci + lysate	18	20
0 min. + 20 min.	9	9
0 min. + lysate	18	19
10 min. + 20 min.	11	11
10 min. + lysate	20	22
20 min. + lysate	25	27
35 min. + lysate	28	26

* Enzyme activity = number of Klett units lysed after 48 hr. at 4°. Input in each tube was 9×10^8 heat-killed strain K_1 cocci (equivalent to 90 Klett units)/ml. Each tube contained a 1/5 dilution of samples; where a mixture was used, a 1/5 dilution of each lysin was present. Mixtures were allowed to remain at room temperature for several hours before assay cocci were added. Samples were additive.

† Sum of two individual samples calculated from above.

Relation between phage formation and enzyme synthesis. Analysis of the contents of cocci removed at intervals in the latent period indicated that the first mature particles appeared within 6 min. and then increased logarithmically until shortly before mass lysis (Fig. 1). The first detectable virolysin generally appeared somewhat later and increased linearly (Fig. 1). The phage assay was much more sensitive than the virolysin assay. With the phage assay, one mature particle could be detected, provided that all free phage was removed after adsorption. We estimate that with the enzyme, under the present conditions of assay, approximately 5×10^6 to 1×10^7 C/ml. must have formed enzyme maximally before its activity can be measured. The first detectable quantity of enzyme/ml. coincided roughly with the time at which 5×10^6 to 1×10^7 P/ml. had appeared in the disrupted cell contents, suggesting that perhaps enzyme appearance is correlated with phage maturation. However, in the absence of knowledge about the distribution of enzyme and phage among individual cocci, this correlation may be more apparent than real.

Absence of virolysin from abortive phage+coccus complexes and from uninfected cocci

While it is clear that virolysin is associated with active phage production, it would be of interest to ascertain whether it is produced by phage-adsorbed cocci which do not form virus, as well as by uninfected cocci grown and lysed under a variety of conditions.

Phage+coccus interactions. (1) *Experiments with phage-sensitized cocci.* It was found (Ralston *et al.*, 1957*a*) that when enough phage particles were adsorbed, the cocci failed to form infective centres (plaques) on agar. They did not lyse by themselves when incubated in broth, but were lysed by externally added virolysin, in contrast to uninfected or phage-producing cocci. The lysates produced in this manner did not contain phage. Cocci in this condition have been called 'sensitized'.

The following experiment was designed to test whether virolysin production could occur in phage-sensitized cocci. Staphylococci of strain K_1, grown for 4 hr. on TP agar at 37°, were resuspended in TP 2 × broth to $2{\cdot}0 \times 10^8$ C/ml. and infected with phage $P_{14}(K_1)$ at phage:coccus ratios from 50 to 2. (The addition of increasing amounts of phage to samples containing a constant number of cocci resulted in increasing numbers of sensitized cocci.) The numbers of infective centres were determined by removing samples at 20 min., treating with phage antiserum for 5 min., and then diluting for plaque count. The numbers of sensitized cocci were estimated from total phage-adsorbed cocci minus numbers of infective centres. The phage+coccus mixtures were incubated for 70 min., by which time complete lysis had occurred in all tubes. The virolysin content of the tubes was determined by assay on heated K_1 staphylococci in presence of antiserum to normal cell autolysin. As shown in Table 4, the yield of virolysin was inversely proportional to the number of sensitized cocci, suggesting that these cocci did not form virolysin. It is presumed that their lysis was brought about by the action of external virolysin released from the cocci which had undergone active infection.

Table 4. *Lack of staphylococcal virolysin production by phage-sensitized staphylococci*

Phage input per coccus	Autolysin and virolysin (total lysin) A	Autolysin (total lysin and virolysin antisera) B	Virolysin A–B	No. of cocci with infective centres*	Relative enzyme yield per infective centre†
	K/min. value				
50	0·0023	0·0012	0·0011	2×10^7	0·0012
25	0·0019	0·0006	0·0013	2×10^7	0·0010
10	0·0058	0·0012	0·0046	6×10^7	0·0010
5	0·0140	0·0035	0·0105	$1{\cdot}4 \times 10^8$	0·0010
2	0·0170	0·0058	0·0112	$1{\cdot}2 \times 10^8$	0·0014

* Total input of cocci = $2{\cdot}0 \times 10^8$/ml.

† Calculated from relative *K*/min. ÷ number infective centres × 10^7.

The conclusion that no virolysin was formed in sensitized cocci is supported by other experiments in which cocci in the logarithmic growth phase were treated with large numbers of phage particles so that virtually the entire population was sensitized. The cocci were incubated at 37° for twice the normal latent period, and

at intervals samples were lysed-from-without by the addition of trace amounts of virolysin. The cell contents were then tested for lysin yield. Under these conditions, a constant amount of lysin was obtained from cocci lysed at each interval, and this was identified as normal cell autolysin.

(2) *Experiments with host-altered phage.* It was previously shown (Ralston & Krueger, 1952, 1954) that a host-controlled alteration occurred with phage P_{14}. Its host range was restricted after passage through staphylococci of strain 145 so that 34 out of 35 particles absorbed to and killed strain K_1 cocci, produced no phage and failed to cause lysis. The following experiment was carried out to test whether such cocci which did not produce phage formed virolysin (Fig. 3).

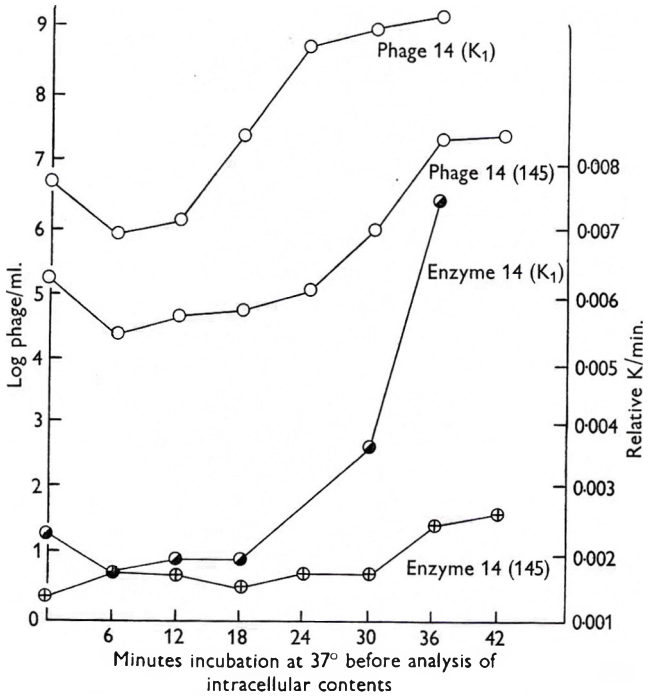

Fig. 3. Phage and enzyme formation by normal and host-altered phage P_{14}.

Strain K_1 cocci were grown on TP agar at 37°, harvested after incubation for 3 hr. (logarithmic phase) and mixed with phage P_{14}, passed previously on host strain K_1 or strain 145 at 9×10^8 P/ml. and $3{\cdot}5 \times 10^8$ C/ml. After 30 min. at 4° the temperature was raised to 37°. Samples were removed at intervals and chilled to stop infection. The intracellular contents were lysed-from-without at 4° by suspending the cocci in samples of a lysing medium as described in Expt. 2. The tubes were then assayed for their relative amounts of phage and virolysin.

In contrast to infection of strain K_1 cocci with phage 14(K_1) the contents of cocci exposed to phage 14(145) (the restricted form) yielded low amounts of virolysin and phage. The low amount of enzyme formed by this infection mixture was accounted for on the basis of the 1 in 35 cocci which produced infective centres. We conclude, therefore, that the remainder of the cocci produced no enzyme.

Attempts to find virolysin in uninfected cocci disrupted in different ways. We previously reported (Ralston *et al.* 1957*b*) that we were unable to detect virolysin in autolysates of strain K_1 cocci grown for 4 hr. and 24 hr. on TP agar and TP broth and autolysed by storage at 4° or by incubation at 37°, under toluene. In the present work further efforts were made to detect virolysin in normal cocci by extending the range of conditions and ages under which uninfected strain K_1 cocci were grown and lysed. These included growth for 1 to 48 hr. on TP agar and TP broth at temperatures from 20° to 37° and at varying rates of shaking. The cultures, ranging from 1×10^8 to 4×10^9 C/ml., were lysed in the following ways: (1) under toluene at 0° and 20°; (2) by adding tyrothricin at 0°–37°; (3) by storing cocci in shallow layers and in tall cylinders at 0°–20°; (4) by disrupting cocci with glass beads; (5) by lysis-from-without by irradiated phage and a high dilution of virolysin; (6) by shaking slowly at 35°; this results in spontaneous lysis at 6 hr. (the late exponential phase of growth). Whenever a lysin was detectable in such lysates, it was identified as normal cell autolysin.

Function of virolysin

We have suggested that virolysin releases phage by acting on a substrate forming the framework of the cell wall (Ralston *et al.*, 1955*b*, 1957*a*). Isolated staphylococcal cell walls, prepared by disintegrating cocci with glass beads, or by trichloroacetic acid extraction and trypsin treatment (Hancock & Park, 1958), are, in fact, dissolved by the action of the enzyme (data to be published). Further evidence to support the view that virolysin is an essential component of phage release is as follows.

Effect of inhibitors on lysis at the end of the latent period. Certain chemicals inhibit the action of external virolysin on phage- and heat-sensitized cocci (Ralston *et al.* 1957*a*, *b*). Since there is a high concentration of virolysin in the coccus at the end of the latent period, these compounds should also inhibit the enzyme if it lyses the infected cocci-from-within (at least if the inhibitor penetrates the coccus). This was shown for $CuSO_4$ (Ralston *et al.*, 1955*b*). In the present work, 22 other compounds, already tested for their action on external virolysin, were tested with infected cocci as follows. Strain K_1 cocci grown on TP agar for 18 hr. were mixed with phage at a phage:coccus ratio of 3:1. Samples (4 ml.) were dispensed into test tubes suitable for reading in a Klett colorimeter. The latent period was approximately 67 min. Just before the 64th min., 1 ml. solution of substance to be tested as inhibitor was added to each tube. Readings were followed turbidimetrically for a further 50 min. at 37°, and the tubes then placed at 4° overnight. The amounts of lysis of infected cocci and of phage release were then determined. Table 5 shows that for each compound, with the exception of virolysin antibody, there was a good correlation between the inhibition of external virolysin and the inhibition of lysis of infected cocci and release of phage. In the case of virolysin antibodies, unlike the other inhibitors (low molecular weight compounds), it is presumed that the antiserum cannot pass across the cell wall or membrane.

Correlation between intracellular lysin content of infected cocci and their rate of spontaneous lysis. Cocci in which virus multiplication had been interrupted by chilling lysed spontaneously on storage at 4°. Figure 2 shows the amounts of lysis after 24 hr. of cocci chilled at intervals during the latent period. Cocci removed early lysed slowly, whereas those removed at later times lysed progressively faster.

Table 5. *Effect of enzyme inhibitors on lysis of phage-infected staphylococci and on phage yield*

Compounds	Final concentration in TP broth	Action on extracellular virolysin*	% lysis of infected cocci when added at end of latent period	Yield of virus/ml.
Na citrate	0·003 g./ml.	None	80	N.t.
NaN_3	0·001 M	None	84	N.t.
$NaAsO_2$	0·001 M	None	95	1×10^8
NaF	0·001 M	None	83	$4{\cdot}5 \times 10^8$
Thiourea	0·001 M	None	90	$4{\cdot}6 \times 10^8$
Semicarbazide	0·001 M	None	80	$4{\cdot}3 \times 10^8$
K oxalate	0·001 M	None	90	N.t.
Na sulphite	0·001 M	None	90	N.t.
KI	0·001 M	None	87	N.t.
Na deoxycholate	0·001 g./ml.	None	90	N.t.
Sulphanilic acid	0·001 M	None	80	$2{\cdot}5 \times 10^8$
$PbCl_2$†	0·0004 M	Variable	26	$1{\cdot}2 \times 10^8$
$K_3Fe(CN)_6$†	0·0033 M	Variable	60	1×10^7
Iodoacetic acid†	0·001 M	Variable	80	N.t.
$HgCl_2$	0·001 M	Inhibitor	0	1×10^4
$AgNO_3$	0·001 M	Inhibitor	0	6×10^6
HCHO	0·001 g./ml.	Inhibitor	0	1×10^2
Duponol	0·001 g./ml.	Inhibitor	0	1×10^6
Thiomersalate	0·001 M	Inhibitor	3	3×10^7
Hg acetate	0·001 M	Inhibitor	0	8×10^3
I_2 in KI	0·01 N in 0·25 % (w/v) KI	Inhibitor	0	1×10^1
Antibody to virolysin	1/25 dilution	Inhibitor	80	N.t.
Control	—	—	80	5×10^8

* Results from Ralston *et al.* (1957*b*) effect of various compounds on lysis of heated or phage-sensitized cocci by virolysin.

† In tests of the action of the soluble enzyme virolysin on heated cocci it was found that the degree of lysis in the presence of these compounds varied from experiment to experiment. One of the important factors appeared to be the age of the organisms used.

N.t. = not tested.

The coccal contents of the early samples yielded no demonstrable virolysin and those of later samples contained increasingly greater concentrations of it. Although the lysis of early samples was accelerated by adding the lysing medium described in Expt. 2, or by the use of tyrothricin at 4° or 37°, no increased amounts of enzyme could be recovered. These facts suggest that intracellular virolysin is not only responsible for the lysis of phage-infected cocci whenever the infection is prematurely interrupted, but also when it is allowed to proceed for the entire latent period.

DISCUSSION

Virolysin appearance in the infected coccus: new synthesis or unmasking of previously formed host enzyme? The available evidence points to the conclusion that the appearance of virolysin represents new enzyme formation following infection. This would follow if (1) the genes directing the synthesis of virolysin are inserted into the coccus

at the time of virus penetration (if this proves to be the case, this system should be a favourable model for studying how genes direct protein synthesis); or (2) the phage introduces an inducer material for a repressed bacterial gene (Pardee, Jacob & Monod, 1958).

Virolysin appearance does not seem to be an activation of a previously formed host enzyme, such as is the case with DNAase in the T_{2r+} and T_{6r+} infections of *Escherichia coli* B (Kunkee & Pardee, 1956; Kosloff, 1953) for the following reasons: (1) DNAase appeared in uninfected organisms on storage, by adding a de-inhibitor, or by changing the pH value; no virolysin has been demonstrated by these devices. (2) Uninfected bacterial extracts in the T_{2r+} system produced inhibition of the DNAase activity of lysates whereas, with our staphylococci, mixing uninfected extracts with samples at different times in the latent period produced no inhibition of virolysin activity. (3) The amount of activated DNAase in the uninfected organism was greater than that in the lysate at the end of the infection cycle; we found no virolysin in uninfected staphylococci but only a low concentration of autolysin which remained constant during infection. Also,there was no antigenic fraction in uninfected cocci which produced neutralizing antibody against virolysin.

A general mechanism for the exit of viruses from host cells. Production of mucopolysaccharide-destroying enzymes may be a general mechanism evolved by viruses to accomplish their release from host cells; phage-induced lysins are known to exist in a taxonomically well-distributed number of bacterium + bacteriophage systems. In several instances it has been shown that they dissolve the bacterial cell wall which is known to contain a rigid framework of mucopolysaccharide. Similarly, the neuraminidase associated with the influenza particle may act to release the virus at the end of the growth cycle in animal cells (Gottschalk, 1958). Viral lysins may also act in penetration of the host cell by the virus particle (Adams & Park, 1956). Since our phage does not cause lysis-from-without and since the virolysin is physically distinct from phage, there is no positive evidence in our system that the lysin acts to effect entrance of the virus into the host. Moreover, other experiments (to be published) indicate that the receptor material is distinct biochemically from the mucopolysaccharide affected by the virolysin. This would suggest that the entrance of phage DNA from one particle into the cell may be accomplished by an entirely different mechanism than the exit of numerous intact particles from the cell. On this basis one might expect the particles to contain a separate enzyme responsible for destruction of a non-rigid wall component (Murphy, 1960).

Factors other than virolysin which are essential to the lysis of phage-infected cocci. The staphylococcal cell wall is resistant to high internal concentrations of lysin as well as to externally added virolysin until the end of the latent period; this suggests that a sensitizing reaction is also necessary for internal virolysin to act. Perhaps sufficient phage particles on the inside can accomplish sensitization just as they do on the outside; however, the addition of large numbers of phage particles does not produce lysis. Both external virolysin and phage are required to lyse infected cocci. These preliminary observations suggest that both virolysin and phage are 'packaged' inside the cell and are prevented from carrying out their functions until an additional mechanism releases them. While virolysin appears to be a key lytic agent, this does not exclude autolysin; but since the latter is present in lesser concentrations, this may play a minor role.

The work reported in this paper was supported by a grant from the Office of Naval Research and by NIH grants nos. E2379 and E 3776.

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J. gen. Microbiol. (1961), 24, 327–334
Printed in Great Britain

A Protein Factor in the Nutrition of *Paramecium caudatum*

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(*Received 7 July* 1960)

SUMMARY

The ciliate *Paramecium caudatum* was cultivated in a medium consisting principally of known chemical compounds, including 17 amino acids, guanylic, adenylic, cytidylic and uridylic acids, sodium acetate and sodium pyruvate, linoleic and oleic acids, a mixture of B vitamins and several inorganic salts. In addition it was necessary to add microgram quantities of a protein concentrate, first obtained from autolysed yeast, but recently by an improved method from dried green peas. Lipids were first extracted from the crude material and the protein was then dissolved and precipitated with 10% trichloroacetic acid. This protein was further purified by paper chromatography to yield a concentrate active in dilutions as low as 10 μg./ml. When the protein was hydrolysed enzymically or by acid or alkali, the hydrolysates were inactive. Sixteen amino acids were qualitatively identified in the hydrolysate. The nutritional role of a protein effective in such small concentrations has not yet been satisfactorily explained.

INTRODUCTION

The ciliate *Paramecium caudatum* has many advantages for microbiological experiments, but a serious limitation on its usefulness has been the difficulty involved in maintaining pure cultures. This became apparent when Glaser & Coria (1933) first succeeded in establishing bacteria-free cultures of this species in a medium containing dead yeast cells, liver extract and kidney tissue. Shortly afterwards the rapid progress in the cultivation of smaller ciliates, particularly *Tetrahymena pyriformis*, led to comparative neglect of the genus *Paramecium* until Johnson & Baker (1942) reported the growth of pure cultures of *P. multimicronucleatum* in a special yeast medium. Van Wagtendonk & Hackett (1949) were successful with cultures of *P. aurelia* in another medium containing yeast. Burbanck (1950) showed that *P. caudatum* was similar in its growth requirements, although his medium contained killed bacteria as well as autolysed yeast. After the development of a chemically defined medium for Tetrahymena by Kidder & Dewey (1951) modifications of that medium were adapted to the requirements of Paramecium by Van Wagtendonk and his students (1953) and by Johnson & Miller (1956), but it was still necessary to add the unknown factor from yeast.

In this laboratory Sterbenz (1956) confirmed the expectation that the media developed for related species would also support growth of *Paramecium caudatum*. It appeared that the three species had a common requirement for the special factor obtained from yeast. Ordinary yeast extracts were not satisfactory and the most important part of the preparation involved a period of autolysis for several

hours; when this step was omitted no growth was obtained. After such autolysis, however, the medium could be autoclaved without loss of growth-promoting activity. Although some progress had been made in purifying the unknown factor from yeast a search for another source of the factor appeared worth while. Earlier Van Wagtendonk & Hackett (1949) had reported that a lettuce infusion inoculated with *Aerobacter aerogenes* and incubated for 24 hr. before sterilization would support the growth of *P. aurelia*. A preliminary search was, therefore, made for various plant infusions which when inoculated with bacteria would yield the factor. The first significant advance was made when it was found that an infusion of split green peas could be used as a source of the factor and that under certain conditions the inoculation with bacteria was unnecessary. Thus it became possible to obtain the factor directly from peas and to prepare the concentrates more active than those previously obtained from the yeast autolysate (Lilly, Klosek & Hartig, 1958). This present report is concerned with attempts at further purification of the factor required by *P. caudatum* and improvement of the test medium in which it is used. While the unknown factor itself still has not been characterized chemically, highly active concentrates have been prepared and additional information has been obtained about its properties.

METHODS

Strains of *Paramecium caudatum* were collected from freshwater ponds on Long Island, New York, U.S.A., but not all of these proved adaptable to artificial media. Suitable strains were obtained by selecting individuals from cultures containing the natural bacterial flora and washing them by a modification of the Parpart (1928) technique previously published (Kidder, Lilly & Claff, 1940). Bacteria-free cultures were first maintained in a two-membered system originally described by Johnson (1946) with the flagellate *Polytomella caeca* as an associate. Later, when large numbers of Paramecium were obtained, they were introduced to the stock culture medium and washed free of the flagellates. This method proved more dependable for the isolation of new strains than did direct attempts at cultivating single specimens under axenic conditions. Stock and experimental cultures were maintained in 5 ml. quantities of medium in screw-capped Pyrex tubes (16 mm. diameter, 125 mm. long). The usual inoculum consisted of 0·5 ml. medium containing approximately 400 organisms from a stock culture. In a few experiments growth was determined by a direct count of the number of ciliates in a sample of 0·1 ml. taken by pipette from duplicate cultures. Maximum growth of 1000–1500 organisms/ml. was attained in the stock medium after 10–14 days, depending on the strain used. This method of direct counting was not practicable for the numerous routine tests of activity of the protein fractions. Evaluation of growth in such tests was based on a visual comparison of the experimental cultures with control cultures in stock medium. Test cultures exhibiting growth comparable with the maximum concentration in the controls were graded $++++$, and growth estimated at 3/4 maximum was graded $+++$. Lower concentrations were graded $++$, $+$, or $-$, but these were not considered significant, Lower rates of growth were sometimes observed when the ciliates required more than 14 days to reach a maximum, but this type of growth could not be compared with controls and was considered negative. Results of experimental cultures were regarded as positive when the rate of growth and final

concentration were within 25 % of that observed in controls. All tests were based on triplicate cultures carried through at least five transfers. Incubation temperature was 25°.

The first medium used for stock cultures was similar to that used by Van Wagtendonk & Hackett (1949). Fleischmann's Activated Dry Yeast was suspended in four times its weight of distilled water. This was first incubated for 4 hr. at 37° with frequent stirring, and then autoclaved for 20 min. at 120°. Most of the solids were removed by centrifugation and the supernatant fluid used as a crude stock medium. Complete removal of all suspended particles resulted in a decrease of the growth rate and of the final concentration of organisms. For stock cultures the supernatant fluid was not filtered. Since older preparations kept at room temperature had less activity than those freshly prepared the unused portion of the crude medium was kept frozen until needed; in this way activity could be preserved for several months.

Table 1. *Stock culture medium*

Preparation of pea infusion

1. 10 g. of dried green split peas suspended in 100 ml. of distilled water.
2. Boil for 15 min.
3. Remove solids by centrifugation or filtration.
4. Inoculate with one loopful of *Aerobacter aerogenes*.
5. Incubate 16 hr.
6. Autoclave at 125° for 15 min.

Preparation of medium

Pea infusion (as prepared above)	50 ml.
Proteose peptone (Difco)	5·0 g.
Cytidylic acid	0·05
Guanylic acid	0·05
Glucose	5·0
Stigmasterol	0·002

Bring volume to 1 l. with distilled water. Adjust pH to 7·0

Later, by substituting the pea infusion for the yeast autolysate a simpler stock culture medium was prepared as given in Table 1. *Aerobacter aerogenes* was introduced to produce an effect comparable to the autolysis of the yeast. By referring to the work done with other species of Paramecium and by testing various modifications on available strains of *Paramecium caudatum* a satisfactory combination of known chemical components with a minimum amount of purified fractions from the peas was eventually developed. This is the experimental test medium as given in Table 2.

RESULTS

Chemically defined components of the medium

The amino acids used were essentially the same as those used for Tetrahymena with the addition of tyrosine. Minor variations of concentrations of individual amino acids similar to the combinations used by other workers for other species of Paramecium did not appreciably affect growth. Omission of any single one or significant lowering of the concentration of any one resulted in cessation of growth. It appeared that as long as the unknown protein component was used with these

Table 2. *Experimental test medium*

All amounts given in μg./ml. of final medium

Chemically defined portion

L-Alanine	110	Linoleic acid	3·75
L-Arginine	206	Oleic acid	1·25
L-Aspartic acid	122		
Glycine	10	*Stigmasterol	2
L-Glutamic acid	233		
L-Histidine	87	*Calcium pantothenate	2
DL-Isoleucine	270	Nicotinamide	4
L-Leucine	244	Pyridoxal HCl	4
L-Lysine	272	Riboflavin	4
DL-Methionine	245	Folic acid	2
L-Phenylalanine	160	Thiamine HCl	12
L-Proline	250		
DL-Serine	394	Sodium ethylenediamine tetra-acetate	20
DL-Threonine	238		
DL-Tyrosine	100		2
L-Tryptophan	76	$MgSO_4.7H_2O$	40
DL-Valine	96	$(NH_4)_2SO_4.6H_2O$	10
		$MnCl_2.4H_2O$	0·1
Guanylic acid	75	$ZnCl_2$	0·02
Adenylic acid	30	$CaCl_2.2H_2O$	20
Cytidylic acid	75	$CuCl_2.2H_2O$	2
Uridylic acid	20	$FeCl_3.6H_2O$	0·5
Sodium acetate	570	K_2HPO_4	570
Sodium pyruvate	570	KH_2PO_4	570

Final pH adjusted to 7·0 with 0·1 N-NaOH

Undefined portion

* Protein factor obtained from peas 50 to 10

* The stigmasterol, the mixture of B vitamins and the protein factor were each sterilized separately and added aseptically to the other components which were mixed and sterilized together.

mixtures, precise quantitative studies on amino acid requirements would not be practicable. As the amount of unknown protein introduced was decreased, however, there was no indication that the amino acid balance was any more critical than previously. Further experiments on the optimum amino acid balance for *Paramecium caudatum* were postponed pending the results of more promising work on purification of the unidentified protein component.

Some of the purine and pyrimidine components of nucleic acid were also necessary for the satisfactory growth of *Paramecium caudatum*. As reported for other species the best mixture tested contained the four nucleotides, guanylic, adenylic, cytidylic and uridylic acids. The optimum proportions of these were not readily determinable while changes were constantly being made in the protein component and, as in the case of the amino acids, it was decided to use the mixture which had given the most satisfactory results in the earlier work.

An additional carbon source was required for the best growth. Glucose was satisfactory in preliminary experiments, but later sodium acetate + sodium pyruvate gave slightly better results. A sterol requirement similar to that reported for the other species was satisfied by the inclusion of stigmasterol in the medium. The

B vitamins were also required in approximately the same concentrations as reported for the other species. A mixture of inorganic salts similar to that used for Tetrahymena was included. The chelating agent sodium ethylenediamine tetra-acetate, although not necessary, was useful in preparing the stock solution of inorganic salts.

The only components of the chemically defined portion of the medium that differed much from those used for the other species were the two fatty acids, linoleic and oleic acid. After some preliminary work with natural oils, particularly corn oil, it was found that these two fatty acids in a three to one ratio promoted the best growth of *Paramecium caudatum*. The fact that in the early work with all three species the yeast autolysates contained both lipids and proteins probably contributed to some of the difficulties encountered at that time. In any event, after the lipids were separated from the pea material by preliminary extraction and replaced in the final medium by the known mixture of fatty acids it was possible to use much less of the chemically undefined material as a source of the protein factor.

Concentration of the protein factor

Progress in purification of the unidentified factor has resulted mainly from several improvements over previous methods. The first promising modification was the use of trichloroacetic acid (TCA) to precipitate the protein. This was originally used with the yeast autolysate when that material was the only source of the factor. Later this protein precipitant was found to be equally effective when the green peas were used as the source. Other methods of separating protein from non-protein material yielded preparations which were active only in much higher concentrations. There was some indication that treatment with TCA actually improved the yield of active material. When some of the native protein was treated with hydrochloric acid a precipitate was obtained that had no activity. When, however, this was dissolved in distilled water and a precipitate obtained with TCA this material proved to be active. This suggested that the native protein either contained an inactive form of the factor or that some sort of inhibition was involved. Another fortunate development coincident with the use of TCA with the crude pea infusion was the finding that no period of enzymic action or autolysis was necessary as had previously been the case when yeast was used. Removal of the TCA was carried out by dialysis against running water since the active factor was non-dialysable. The resulting product was a slightly hygroscopic white powder which could be further purified. Figure 1 outlines the method of preparation. The most active concentrates tested so far were obtained from this material by descending chromatography on Whatman no. 1 paper with 1 % (v/v) ammonia (sp.gr. 0·900) in 50 % (v/v) aqueous ethanol. Under these conditions the R_F value was 0·84. Larger quantities of concentrates were obtained by use of the same system as applied to the chromatopile described by Mitchell & Haskins (1949). When used with the chemically defined portion of the medium the concentrate was active in dilutions of 10 μg./ml.

Before chromatography the preparation, even though active in dilutions of 20 μg./ml., probably contained considerable amounts of impurities. Ultraviolet and infrared absorption spectra of this relatively crude material did not reveal any characteristic patterns of maximum absorption. After chromatography, however,

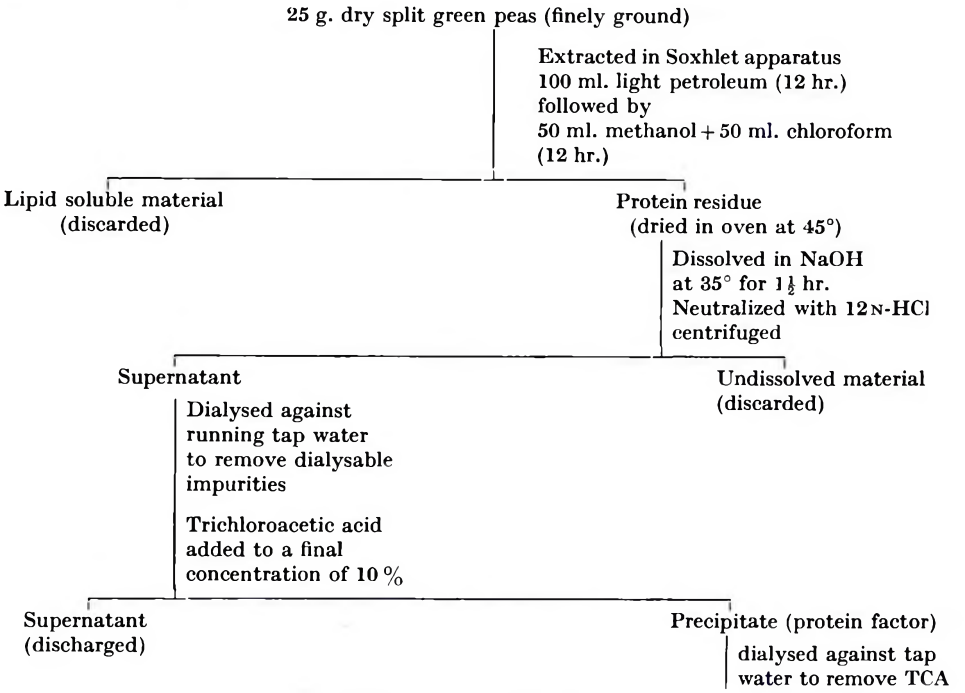

Fig. 1. Preparation of protein factor.

the active material did yield reproducible spectra showing maximum absorption at 2660 Å. in the ultraviolet and more consistent patterns in infrared spectroscopy. Apparently some of the impurities contributing to the non-specific absorption had been eliminated by the chromatography.

Since all the evidence indicated that the active principle was protein in nature some experiments were done to gain information about its constitution. Firstly, most of the proteolytic enzymes, including pepsin, chymotrypsin, trypsin, papain and ficin, when allowed to act for a sufficient length of time destroyed all growth-promoting activity of the material. All enzyme preparations used were obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. In the experiments with pepsin 3 mg. of the commercial preparation were dissolved in 3 ml. of 0·01 N-HCl. The mixture was incubated 24 hr. at 37°. The pepsin was then inactivated by boiling and the solution evaporated to dryness *in vacuo*. In the experiment with chymotrypsin 23 mg. of the commercial preparation were added to 50 ml. of a solution containing 500 mg. of the pea protein at pH 7·8. After incubation at 25° for 24 hr. the solution was brought to pH 3 by the addition of HCl and evaporated to dryness *in vacuo*. In a similar test 12 mg. of crystalline trypsin was dissolved in 12 ml. of water and added to a solution of 250 mg. of the pea protein in 25 ml. of water at pH 7·5. In this case the incubation temperature was 37°. The above methods for the three common proteolytic enzymes were based on those described by Sanger & Tuppy (1951).

In the experiments with papain and ficin the methods were based on those published by Petermann (1946), and by Kimmel & Smith (1957). A stock solution of 200 mg. of the commercial enzyme in 25 ml. of 0·9 % NaCl solution was first pre-

pared. To 2·5 ml. of this stock preparation 1 ml. of 0·5 M-cysteine solution and 1·5 ml. of 0·1 M-sodium citrate buffer (pH 6) were added. Of this enzyme + cysteine solution 1 ml. was added to 120 mg. of the pea protein dissolved in 30 ml. of water and buffered with 0·3 ml. of the same sodium citrate solution used above. The mixture was incubated for 24 hr. at 37°.

When the active material was subjected to acid or alkaline hydrolysis the activity progressively decreased as the hydrolysis continued. After complete hydrolysis when the biuret test was negative, there was no growth-promoting activity. Acid hydrolysis was accomplished by refluxing one part of the protein with 5 parts of 6 N-hydrochloric acid for 24 hr. The hydrochloric acid was then removed by distillation under reduced pressure. Alkaline hydrolysis was carried out by refluxing one part of the protein with five parts of 6 N-barium hydroxide for 10 hr. The barium was removed with sulphuric acid.

The hydrolysates thus obtained were analysed chromatographically for their amino acid content by the two-dimensional methods described by Redfield (1953). The following were qualitatively identified: alanine, arginine, cystine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. No evidence was obtained for the presence of either aspartic or glutamic acid either by the above methods or by the one-dimensional methods described by Hardy, Holland & Nayler (1955). Otherwise the protein involved appeared to be complete and not unusual in its amino acid composition. The possibility that more than one type of protein might have been present in the original sample of concentrate before hydrolysis was not eliminated by this preliminary study.

DISCUSSION

The fact that *Paramecium caudatum* is so common in nature suggested the extension of nutritional studies to this species. Up to the present no striking differences have been found from the known requirements of related species. The chief advance has been the discovery of a new and more convenient source of the unknown protein factor which has been the principal obstacle to formulation of a completely defined medium. Now that it is possible to obtain from dried green peas a highly active protein material by relatively simple chemical methods and without preliminary enzymic processing, more rapid progress can now be expected leading to the identification of this factor. It should be emphasized that it is by no means established that the protein obtained from the peas is necessarily identical with the material previously obtained from autolysed yeast, or that the active component of either of these artificial products enters into the nutrition of Paramecium under natural conditions. In the present state of knowledge it is difficult to conceive of a protein or a polypeptide having a vitamin-like activity, yet these latest preparations are active in microgram quantities. One possibility that should not be overlooked is that the difficulty in developing a defined medium may be due to some imbalance of the known components so that some type of toxicity or inhibitory effect on growth occurs. The role of the unknown component may be to detoxify the medium or to annul this inhibition. It is conceivable that several different types of compounds might have such an effect, comparable to the 'protective action of proteins' in other situations. In any event, even if one such compound could be

isolated in pure form and its structure determined this would be a step towards understanding the mode of action.

This research was supported by grant E-1506 from the National Institutes of Health of the U.S. Public Health Service.

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Printed in Great Britain

Some Aspects of the Genetics of Methionineless Mutants of *Salmonella typhimurium*

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SUMMARY

Forty-six methionineless mutants of *Salmonella typhimurium* were arranged in three phenotypic groups according to their growth responses to potential precursors of methionine. The results of syntrophism tests led to the recognition of two more phenotypic groups and permitted the arrangement in sequence of the metabolic steps in which mutants of each of the five groups were deficient. Transduction experiments indicated that each of these groups comprised mutants whose sites of mutation were closely linked within a complex locus. Attempts to map the sites of mutation of 12 mutants within one locus were unsuccessful. One of the mutants, probably a deletion, failed to recombine with the other 11 and behaved differently from them in linked transduction. A group of three and one pair of the 11 mutants could not be separated by recombination. Linkage was detected by transduction between only 2 of the 5 loci; these were concerned with non-sequential steps in the biosynthesis of methionine. No linkage was detected between the methionine loci and any of a number of other loci, including those controlling the biosyntheses of cysteine and tryptophan. These results were only partly in accordance with a previously suggested linkage map.

INTRODUCTION

The transfer of genetic fragments between strains LT-2 and LT-7 of *Salmonella typhimurium* and their derivatives by the temperate phage PLT-22 (transduction) was first reported by Zinder & Lederberg (1952). This was later defined as a system of common transduction in that, apparently, any marker could be transferred (Clowes, 1960). Since 1952, this system has been applied to the study of fine genetic structure by Demerec and his co-workers who used large numbers of auxotrophic, fermentative and antibiotic-resistant mutants. The genetic subdivisibility of the functional gene (locus) has been demonstrated (Hartman, 1956; Demerec & Hartman, 1956). Genes concerned with related functions were often found to be closely linked (Demerec, 1956*a*). A complementation test (abortive transduction) has permitted the definition of a functional unit (Demerec & Ozeki, 1959). Studies of the fine structure of certain loci have led to the construction of detailed linkage maps revealing, for example, the probable occurrence of deletions (Demerec, Blomstrand & Demerec, 1955; Clowes, 1958; Hartman, Loper & Šerman, 1960) and leading to an understanding of the nature of suppressor activity (Yura, 1956*b*; Gots, 1956; Howarth, 1958). Evidence has also been obtained that recombination occurs in transduction by crossing-over (Demerec & Demerec, 1955) and a copy-choice model has been suggested (Demerec, Goldman & Lahr, 1958). Fragmentation of the donor

chromosome in the production of fragments incorporated into transducing phage is probably not random (Ozeki, 1959).

Previous work with 32 methionineless (*met*) mutants led to the recognition of 5 loci (*metA*, *metB*, *metC*, *metE*, *metF*) based primarily on the frequency of transduction between mutants (Glover, 1955, 1956). Growth responses to precursors of methionine, syntrophism tests and chromatographic analyses of substances accumulated by representative strains indicated that each locus comprised mutants with identical biochemical properties which were different from those of mutants of the other loci (Glover, 1956, 1958). Linkage studies (Glover, 1955, 1956) led to the construction of a map in which all the *met* loci, the 4 linked tryptophan loci (Demerec & Hartman, 1956) and 4 loci controlling cysteine synthesis were linked together.

It was originally intended to examine whether each of a number of newly-isolated *met* mutants could be assigned to an appropriate *met* locus and to investigate the fine structure of one of these loci by transduction. Nineteen new mutants and 27 of those previously used were classified and the fine structure of a locus containing 12 mutants examined. Incidental to this work was a re-investigation of linkage between the *met* loci and between these and other loci.

The system of nomenclature used is that of Demerec (1956*b*) and elaborated in the *Microbial Genetics Bulletin*, 1958, No. 16. Each type of mutant is given a symbol, e.g. *met* for methionine requirement, a number according to its chronological isolation, e.g. *met-2*, and a letter (e.g. *E*) if subsequently assigned to a locus, e.g. *metE-2*. In transduction, there is a transfer of genetic fragments from one strain of bacterium (donor) from which phage is prepared, to another strain (recipient) infected with this phage. A cross is written: recipient (bacterium) ($\times$) donor (phage), e.g. *metE-2*($\times$) *metC-30*, to designate the transduction resulting from the infection of *metE-2* bacterium with phage prepared on *metC-30*. The terminology is in accordance with Demerec (1956*b*). A 'gene' is considered to occupy a linear section of a chromosome called a 'locus' concerned with the control of one function. This linear section is subdivisible into units termed 'sites' separable by crossing-over. 'Allele' is defined as one of several forms in which a gene exists. Two alleles are 'non-identical' when due to mutation at different sites, 'identical' when homologous sites are involved.

METHODS

Organisms. The wild-type strain LT-2 of *Salmonella typhimurium* (Zinder & Lederberg, 1952) and 46 different methionineless (*met*) mutants of it or of other auxotrophic derivatives from it, were used (Table 1). All the mutants except *metC-30* were obtained (in the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, N.Y., U.S.A.), either as spontaneous or ultraviolet-induced mutants by using the penicillin selection technique (Davis, 1948; Lederberg & Zinder, 1948). *MetC-30* was a spontaneous mutant isolated by Dr N. D. Zinder (personal communication). Twenty-seven mutants (*met-2* to *met-45* inclusive) had been used by Glover (1955, 1956, 1958) in his biochemical and genetical studies. The remaining 19 strains (*met-47 to met-68* inclusive) were newly isolated.

Stock cultures of these strains were maintained as stabs in 0·7 % Bacto agar in small corked tubes which had been incubated overnight, sealed with paraffin wax and then kept at room temperature. Viability was retained during $2\frac{1}{2}$ years. For day to

day use, strains were kept at 0–5° on nutrient agar slopes and subcultured every 6 weeks. Their nutritional requirements were tested periodically by an auxanographic technique.

Table 1. *Methionineless* (met) *mutants of* Salmonella typhimurium

Mutant	Group	Other markers	Mutant	Group	Other markers
*met- 6**	*A*	.	*met-45*	*B*	*adeE-11*
*-7**	*A*	*cysA-21*	*-49*	*B*	.
*-10**	*A*	*cysB-12*	*-66*	*B*	.
*-15**	*A*	.	*-30*	*C*	.
-18	*A*	.	*-32*	*C*	*adthA-2*
-19	*A*	*tryA-8*	*-35*	*C*	*adthA-2*
-21	*A*	*tryA-8*	*-37*	*C*	*adthA-2*
-22	*A*	*tryB-2*	*-50*	*C*	.
-40	*A*	*adthA-2*	*-52*	*C*	.
-43	*A*	*adeE-11*	*-56*	*C*	.
-44	*A*	*adeE-11*	*-57*	*C*	.
-48	*A*	.	*-58*	*C*	.
-53	*A*	.	*-65*	*C*	.
-54	*A*	.	*- 2**	*E*	*cysC-7*
-55	*A*	.	*-47*	*E*	.
-16	*B*	*cysB-16*	*-51*	*E*	.
-17	*B*	*cysB-16*	*-60*	*E*	.
-20	*B*	*tryA-8*	*-61*	*E*	.
-23	*B*	.	*-68*	*E*	.
-33	*B*	*adthA-2*	*-31*	*F*	*adthA-2*
-34	*B*	*adthA-2*	*-31r***	*F*	.
-36	*B*	*adthA-2*	*-59****	*F*	.
-39	*B*	*adthA-2*	*-64****	*F*	.
-41	*B*	*adeE-11*	.	.	.

ade = adenineless; *adth* = adenine and thiaminless; *cys* = cysteineless; *try* = tryptophaniess; * Obtained after ultraviolet irradiation; ** an *adth*$^+$ reversion derived from *metF-31*; *** high frequency of reversion.

In all experiments incubation was at 37°. All liquid cultures were aerated either by bubbling air through them or by agitation at 60–70 rev./min. in tubes held at an angle of 30°. Sedimentation by centrifugation was at approximately 2000 *g* for 15–20 min.; washing was carried out with 0·5% (w/v) NaCl solution or 33 mM-phosphate buffer (KH_2PO_4) adjusted to pH 7 with NaOH.

Phage. The temperate phage PLT-22 (Zinder & Lederberg, 1952) was used for transduction. Donor lysates were prepared by the infection, at a multiplicity of 0·05–0·1, of log-phase cultures of bacteria with phage initially propagated on wild-type LT-2. Incubation was continued for 6 hr. Phage was then sedimented by centrifugation at 78,000 *g* for 1 hr. The deposited phage was resuspended in $\frac{1}{20}$th volume T2 buffer (adsorption medium; Hershey & Chase, 1953) and re-centrifuged at normal speed, the opalescent supernatant fluid then being retained. The phage preparations were sterilized by shaking with $\frac{1}{10}$th volume of chloroform for 30 min. at room temperature and then removing the chloroform by bubbling with air at 37° or room temperature. The layer-plate method (Adams, 1950) was used for phage assay, two separate 1×10^{-10} dilutions of each preparation being plated in triplicate or quadruplicate. The titres of the phage suspensions ranged from 3×10^{11} to 2×10^{12} particles/ml. and were constant during many months of storage at 0–5°.

Transduction. In early experiments, phage from donors was added to overnight

broth cultures of recipient strains at 37° at a multiplicity of 5. After 6 min. adsorption, 0·1 ml. samples of the mixtures were plated on selective minimal media. Phage and recipient bacterial controls were plated at the same time. The number of prototrophic colonies was counted after incubation up to at least 40 hr. The frequency of transduction obtained with *met* mutants by this method (about 1 per 10^7–10^8 donor phage particles) was low compared with that obtained with other auxotrophs of *Salmonella typhimurium*. A 10–100-fold increase in frequency of transduction with the *met* mutants as recipients was obtained by adding phage at 4×10^{10} particles/ml. to cultures in the middle of the log phase. Cultures of suitable titre were obtained by diluting overnight broth cultures 1/20 in broth, incubating for 2 hr., centrifuging and then resuspending in $\frac{1}{4}$ volume of broth. This yielded a culture containing $2–4 \times 10^9$ organisms/ml. The multiplicity of infection was therefore at least 10. In some experiments it was necessary to have plates with 100–200 transduced colonies. After samples had been removed for plating, transduction mixtures were stored at 0–5° for 24 hr. for replating, when required, using new appropriate dilutions.

Media. The nutrient medium used was either 0·8 % (w/v) Difco Beef Peptone in 0·5 % (w/v) NaCl in water or in the tryptic meat digest broth (TMB) made in this Establishment. The addition of agar at 2 % (w/v) provided a plating medium (TMA). The minimal medium (MM) was that of Davis (see Lederberg, 1950) with the K_2HPO_4 and KH_2PO_4 concentrations adjusted to 10·5 g. and 4·5 g./l., respectively. Plating medium (MA) was obtained by the addition of agar (1·5 %, w/v). The following growth factors (μg./ml.) were added as required: adenine HCl, 20; L-cysteine HCl, 80; DL-homocysteine thiolactone HCl, 100; DL-methionine, 20; thiamin (aneurin) HCl, 1; DL-tryptophan, 20; vitamin B_{12}, 0·1. Plating medium MA was occasionally enriched with 0·01 % or 1·25 % (v/v) broth (eMA and EMA, respectively).

Purity of chemicals. The purity of the preparations of DL-cystathionine HCl, L-cysteine HCl, L-homocystine, DL-homocysteine thiolactone HCl, DL-methionine and vitamin B_{12} used in the growth response experiments was tested by means of one-dimensional paper chromatography with collidine as the solvent (Dent, 1948). The preparations of cystathionine, homocystine, vitamin B_{12} and methionine were pure; those of cysteine and homocysteine thiolactone were not. The identification of three impurities besides cystine in the cysteine was not attempted. The preparation of homocysteine thiolactone contained homocystine and probably homocysteine; these may have resulted from slow hydrolysis of the thiolactone (Dubnoff, 1952).

RESULTS

The utilization of methionine precursors on the known pathway of methionine biosynthesis in *Neurospora crassa* (Horowitz, 1947), and of vitamin B_{12}, known to be utilized by some methionineless mutants of *Escherichia coli* (Davis & Mingioli, 1950) was tested by surface inoculation of all mutants to supplemented MA plates as in the normal auxanographic technique (Pontecorvo, 1949). Plates supplemented with different concentrations of cystathionine, cysteine, homocystine, homocysteine thiolactone, methionine or vitamin B_{12} were inoculated with drops, each containing about 2×10^4 organisms, from phosphate buffer dilutions of washed organisms from overnight broth cultures of each mutant, by using a multiple inoculation

device (Smith, unpublished) and incubated for 66 hr. The growth responses observed were similar to those obtained by Glover (1956, 1958); his nomenclature will be used. All mutants responded to methionine; none to cysteine. Thirty-seven mutants responded to either cystathionine, homocystine or homocysteine thiolactone (*metA* + *metB* + *metC* mutants), of which 10 (later designated *metC* by syntrophism experiments), grew slowly on MA medium and were considered to be 'leaky'. Six other mutants responded to vitamin B_{12} (*metE* mutants) and three others to methionine only (*metF* mutants). Thus, on the basis of growth responses alone, three phenotypic groups were recognizable, comprising the *metA* + *metB* + *metC*, the *metE* and the *metF* mutants.

The lowest concentrations of each substance and the times of incubation for maximum growth responses were recorded. Growth responses of mutants (other than the leaky mutants) to methionine (5–10 μg./ml.), homocystine (20 μg./ml.), homocysteine thiolactone (50 μg./ml.) and vitamin B_{12} (0·001 μg./ml.) were maximal after incubation for 42 hr. and to cystathionine (50–100 μg./ml.) after incubation for 66 hr. The leaky mutants responded to methionine, cystathionine, homocystine or homocysteine thiolactone at concentrations of one-half to one-tenth of those necessary for *metA* and *metB* mutants.

The slow growth response of *metA* + *metB* + *metC* mutants to cystathionine at high concentration was further investigated by chromatographic examination of solutions of the substance in sterile distilled water and MM medium after incubation for 66 hr. The former was pure but the latter contained small quantities of two impurities, one of which was homocystine, which might well have been responsible for the growth in the presence of cystathionine. However, the lag in growth response to cystathionine still persisted after inoculation on to MA medium + cystathionine at 50 μg./ml. pre-incubated for 66 hr.

Indication of the sequence of metabolic blocks with which each group of mutants was associated

This was determined by means of syntrophism tests. Two methods gave readily reproducible results.

Parallel streaking. Loop inocula from saline dilutions (1/1000) of washed overnight broth cultures of mutants were streaked 0·5–1 cm. apart on EMA medium, each pair of inocula being isolated either in separate 4 cm. diameter Petri dishes or on sectors of solid medium in 9 cm. diameter Petri dishes. Control streaks of homologous pairs and mutants alongside wild-type were always made. Syntrophism was indicated by relatively denser growth of the edge of a streak adjacent to one releasing a diffusible compound (accumulant) and was visible after incubation for 60 hr. but more clearly after 85–90 hr.

All paired combinations of the strains *metA-6*, *B-16*, *C30*, *E-2* and *F-31*, representative of Glover's 5 groups, were tested in this way. Strain *metC-30* fed strains *metA-6* and *B-16* but not strains *metE-2* or *F-31*. No other feeding was clearly demonstrated. The other 9 leaky strains behaved like strain *metC-30* in similar experiments. These results indicated that the 10 leaky mutants comprised a fourth phenotypic group (*metC*) and that the metabolic blocks in the biosynthesis of methionine associated with the *metA* and *B* mutants preceded, and those of the *metE* and *F* mutants followed, those of *metC*.

Replica plating. Inocula (0·1 ml.) from washed overnight broth cultures of strains *metA-15*, *B-23*, *C-30*, *E-47* and *F31r*, were spread on single EMA medium plates (eMA medium for *C-30*) which, after drying, were stored overnight in the refrigerator. Loop inocula from each culture and a similar culture of wild-type were also spread over well-separated circular areas of about 1 cm. diameter on each of 6 TMA plates, over a template. After overnight incubation, replication was carried out (Lederberg & Lederberg, 1952) from each of these plates to single different refrigerated lawn plates and to an additional uninoculated plate. These plates were then observed during incubation for 96 hr.

Table 2. *Syntrophism of representative* met *mutants of* Salmonella typhimurium

Samples (0·1 ml.) of washed organisms from overnight broth cultures of each mutant were spread as lawns on single EMA medium plates (*eMA* medium for *metC-30*) which were refrigerated overnight. Loop inocula of each mutant and wild-type were spread over areas about 1 cm. diameter on each of 6 TMA medium plates, over a template. After overnight incubation, replication was made from each of these plates to a different refrigerated plate and to an uninoculated EMA medium plate. These plates were then incubated for at least 96 hr.

	Lawns				
Replicate areas	*metA-15*	*B-23*	*C-30*	*E-47*	*F-31r*
metA-15	−, 2	−, 6	−, 6	−, 6	−, 6
metB-23	+, 2	−, 2	−, 6	−, 6	−, 6
metC-30	+, 4	+, 2	−, 5	−, 6	−, 7
metE-47	±, 6	−, 6	±, 7	−, 1	−, 2
metF-31r	±, 6	−, 5	±, 6	−, 1	−, 1
Wild-type	−, 8	−, 8	±, 8	−, 8	−, 8

+ = presence of halos of growth *around* replicated area; − = absence of halos of growth *around* replicated area; ± = poor halos of growth *around* replicated area; 1–8, relative density of growth *within* replicated area.

Syntrophism was evident from the growth response of organisms in the surface lawns as halos around replicated areas, and from the relative density of growth within different replicated areas on each lawn (Table 2). Halos indicated the diffusion of an accumulant to which organisms in the lawns responded. Thus it was inferred that the metabolic block of strain *metA-15* preceded that of *B-23* and that both the *metA-15* and *B-23* blocks preceded that of *metC-30*. There was also a suggestion that the *metA-15* and *C-30* blocks preceded those of *metE-47* and *F-31r*. Allowing for poorer growth within areas surrounded by halos, and the leaky growth within *metC-30* areas, the relative density of growth within other areas confirmed the inferred sequence of metabolic blocks *metA-15–metB-23–metC-30*. No syntrophism of *metE-47* and *F-31r* was observed, but syntrophism of *metA-15*, *B-23* and *C-30* with both *metE-47* and *F-31r* did occur, although the direction of feeding was uncertain. Fourteen of the other 25 (*metA* and *metB*) mutants behaved exactly as *metA-15* and 11 exactly as *met-B23* in further similar experiments. This indicated that these mutants might be divided into two phenotypic groups, comprising 15 *metA* and 12 *metB* mutants.

To find the direction of feeding in syntrophism of *metA*, *metB* or *metC* mutants with *metE* or *metF* mutants, the relative numbers of organisms of each phenotype in replicate areas showing syntrophism of *metA-15*, *metB-23* or *metC-30* with *metE-47* or *metF-31r* was determined, assuming that fed organisms would pro-

liferate more than feeders. Loopfuls of growth from these areas were suspended in 0·5 ml. phosphate buffer and appropriate dilutions plated on TMA medium to yield about 100 single colonies/plate. After overnight incubation replication to MA, MA + homocysteine thioloactone, MA + vitamin B_{12} (where *metE* organisms were involved) and TMA media was carried out and the plates incubated for 4 hr. at 37° and 17 hr. at room temperature.

Between 200 and 500 colonies derived from each area were screened. In all cases, colonies with the *metA*, *B* or *C* phenotype predominated. They comprised an average of 91 % of those from organisms in areas on lawns of *metA-15*, *met-B23* or *metC-30* and 82 % from areas on lawns of *metE-47* or *metF-31r*. It was concluded that the *metA*, *metB* and *metC* metabolic blocks preceded those of *metE* and *metF*.

In summary, the results of the syntrophism tests indicated the existence of 5 phenotypic groups amongst the methionineless mutants (*metA*, *metB*, *metC*, *metE*, *metF*) and that the probable sequence of the metabolic blocks in the biosynthesis of methionine was: *metA–metB–metC–*(*metE–metF*). The arrangement of the *metE* and *metF* blocks in sequence was not possible because of the lack of syntrophism of *metE* and *metF* mutants.

The relationship between phenotypic and transduction groups

This was tested by determining the frequency of transduction to produce prototrophs between all mutants of each phenotypic group and single representative mutants of other groups as recipients with appropriate single representative strains of each of Glover's (1956, 1958) five groups and the wild-type as donors. A lower frequency of transduction between mutants indicated close linkage and membership of the same transduction group (Demerec, Blomstrand & Demerec, 1955). The experiments were carried out in three stages.

The transduction groups amongst the *metA* and *metB* mutants were determined by using the original transduction technique. The selective medium was EMA. From the results recorded in Table 3 (*a*) it can be seen that relatively few if any prototrophs resulted from transduction between all 15 of the *metA* mutants and *metA-6*, or between the 12 *metB* mutants and *metB-17*, thus indicating two transduction groups.

Transduction involving all of the *metC* strains as recipients was carried out in T2 buffer instead of broth; plating was on eMA medium. This controlled residual growth of the leaky *metC* strains and made prototrophic colonies easier to count. The improved transduction technique was used. The results in Table 3 (*b*) indicated that 9 of the 10 *metC* mutants comprised one transduction group. A high frequency of reversion did not permit the use of *metC-57* as a recipient or donor.

Transduction groups amongst the *metE* and *metF* mutants were determined at the same time. Of the three *metF* mutants (Table 1), *metF-59* and *metF-64* showed high frequencies of reversion. That of *metF-59* was too high for its use as donor or recipient but that of *metF-64* (approximately 1 in $1{\cdot}25 \times 10^6$ organisms) permitted its use as a donor. The improved transduction technique was used, with plating on MA medium. All of the *metE* mutants appeared in one group and *metF-31* and *metF-64* in another (Table 3 (*c*)). The decreased frequency of transduction in the *metB-23* (×) *metF-64* cross may indicate linkage between these two mutants. Although all possible crosses between methionineless mutants of the same

Table 3. *The numbers of prototrophs obtained in transduction between* Salmonella typhimurium met *mutants of different phenotypic groups*

Each figure represents the total number of colonies obtained on three enriched MA plates (EMA or eMA media) less the total number of colonies on three recipient control plates after 41 hr. of incubation. Each plate was inoculated with about 2×10^8 organisms from overnight broth cultures (centrifuged and resuspended in T2 buffer where *metC* mutants were recipients) and 1 or 4×10^9 phage particles which had been incubated together for 6 min.

(*a*) *metA* and *metB* mutants

	Donor				Donor		
Recipient	*metA-6*	*metB-17*	Wild-type	Recipient	*metA-6*	*metB-17*	Wild-type
metA-6	0	379	211	*metB-16*	81	0	128
A-7	0	175	122	*B-17*	115	0	114
A-10	0	476	240	*B-20*	83	0	156
A-15	0	41	38	*B-23*	140	5	138
A-18	0	120	62	*B-33*	213	0	195
A-19	10	81	44	*B-34*	91	0	111
A-21	2	148	81	*B-36*	212	0	308
A-22	3	109	82	*B-39*	93	9	147
A-40	7	419	170	*B-41*	72	0	121
A-43	0	445	268	*B-45*	168	26	201
A-44	19	210	102	*B-49**	20	0	14
*A-48**	2	125	64	*B-66**	142	21	176
*A-53**	3	146	83	*C-32*	96	259	137
*A-54**	4	139	74	*E-2*	91	92	86
*A-55**	0	143	65	*F-31*	110	213	142

* New mutants assigned to their transduction groups.

(*b*) *metC* mutants

	Donor			Donor	
Recipient	*metC-30*	Wild-type*	Recipient	*metC-30*	Wild-type*
metC-30	0	3840	*metC-57*	High frequency of reversion	
C-32	590	5850	*C-58*	0	1445
C-35	207	6705	*C-65*	9	5245
C-37	72	2950	*A-15*	3554	1615
C-50	24	1725	*B-23*	4040	3050
C-52	721	3255	*E-2*	3977	2955
C-56	712	3115	*F-31*	6135	5615

* Counts of platings of 1/5 dilutions × 5.

(*c*) *metE* and *metF* mutants

	Donor			
Recipient	*metE-2*	*metF-31*	*metF-64*	Wild-type*
metE-2	0	6459	3719	4180
E-47	798	X	X	6910
E-51	38	X	X	695
E-60	295	X	X	4970
E-61	124	X	X	2310
E-68	108	X	X	5720
F-31	11010*	0	604	8710
*A-15**	7170	8630	6550	5910
*B-23**	6190	5320	1750	5800
*C-30**	3920	5010	2790	3840

* Counts of platings of 1/10 dilutions × 10.
X = no crosses made.

phenotypic groups were not made, the results of the above experiments were considered to show that the transduction and phenotypic grouping of these mutants was coincident.

Attempts to detect abortive transduction

Attempts to detect abortive transduction between representative mutants of each *met* group and wild-type were carried out at different times in several different ways. Both mid and late log-phase recipient cultures were used. Plating from T2 buffer as well as broth to MA, eMA, EMA, MA+ neutralized vitamin-free casein hydrolysate at 0·2 % and MA+ casamino acid at 0·1 % (Demerec *et al.* 1957) media was tested. Observations were made during incubation for 7 days, either continuously at 37° or for 1 day at 37° and then 6 days at room temperature as suggested by Dr H. Ozeki (personal communication). In no case were colonies characteristic of abortive transduction obtained (Ozeki, 1956) although Ozeki (1959) reported abortive transduction in the cross *metC-50* (×) wild-type.

Mapping the sites of mutation of the metB *mutants*

This was attempted by determining the frequency of transduction to produce prototrophs in a series of crosses between them. The improved transduction technique was used and plating was on EMA medium. The same phage preparations were used throughout and estimates of donor and recipient efficiency obtained by transductions using *cysC-7* as recipient with all the *metB* strains as donors, and all of these strains as recipients with phage from the wild-type donor strain. To facilitate counting, 1/10 broth dilutions of these control transduction mixtures were plated. From the results (Table 4) three transduction subgroups within which

Table 4. *The numbers of prototrophs obtained in transductions between* Salmonella typhimurium *mutants of the* metB *group*

Numbers as in Table 3. Plating was on EMA medium.

Recipient	Donor												
	metB												Wild-* type
	-16	*-17*	*-20*	*-23*	*-41*	*-66*	*-33*	*-34*	*-49*	*-36*	*-39*	*-45*	
metB-16	0	0	0	314	613	393	307	239	132	0	453	542	7170
-17	0	0	0	277	404	392	363	155	118	0	444	457	7040
-20	0	0	0	56	20	66	110	8	44	0	69	137	9200
-23	267	218	33	0	9	11	161	73	123	0	241	78	6300
-41	299	126	80	13	0	18	168	35	96	0	156	157	4610
-66	248	144	87	6	27	0	153	40	98	0	81	147	4930
-33	379	192	132	115	220	256	0	44	0	0	135	541	8230
-34	125	112	0	36	28	130	21	0	0	0	147	240	9220
-49	1073	466	140	322	475	622	0	35	0	0	212	856	10260
-36	0	0	0	0	0	0	0	0	0	0	0	0	1350
-39	360	179	221	159	153	158	646	67	56	0	0	484	8580
-45	1233	535	532	136	260	129	975	265	316	0	578	0	13040
*cysC-7**	14710	12570	5210	4990	7020	16880	5760	4780	4950	15250	6590	6890	13380

* Counts of 1/10 dilutions × 10.

transduction occurred at low frequency or not at all were apparent. They comprised *metB-16*, *metB-17* and *metB-20*; *metB-23*, *metB-41* and *metB-66*; *metB-33*, *metB-34* and *metB-49*, respectively. No transduction occurred between the members of the first sub-group or between *metB-33* and *metB-49* in the third subgroup. On the basis of frequency of reversion and behaviour in crosses with all other *metB* mutants, *metB-16* and *metB-17* were similar to each other but different from *metB-20*. However, *metB-33* and *metB-49* were dissimilar in these respects.

No transduction was obtained in reciprocal crosses between *metB-36* and all other *metB* mutants, and transduction between *metB-36* and wild-type occurred at a lower frequency than that between other *metB* mutants and wild-type. As a donor in crosses with mutants of other *met* groups, *metB-36* was as efficient as other *metB* mutants. No reversions were detected on *metB-36* control plates even when each of 10 plates was inoculated with 10 times the number of organisms normally plated in transduction experiments.

The construction of a linkage map from the data of Table 4 was attempted. To correct for donor and recipient efficiency, the frequency of transduction for each cross was multiplied by both the ratios of the frequencies of transduction in the crosses *cysC-7* (×) wild-type/*cysC-7* (×) donor, and *cysC-7* (×) wild-type/recipient (×) wild-type, respectively. As an initial step, linear arrangement of the three transduction subgroups was tried. From the results of an analysis of corrected data from reciprocal crosses between different sets of single representative mutants of each subgroup it was not possible to construct a linear map. The same difficulty was encountered by using the sum of reciprocal transduction between pairs of *met* strains (after Hartman, 1956).

Linkage between the metA, B, C, E *and* F *groups*

This was tested by transduction between the representative strains *metA-15*, *B-23*, *C-30*, *E-47* and *F-31* in a series of donor phenotype selection experiments similar to those used by Clowes (1958) with cyst(e)ineless mutants. If donor and recipient differ phenotypically and it is possible to detect the donor phenotype amongst large numbers of organisms of the recipient phenotype, then the frequency with which transduction between mutants results in organisms which are wild-type for the recipient marker but mutant for the donor marker, should indicate the proximity of the sites of mutation concerned.

Three donor phenotypes were potentially detectable in transduction between pairs of mutants belonging to the different *met* groups. They were: the ability to utilize homocysteine thiolactone (met^{homth}) or vitamin B_{12} ($met^{B_{12}}$) as an alternative to methionine; attributes of *metA*, *metB* or *metC* and *metE* mutants, respectively; and leaky growth on MA medium when plated from broth (met^{leaky}) coupled with feeding of *metA* and *metB* mutants, attributes of *metC* mutants. The crosses in which each phenotype might have been detected are indicated in Table 5.

The ability to differentiate colonies with the different *met* phenotypes from wild-type arising after transduction was established in a series of reconstruction experiments under the conditions summarized in Table 6. All the permissible types of crosses (Table 5) were made by using the improved transduction technique, suitable dilutions of the transduction mixtures being made to give 100–200 transduced colonies/plate. Infected recipient cultures of *metC-30* were diluted in T2 buffer rather than

in broth, to avoid too heavy background growth on the selective medium. Approximately 2000 (range 1257–3025) colonies from each cross were screened for donor phenotype. Transduction of donor phenotype was detected only in the *metF-31* (×) *metB-23* cross in which 1139 out of 3025 (38 %) transduced colonies were of the *met*^{homth} phenotype.

In these experiments, linkage between all pairs of mutants from the different groups, except between those of *metA* and *metB*, was tested in crosses involving transduction in at least one direction. Linkage between *metA* and *metE*, *metB* and *metE* and *metC* and *metE* was tested in both directions (Table 5). It was concluded that no linkage was detectable except between *metB* and *metF*. Assuming a linear arrangement of sites of mutation and linkage of *metB* and *metF*, *metA* could either be between *metB* and *metF* or distal to *metB* on the same side of *metF* as *metB*. If the first situation prevailed, greater than 38 % linked transduction between *metA* and *metF* should have been obtained. In fact no *metA-metF* linkage was detected. Allowing for a 2 % error in the replication technique (Clowes, 1958) and assuming that the production of transducing phage did not involve fragmentation between *metA* and *metB* (Ozeki, 1959), it was concluded that, if *metA-metB* linkage existed, the distance between *metA* and *metB* was at least 19 times that between *metB* and *metF*.

Table 5. *Donor phenotypes* Salmonella typhimurium *met in mutants detectable in inter-group transduction*

Recipient	Donor (phage)				
	metA	*B*	*C*	*E*	*F*
metA	0	X	*met*^{leaky}	*met*$^{B_{12}}$	X
B	X	0	*met*^{leaky}	*met*$^{B_{12}}$	X
C	X	X	0	*met*$^{B_{12}}$	X
E	*met*^{homth}	*met*^{homth}	*met*^{homth}	0	X
F	*met*^{homth}	*met*^{homth}	*met*^{homth}	*met*$^{B_{12}}$	0

met^{homth} = utilization of homocysteine thiolactone; *met*$^{B_{12}}$ = utilization of vitamin B_{12}; *met*^{leaky} = leaky growth on MA and feeding of *metA* and *B* strains; X = donor phenotype not detectable; 0 = homologous crosses.

In conjunction with the previously described attempts to map the sites of mutation of *metB* mutants (Table 4) the frequency of transfer of donor phenotype in crosses between *metF-31* and each of the *metB* mutants was determined. Transduction was carried out under optimum conditions for the detection of the *metB* *met*^{homth} phenotype (Table 6) except that only about 1000 colonies/cross were screened for donor phenotype. An average of 27·5 % (range 21·6–34·1 %) transfer of donor phenotype was obtained in crosses involving 11 of the 12 *metB* mutants as donors. The exception was *metB-36* with which only 3·6 % transfer was obtained. These results indicated that all the *metB* sites were linked to *metF-31* but that 11 of them were clustered together closer to *metF-31* than to *metB-36*.

Linkage between met *mutants and certain other markers*

Linkage was tested by transduction in two ways.

By donor phenotype selection. This was carried out in transduction between the 5 representative *met* mutants used in the intergroup crosses above (recipients) and the six double-auxotrophs *metA-7cysA-21*, *metB-16cysB-16*, *metE-2cysC-7*,

Table 6. *Optimum conditions for the detection of the different donor phenotypes* of *met* mutants of Salmonella typhimurium *in intergroup transduction*

Donor phenotype	Recipient	Initial selection: Medium	Initial selection: Time of incubation	Replication: Media	Replication: Time of incubation	Characteristics of donor phenotype
met^{homth} (*metA, B* and *C*)	*metE* and *F*	EMA + homth	41 hr.	MA and MA + homth	17 hr.	Growth on MA + homth; not on MA
met^{leaky} (*metC*)	*metA* and *B*	MA	6 days	.	.	Microcolonies (1–1·5 mm. diam.) + feeding of background
met$^{B_{12}}$ (*metE*)	*metA* and *B*	EMA + B_{12}	41 hr.	MA and MA + B_{12}	17 hr.	Growth on MA + B_{12}; not on MA
	metF	EMA + B_{12}	28 hr.			
	metC	MA + B_{12}	41 hr.			

homth = homocysteine thiolactone at 100 μg./ml.
B_{12} = vitamin B_{12} at 0·01 μg./ml. (i.e. 1/10th of concentration used in growth response experiments)

metB-20 tryA-8, *metF-31 adthA-2* and *metB-41 adeE-11* (donors). The initial selective medium was supplemented with cysteine, tryptophan, adenine and thiamin or adenine where appropriate, to facilitate growth of colonies with donor phenotype. The screening of about 1000 colonies from each cross was attempted. Initial selection plates were incubated for 24–65 hr. according to the cross, until all of the transduced colonies were about the same size (1–2 mm. diameter) before replication to unsupplemented and supplemented media. The replicated colonies were observed

Table 7. *Attempts to detect the transfer of donor phenotype in transduction between representative mutants of the five* met *groups and certain other mutants of* Salmonella typhimurium

Transduction was as described in Table 3. The MA medium for each cross was supplemented with either cysteine, tryptophan, adenine or adenine + thiamin according to the additional nutritional requirement of each donor strain. The initial incubation was for 24–65 hr. After replication to unsupplemented and supplemented MA medium the plates were incubated for 17 hr.

Recipient	Donor: *metA-7** *cysA-21*	*metB-16** *cysB-16*	*metE-2** *cysC-7*	*metB-20** *tryA-8*	*metF-31* *adthA-2*	*metB-41* *adeE-11*
	No. of colonies screened without detecting donor phenotype					
metA-15	0	1197	1107	1024	1060	1034
B-23	1140	841	730	0	1047	0
C-30	603	1279	716	1056	975	1288
E-47	1355	1533	941	1132	851	820
F-31	1665	1401	1316	847	×	**

* No linked transduction between these double auxotrophs and wild-type (see Table 8).
** Not tested, since selection of donor phenotype *ade* impossible because the additional marker of *metF-31* is *adth*.
× Homologous cross.

after incubation for 17 hr. No colonies with donor phenotype resulted from any of these crosses (Table 7). Three crosses yielded no transduction and the donor phenotype could not be selected in another so that linkage between *metA* and *cysA*, *metB* and *tryA* or *adeE* and *metF* and *adthA* could not be tested. There therefore appeared to be no linkage between: *metA* and *cysB*, *cysC*, *tryA*, *adthA* and *adeE*; between *metB* and *cysA*, *cysB*, *cysC* or *adthA*; between *metC* and *cysA*, *cysB*, *cysC*, *tryA*, *adthA* or *adeE*; between *metE* and *cysA*, *cysB*, *cysC*, *tryA*, *adthA* or *adeE*; or between *metF* and *cysA*, *cysB*, *cysC* or *tryA*.

By transduction of doubly auxotrophic recipients with phage from wild-type donor

This involved the attempted transduction of both markers simultaneously. Crosses of all of the double auxotrophs used above, with *metA-19 tryA-8* substituted for *metF-31 adthA-2*, were made by the improved transduction technique. Each transduction mixture was plated on MA medium, MA + methionine medium and MA + cysteine, tryptophan or adenine medium, according to the alternative nutritional requirement of the recipient. Colonies on the supplemented media were counted after incubation for 41 hr. Final observations of all plates were made after incubation for a further 48 hr. Replication was then made from the supplemented plates to MA medium. The replica plates were observed for wild-type colonies during incubation for 48 hr. The results of these experiments (Table 8), confirmed the absence of linkage between *metA* and *tryA*, *metB* and *cysB* and *metE* and *cysC*, and also indicated its absence between *metA* and *cysA* and *metB* and *tryA* or *adeE*.

Table 8. *Attempts to detect linked transduction between certain* Salmonella typhimurium met *mutants and other loci, by the use of double auxotrophs*

Numbers as in Table 3 except that plating was on MA medium. Where the MA medium was supplemented, platings were made from 1/10 dilutions of the transduction mixtures in addition to those from the concentrated suspensions.

	Recipient					
	metA-7 *cysA-21*	*metB-16* *cysB-16*	*metE-2* *cysC-7*	*metA-19* *tryA-8*	*metB-20* *tryA-8*	*metB-41* *adeE-11*
Media of selection	No. of colonies resulting from transductions using wild type donor					
MA	0	0	0	0	0	0
MA + methionine	4500*	1484	3000*	9000*	14000*	1968
MA + cysteine, tryptophan or adenine	544	1214	568	3580*	4070	2765

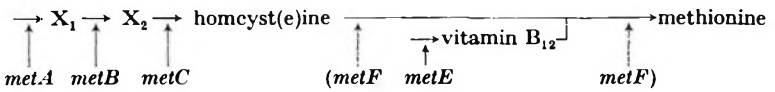
* Approximate counts.

DISCUSSION

Metabolic blocks in a possible pathway of methionine biosynthesis

Homocysteine is believed to be a precursor of methionine in several different micro-organisms, e.g. *Neurospora crassa*, *Bacillus subtilis*, *Escherichia coli* (Young & Maw, 1958) and *Streptococcus lactis* (Kizer, Speck & Aurand, 1955) and is implicated in the synthesis in *Aerobacter aerogenes* (Shapiro, 1955). Davis & Mingioli (1950) reported methionineless mutants of *E. coli* W which showed similar growth

responses and syntrophism to those of the *Salmonella typhimurium metE* and *metA*, *B* and *C* mutants, and postulated that vitamin B_{12} was a component of a coenzyme concerned with the methylation of homocysteine. This function of vitamin B_{12} seemed to be supported by studies of the synthesis of methionine with cell suspensions and cell-free extracts of at least one of these mutants (Gibson & Woods, 1952; Helleiner & Woods, 1956). The growth response of members of the *S. typhimurium metA*, *B* and *C* phenotypic groups to homocystine and homocysteine thiolactone suggested that homocystine or homocysteine (homocyst(e)ine) was also on the pathway of methionine biosynthesis in *S. typhimurium* and that these mutants were blocked in metabolic steps before this intermediate, and *metE* and *F* mutants after it. This sequence is supported by the results of the syntrophism experiments and the existence of three metabolic blocks associated with *S. typhimurium metA*, *metB* and *metC* mutants in that sequence established (Fig. 1).



X = unknown precursor of methionine; ↑ = possible alternative positions of metabolic block; ( ) = indeterminate sequence of blocks.

Fig. 1. A suggested pathway of methionine biosynthesis in *Salmonella typhimurium*.

Since *metE* and *metF* mutants fed *metA*, *B* and *C* mutants but themselves failed to show syntrophism, it was concluded that their metabolic blocks involved inability to carry out some (probably the same) biosynthetic step between homocyst(e)ine and methionine. It is suggested that the function of vitamin B_{12} in the biosynthesis of methionine is the same in *Salmonella typhimurium* and *Escherichia coli* and that the *S. typhimurium metE* mutants lack the coenzyme concerned with the methylation of homocysteine of which vitamin B_{12} (or a derivative of it) is a component. *S. typhimurium metF* mutants may be considered to lack the enzyme necessary for this metabolic step.

The results of these biochemical studies are in general agreement with those outlined by Glover (1956, 1958) who used some of the same mutants, except that the growth response of *Salmonella typhimurium metA* and *B* mutants to cystathionine was not clearly confirmed. However, cystathionine should not perhaps be excluded as an intermediate, for Wijesundera & Woods (1953) showed that 8 out of 10 mutants of *Escherichia coli* which possessed cystathionase were able to convert exogenous cystathionine to homocysteine but only synthesized small quantities of methionine. They suggested that other enzymes might compete for homocysteine; perhaps the differences between cultural conditions for *S. typhimurium metA* and *B* mutants in Glover's experiments and those reported in this paper, affected the relative activity of these competing enzymes so that a definite growth response to cystathionine was obtained in the former but not in the latter.

Correlation of phenotypic and transduction groups

Members of the same transduction group of *Salmonella typhimurium met* mutants possessed identical biochemical characteristics so far as they were tested and each can be regarded as an allele of one of 5 complex gene loci (Demerec, 1956*b*) namely

metA, *metB*, *metC*, *metE* and *metF*. In the absence of a complementation test (abortive transduction) it was not possible to decide whether each locus was a functional unit. Demerec & Ozeki (1959) gave examples of complex loci defined in the same way as the *met* loci but subsequently shown to comprise more than one closely linked functional unit. They also reported, incidentally, that complementation might not always serve to differentiate such loci and that abortive transduction was not detectable with some mutants.

The metB *locus*

The attempts to map the sites of mutation of the *metB* locus in *Salmonella typhimurium* were subject to limitations, because, even with the improved transduction technique, the efficiency of transduction of *met* mutants was less than that of most other mutants of *S. typhimurium* and hence the chances of recombination were decreased. The resolving power within *met* loci might well have been as low as 1% of that within the *his* locus (Hartman, Loper & Serman, 1960) and 10% of that within the *try* locus (Demerec & Hartman, 1956) respectively. It was perhaps not surprising that a linear map of the *metB* locus could not be constructed. For accurate location of sites three- or four-point linkage tests are a great advantage, especially when the efficiency of transduction is low (compare Clowes's (1958) studies of the *cysB* locus). Unfortunately, no linkage was detected between any of the *met* loci and a number of others suitable for such tests.

The properties of *Salmonella typhimurium metB-36* indicate that it is a multiple-site mutant (Demerec, 1956*a*) in that it failed to recombine in reciprocal transduction with a number of closely-linked mutants, that it was the only mutant of the 12 *metB* mutants to do this, and that it failed to revert. Definition of the region of the chromosome covered by the *metB-36* mutation is not possible at present. It does not cover the *metF* locus to which the *metB* locus is linked, but may cover an undiscovered locus (or loci) between *metB* and *metF* or on the other side of *metB*, or may even cover loci on both sides of *metB*. The failure to obtain transduction between one group of three (*metB-16*, *B-17* and *B-20*) and one pair (*metB-33* and *B-49*) of the *metB* mutants other than *metB-36* may have been due to identical-site mutation at two sites (Demerec, 1956*a*). There is evidence that some identical-site mutants show similar frequencies of reversion (Demerec, 1956*a*), and similar behaviour in intra-locus transduction might be anticipated. For *metB-16*, *B-17* and *B-20* and *metB-33* and *B-49* incidental observations of their reversion frequency and the data of Table 4 revealed that only *metB-16* and *B-17* were similar in these respects. It was concluded that the sites of mutation of *metB-16* and *B-17* might well be identical, but *metB-20* differed from these two and *metB-33* and *B-49* from each other, in that mutation in these mutants involved overlapping but probably different numbers of sites on the chromosome. The speculation could be made that each mutation in the *metB* locus other than *metB-36* (probably a deletion) is a multiple-site change involving different small lengths of the chromosome. This would perhaps explain mapping difficulties within the locus.

Behaviour of metB-36 *in transduction*

In the *metF-31* (×) *metB-36* cross only 3·6% donor phenotype transfer was obtained as compared with an average of 27·5% with other *metB* mutants as donors.

From the results of transduction between all the *metB* mutants (Table 4), *metB-36* appears to be a multiple-site mutant, probably a deletion. Interference with synapsis seems the most likely explanation of the decreased frequency of donor phenotype transfer with *metB*-36 and, incidentally, the decreased frequency of transduction in the cross *metB-36* (×) wild-type in Table 4. In the former case this could have resulted from a reduction in size of the donor transducing fragments. If fragmentation of the donor chromosome in the preparation of phage lysates is not random (Ozeki, 1959), the formation of chromosomal fragments from *metB-36* carrying the *metB* and *F* loci should include a deletion and therefore be shorter.

Linkage relationships of the met *loci*

The linkage between two of the 5 *met* loci is a further example of linkage between loci concerned with related function in *Salmonella typhimurium* (Demerec, 1956*a*). Detection of linkage of other *met* loci may have been impossible because of fragmentation of the donor chromosome between them in the preparation of transducing phage (Ozeki, 1959). In some instances the linear linkage sequence of functionally related loci of *S. typhimurium* was the same as that of the sequence of metabolic blocks associated with each locus, i.e. an 'assembly line' was formed. Five of the 7 loci concerned with histidine (Hartman, Loper & Šerman, 1960) and all the 4 loci concerned with tryptophan biosynthesis (Demerec & Hartman, 1956) were shown to be arranged like this. Tryptophan biosynthesis in *Escherichia coli* involves the same pathway and is controlled by similarly linked loci (Yanofsky & Lennox, 1959). Examples of less perfect associations prevail amongst the cysteine (Clowes, 1958) and proline loci (Demerec *et al.* 1958) of *S. typhimurium*, where one pair out of 5 and one pair out of 4 loci, respectively, were linked and associated with consecutive metabolic steps. In any case, with the possible exception of the purineless mutants (Yura, 1956*a*; Demerec *et al.* 1956), whenever linkage between functionally related loci was demonstrated, the equivalent metabolic blocks appeared to be consecutive (Demerec *et al.* 1955, 1958). The sequence of metabolic blocks in the biosynthesis of methionine is *metA–metB–metC–*(*metE metF*) so that the linkage of the *metB* and *metF* loci constitutes an example of linkage between functionally-related loci not concerned with consecutive metabolic blocks.

The selective advantage of the assembly line of genes has been stressed both from the functional (Demerec & Demerec, 1955; Demerec & Hartman, 1956) and genetical (Hartman, Loper & Šerman, 1960) points of view. Linkage between *metB* and *F* indicates that at least two of the *met* loci are not arranged in an assembly-line sequence. However, within intact bacteria, folding and interfolding of the chromosome may ensure that functionally related loci are arranged in such a sequence (Pontecorvo, 1958). On the other hand, general acceptance of the genetical selective advantage of assembly lines to organisms with systems of partial genetic transfer should obviously be avoided.

Methionineless mutants of a number of different micro-organisms have been reported. However, appropriate biochemical and linkage data for comparison with the *met* mutants of *Salmonella typhimurium* are at present limited to strains of *Escherichia coli*, *Neurospora crassa* and *Streptomyces coelicolor*.

The information available on the methionineless mutants of *Escherichia coli* K12 suggests similarities with *Salmonella typhimurium* (Clowes & Rowley, 1954;

Ruebner, 1956; Dr F. Jacob, personal communication). On the basis of growth responses, three phenotypic groups are recognizable. They are equivalent to the *metA*+*metB*+*metC*, the *metE* and the *metF* mutants of *S. typhimurium*, respectively. In conjugation experiments one mutant of the first group (comprising 7 mutants) is linked to the only available representative of the last group (the M1 and M2 mutants of Clowes & Rowley, 1954). The other 6 mutants of the first group are closely linked together and located some distance from M1 and M2 on the circular linkage map of Pardee, Jacob & Monod (1959). Mutants of the second group are closely linked together but unlinked to any of the other methionineless mutants. It is interesting to note that the only linkage between phenotypically different methionineless mutants of *E. coli* involves mutants similar to those of the *metB* and *metF* loci of *S. typhimurium* which were the only *met* loci of this organism shown to be linked.

Seven mutants of *Neurospora crassa* associated with at least four different blocks in methionine biosynthesis have been recognized. Six of these have been mapped (Barratt, Newmeyer, Perkins & Garnjobst, 1954; Drs Catcheside & Murray, personal communication). None of these is closely linked although three are located on the same chromosome. Hopwood (1959) recognized 4 loci concerned with methionine biosynthesis in *Streptomyces coelicolor* but none of these showed close linkage to the others. Three linkage groups were suggested; 2 of the loci were situated in one and one each in the other two.

The following linkage map for some loci of *Salmonella typhimurium*, including the 5 *met* loci, was suggested by Demerec & Demerec (1955) and Glover (1955, 1956):

tryD–tryC–tryB–tryA–cysB–metE–cysA–metC–metA–metB–metF–(*cysC cysD*)

From the transduction studies reported in the present paper, the inter-locus donor phenotype selection data support the suggestion of linkage between *metB* and *metF* but not between *metC* and *metA* or *metA* and *metB*. Failure to detect donor phenotype transfer in transduction involving certain double auxotrophs as donors (Table 7) and the absence of prototrophs results from transduction between certain double auxotrophs and wild type (Table 8), provide no evidence for linkage between *tryA* and *metE*, *cysB* and *metE*, *metE* and *cysA*, *cysA* and *metC* and *metF* and *cysC*. These results and those of similar experiments by Clowes (1958, and unpublished) do not support the linkage map of Demerec and his colleagues, at least for the region to the right of *cysB*.

This work was begun in the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harber, Long Island, N.Y., U.S.A. during the tenure of an English Speaking Union King George VI Memorial Fellowship for 1955–6. I am indebted to Dr M. Demerec and his colleagues in this Department for the provision of mutants. I wish to thank Dr N. Horowitz (California Institute of Technology) for preparations of cystathionine and homocysteine thiolactone. At the Microbiological Research Establishment, Porton, thanks are due to Mr J. R. Slade for additional preparations of these compounds, to Mr H. E. Wade for carrying out chromotographic analyses and to Dr T. W. Burrows for helpful discussion. Much excellent technical assistance was given by Mr L. W. Stott. Some of this work formed part of a Ph.D. thesis submitted to the University of Reading in 1959.

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J. gen. Microbiol. (1961), 24, 355–367
With 2 plates
Printed in Great Britain

Phenotypic Alterations Associated with the Bacteriophage Carrier State of *Shigella dysenteriae*

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(*Received* 19 *August* 1960)

SUMMARY

Shigella dysenteriae strain 136-R4 is lactose-negative, mannitol-negative (Lac^- $Mann^-$) and is sensitive to bacteriophage T7. Carrier clones of strain R4 (contaminated with T7) were found to maintain their association with phage T7 through as many as fifty consecutive single-colony isolations (from an ancestral colony which had survived lysis by phage T7). All carrier cultures (so-called pseudolysogenic strains) were found to be lactose-positive and mannitol-positive (Lac^+ $Mann^+$). Passage of Lac^+ $Mann^+$ bacteria through media containing antiserum directed against phage T7 resulted in a change back to Lac^- $Mann^-$ and in the complete elimination of phage T7.

Biochemical, genetic and immunochemical evidence indicates that the change from Lac^- $Mann^-$ to Lac^+ $Mann^+$ is the result of a phage-controlled alteration in the phenotype of *Shigella dysenteriae*. This dysentery bacillus is cryptic with respect to the expression of Lac^+ $Mann^+$ and the crypticity is attributable to surface structures (which are probably not a part of the Y or permease system). Under appropriate conditions decryptification may be brought about by phage-associated endolysin. Similar phenomena were observed in carrier strains of certain other members of the Enterobacteriaceae.

INTRODUCTION

In a previous communication (Barksdale, 1959) it was suggested that phage T7 is capable of converting Lac^- *Shigella dysenteriae* to Lac^+ *S. dysenteriae* and that this happens whether the phage T7 stocks have been derived from Lac^- or Lac^+ bacteria. In other words, an inherent property of bacteriophage T7 is responsible for the change from Lac^- to Lac^+. This phage conversion at first attracted our attention because we assumed *S. dysenteriae* to be genotypically Lac^- (Z^-) as had Luria & Burrous (1957*a*, *b*) and Cohn, Lennox & Spiegelman (1960). From the data to be presented here it is evident that some strains of *S. dysenteriae* are phenotypically Lac^- (cryptic) but genotypically Lac^+ (Z^+). Bacteriophage T7 produces an endolysin capable of decryptifying Shiga's bacillus (*S. dysenteriae*). This 'unmasking' is non-specific in that all strains which are Mal^- (maltose-negative), $Mann^-$ (mannitol-negative) and Lac^- become in the presence of phage (under suitable conditions) Mal^+ $Mann^+$ Lac^+. (Rough strains of *S. dysenteriae* grow feebly on maltose and on maltose MacConkey plates these organisms form pale pink colonies which are easily distinguishable from the red large colonies formed by phage-carrying clones.)

The association between phage and bacterium which makes possible these alterations in phenotype has been termed the carrier state or pseudolysogenic condition (Lwoff, 1953; Jacob & Wollman in Adams, 1959). The fact that the pseudolysogenic condition carries with it pseudogenetic effects is of some consequence for investigations concerned with bacterial and bacteriophage genetics. Decryptification by phage offers a useful tool for quickly determining the presence of hidden properties of bacteria not detectable in the phage-free state.

METHODS

Organisms. Aside from the strains of *Shigella dysenteriae* described herein, subcultures of the old rough strain Sh, originally used by Lisbonne & Carrère (1923) were kindly supplied by Dr G. Bertani (Sh Bertani) and by Dr S. Spiegelman (for E. S. Lennox) and by Dr S. E. Luria (Sh 15 and Sh 16). Strain Sh 60 was sent to us by Dr W. E. van Heyningen.

Escherichia coli strains of established genetic constitution with regard to the β-galactoside system were supplied by Dr J. Monod. We are indebted to Dr N. Zinder for his strain(s) of *Salmonella typhimurium.*

Media. MacConkey's agar base was compounded from (g.): Bacto-peptone, 17; Proteose-peptone, 3; NaCl, 5; Bacto-agar, 13·5; Bacto Neutral red, 0·025; water to 1 l., final pH 7·1. Sterilized by autoclaving at 120° for 20 min. Carbohydrate supplements were added as 20% (w/v) solution in distilled water, sterilized by filtration, sufficient being added (aseptically) to the molten agar base to yield 1% (w/v). Plates were poured to desired thickness.

Neopeptone broth consisted of Neopeptone Difco + 50% (v/v) fresh beef infusion. Neopeptone agar consisted of Neopeptone Agar Difco + 50% (v/v) fresh beef infusion.

PGT medium is the casein hydrolysate medium of Mueller & Miller (1941) as modified by Barksdale & Pappenheimer (1954).

Bacteriophage. All phages of the T series were derived from stocks maintained by the late Mark H. Adams. Phage PLT22 *vir* (virulent) was kindly supplied by Dr N. Zinder (Rockefeller Institute). Methods and materials for phage assay were as described by Adams (1959).

Preparation of acetone powder of Shigella dysenteriae. Organisms from an actively growing culture were twice washed in saline in the cold (4°) and subsequently washed four times with eight volumes of cold acetone. Acetone from the final wash was decanted and the organisms dried to a white powder in vacuum. Plates for endolysin assay were prepared by adding 0·5 ml. of powder (resuspended in saline to optical density 5, at 590 mμ as measured in a Bausch & Lomb Jr. spectrophotometer) to 2 ml. molten soft agar and plating as a soft agar layer according to the method described by Adams (1959).

Assay for β-galactosidase. For the assay of this enzyme the method of Pardee, Jacob & Monod (1959) was modified as follows. Toluenized extracts were not prepared because, except for organisms grown in maltose, toluenized extracts of the shigella strains used here proved to have less activity than unextracted organisms. Instead, organisms were mixed with 10^{-3} M-*o*-nitrophenyl-β-D-galactoside in 0·25 M-sodium phosphate buffer and incubated for 10 min. at 28°. The reaction was then halted by the addition of 4 ml. cold M-Na_2CO_3 and the organisms removed by

centrifugation in the cold. The supernatant fluid was decanted and its optical density read in the Zeiss spectrophotometer at 420 mμ. The unit of enzyme was that amount which yielded 1 mμmole-*o*-nitrophenol/min. at 28°.

RESULTS

Shigella dysenteriae wild type (strain Sh 136-T) was isolated by one of us (L.B.) from a case of Shiga dysentery in Tokyo in 1947. Strain 136-T forms smooth translucent colonies on Neopeptone agar; it possesses a characteristic surface antigen and exhibits a low but definite virulence for mice (Barksdale, Okabe & Li, unpublished). It is resistant to the phages T3, T4, T5 and T7. It does not ferment lactose, mannitol or maltose. A commonly encountered mutant of strain Sh 136-T forms opaque colonies (strain Sh 136-0) on Neopeptone agar, is antigenically distinguishable from wild-type, is of a lower order of virulence for mice and can support the multiplication of phage T7. From strain Sh 136-0 a number of rough mutants have been derived. Those important to this report are Sh 136-R1, Sh 136-R2 and Sh 136-R4. Those are all less virulent for mice than strains 136-T and 136-0. They belong to the class of rough *Shigella dysenteriae* and therefore have much in common with the rough strains: Sh (Bertani), Sh 15 of Luria (see Adams & Luria, 1958) and Sh 60 of Van Heyningen (1955). It can be seen from Table 1 that rough strains were capable of adsorbing and supporting the growth of more different phages of the T series than was the wild-type 136-T. For example, strain 136-T is resistant to phage T7, whereas strain 136-R4 is sensitive to T7.

Table 1. *Efficiency of plating* of coliphages on* Shigella dysenteriae

	Bacteriophage							
	T1	T2	T3	T4	T5	T6	T7	$T7^{Sh}$
Strain	Efficiency of plating (%)*							
Sh 136-T	84	1	r	r	r″	5	r	0·3
Sh 136-0	180	39	r	r	r″	0·01	1·8	1·0
Sh 136-R1	100	85	97	r	46	96	195	150
Sh 136-R2	127	93	50	r	81	22	110	350
Sh 136-R4	103	86	93	r″	71	81	432	520
Sh (60)	r	73	95	0·001	r	1	126	33
Sh (Bertani)	102	0·01	14	100	180	100	104	67
Sh 15	93	90	0·5	26	67	54	98	130

* $\text{EOP} = \dfrac{\text{Number of plaques formed on Shigella}}{\text{Number of plaques formed on } E.\ coli\ \text{B}} \times 100.$

r = resistant, as indicated by failure of plaque formation in any dilution of stocks containing 10^{10} particles/ml.

r″ = resistant but lysed by phage endolysin (this category includes lysis from without).

Though sensitive to phage T7, clones of 136-R4 were found to enter a carrier relationship with this bacteriophage, so that the virus persisted with the bacteria through many generations. Our carrier strains were first isolated as supposed bar mutants of 136-R4 resistant to phage T7 (136-R4/7). Drops (approximately 0·025 ml.) of phage T7 (5×10^{10} particles/ml.) were placed on lawns of 136-R4.

After several days of incubation at 30° there appeared in the middle of the lysed area, colonies of resistant bacteria (see Demerec & Fano, 1945). Some of these were picked and streaked on agar plates to obtain isolated clones free from contaminating phage. A number of the colonies derived from this first plating exhibited a nibbled appearance. Restreaking of entire (non-nibbled) colonies yielded some nibbled and some entire colonies. After fifty successive restreakings of entire colonies, nibbled colonies and entire colonies continued to segregate. Since T7 is a virulent phage, it seemed unlikely that it had been perpetuated as prophage in the cultures. Cultivation of phage-carrying clones in anti-T7 serum resulted in a complete elimination of the virus. The phage-free bacteria so treated were again sensitive to T7.

Presence of phage associated with Lac$^+$ condition

When the streaking which led to the segregation of entire and nibbled colonies was carried out on lactose agar plates containing neutral red as an indicator (MacConkey's agar), red colonies (lactose-positive) and colourless colonies (lactose-negative) developed on the plates. Some of the red colonies were obviously nibbled and some were entire. When 10 red colonies were suspended in 1 ml. broth, centrifuged and the supernatant fluid assayed for phage, phage was present in each of the 10; none of 10 colourless (Lac$^-$) colonies, so examined, contained phage. When the bacteria from a Lac$^+$ colony were suspended in anti-T7 serum and subsequently plated on lactose plates, only Lac$^+$ colonies grew out. Thus it appeared that Lac$^+$ colonies always carried virus and, on subculture, always segregated Lac$^+$ and Lac$^-$ colonies. Furthermore it became apparent that Lac$^+$ was a more reliable index to the presence of virus in a colony than was the state of the margin of the colony (nibbled *versus* entire). The first question these observations raised was 'where is the lactose-positiveness coming from?' The phage T7 preparations were originally grown on *Escherichia coli* B, a lactose-positive organism. So, starting from a single plaque of T7 on 136-R4 (a supposed lactose-negative organism) a stock of T7 was prepared by growth on this shigella. The preparation of phage so obtained was designated T7^{8h}. It differed from T7 slightly (see Table 2) but was able satisfactorily to establish the carrier condition with 136-R4. The carrier clones were Lac$^+$.

Table 2. *Host ranges of phages T7 and T7^{8h}*

	Lysis by	
Bacterium	T7	T7^{8h}
Shigella 136-T	−	+
Shigella 136-R4	+	+
Coli W-2241	+	−

One interesting fact about the phages T7 and T7^{8h} multiplying on Shigella 136-R4 is worthy of note. The one-step growth curves obtained from the growth of T7 and T7^{8h} on strain *Escherichia coli* B were quite different from those obtained on shigella 136-R4. In the case of strain 136-R4 there appeared to be two separate bursts: one occurred from the 19th to the 30th minute; the other, from the 35th to the 52nd minute. The greatest increment of phage came from the late-lysing

population. This effect was observed only under standard conditions of phage growth (Adams, 1959) where nutrient broth or Neopeptone broth were employed. Experiments carried out in the semi-defined PGT medium (casein hydrolysate) yielded data similar to that shown in Fig. 1*a*.

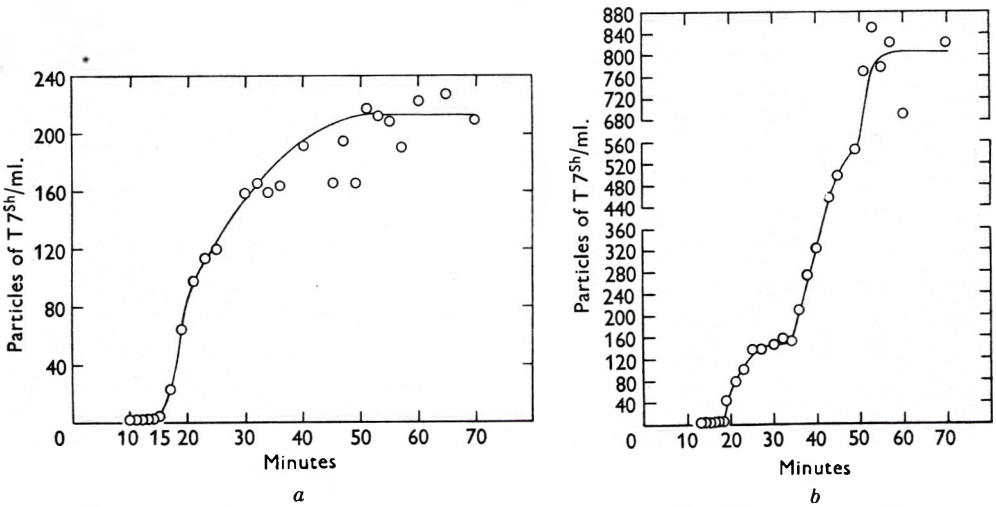

Fig. 1*a*. One-step growth curve of $T7^{Sh}$ grown on *Escherichia coli* B. Multiplicity of phage to bacterium = 0·1. Multiplicity was calculated from titre as determined on *E. coli* B (concentration of bacteria: $1{\cdot}5 \times 10^8$ bacteria/ml.).

Fig. 1*b*. One-step growth curve of $T7^{Sh}$ on *Shigella dysenteriae* 136-R4. Multiplicity of phage to bacterium = 0·1. Multiplicity was calculated from titre as determined on Sh. 136-R4 (concentration of bacteria $1{\cdot}5 \times 10^8$ bacteria/ml.).

The carrier colony

Carrier colonies which grew out on MacConkey's agar developed as completely red (lactose-positive) colonies or as sectored colonies (lactose-positive sectors alternating with lactose-negative sectors). As the sectored colonies aged, they became completely red. Restreakings of sectored colonies yielded Lac^+ and Lac^- colonies. Microscopic examination of organisms from Lac^- colonies revealed short rod-shaped bacteria. Organisms from Lac^+ colonies were swollen spheroplast-like structures; sometimes with pieces of material adhering to their surfaces, much as portions of cell wall adhere to the membranes of freshly prepared spheroplasts (See drawings in Lederberg, 1956, which show a similar appearance). Occasionally, these swollen structures occurred in short chains of three to five units flattened at their abutting sides. Larger fragments of cell wall appeared to be retained by organisms in chains than by single units. These spheroplast-like structures (demi-spheroplasts) were osmotically fragile. They were not observed on Gram-stained preparations but were readily visible in wet mounts prepared in 20% (w/v) sucrose. When the bacterial mass was taken from one colony, suspended in sucrose broth, diluted in sucrose broth and plated, the number of viable units was about 5×10^5. This is one hundred to one thousand times fewer viable units than one would expect from an uninfected bacterial colony of similar size.

Osmotic fragility of carrier clones

The osmotic fragility of a carrier colony was tested by suspending the colony in sucrose, diluting half of the suspension in sucrose broth and half in plain broth and comparing the viable units obtained by plating on MacConkey's agar. Eighty % more survivors occurred on the platings from sucrose than on those from broth. Seventy-five % of the sucrose survivors were Lac$^+$ whereas only 12 % of the broth survivors were Lac$^+$, thus suggesting that the organisms likely to give rise to Lac$^+$ clones were osmotically more fragile than those likely to give rise to Lac$^-$ clones. A probable explanation for the extreme fragility of these organisms was found. Phages T7^{8h} and T7, like so many bacteriophages, produce plaques with a central clear area surrounded by a halo (opaque zone) in which the appearance of the bacterial mass is different from that existing beyond the plaque and comprising the unaffected lawn. While the bacteria in the halo have an obvious alteration in their optical properties, they are not infected with phage; no phage can be obtained from them. The endolysin responsible for their appearance is also capable of bringing about the lysis of dead bacteria. In Pl. 1, figs. 1, 2, is shown a hole formed in a lawn of dead dysentery bacilli (acetone-powder) by phage T7 endolysin.

It is very well established that endolysins are capable of removing specific phage receptors (Adams & Park, 1956; Adams, 1959). Bacteria which have lost their receptors, either by enzymic action or as a result of mutation, are immune to attachment by the whole phage (Spizizen, 1957). Thus the bacteria in a halo are immune to phage much as are spheroplasts. The bacteria in a *Shigella dysenteriae* carrier strain presumably owe their immunity to endolysin generated by the small number of bacteria in which phage succeeds in multiplying.

How does the first bacterium of a carrier clone survive infection? In Table 3 is shown the incidence of Lac$^+$ survivors at various multiplicities of phage to bacteria. Each of these Lac$^+$ clones must have been initiated by a bacterium which had escaped lysis. Fraser (1957) in experiments carried out in microdrops established that certain mutants of phage T3 gave rise to lasting complexes with *Escherichia coli* B. 'The complexes' (bacteria infected with phage) 'can increase in length, divide and give phage-sensitive uninfected segregants before lysis'. We found that phage T3 gave Lac$^+$ carrier clones with both *Shigella dysenteriae* and *S. sonnei*. Whilst we have carried out no single-cell studies, we feel that Fraser's findings offer a reasonable explanation for the origin of our carrier clones: the initially infected ancestral bacterium fails to lyse, reaches a spot on an agar plate, divides to give rise to an infected and uninfected daughter. This process may be repeated several times before lysis actually occurs. (Of course a few bacteria in the population might very well have their receptors destroyed and escape infection and arrive on the plating medium in a receptorless state, etc.). With lysis there is liberation of phage and endolysin. Bacteria geographically so located that they absorb phage may subsequently lyse. Bacteria bathed in endolysin become immune as they lose their receptors. Eventually, an equilibrium is reached between phage, susceptible bacteria and immune bacteria. Susceptible bacteria are necessary for continued generation of endolysin. This equilibrium may be destroyed by anti-phage serum or by dilution into broth or other media having little or no osmotic buffering capacity.

Table 3. *Incidence of carrier clones* (136-*R*4.*T*7^{sh}) *when* 1×10^8 *organisms of* Shigella dysenteriae 136-*R*4 *were infected with various multiplicities of phage* *T*7^{sh}

Ratio of phage to bacteria	Dilution of infected culture which was plated	No. of Lac$^+$ survivors	Lac$^+$ survivors as % of total survivors
50	10^{-1}	0	—
	10^{-2}	2	33
	10^{-3}	27	28
	10^{-4}	2	67
10	10^{-1}	0	—
	10^{-2}	8	12
	10^{-3}	20	33
	10^{-4}	2	40
1·0	10^{-1}	0	—
	10^{-2}	30	23
	10^{-3}	3	60
	10^{-4}	0	—
0·1	10^{-1}	0	—
	10^{-2}	10	5
	10^{-3}	1	2
	10^{-4}	0	—

Evidence for the presence of β-galactosidase in Shigella dysenteriae

*The effect of methyl-thio-β-*D*-galactoside* (*TMG*) *on the degree of β-galactosidase activity in culture of* 136-*R*4. Following a discussion with Dr H. Rickenberg about the possible presence of *β*-galactosidase in *Shigella dysenteriae*, we found that 136-R4 organisms grown in PGT medium produced appreciable amounts of enzyme when methylthio-*β*-D-galactoside (TMG) was present. In Table 4 are shown the results of growing strain 136-R4 in PGT medium with no added carbohydrate, with 2% maltose and with 10^{-3} M-TMG.

Table 4. *β-Galactosidase synthesis by* Shigella dysenteriae 136-*R*4 *Grown in PGT medium*

	+ Maltose	+ TMG	No addition
Optical density*	3·1	2·6	2·75
Units of enzyme	0·11	11·6	0·026
$\frac{\text{Units enzyme}}{10^9 \text{ bacilli}}$	0·035	4·4	0·009

* O.D. 1·0 = 1×10^9 136-R4 organisms/ml.
Similar results were obtained with Shigella strain Sh 60. Strain Sh 15, however, showed about $\frac{1}{200}$th the activity of shigella 136-R4.

The occurrence of Lac$^+$ mutants of 136-*R*4. When bacteria were plated so that about 5 to 10 well-isolated colonies developed on a single plate the diameter attained by each colony (with prolonged incubation) might be as much as 10–15 mm. (so-called giant colonies). Thick MacConkey's agar plates (lactose) containing giant

colonies of strain 136-R4 were sealed with masking tape and incubated for 8 weeks at 30°. During this time several red papillae appeared on the colonies of 136-R4. Four bacterial lines developed from an equal number of papillae (136-R4-L-1, -L-2, -L-3 and -L-4) all proved to give positive fermentation reactions in standard lactose fermentation tubes. When one of these mutants was tested for enzyme activity, its basal level was found to be much greater than that of 136-R4 (see Table 5).

Table 5. *β-Galactosidase production by mutant shigella* 136-*R*4-*L*-1 *when grown in PGT medium with the indicated additions*

	No addition	Maltose 2%	Glycerol 2%	Glucose 2%	Lactose 1%	TMG* 10^{-3}M	IPGT† 10^{-4}M
Optical density	2·8	3·3	2·2	1·4	2·8	1·8	2·8
Units β-galactosidase‡ / 10^9 bacilli	5·8	6·3	3·3	2·9	15·1	5·3	11·2

* The presence of TMG in the medium resulted in a 'stickiness' of the bacteria, i.e. on standing they sedimented to form a tightly adhesive mass. This phenomenon was observed only in the case of organisms grown with TMG.

† Isopropyl-thio-β-D-galactoside was kindly provided by Dr S. E. Luria from a sample from Dr D. Türk (Institut Pasteur, Paris).

‡ 1 unit enzyme is that amount which produces 1 mμmole *o*-nitrophenol/min. at 28°, pH 7·0 (see Pardee *et al.* 1960).

The occurrence of Lac⁺ recombinants in the cross Hfr (Z⁻) × 136-*R*4. It was previously established (Barksdale, 1959) by radioisotope experiments carried out with Dr M. J. Osborn of this department that, under conditions where *Escherichia coli* ML30 showed normal uptake of labelled ^{35}S thio-β-D-digalactoside, 136-R4 was unable to concentrate this substrate. If 136-R4 is truly a cryptic strain, then one should be able to eliminate its crypticity by bringing about a genetic alteration of its surface. Mating between shigella and *E. coli* strains was first accomplished by Luria & Burrous (1957*b*). We have for some time been using this ability to mate as one of several means of characterizing various strains of *Shigella dysenteriae* and we find that rough strains undergo mating and recombination with various Hfr strains of *E. coli*. Dr S. E. Luria kindly supplied us with a strain of Hfr characterized as: Hfr (λ) M⁻Pr⁻ Lac 72 (extreme Z⁻ type deletion). The genetic designations Y (permease), Z (β-galactosidase) and i (inducibility) refer to that *Escherichia coli* chromosomal segment governing the β-galactoside system. By using a streptomycin-resistant mutant of strain 136-R4 (136-R4 S^r) as an F⁻ (acceptor) parent, a mating was carried out: 1×10^8 organisms of 136-R4 S^r Lac⁻ *non-motile* were combined with 3×10^9 organisms of *Hfr S^s Lac⁻* (*Z deletion*) *motile* and incubated with gentle shaking for 100 min. Dilutions of the mating mixture were then plated on MacConkey's lactose agar containing 1 mg. streptomycin/ml. Control platings were made of the donor strain and of the acceptor strain. After incubation for 48 hr. at 37° Lac⁺ colonies were found on platings from the lower dilutions of the mated mixture. There were no Lac⁺ colonies on the acceptor control plates; there was no growth on the donor control plates. Following restreaking and 'purification', the recombination clones proved to be non-motile, S^r and frankly lactose-positive organisms. Since both parental organisms were Lac⁻ and since it is well established that the donor strain harbours a deletion with regard to Z⁺, it seems evident that Z⁺

of the recombinants must have been furnished by the shigella parent. The Lac$^+$ condition (arising from the cross of Lac$^-$ × Lac$^-$) in all probability resulted from the influence of donor genes upon the surface properties (permeability to β-galactosides) of the recombinant. In this connexion it should be pointed out that some years ago Luria & Burrous (1957*a*) found a correlation between alteration in serotype and lactose-positiveness in crosses between *E. coli* and *Shigella flexneri*. They were so impressed with this apparent relationship (between serological type and Lac$^+$) that they stated: 'The new serotype is controlled by a genetic factor closely linked to, but not identical with, the Lac locus.'

The relationship of Escherichia coli *β-galactosidase to the β-galactosidase of* Shigella dysenteriae

We are much indebted to Drs M. Cohn, S. Spiegelman and E. Lennox for a generous supply of purified *Escherichia coli* β-galactosidase and of its antiserum (rabbit). Without these we should not have attempted to establish the immunochemical identity of the β-galactosidase derived from *E. coli* and that derived from *Shigella dysenteriae* strain 136-R4. The simplest method of making an immunochemical survey of the various enzymes, toxins and other antigens produced by micro-organisms is through the use of crude lysates and purified known antigens and antibodies reacting in an agar medium. (It was while experimenting with phage T7 endolysin as an agent for releasing Shiga neurotoxin that we observed the Lac$^+$ carrier clones of *Shigella dysenteriae*.) In Pl. 2, fig. 4 is shown the reaction of β-galactosidase (released by a T7^{Sh}.136-R4 carrier clone) from lysing *S. dysenteriae* with anti-serum against *E. coli* β-galactosidase. It can be seen that the lines formed by the reaction shigella β-galactosidase *v.* coli anti-β-galactosidase are continuous with the lines resulting from the reaction of purified coli β-galactosidase *v.* coli anti-β-galactosidase. The plate shown in Pl. 2, fig. 4 was set up in the following way. Plates of MacConkey's agar base (without neutral red) containing 10^{-3} M-TMG as the only added 'carbohydrate' were poured to yield thick layers of agar. Upon each plate were placed 3 disks of Whatman no. 50 filter paper (diam. 13 mm.) as shown in Pl. 2, fig. 4. To disks 1 and 3 were added 0·03 ml. undiluted rabbit antiserum directed against a purified β-galactosidase. To disk number 2 was added 0·02 ml. containing 360 μg. (protein) of purified β-galactosidase. To position 4 was added 0·01 ml. of a freshly prepared lysate of strain 136-R4 resulting from the action of bacteriophage T7^{Sh} (5×10^9 T7 particles/ml. in combination with 1×10^9 136-R4 organisms/ml.). After incubation for 7 days at 30° definite single lines developed between the β-galactosidase (position 2) and the anti-β-galactosidase (positions 1 and 3) and these fused with the major component which developed between the carrier clone and the anti-β-galactosidase (of positions 1 and 3). There seems little doubt, then, that the β-galactosidase of *E. coli* and *S. dysenteriae* 136-R4 are immunochemically identical. Similar results obtained with strains Sh 60 and Sh 15. The latter strain produce very small amounts of enzyme.

Non-specific nature of the effect of bacteriophage on the phenotype of Lac$^-$ strains

The key character upon which depends the taxonomic delineation of subgroups in the genus *Shigella* is that of mannitol fermentation (+ or −). *S. dysenteriae* is

mannitol-negative. It seemed evident to us that if the effect of phage T7 on the phenotype 136-R4 was merely an alteration of permeability by endolysin action, then permeability to some other sugars not used by *S. dysenteriae* should also be altered in clones of 136-R4 carrying phage $T7^{8h}$. This would be true were *S. dysenteriae* cryptic with respect to enzymes needed for the dissimilation of certain other sugars. MacConkey agar plates containing salicin, mannitol or maltose were prepared and Lac^+ carrier clones from lactose plates (MacConkey agar) were transferred to these. All Lac^+ clones proved to be maltose-positive and mannitol-positive but not salicin-positive. As indicated earlier, on maltose plates *S. dysenteriae* formed slightly pink colonies; the carrier clones yielded bright red colonies which attained a larger size than uninfected colonies.

The universality of the effect of bacteriophage on the phenotype of carrier clones

When the Zinder strain of *Salmonella typhimurium* (stm) becomes infected with a virulent mutant of phage PLT22 (Zinder, 1957) the carrier state is established. These carrier clones show no alteration in their fermentative properties on lactose plates. However, on maltose and on mannitol agar the infected populations formed deep red colonies in contrast to the pink ones formed by uninfected populations. Passage of infected populations through anti-PLT22 serum (kindly supplied by Dr Zinder) restored the native state of *S. typhimurium*.

Escherichia coli K12 W2241 ($Y^-Z^+i^+Mal^+$) when infected with phage $T7^{8h}$ at a multiplicity of 5 yielded 2·1 Lac^+ survivors/10^7 bacteria. Among Lac^+ colonies developing on MacConkey medium plates some were carriers and some contained Lac^+ back mutants. Plating of the same material on mannitol plates, however, clearly distinguished between red carriers and colourless uninfected survivors. Neutralization of red clones with T7 antiserum resulted in a return to the 'colourless' state.

In the case of the formation of carrier colonies between phage $T7^{8h}$ and *Escherichia coli* K12 W2242 ($Y^+Z^-i^+Mal^+$) and *E. coli* K12 W2244 ($Y^+Z^-i^+Mal^+$), no Lac^+ clones were detected. Red phage-infected colonies did develop on mannitol plates and on maltose plates, however, and this condition was reversed by passage in anti-T7 serum. Carrier clones of Hfr Z^- (W-4032) were also lactose-negative. *E. coli* ML3 ($Y^-Z^+i^+$) and ML3080 ($Y^-Z^-i^-$) were both resistant to phages T7 and $T7^{8h}$. When combined with either of these phages and plated on lactose plates, no Lac^+ clones developed. The high rate at which these strains mutated to Lac^+ made it necessary to select a fresh Lac^- colony for each experiment.

DISCUSSION

It is a common practice among geneticists to characterize mutant organisms over the common denominator of their ancestral wild type. There is always some variation among wild strains and the taxonomists in selecting type strains (ideally) attempt to choose one that is a happy medium of those extremes found in nature. Some years ago we examined several wild type strains of *Shigella dysenteriae* in an effort to find out what, if anything, distinguishes this organism, freshly isolated from cases of dysentery, from various laboratory strains designated as *S. dysenteriae*. Wild type *S. dysenteriae* is a smooth organism with specific immunochemical, biochemical and pharmacological properties. From several wild types isolated from

cases of Shiga dysentery, we have selected strain 136T as a typical Shiga bacillus (and maintained it as lyophilized 'copies' all 'fresh from nature'). The series of stepwise mutations presented here, from 136-T Lac$^-$ to 136-0 Lac$^-$ to 136-R1 Lac$^-$ to 136-R4 Lac$^-$ are probably the same steps by which strains Sh15, Sh60 and other rough strains, considered in this paper, came into being. While the wild type is resistant to several of the coliphages, various colony-form mutants, which exhibit different surface antigens, are sensitive to a wide range of coliphages. Mutant rough strains, several steps removed from the wild type, are sensitive to all of the standard coliphages. It is from this latter group of extreme-rough organisms that one isolates Lac$^+$ *S. dysenteriae*. Considered as a mutant of the wild type, a Lac$^+$ mutant represents then a very rare event.

Our evidence seems to indicate that *Shigella dysenteriae* is cryptic with respect to β-galactosidase (and to the enzymes involved in maltose and mannitol utilization). This 'walled off' state is attributable to surface properties peculiar to Shiga's bacillus and has, probably, only an apparent relation to β-galactoside system. The lactose utilization system in *Escherichia coli* involves three effects which, genetically, are closely linked: β-galactoside permease (Y), β-galactosidase (Z) and the inducible condition (i) (see Pardee *et al.* 1959). While the β-galactosidase of *S. dysenteriae* is immunochemically identical with that of *E. coli*, there is as yet no evidence that the two organisms harbour identical transport (permease) systems. If they do, then other surface structures in the case of Shiga's bacillus serve to block the permease (Y) thus rendering the organism phenotypically Y$^-$. Such a masking of phenotype would be analogous to the state of the mucoid mutant of a phage-sensitive bacterium which is itself resistant to phage, not because it lacks phage receptors but because its phage receptors are covered by a surface layer of polysaccharide.

It is not surprising that *Shigella dysenteriae* has an enzyme in common with *Escherichia coli*, for interfertile populations usually have much in common. As a matter of fact the number of cross-reacting antigens shared by *S. dysenteriae* and *E. coli* B, for example, appears to be considerable, for when lysates of these organisms are placed on areas of agar equidistant from Shiga antitoxin, several common antigen-antibody lines are formed. These lysates (complexes of phage and bacterium) offer a simple and effective means of releasing for immunochemical identification various biologically-active macromolecules not ordinarily detected in micro-organisms or as their extracellular products. It is possible by removing borings of agar (with a capillary pipette) at various distances from the phage + bacterium complex on TMG plates, to obtain samples having different concentrations of β-galactosidase. By this means β-galactosidase activity can be detected in strains producing only slight amounts of active enzyme. In examining carrier clones of the Z$^-$ coli strains furnished by Dr J. Monod we observed one strain which produced an impressive β-galactosidase anti-β-galactosidase line in TMG MacConkey agar but which showed only an extremely small amount of β-galactosidase activity. Presumably, most of the β-galactosidase produced by this strain was inactive (see Perrin *et al.* 1959). We mention this here because it has some bearing on the quality and amounts of β-galactosidase to be expected in other micro-organisms.

Shigella dysenteriae strain Sh15. By conventional methods of examination the Sh15 strain of *Shigella dysenteriae* would appear to be Z$^-$. We were unable to obtain Lac$^+$ mutants of it under the conditions here described for selecting Lac$^+$ mutants

of 136-R4. It is possible, however, to prepare Lac$^+$ complexes between strain Sh15 and phage T7^{Sh}. The fusion of faint but definite antigen-antibody lines, formed between these complexes (T7^{Sh}, 136-R4) and anti-β-galactosidase (rabbit serum), with bona fide β-galactosidase anti-β-galactosidase lines has been observed. This indicates that the Z-region is present in strain Sh 15. Dr S. Luria (personal communication) has additional evidence which suggests that strain Sh15 is Z$^+$. We pointed out (Table 5) that strain Sh15 produced minute amounts of active enzyme. In addition, its output of material immunochemically reactive with anti-β-galactosidase was low. It would seem, therefore, that though strain Sh15 is Z$^+$, the Z-region of its genome is much impaired.

We are indebted to Drs E. S. Lennox and M. J. Osborn for reading the manuscript and for their general interest in the subject with which this paper is concerned. This work was carried out under a grant from the Allergy and Infectious Diseases section of the National Institutes of Health, the U.S. Public Health Service.

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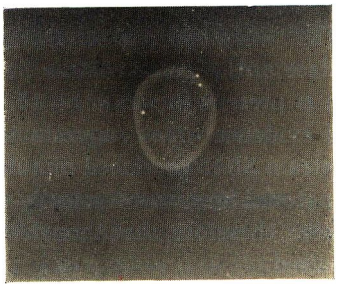

Fig. 1

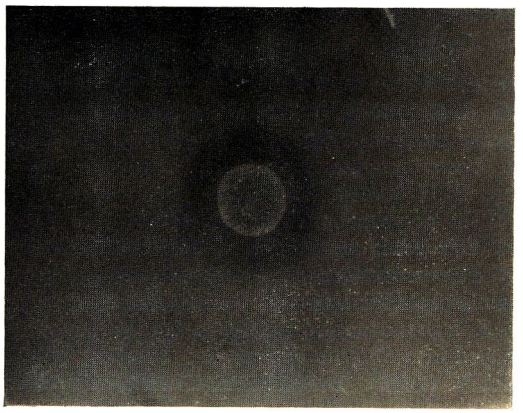

Fig. 2

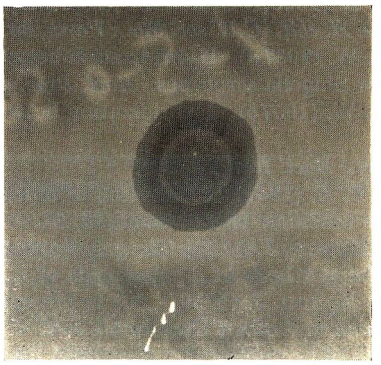

Fig. 3

Fig. 4

EXPLANATION OF PLATES

Plate 1

Figs. 1 and 2 are photographs of holes produced in a lawn (prepared from an acetone powder of *Shigella dysenteriae* 136-R4) by a carrier clone (lysate) of 136-R4.T7^{8h}.

Fig. 3. A photograph of the lysis of living 136-R4 caused by the diffusion of phage and endolysin from carrier clone (lysate) of 136-R4.T7^{8h}. Note the differentiation of the lysed area into clear inner portion and outer halo.

Plate 2

Fig. 4. The fusion of antigen-antibody lines formed between the β-galactosidase released by carrier clone 136-R4 (Lac$^-$).T7^{8h} (position 4) with antigen-antibody lines formed between purified β-galactosidase (position 2) and rabbit antiserum directed against β-galactosidase (positions 1 and 3).

J. gen. Microbiol. (1961), **24**, 369–377
Printed in Great Britain

Stimulation of Streptomycin-Resistant Bacteria in the Rhizosphere of Leguminous Plants

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(*Received* 29 *September* 1960)

SUMMARY

Bacteria which were resistant to streptomycin and rose bengal were preferentially stimulated in the rhizospheres of several plant species as compared with those in the soil away from the rhizosphere. Leguminous plants were the most effective and clover plants as young as 6 days had some effect. The separate and combined effects of rose bengal and streptomycin in the isolation medium were examined. Rose bengal alone had little effect on bacterial numbers, whereas streptomycin alone decreased them. The two substances together affected rhizosphere numbers strikingly but inconsistently. The bacteria from rhizosphere and soil were grouped according to their nutritional requirements. Chromogenic bacteria with simple requirements were the most abundant forms resistant to streptomycin + rose bengal in the rhizosphere and a species of *Flavobacterium* was especially favoured.

INTRODUCTION

Media for the isolation of soil fungi usually have antibacterial agents added, otherwise bacterial growth spreads over the fungal colonies. Several such agents have been used. Martin (1950) found that a mixture of streptomycin + rose bengal at 30 and 15 mg./l., respectively, was very effective. Rose bengal restricts fungal colony size and is also bacteriostatic; streptomycin inhibits only the bacteria. Martin found that the few bacteria which did develop in media containing these substances did not interfere with or decrease fungal counts. Bakerspigel & Miller (1953) used streptomycin in media for the isolation of *Heterosporium iridis* and found that it inhibited equally bacteria from soil and from the rhizospheres of iris, potato and bean. In contrast Peterson (1957), with media containing streptomycin + rose bengal found enough bacteria on plates prepared from the rhizosphere of clover plants to interfere with fungal isolations, but not on plates prepared from the corresponding non-rhizosphere soil. There was thus a preferential stimulation of the resistant bacteria in the rhizosphere; sugar beet and wheat had a similar effect but less so than the leguminous species. Such preferential stimulation does not seem to have been recorded previously and further investigation appeared worth while.

METHODS

Rothamsted clay loam soil (Pastures field; pH 6·6) was used. The plants were grown in a greenhouse in 4 in. glass pots of soil, watered as required; they were not watered during 24 hr. before sampling. Rhizosphere samples were obtained by

carefully lifting the plants and shaking them to remove most of the soil adhering to the roots, which were then cut off at the crown and dropped into a screw-capped bottle containing 100 ml. sterile distilled water; enough roots were used to provide about 1 g. rhizosphere soil. The bottle was then shaken on a machine for 10 min. at 150 oscillations/min. with a horizontal excursion of 1 in. Serial ten-fold dilutions were prepared and three replicate plates were made from suitable dilutions. After removing roots the contents of the bottle and the subsequent dilution were evaporated to dryness to find the dry weight of soil. The numbers of bacteria were finally calculated per g. dried soil adhering to the roots.

Non-rhizosphere samples were taken from unseeded pots of soil which had been treated similarly to the seeded ones. The surface quarter inch of soil was removed with a sterile spatula and about 1 g. soil taken from the upper two inches and put in a bottle containing 100 ml. sterile distilled water. From this point the procedure was like that described for the rhizosphere samples.

Media for making colony counts. The numbers of bacteria in rhizosphere and non-rhizosphere soils were estimated from colony counts after incubation for 7 days at 25° on glucose peptone agar (glucose, 10·0 g.; peptone, 5·0 g.; KH_2PO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; agar, Difco, 20·0 g.; tap water to 1 l.; pH 7·0). The numbers of bacteria resistant to streptomycin + rose bengal were estimated from colony counts made on the above medium to which streptomycin to 30 mg./l. was added just before pouring the plates and rose bengal to 33 mg./l. added during the preparation of the medium (GPStRb) agar. Bacterial numbers were also estimated from colony counts after incubation for 7 days at 25° on soil-extract agar (K_2HPO_4. 0·2 g.; agar, Difco, 20·0 g.; soil-extract, 1000 ml., prepared by autoclaving 1 kg. soil with 1 l. distilled water for 30 min., filtering after adding a little $CaSO_4$, and making filtrate up to 1 l.; pH 7·0).

Nutritional grouping. The bacteria from the rhizospheres and soil samples were grouped according to the method of Lochhead & Chase (1943) by testing whether they would grow on the following media:

(*a*) Basal medium: glucose, 1·0 g.; K_2HPO_4, 1·0 g.; KNO_3, 0·5 g.; $MgSO_4.7H_2O$, 0·2 g.; $CaCl_2$, 0·1 g.; NaCl, 0·1 g.; $FeCl_3$, 0·01 g.; distilled water to 1 l. Heat to 100°, filter, and adjust to pH 6·8.

(*b*) Basal medium + amino acids: basal medium + 0·05 g./l. each of cysteine, alanine, proline, asparagine, glutamic acid, aspartic acid, arginine, leucine, glycine and lysine; pH 6·8.

(*c*) Basal medium + yeast extract: basal medium + yeast extract (Difco), 1·0 g./l.; pH 6·8.

(*d*) Basal medium + yeast extract + soil extract: 750 ml. basal medium + 250 ml. soil extract (prepared as above) + yeast extract 1·0 g./l.; pH 6·8.

RESULTS

Experiments were made to test for rhizosphere stimulation of bacteria resistant to streptomycin + rose bengal with a wide range of plant species. The plants were all grown in Pastures field (Rothamsted) soil and sampled first when they were well developed and at intervals afterwards. Table 1 expresses the results in terms of the ratio number of bacteria in rhizosphere to number of bacteria in corresponding soil

(R/S), and shows that antibiotic-resistant bacteria were preferentially stimulated in the rhizospheres of all the plants tested, but most markedly in the rhizospheres of legumes. Also, whereas legumes showed this effect as early as 6 days, plants from other families only showed it when they were quite old (53 days). *Pisum sativum* and *Trifolium repens* were selected for later experiments.

Table 1. *R/S ratios for resistant and non-resistant bacteria for different plant species*

R/S ratio = ratio of number of bacteria in rhizosphere to number of bacteria in corresponding soil. GP = glucose peptone agar. GPStRb = glucose peptone agar + streptomycin + rose bengal.

Age of plant (days)	Plant species	R/S ratio obtained from count on GP agar	R/S ratio obtained from count on GPStRb agar
12	*Phaseolus angularis*	2·8	29·0
12	*P. vulgaris*	19·4	27·0
12	*Vigna unguiculata*	6·4	37·0
6	*Trifolium repens*	6·0	20·0
21	*T. repens*	9·6	57·0
20	*Glycine max*	21·0	866·0
20	*Vicia faba*	16·8	1716·0
12	*Pisum sativum*	4·0	16·0
29	*P. sativum*	61·0	322·0
29	*Triticum vulgare*	32·0	28·0
53	*T. vulgare*	35·0	133·0
29	*Lactuca sativa*	1·8	0·02
53	*L. sativa*	10·0	34·0
29	*Lycopersicum esculentum*	7·7	2·2
53	*L. esculentum*	6·0	21·0
29	*Raphanus sativus*	27·0	23·0
53	*R. sativus*	50·0	270·0

Sources of variation in the examination of rhizosphere and non-rhizosphere samples of soil

Before studying the stimulation of the streptomycin resistant flora in the rhizosphere in detail, variations due to technique and to plant variability were examined.

Replicate variance and validity of the control samples. There were too few replicates in the previous experiments to estimate the sampling variance, and the control samples were from unplanted pots and, therefore, had a different moisture régime from the planted pots. To examine these possible sources of variation, replicate pots of Pastures field soil were each seeded with one pea plant for rhizosphere samples and unseeded pots of soil were used for controls. To obtain control samples from seeded pots, cylinders of fine-mesh heavy-weight Terylene gauze were placed in each pot before sowing. The pea roots did not penetrate the gauze, but the soil in the cylinders was subjected to the same variation in moisture conditions as the soil around the pea roots. The soil samples were taken 30 days after seeding and dilution series prepared and platings made on glucose peptone agar and GPStRb agar. Table 2 shows both the numbers of bacteria and the R/S ratios. The R/S ratio of the resistant bacteria was everywhere higher than that of the total population.

Table 2. *Mean number of resistant bacteria and total bacterial population/g. dry weight soil in rhizosphere and in two sets of control samples*

Plant	Counted on medium: GP agar — Rhizosphere	GP agar — Pot soil	GP agar — Cylinder soil	GPStRb agar — Rhizosphere	GPStRb agar — Pot soil	GPStRb agar — Cylinder soil
	Bacteria $\times 10^{-6}$/g. dry soil					
1	194	27·8	17·4	32	0·63	0·17
2	103	15·4	20·0	28	0·50	0·79
3	213	25·0	20·0	29	0·38	0·28
4	261	14·1	17·1	39	0·55	0·26
5	531	15·2	19·1	127	0·35	0·66
6	246	18·5	9·9	41	0·46	0·14
Average	258	19·3	17·2	49·3	0·48	0·38

Plant	R/S ratios from colony counts on: GP agar — Pot soil	GP agar — Cylinder soil	GPStRb agar — Pot soil	GPStRb agar — Cylinder soil
	Bacteria $\times 10^{-6}$/g. dry soil			
1	6·9	11·1	50·8	188·2
2	6·7	5·1	56·0	35·4
3	8·5	10·6	76·3	103·6
4	18·5	15·3	70·9	150·0
5	34·9	27·8	362·8	192·4
6	13·3	24·8	89·1	292·8
Average	14·8	15·7	117·6	160·4

GP = glucose peptone; GPStRb = GP + streptomycin + rose bengal.

For statistical analysis the stimulation of resistant bacteria in the rhizosphere was expressed as the difference between the log values of the rhizosphere and control count on GPStRb agar and the log values of the rhizosphere and control count on glucose peptone agar. These differences were calculated for each of the six replicates and from these the mean difference was obtained. The mean difference with unseeded pots as controls was $+0{\cdot}43 \pm 0{\cdot}031$ and with soil from the Terylene gauze cylinder as control $+0{\cdot}49 \pm 0{\cdot}031$. These differences are individually highly significant, confirming the preferential stimulation of resistant bacteria in the rhizosphere of the pea plants. There was no significant difference in the numbers of either type of bacteria in the two sets of controls, showing that unseeded pots of soil could be used as controls, although the soil was subjected to moisture conditions different from the seeded pots.

The effect of streptomycin and rose bengal separately and together in the medium

In two experiments the effects of streptomycin and rose bengal were examined separately and together. Pea seeds were sown in Pastures field soil and unseeded pots of soil were used as controls. After 30 days, three rhizosphere and three control

samples were taken and soil dilutions prepared. Suitable dilutions were then plated with the following agars: glucose peptone; glucose peptone + streptomycin (GPSt); glucose peptone + rose bengal (GPRb); glucose peptone + streptomycin + rose bengal (GPStRb). In another experiment two sets of plants and controls were examined similarly after 22 days. Table 3 shows the results.

Table 3. *The effects of streptomycin and rose bengal, separately and together, on counts of bacteria from rhizosphere and from control soils*

Peas were grown in Pastures field soil; samples taken after 30 days (Expt. 1) and 22 days (Expt. 2). R/S ratio = ratio of number of bacteria in the rhizosphere to number of bacteria in corresponding soil

Sample	GP agar	GPRb agar	GPSt agar	GPStRb agar
	Numbers of bacteria $\times 10^{-6}$/g. dry soil as estimated from colony counts on media			
Expt. 1				
Rhizosphere	478	409	229	55
Control	15·3	14·6	0·8	0·8
	R/S ratio			
	31	28	263	68
	Count $\times 10^{-6}$/g. dry soil			
Expt. 2				
Rhizosphere	284	230	35	70
Control	7·2	5·1	0·5	0·2
	R/S ratio			
	39	45	70	350

GP = glucose peptone; GPRb = GP + rose bengal; GPSt = GP + streptomycin; GPStRb = GP + streptomycin + rose bengal.

The presence of rose bengal alone in the medium had little effect upon the bacterial numbers, either in the soil or rhizosphere, whereas streptomycin alone prevented the development of an appreciable proportion of the population. With streptomycin + rose bengal present the numbers of bacteria/g. dry soil, as estimated from the plates made from soil samples, were affected differently in the two experiments; in Expt. 1 the numbers were the same as with streptomycin alone ($0{\cdot}8 \times 10^6$/g.), but in Expt. 2 they were decreased from $0{\cdot}5 \times 10^6$ to $0{\cdot}2 \times 10^6$/g. Also there was a marked and irregular effect on the rhizosphere count; in Expt. 1 it was decreased from 229×10^6 to 55×10^6, but in Expt. 2 it was increased from 35×10^6 to 70×10^6/g. No explanation can be offered for these divergent effects of supplementary rose bengal.

The fact that the bacteria were unaffected by rose bengal alone indicates that streptomcyin was the operative substance; this was further substantiated by the following tests in which the effects of streptomycin and rose bengal were separately examined on a range of streptomycin-resistant bacteria isolated from GPStRb agar plates. Glucose peptone agar plates were seeded with cultures of the various resistant bacteria and assay cylinders placed on the surface of the plates. Different concentrations of rose bengal (mg./l., 6600, 66, 33, 6·6) were added to one series of cylinders and different concentrations of streptomycin (mg./l., 12,000, 120, 30, 12)

added to another series of cylinders. The plates were incubated at 25° for 7 days. All the bacteria were completely inhibited by rose bengal at 6600 mg./l. and some by streptomycin at 12,000 mg./l.; lower concentrations of either substance had no effect. Thus, whatever may be the nature of the action of the streptomycin and rose bengal when together, these bacteria are resistant to both substances and it is reasonable to call the population streptomycin-resistant. The use of rose bengal in the medium may have the disadvantage of increasing the error of estimating the streptomycin-resistant population, but this is offset by its great practical value. Its function in the medium is primarily to restrict fungal growth; without it colonies are exceedingly difficult to count because of fungal overgrowth, particularly at low dilutions. For this reason rose bengal was included in all the media used later.

The selective effect of the basal medium

The glucose peptone agar used in the present work was somewhat selective and, should the streptomycin-resistant population be favoured in preference to others, the preferential stimulation of resistant types would be over-estimated. To examine the effect of the basal medium, rhizosphere and control samples from two sets of pea plants were plated with soil-extract agar, with and without the antibacterial agents; also with glucose peptone agar and GPStRb agar. Soil-extract agar was chosen for this comparison as it was the least selective of the media generally used. Table 4 shows that, as expected, the total count on the soil-extract agar without the antibacterial agents was larger than on glucose peptone agar, but the resistant population counts were similar on both media. Thus the preferential stimulation of resistant bacteria was evident with both media but appeared greater with GPStRb agar because the control resistant-count was lower. Glucose peptone agar was used as the basal medium in all further experiments because the colonies grew better and were easier to count.

Table 4. *Average number of bacteria per g. dry weight of soil as estimated from soil extract and glucose peptone agars with and without antibacterial agents*

	Bacteria $\times 10^{-6}$/g. dry soil					
	Total population			Resistant population		
Medium	Rhizosphere	Control	R/S	Rhizosphere	Control	R/S
Soil	400	10·0	40	62	0·6	103
extract	586	8·0	73	61	0·7	87
Mean	493	9·0	55	61·5	0·65	95
Glucose	238	8·0	30	57	0·2	285
peptone	330	6·3	52	83	0·2	415
Mean	284	7·1	40	70	0·2	350

The properties of the bacteria

Lochhead & Chase (1943) devised a system of grouping soil bacteria according to certain physiological criteria based on their nutritional requirements, rather than classifying them by biochemical tests, which were inadequate for any 'rational grouping helpful to an understanding of the activity or significance of these bacteria in soil'. They divided the bacteria into seven groups based on their growth in seven

media, ranging from those organisms which could grow in a simple basal medium to those requiring unidentified substances present in both yeast and soil extracts. Lochhead & Thexton (1947) showed that those bacteria which grew well in the simple medium were relatively more abundant in the rhizosphere, also that there was an increased incidence of bacteria requiring amino acids for maximum growth or which were stimulated by amino acids. Thus certain nutritional groups of bacteria were preferentially stimulated in the rhizosphere.

It was considered of interest in the current work to know whether the streptomycin-resistant bacteria in the rhizosphere had similar nutritional requirements and could be placed in groups known to be preferentially stimulated, or whether they had widely divergent requirements. They were compared with non-resistant bacteria in the rhizosphere and resistant and non-resistant bacteria in the corresponding soil. Lochhead & Chase's method was used but growth in four media only was examined. These were the basal medium, basal medium + amino acids, basal medium + yeast extract and basal medium + soil extract + yeast extract. Colonies of bacteria were systematically picked, about 60 for each sample, so that all on a plate or sector were taken, and inoculated on to glucose peptone agar and checked for purity. The various nutritional media were then inoculated and after incubation for 5 days at 25° the growth response of each isolate recorded by measuring the turbidity. The relative incidence of the various nutritional groups of bacteria and the estimated number of bacteria of the different groups per g. soil are shown in Table 5.

As expected there was an increased percentage in the rhizosphere of non-resistant bacteria with simple requirements and those needing amino acids. An interesting feature was that the resistant bacteria in the rhizosphere showed a greater percentage which had simple needs and a lower percentage requiring amino acids than did the non-resistant bacteria. This preferential stimulation of bacteria with simple needs was also reflected in the R/S ratio: 125 with resistant bacteria as compared with 30 with non-resistant bacteria. Thus it seems that most of the preferentially stimulated resistant bacteria belonged to one nutritional group.

In addition to this property 85 % of the resistant bacteria were chromogenic, producing a yellow pigment; half of these were identified as a *Flavobacterium* species (*Bergey's Manual*, 1957); the specific identity was not determined. The organism was a Gram-negative non-motile rod which produced a yellow pigment, the hue depending on the medium, being particularly bright on gelatine. Litmus milk was made alkaline; there was no proteolytic activity. Growth occurred in a wide range of carbohydrate media, sometimes with the production of acid, but never of gas; starch was not hydrolysed. The bacterium had simple nutritional requirements. The other chromogenic bacteria were also probably flavobacteria, producing bright yellow-orange pigments, which did not change colour with the medium. The specific identities were not determined. Three strains of these bacteria were found, the first having simple nutritional needs, the second required amino acids and the third required unidentified substances from yeast extract.

Of the non-resistant rhizosphere bacteria, only 33 % were chromogenic and were identified as *Flavobacterium* spp., but with different properties from the resistant types. In the soil 19 % of the resistant organisms and 12 % of the non-resistant types were chromogenic. These were identified as *Flavobacterium* spp.

Table 5. *Nutritional groups of bacteria in rhizosphere and control soil*

	Resistant bacteria				
	Rhizosphere		Control		R/S
	Plate count ($\times 10^{-6}$/g.)				
	3·3		0·36		91
Nutritional groups	% Colonies	Estimated no. ($\times 10^{-6}$/g.)	% Colonies	Estimated no. ($\times 10^{-6}$/g.)	R/S
Grow in basal medium	61	2·0	44	0·016	125
Require amino acids	20	0·7	56	0·02	35
Require yeast extract	18	0·6	—	0	—
Require yeast + soil extracts	—	0	—	0	—
	Non-resistant bacteria				
	Rhizosphere		Control		R/S
	Plate count ($\times 10^{-6}$/g.)				
	184		7·1		26
Nutritional groups	% Colonies	Estimated no. ($\times 10^{-6}$/g.)	% Colonies	Estimated no. ($\times 10^{-6}$/g.)	R/S
Grow in basal medium	50	93	43	3·1	30
Require amino acids	25	46	12	0·9	51
Require yeast extract	20	38	31	2·2	17
Require yeast + soil extracts	4	7	12	0·9	7

DISCUSSION

These experiments showed that bacteria resistant to streptomycin + rose bengal were stimulated to a greater degree than non-resistant bacteria in the rhizosphere of a variety of plant species. The stimulation of resistant bacteria by legumes was greater than that produced by non-legumes, a result which was consistent with present knowledge concerning the legume rhizosphere. Also, this stimulation occurred with younger plants than with non-legumes, which might be related to the faster release of stimulatory substances; for example, Rovira (1956) showed that pea roots excreted greater amounts of amino acids during the first 21 days of growth than did oat roots. However, most of the streptomycin-resistant organisms had simple nutritional requirements, which suggested that, although amino acids might stimulate, they were not obligatory for these bacteria and the preferential stimulation of this group was not entirely dependent upon the presence of amino acids in the rhizosphere. Lochhead (1948) noted that there was a preferential stimulation of chromogenic bacteria in the rhizospheres of a variety of crop plants; in the current work, also, the streptomycin-resistant bacteria were mostly chromogenic, the predominant form being a *Flavobacterium* species. A possible reason for the stimulation of streptomycin-resistant bacteria in the rhizosphere might lie in their property of antibiotic resistance. Bacteria, fungi and actinomycetes are all stimulated in the rhizosphere and species of all these can produce antibiotics; it has been suggested (Brian, 1957) that the rhizosphere is a region where such production could take

place. Thus bacteria resistant to antibiotics would have a competitive advantage over those which were sensitive, and such a group could be preferentially stimulated. Further examination of such a relationship between the various groups of soil micro-organisms in the rhizosphere is needed.

I wish to thank Dr P. S. Nutman for his interest in this work and for criticizing the manuscript and the Statistics Department, Rothamsted, for their advice.

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Printed in Great Britain

Chemical and Metabolic Properties of Various Elements Found in Cultures of a Stable Proteus L Form

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(*Received* 20 *September* 1960)

SUMMARY

The microscopic elements which constituted cultures of a stable L form (Proteus L9) were found to be highly heterogeneous with respect to morphological, chemical and metabolic properties. The smallest elements found in the cultures studied (diameter $< 0{\cdot}3\mu$) contained more lipid-phosphorus (lipid-P), but less ribonucleic acid-phosphorus, deoxyribonucleic acid-phosphorus and protein-nitrogen, than whole cultures consisting predominantly of bodies of diameter $> 1\mu$. The small elements respired at about the same rate as whole cultures, but they showed low, if any, biosynthetic activity. The small elements probably possess a structural organization similar to that of L bodies of larger sizes, but most of them contained little, if any DNA.

INTRODUCTION

The bacterial L forms (reviewed by Dienes & Weinberger, 1951; Tulasne, 1955; Kandler & Kandler, 1960; Kleineberger-Nobel, 1960) are highly pleomorphic. Vesicular and granular elements are most frequent in cultures of L forms, but various irregular structures may also be found. Mandel and his associates have published a series of papers describing the fractionation of L cultures into fractions characterized among other things by the size of the elements present in them (Mandel, Terranova & Sensenbrenner, 1957; Mandel, Feo, Sensenbrenner & Terranova, 1959; Mandel, Terranova, Sensenbrenner & Feo, 1959; Mandel *et al.* 1959). The fractionation was performed by means of differential centrifugation. This technique has been used in most of the experiments described in the present paper. A stable L form derived from *Proteus mirabilis* was investigated.

METHODS

Organism. The Proteus L form used was obtained from Dr E. Klieneberger-Nobel (Lister Institute of Preventive Medicine, London). It has not shown any signs of reversion during its history and has been designated as strain L9 (Klieneberger-Nobel, 1956). Tests performed in our laboratory have shown that the normal Proteus strain from which Proteus L9 was derived does not produce acid and gas from maltose, and does not produce indole; this strain thus exhibits the properties of *Proteus mirabilis* (see Taubeneck, 1956).

Growth conditions and harvesting of the cultures. Samples of the L form were obtained for experimental purposes from stock cultures grown in 250 ml. Erlenmeyer flasks containing 50 ml. of the liquid serum-free medium described by

Abrams (1955). No penicillin, however, was included in the medium used in the present work. The culture were incubated for 24 hr. at 30° on a rotary shaker (100 rev./min.) and stored at room temperature. Subcultures were made every week.

To obtain large amounts of L form grown in a liquid medium, 3 l. Erlenmeyer flasks containing 500 ml. medium were used. The flasks were inoculated with 50 ml. of an overnight culture of Proteus L9, obtained as described above.

For the growth of Proteus L9 on solid medium, Petri dishes (diam. 20 cm.) were used containing Abram's liquid medium supplemented with 0·8 % (w/v) Difco agar. The plates were heavily inoculated with a liquid culture of Proteus L9 and incubated for 48 hr. at 30°. For harvesting the L bodies, a liquid culture of Proteus L9 was centrifuged for 20 min. at 78,000 *g*. The supernatant fluid was used for suspending the growth present on the Petri plates.

Differential centrifugation. The centrifugations were made with a Spinco model L preparative ultracentrifuge equipped with a no. 30 rotor (tube diameter 2·54 cm., tubes inclined 26° from axis of rotation). In each fractionation experiment the centrifugations were conducted in two centrifugal fields, as outlined in Fig. 1. All centrifugations lasted for 20 min., plus the time required for stopping the rotor with the magnetic brake.

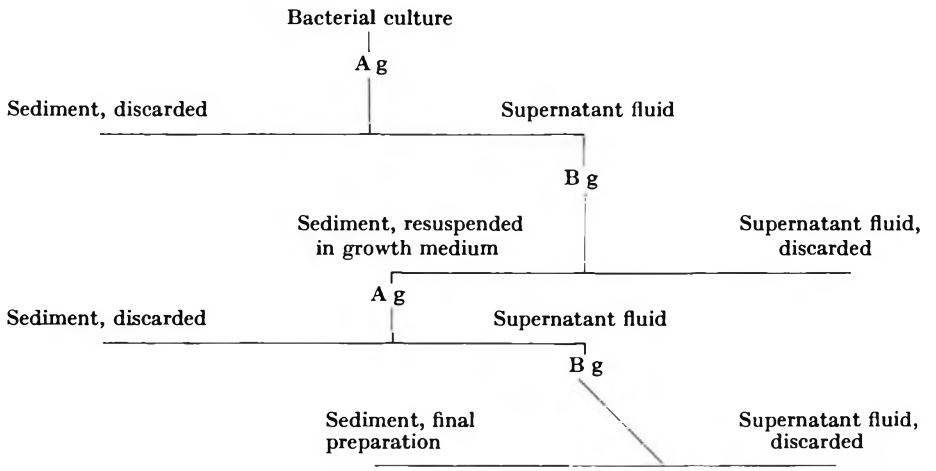

Fig. 1. Fractionation of L elements by means of differential centrifugation. The symbols 'A g' and B g' stand for the centrifugal fields used.

Chemical analyses. For the determination of lipid-phosphorus, nucleic acids and protein, samples were subjected to the Schneider fractionation procedure (Schneider, 1945; Weibull & Beckman, 1960). Total phosphorus in the lipid fraction (lipid-P) was determined according to Allen (1940). Ribonucleic acid-phosphorus (RNA-P) was determined as described by Schneider (1945) and deoxyribonucleic acid-phosphorus (DNA-P) according to Burton (1956). The Kjeldahl method was used to estimate protein nitrogen (protein-N). The dry weight of bacterial material was determined as described by Weibull & Beckman (1960).

Enzyme assays and measurements of respiration. Succinic dehydrogenase was determined according to Storck & Wachsman (1957). Catalase was estimated by

the method described by Bonnichsen, Chance & Theorell (1947). These measurements were made at room temperature. The conventional Warburg technique was used for respiration measurements. The experiments were conducted at 30°.

Isotope experiments. ^{32}P was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Activities were measured with a Tracerlab TGC-2 Geiger tube, connected to a Tracerlab SC33A '1000' Scaler. Samples were measured either wet (volume of sample 1 ml.) or after drying at 100°.

RESULTS

Fractionation by means of filtration

The experiments described in this section were performed with Proteus L9 grown in Abram's liquid medium. Microscopical inspection revealed spherical bodies of various sizes as predominant constituents of these cultures (see Weibull & Beckman, 1960); minor amounts of morphologically ill-defined material were also seen. The spherical bodies were single, or formed small aggregates, each consisting usually of less than 10 individual spheres. When the L cultures were centrifuged at high speed in the Spinco centrifuge, a sediment consisting of two rather well-defined layers was usually formed. The upper one was transparent, the lower one opaque. However, when the two layers were resuspended separately in the growth medium, the suspensions obtained could not be clearly distinguished from each other when viewed by phase-contrast microscope.

Up to about half of the bacterial material of a Proteus L9 culture did not pass through a Munktell 1F filter paper (manufactured by Grycksbo Pappersbruk, Ltd, Grycksbo, Sweden). When the filtrate was centrifuged, the opaque material mentioned above was almost completely absent from the centrifugal pellet. The filtered bacterial material always contained more lipid-P and nucleic acid phosphorus per mg. dry weight than the corresponding unfiltered material. On the other hand, the content of protein-N was about the same in the two kinds of material. Thus the ratio content of lipid-P, RNA-P, DNA-P, protein-N, in 1 g. dry wt. filtered material to content of same substance in 1 g. unfiltered material was found to be: lipid-P, 1·36; RNA-P, 1·36; DNA-P, 1·41; protein-N, 0·94. These figures represent average values obtained from four separate experiments.

The respiration of filtered L cultures was found to be about 30 % more vigorous than that of unfiltered material, calculated per mg. bacterial dry weight.

Fractionation by means of differential centrifugation

The main purpose of these experiments was to isolate from L cultures small elements similar to those described by Tulasne (1955) and to study the chemical and metabolic properties of these elements as compared with those of the unfractionated cultures. Cultures grown in a liquid medium and on a solid medium (see Methods) were investigated. The type of medium did not influence the results markedly. However, larger yields of bacterial material were obtained when Proteus L9 was grown on a solid medium. When not otherwise mentioned, the data reported below refer to experiments carried out with solid medium.

Morphological studies. As with L cultures grown in liquid medium, unfractionated cultures grown on solid medium consisted essentially of spherical elements

of various sizes. Electron microscopical observations showed that the fractions obtained by differential centrifugation contained essentially elements having a diameter $< 0{\cdot}3\mu$.

Chemical studies. Unfractionated and fractionated L cultures were analysed for lipid-P, protein-N, RNA-P and DNA-P. Table 1 gives data from a number of independent experiments. The gravitational fields applied for fractionation of the L cultures differed from experiment to experiment, ranging from 5500 to 78,000 *g*. The fractions were examined by oil-immersion phase-contrast microscopy; this revealed granular elements of a size near the resolving limit of the microscope as predominant elements in all the fractions obtained.

The figures collected in Table 1 show that, on a dry-weight basis, the unfractionated L bodies contained on an average more RNA-P, DNA-P and protein-N than the small elements isolated from them, but less lipid-P. The wide variations in the DNA-P content of the small bodies should be emphasized; the maximal and minimal values differed by a factor of 64. The corresponding factor for the other compounds assayed varied between 1·2 and 4·8.

Table 1. *Chemical composition of unfractionated L cultures and of fractions of such cultures obtained by differential centrifugation at* 5500–78,000 *g*

All data are based on results obtained from 5 to 10 independent experiments. The ± sign indicates standard error.

Substance assayed		Unfractionated cultures	Small elements (diameter $< 0{\cdot}3\mu$) obtained by differential centrifugation
		(% dry wt. of material analysed)	
Lipid-P	Max. and min. value	0·51, 0·71	0·72, 1·24
	Average	0·57 ± 0·04	1·01 ± 0·11
RNA-P	Max. and min. value	0·55, 0·83	0·21, 1·00
	Average	0·71 + 0·06	0·45 ± 0·07
DNA-P	Max and min. value	0·35, 0·43	0·01, 0·64
	Average	0·39 ± 0·02	0·18 ± 0·06
Protein-N	Max. and min. value	8·18, 9·79	4·90, 6·91
	Average	8·61 ± 0·31	6·32 ± 0·41

The pronounced fluctuations in the DNA content of the fractionated L cultures suggest that at least part of the DNA did not form an integral part of the spherical bodies in these preparations. To test this hypothesis crystalline DNAse (Mann Research Laboratories Inc., New York 6, N.Y., U.S.A.) was added to a suspension of small L elements in the growth medium. The mixture was incubated for 2 hr. at 30° and then analysed for lipid-P, RNA-P and DNA-P; an unfractionated culture containing mainly elements having a diameter $> 1\mu$ was similarly treated. This culture had been grown in liquid medium, since L bodies grown on solid medium have a tendency to lyse when suspended in a fluid and then probably release DNA.

Table 2 shows the results of the digestion experiment. It can be seen that the chemical composition of the unfractionated culture was not influenced by the

enzymic treatment. The content of lipid-P and RNA-P in the small elements remained practically constant during the treatment with DNAse. The DNA-P content, on the other hand, diminished by about 70 %.

Table 2. *Digestion with DNAse of an unfractionated L culture and of a fraction of such a culture obtained by means of differential centrifugation*

The fractionated preparation contained only small elements (diameter < 0·3μ); the unfractionated culture mainly elements of diameter > 1μ. The digestion took place in the growth medium at 30° for 2 hr. The concentration of DNAse was 0·5 mg./ml., that of the L elements about 1 mg. dry wt./ml.

	Unfractionated L culture		Fraction containing small L elements	
Substance assayed	Before digestion	After digestion	Before digestion	After digestion
	% dry wt. material analysed			
Lipid-P	0·60	0·60	1·15	1·14
RNA-P	0·68	0·69	0·41	0·42
DNA-P	0·36	0·36	0·28	0·09

As mentioned in Table 1 the data about small L elements refer to batches obtained by centrifugation at different centrifugal fields. To test the relationship between the centrifugal fields applied and the chemical composition of the fractions obtained, some L cultures were fractionated into two or three fractions. Each fraction was isolated as outlined in Fig. 1 and was analysed chemically. Table 3 shows the results of three experiments of this kind. It can be seen that the RNA-P content of elements collected by using high centrifugal fields tended to be lower than that of elements obtained by centrifugation at lower speeds. The content of DNA-P, lipid-P and protein-N in the fractions analysed varied less, or in an irregular manner.

Table 3. *Chemical composition of L elements isolated by means of differential centrifugation at various centrifugal fields*

The centrifugations were carried out as outlined in Fig. 1.

Expt.	Applied centrifugal fields (*g*)	Lipid-P	RNA-P	DNA-P	Protein N
		(% dry weight of the bacterial material analysed)			
I	2,100, 5,500	—	0·54	0·016	—
	5,500, 19,600	—	0·43	0·020	—
	19,600, 78,000	—	0·21	0·008	—
II	2,100, 5,500	—	0·39	0·068	—
	5,500, 19,600	—	0·28	0·038	—
III	2,100, 5,500	0·65	0·50	0·17	6·91
	5,500, 19,600	0·80	0·51	0·20	6·68

To compare the structural and macromolecular organization of L bodies of various sizes, unfractionated and fractionated L cultures were shaken in a Mickle disintegrator (Mickle, 1948) for 2 min. After this treatment only bodies or fragments of a size near the resolving power of the light microscope were seen in the cultures. The broken material was centrifuged at 103,000*g* for 20 min. The supernatant liquid

obtained was centrifuged for a further 120 min. at 103,000*g*, transferred to fresh tubes and again centrifuged at the same speed for a further 120 min. Table 4 shows the distribution of lipid-P, RNA-P and DNA-P in the fractions obtained by this centrifugation procedure. It can be seen that the greater part of the lipid-P of both whole L cultures (mainly containing bodies with a diameter $> 1\mu$) and small L bodies (diameter $< 0{\cdot}3\mu$) was sedimented when the disintegrated L bodies were centrifuged at 103,000*g* for 20 min. Most of the DNA-P was sedimented after prolonged centrifugation at the same speed. The RNA-P of the whole L cultures and the small L bodies behaved differently when the disintegrated cultures were centrifuged. Thus about 80 % of the RNA-P of the small L bodies was sedimented after centrifugation at 103,000 *g* for 20 min., whereas about half of the RNA-P of unfractionated cultures was not sedimented even after centrifugation at the same speed for 260 min.

Table 4. *Distribution of lipid-P, RNA-P and DNA-P in fractions of L bodies obtained by disruption of these bodies in a Mickle disintegrator and differential centrifugation of the disintegrated material*

Each figure represents an average value from two independent experiments.

		Cell material sedimented by centrifugation at		Cell material, not sedimented by centrifugation at 103,000 g for 260 min.
		103,000 *g* for 20 min.	103,000 *g* for 260 min. (20+120+120)	
		% of that found before centrifugation		
Lipid-P	Whole L cultures	75·3	15·6	10·1
	Small L bodies (diameter $< 0{\cdot}3\mu$)	82·2	16·0	5·2
RNA-P	Whole L cultures	21·4	38·1	41·9
	Small L bodies (diameter $< 0{\cdot}3\mu$)	81·4	15·0	6·5
DNA-P	Whole L cultures	16·6	72·1	16·4
	Small L bodies (diameter $< 0{\cdot}3\mu$)	11·4	64·0	28·4

Enzyme studies. Many enzymes of the bacterial cell are undoubtedly located in definite subcellular structures (Marr, 1960). To elucidate the nature of the small bodies (diameter $< 0{\cdot}3\mu$) isolated from whole L cultures by means of differential centrifugation, some enzymic activities of these bodies were studied and compared with those of unfractionated cultures. Succinic dehydrogenase and catalase were the two enzymes chosen for this investigation since they are located differently, at least in some bacteria (Alexander & Wilson, 1955; Weibull, Beckman & Bergström, 1959). The amounts of succinic dehydrogenase and catalase found in unfractionated cultures of Proteus L9 grown on a solid medium, and of fractions of such cultures obtained by means of differential centrifugation were: succinic dehydrogenase, $20{\cdot}8 \pm 1{\cdot}6$ and $35{\cdot}0 \pm 2{\cdot}1$ units; catalase $70{,}000 \pm 3200$ and $20{,}100 \pm 2600$ units (the enzymic activities are expressed as μmole of substrate oxidized or split per min. and per l. of an incubation mixture containing 1 g. dry weight bacterial material).

Thus the unfractionated L bodies contained more catalase but less succinic dehydrogenase than the fractionated material.

The localization of succinic dehydrogenase and catalase in L bodies of various sizes was investigated by treating unfractionated and fractionated L cultures in a Mickle disintegrator and centrifuging the broken material in the manner used for characterization of the macromolecular organization of the L bodies by means of chemical analyses (see the preceding section of this paper); Table 5 shows the results of these experiments. It can be seen that both the catalase and the succinic dehydrogenase of the L bodies were distributed in much the same manner in unfractionated L cultures (containing mainly elements having a diameter $> 1\mu$) and in small L bodies (diameter $< 0{\cdot}3\mu$). The succinic dehydrogenase was associated with sedimentable matter, whereas the major part of the catalase was not sedimented even after prolonged centrifugation at high gravitational fields.

Table 5. *Distribution of succinic dehydrogenase and catalase in fractions of L bodies obtained by disruption of these bodies in a Mickle disintegrator and differential centrifugation of the disintegrated material*

The results are expressed as per cent of total enzymic activity of the material before the centrifugation procedure

		Cell material sedimented by centrifugation at		Cell material not sedimented by centrifugation at 103,000 *g* for 260 min.
		103,000 *g* for 20 min.	103,000 *g* for 260 min. (20 + 120 + 120)	
Succinic dehydrogenase	Whole cultures	72·1	< 1	< 1
	Small L bodies (diameter < 0·3 μ)	69·0	< 1	< 1
Catalase	Whole L cultures	1·0	30·4	52·0
	Small L bodies (diameter < 0·3 μ)	5·0	29·6	48·0

Respiration experiments. Measurements were carried out on unfractionated L cultures grown in liquid and on solid media. Fractions of L cultures, obtained by differential centrifugation and containing as predominant elements bodies of a diameter $< 0{\cdot}3\mu$, were also studied. The growth medium served as substrate. The Q_{O_2} values obtained were: unfractionated L culture, grown in liquid medium, 410 ± 52; similar culture, treated for 2 min. in a Mickle apparatus, < 5; unfractionated L culture, grown on solid medium, 233 ± 31; fractionated L elements, 209 ± 26. Thus unfractionated L cultures grown in liquid medium respired about twice as vigorously as L bodies grown on solid medium. On the other hand, fractions obtained by differential centrifugation respired at about the same speed as did the whole cultures from which they were prepared (the fractionated material was obtained from cultures grown on a solid medium). Mechanical disintegration abolished the respiration of the L bodies almost completely. The same result was obtained when L bodies were ruptured by suspending them in distilled water. No oxygen consumption was observed when L bodies were suspended in Krebs–Ringer

solution containing glucose (see Mandel, Terranova, Sensenbrenner & Feo, 1959). No certain relationship was detected between the chemical constitution of fractionated L cultures and their respiratory activity. In Fig. 2 the circles in the diagram indicate the DNA-content of several batches of fractionated material and the corresponding Q_{O_2} values.

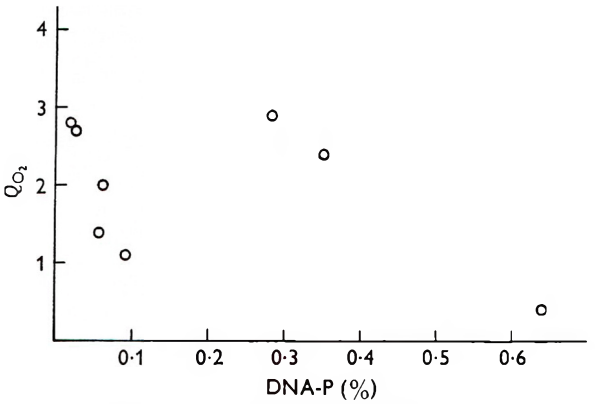

Fig. 2. Q_{O_2} values and DNA-P content of fractionated L cultures, predominantly consisting of spherical bodies of a diameter smaller than 0·3μ. The fractionation was carried out by means of differential centrifugation.

Estimations of the number of viable elements in unfractionated and fractionated L cultures. These estimations to be described in detail elsewhere were made by the pour-plate method. The nature of the growth medium used was of decisive importance for the outcome of the experiments. Thus in the case of unfractionated L cultures, no growth was obtained in pour plates when fresh Abram's medium was used for diluting the cultures and for pouring the final dilutions. However, a growth medium which gave viable counts not very much lower than the corresponding total counts as determined according to Weibull (1960), was obtained by centrifuging an overnight culture of Proteus L9 and sterilizing the supernatant fluid by heating at 56° for 1 hr. The inclusion of 10 % (v/v) horse serum in this heated supernatant fluid further slightly increased the viable counts.

When fractionated L cultures, containing as predominant constituents bodies of diameter $< 0{\cdot}3\mu$, were tested for viable counts on the serum-containing medium described above, at the most 5×10^5 colonies were obtained per mg. dry wt. fractionated material. It can be calculated that this material must have contained more than 10^{11} individual L bodies (diameter $< 0{\cdot}3\mu$). This implies that the number of non-viable bodies would exceed that of the viable ones by a factor greater than 10^5. When fractionated L cultures were investigated the viable counts obtained could thus be attributed to large L elements, present as contaminants. Because of their scarcity such elements might easily be overlooked in microscopic preparations.

The biosynthetic activity of small L bodies

The experiments described in the preceding section showed that of the small L bodies (diameter $< 0{\cdot}3\mu$) isolated by differential centrifugation, at the most very few ($< 0{\cdot}001$ %) were able to grow, divide and finally to form visible colonies under

the experimental conditions used. It might be argued, however, that these bodies might exhibit less extensive biosynthetic activity. To test for this, small L elements were incubated in a growth medium for 2–4 hr. at 30°. At the beginning of the experiment, ^{32}P was added to the incubation mixture at a final concentration of about 15μC/ml. Samples were taken during the incubation and fractionated according to the Schneider procedure. The incorporation of ^{32}P in the nucleic acid fraction of the L elements during the incubation was followed by radiochemical analyses. The net changes in the content of lipid-P, RNA-P, DNA-P and protein-N of the L elements were followed by conventional chemical analyses (Schneider, 1945; Burton, 1956).

Three experiments of this kind were performed with different growth media. In the first experiment (indicated by the Roman numeral I in Fig. 4) fresh Abrams's medium (modified as described in the next paragraph) was used, supplemented by about half its volume of a batch of Abrams's medium which had been previously used for growing L bodies and then centrifuged and sterilized (this medium will subsequently be called 'old' Abrams's medium). In the second experiment (II)

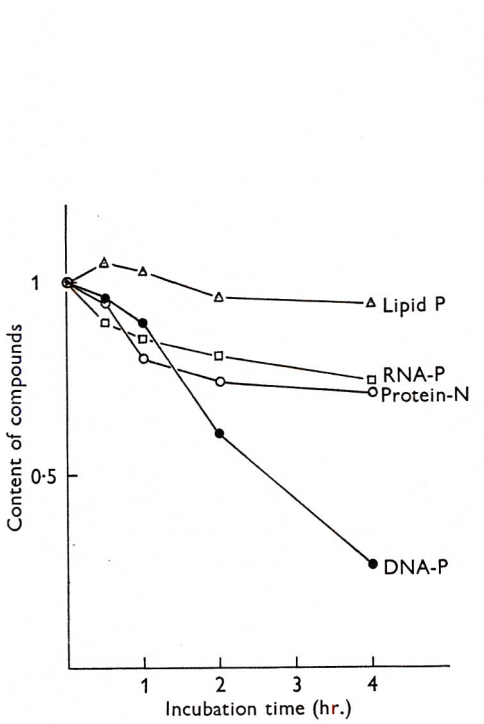

Fig. 3

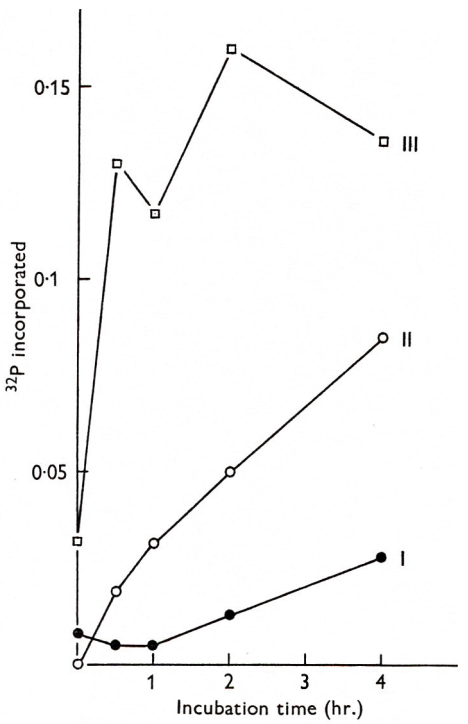

Fig. 4

Fig. 3. Content of lipid-P, protein-N, RNA-P and DNA-P in a fractionated L culture incubated in Abrams's medium supplemented with serum. The content of each compound in the L culture at the beginning of the experiment is given as being equal to unity.

Fig. 4. Incorporation of ^{32}P in fractionated L cultures, containing as predominant elements spherical bodies of diameter < 0·3μ. The amount of incorporated ^{32}P is expressed as % of nucleic acid-P of the whole culture. The results of three independent experiments (I–III) are given. For experimental details see text.

only 'old' Abrams's medium was used; in the third experiment (III) 'old' Abrams's medium, +10% (v/v) horse serum inactivated by heating for 30 min. at 56°. Thus media were used which gave high viable counts for unfractionated L cultures (see the preceding section).

In all three experiments the phosphate content of the medium was reduced to 2% of the figure given by Abrams (1955) to minimize the amount of added ^{32}P. The tonicity of the medium was restored by the addition of 11·4 g. KCl/l. medium.

In none of the experiments did chemical analysis show any steady net increase in the content of lipid-P, RNA-P, DNA-P or protein-N in the small L bodies during the incubation (4 hr.). Instead, a decrease in the content of high molecular weight compounds in these bodies was generally noticed. Fig. 3 shows the results of an experiment carried out with serum added to the medium. When a similar experiment was made with an unfractionated L culture the content of nucleic acid-P and lipid-P in the culture increased 2–3 times.

Before measuring the radioactivity of the nucleic acid-containing extracts of the fractionated L elements, 0·5 ml. 10N-sulphuric acid, 0·5 ml. 10% (w/v) ammonium molybdate and 3 ml. of a mixture of equal volumes of isobutanol and benzene was added to 2 ml. samples of these extracts. The resulting two-phase systems were shaken for 15 sec. In this way, any inorganic phosphate originally adsorbed to the L bodies would be removed from the aqueous extracts (see Lindberg & Ernster, 1956). At the same time any inorganic phosphate formed by acid hydrolysis of RNA would also be removed from these extracts. The remaining organic phosphate would, however, correspond to all of the DNA-P and about 50% of the RNA-P of the bacterial material analysed (Loring, 1955).

The results of the radiochemical measurements performed are given in Fig. 4. It can be seen that the ^{32}P incorporated in the L elements during the incubation corresponded at the most to between 0·1 and 0·2% of the nucleic acid-P in the L cultures investigated. The possibility cannot be excluded that this small amount of incorporated phosphorus could be attributed to large L bodies present as contaminants in the fractionated L cultures (compare the determinations of viable counts for fractionated L cultures, described in the preceding section).

DISCUSSION

The present work shows the marked heterogeneity of bacterial L cultures. Our fractionation experiments, carried out by means of filtration or differential centrifugation, showed that various fractions characterized by different morphological, chemical and metabolic properties, could be prepared from the cultures of Proteus L9 studied. Most of the fractions prepared contained spherical bodies of different sizes as predominant elements. In the present work most attention was paid to L bodies which had a diameter $< 0{\cdot}3\mu$, which we separated from other elements in cultures of Proteus L9 by differential centrifugation. Light microscope and electron microscope observations showed that larger elements were only occasionally found in the fractionated material thus obtained.

In some bacteria at least the main part of the phospholipid is bound to particulate matter, located in the cytoplasmic membrane (cell envelope) of the intact organism (Mitchell & Moyle, 1951; Marr & Cota-Robles, 1957; Weibull, 1957). A

similar distribution seems to characterize the bacterial succinic dehydrogenase (Mitchell & Moyle, 1956; Storck & Wachsman, 1957; Weibull, Beckman & Bergström, 1959). Catalase, on the other hand, generally seems to be a soluble enzyme, probably located within the bacterial cell (Alexander & Wilson, 1955; Few, Frazer & Gilby, 1957; Weibull, Beckman & Bergström, 1959). Most of the RNA of the bacterial cell is also found in the non-peripheral part of the cytoplasm, the cytoplasmic membrane being almost free from this substance (Marr & Cota-Robles, 1957; Gilby, Few & McQuillen, 1958; Weibull, Beckman & Bergström, 1959).

Those of our chemical and enzymic studies which were carried out with mechanically disintegrated L bodies (see Tables 4 and 5) showed that also in Proteus L9 the phospholipid and the succinic dehydrogenase were largely bound to particulate matter and that the major part of the catalase behaved as a soluble enzyme. This was true of unfractionated and fractionated L cultures. Thus at least in some respects the L form investigated seems to possess a structural organization similar to that found in several normal bacteria.

The RNA of small L bodies (diameter $< 0{\cdot}3\mu$) was much more readily sedimented than that of unfractionated cultures consisting mainly of bodies of diameter $> 1\mu$ (see Table 4). Judging from the digestion experiment with DNAse (Table 2), the small L bodies seemed to contain little if any DNA. In other respects, results of the fractionation and disintegration experiments suggest that the structure of the L bodies was about the same, irrespective of size. Thus the small L bodies contained about double the amount of lipid-P than did the unfractionated cultures, and about 40 % less RNA-P (Table 1). Furthermore, our enzyme studies showed that the small L bodies contained more succinic dehydrogenase and less catalase than unfractionated cultures of Proteus L9. Taking into account the greater surface/volume ratio of the small elements as compared with the larger ones, these facts could be explained at least qualitatively if it were assumed that the L bodies consisted of a peripheral layer (the cytoplasmic membrane) containing the major part of the phospholipid and succinic dehydrogenase, and a central part containing the RNA and catalase.

The respiratory experiments showed that the small L bodies respired as vigorously as the unfractionated ones from which they were derived, and that the respiration rate of L bodies grown on solid medium was about 50 % lower than that of L bodies grown in liquid medium.

The work thus demonstrates that L bodies of different sizes differed in their chemical and metabolic properties. This is in agreement with the results of Mandel and co-workers (Mandel, Feo, Sensenbrenner & Terranova, 1959; Mandel *et al.* 1959). Our work has shown, however, that even small L bodies respire under appropriate conditions (cf. Mandel, Terranova, Sensenbrenner & Feo, 1959).

More fundamental differences between L bodies of different sizes than those discussed above appear when the biosynthetic activities are compared. The viable counts were very low for fractionated L cultures which contained as predominant elements small bodies of diameter $< 0{\cdot}3\mu$, the counts amounting to about 0·001 % of the total number of individual elements in the samples studied. Probably the viable elements in the fractionated material consisted of large L bodies, undetected in microscopic preparations of this material because of their scarcity.

Judging from the incorporation experiments performed, the synthesis of nucleic

acids in small L elements suspended in a growth medium and incubated for 4 hr. amounted at most to about 0·1–0·2 % of the total nucleic acid content of these elements. Under similar conditions the nucleic acid content of unfractionated L cultures increased 2–3 times. Thus the nucleic acid synthesis in the small L bodies was more than 1000 times less than in whole L cultures; however, the role of contaminating L bodies of larger sizes has to be taken into account. It then seems possible that all the nucleic acid synthesis which occurred in the fractionated material might be attributed to such contaminants. The possible occurrence of exchange reactions should also be borne in mind. The biosynthetic activity of the small L elements seems therefore questionable, as judged from the data presented here. Consequently their role in the life cycle of the L cultures, as described by Tulasne (1955), seems doubtful. On the other hand, these small bodies seems to possess a certain degree of structural organization, and they can hardly be regarded merely as protoplasmic debris. This is especially evident from the fact that mechanically prepared debris of Proteus L9 showed at most a very low degree of respiratory activity, whereas small L bodies isolated by differential centrifugation respired about as intensely as unfractionated L cultures. The chemical and enzymic composition of the small L bodies also suggests a certain degree of structural organization. Earlier work about the structure of small L elements as seen by the electron microscope should also be mentioned in this connexion (Thorsson & Weibull, 1958).

This investigation was part of a programme on the submicroscopic structure of the bacterial cell financially supported by the Swedish Natural Science Research Council.

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Printed in Great Britain

The Effect of Temperature on the Production by Coagulase-Positive Staphylococci of Staphylokinase and other Extracellular Substances

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(*Received* 14 *October* 1960)

SUMMARY

Three strains of coagulase-positive staphylococci, grown at temperatures between 35° and 43°, showed no significant change in mass of growth or in yield of α-haemolysin and the Panton-Valentine (PV) leucocidin. Staphylokinase production, however, decreased by 18 % (per mg. dry weight) for each degree rise in temperature. The amount of hyaluronidase produced at 37° was five to eight times greater than that produced at 41°.

INTRODUCTION

The optimal temperature for growth of a pathogenic organism is usually that of its host. The effects of different temperatures during the growth of numerous micro-organisms has been studied. For example, toxin production by *Corynebacterium diphtheriae* is best at 34°. *Escherichia coli* grown at progressively higher temperatures has its generation time progressively increased (Barber, 1908). *Mycobacterium ulcerans* shows little or no growth above 33° (MacCallum, Tolhurst, Buckle & Sissons, 1948). *Pasteurella pestis* at temperatures above 32° has its synthesis of metabolites markedly depressed (Hills & Spurr, 1952). *P. pseudotuberculosis*, which is strongly motile and possesses good flagellar antigens at 22° loses these at 37° (Preston & Maitland, 1952). *Listerella monocytogenes* produces better flagellar antigens at 25° than at 37° (Paterson, 1939, quoting McGaughey). Type III strains of Pneumococcus which produce only local lesions in rabbits do not survive when grown at 41°, whereas those strains which withstand this growth temperature cause fatal infections (Enders & Shaffer, 1936; Rich & McKee, 1936). It was of interest, therefore, to see what effects, if any, the incubation temperature had with three strains of Staphylococcus on the crop of organisms and yield of staphylokinase, α-haemolysin, Panton-Valentine (PV) leucocidin (Panton & Valentine, 1932) and hyaluronidase.

METHODS

Organisms. Three coagulase-positive strains of Staphylococcus were used in these investigations: P80, a phage-type 80 strain (from the late Dr E. S. Duthie); V8, isolated from a case of chronic furunculosis (Gladstone & Van Heyningen, 1957); the Oxford strain used in the assay of penicillin. The organisms for inoculation were grown on Difco heart infusion agar slopes overnight at 37°. These slopes were kept at room temperature and used during about 4 weeks, after which new slopes were used.

Inoculum. In all cases a 10 % (v/v) inoculum was used, grown in the same medium and at the same temperature as in the test. An overnight culture on a Difco agar slope was inoculated into 10 ml. broth, grown overnight and diluted 1/10 in the test medium.

Production of staphylokinase, PV leucocidin and hyaluronidase. Preliminary tests showed that the same media and cultural conditions were suitable for the production of these substances, but different times of incubation were required. The medium (CCY) was Woodin's modification (Woodin, 1959) of the yeast casein medium of Gladstone & Fildes (1940), namely: Difco Casamino acids, 20 g., Na lactate 70 % syrup, 14·4 ml.; Na glycerophosphate, 20 g.; $MgSO_4.7H_2O$ solution (16 %, w/v), 0·25 ml.; $MnSO_4.4H_2O$ solution (6·4 %, w/v), 0·125 ml.; $FeSO_4.7H_2O$ solution (0·32 %, w/v), citric acid solution (0·32 %, w/v), 2 ml.; KH_2PO_4, 0·41 g.; $Na_2HPO_4.2H_2O$, 3·1 g.; Oxoid yeast diffusate from 20 g. (this yeast diffusate was prepared by dialysing 100 g. yeast extract (Oxoid) dissolved in 100 ml. distilled water against 900 ml. distilled water for 24 hr. in the cold). Distilled water was added to make the final volume of the medium to 1 l. after adjustment to pH 7·6. The medium was distributed in 10 ml. volumes in ⊥-tubes (Van Heyningen & Gladstone, 1953) and autoclaved for 15 min. at 120°. Cultures were shaken exposed to air in a water-bath at a known constant temperature ($\pm 0{\cdot}1°$) and were harvested, unless otherwise stated, at 6½ hr. for staphylokinase (time for optimal production; see Results), 14 hr. for hyaluronidase and 18 hr. for PV leucocidin.

Production of α-haemolysin. Preliminary tests showed that 2 % (w/v) Difco heart infusion broth, without the addition of 0·2 % (w/v) agar, produced good yields of α-haemolysin. This medium was distributed in 15 ml. volumes in 4 oz. medical flats and autoclaved at 120° for 15 min. After inoculation, the cultures were gassed with a mixture of 20 % (v/v) CO_2 + 80 % (v/v) air, closed with screw caps and incubated in a horizontal position in a water-bath at known temperature for 48 hr.

Estimation of growth. Growth was estimated in a Hilger Spekker spectrophotometer calibrated in terms of bacterial dry weight per ml. culture.

Harvesting of cultures. After incubation for the appropriate time, the cultures were centrifuged at 2500 rev./min. for 10 min.; the supernatant fluids were retained and used without filtration, and the deposits examined by Gram staining to test for purity. For the estimations of staphylokinase, 1/100,000 thiomersalate was added to prevent further growth.

Assay of staphylokinase

Staphylokinase was assayed on a semi-microscale, by a combining test with antibody, as follows. Glass coverslips were thoroughly cleaned in chromic acid (40 g. potassium dichromate in 1 l. concentrated sulphuric acid) rinsed in tap water and distilled water and stored in ethanol until required. All tubes used for dilutions were cleaned in a similar manner. Four small wax circles were made on each coverslip and into each circle was pipetted a solution of 0·01 % (w/v) fibrinogen (Blood Products Laboratory, Lister Institute) in 0·5 % (w/v) gelatin saline (pH 7·6) using a microsyringe. To each circle was added about 0·002 ml. of a 0·2 % (w/v) solution of thrombin (Blood Products Laboratory, Lister Institute) in gelatin saline containing 0·01 ml. Hucker–Conn fibrin stain/ml. As each drop was added,

the two solutions within a circle were mixed with a small glass rod. These quantities were found to provide a firm clot after incubation at 37° for 10 min. in moist chambers.

The minimum lytic dose (MLyD) of the staphylokinase preparation was first determined as follows. The culture supernatant fluid was serially diluted at 100 % differences in gelatin saline. A volume of 0·01 ml. of each dilution, ranging from undiluted supernatant fluid to 1/1024 dilution, was pipetted on to a preformed fibrin clot, prepared as above, by using a microsyringe. As a control, 0·01 ml. of 1/10 dilution of a preparation of soya bean trypsin inhibitor (from Worthington Biochemical Sales Co., New Jersey) was mixed with the undiluted supernatant fluid before addition to a clot. The clots were then incubated for 18 hr. at 37°, after which a small volume of gelatin saline was pipetted on to each coverslip. Where the fibrin had been digested, the end-point was clearly seen by the absence of the mauve clot and the appearance of dye in the fluid. The dilution of supernatant fluid which contained 1 MLyD was thus easily estimated.

The L+ dose, defined in the usual way as the smallest amount of staphylokinase which, when mixed with one unit of antibody, results in dissolution of the clot, was then determined. The usual procedure in which serial dilutions of the test toxin are mixed with a constant unitage of standard antitoxin was reversed, serial dilutions of a standard antiserum (Wellcome Laboratories, CPP 76/63, given an arbitrary value of 100 units anti-staphylokinase/ml.) at 100 % differences being mixed with a constant amount of the staphylokinase preparation containing at least 4 MLyd. The technique was as follows: 0·01 ml. of each dilution of antiserum and 0·01 ml. of supernatant fluid containing 4 MLyD/ml. were mixed and placed on preformed fibrin clots. The preparations were incubated at 37° for 18 hr. and the results read as before. A more accurate determination of the L+ dose was then made by decreasing the differences between the dilutions of antiserum to 20 %. The amount of staphylokinase/mg. bacterial dry wt. was then calculated and expressed as L+ units of staphylokinase/mg. bacterial dry wt.

Assay of α-haemolysin

The L_H dose was determined by mixing serial dilutions of standard antitoxin at 20 % differences with a constant amount of culture supernatant fluid and adding 3 % (v/v) washed rabbit red cells, using the semi-micromethod of Gladstone & Glencross (1960). The results were related to bacterial growth and expressed as L_H/mg. bacterial dry wt.

Assay of S and F components of PV leucocidin

Woodin (1959, 1960) showed that PV leucocidin contained two components, S and F. These were estimated by the method of Gladstone & Glencross (1960) in which serial dilutions of antitoxin containing known arbitrary unitages of anti-F and anti-S at 50 % differences were mixed with a constant volume of supernatant fluid in the presence of excess of purified S or F components respectively, and placed on preparations of human leucocytes which were examined microscopically under phase contrast. The results were expressed as L+ units of F and S/mg. bacterial dry wt.

Assay of hyaluronidase

Estimations of hyaluronidase were made by a modification of the turbidity-reduction method of Pearce (1953). A standard curve was prepared by measuring the turbidity produced by different quantities of a solution of hyaluronic acid (from Dr D. Riding, Evans Biological Institute) dissolved in 0·1 M-acetate buffer (pH 6·0) containing 0·15 N-NaCl, made up to 1 ml. with 0·2 % (w/v) gelatin acetate buffer (pH 6·0) 5 min. after the addition of 5 ml. acidified protein reagent (0·1 %, w/v, bovine plasma fraction V, Armour, in 0·1 M-acetate buffer pH 4·2, adjusted to pH 3·75 with 4 N-HCl). All estimations were done in duplicate and the optical density at hyaluronidase concentrations of 50, 100, 150 and 200 μg./ml. measured before each experiment to check the slope and position of the curve.

The culture supernatant fluids were adjusted to pH 6·0 with 2 N-HCl and dilutions prepared in 0·2 % (w/v) gelatin acetate buffer (pH 6·0). To 0·5 ml. of each dilution was added 0·5 ml. of the solution of hyaluronic acid containing 400 μg. hyaluronic acid/ml. in spectrophotometer tubes. These were incubated at 37° for exactly 10 min. and then transferred to a 70° water-bath for 30 min. to inactivate hyaluronidase. After the solutions had cooled to room temperature, 5 ml. acidified protein reagent were added to each tube at 1 min. intervals, and the turbidity read after exactly 5 min. The amount of hyaluronic acid left was read from the standard curve and the amount of hyaluronidase present calculated in turbidity reducing (t.r.) units. One t.r. unit is defined as the amount of hyaluronidase which will decrease the turbidity given by 200 μg. hyaluronic acid to that given by 100 μg. hyaluronic acid. The results were expressed as t.r. units/mg. bacterial dry wt.

RESULTS

Experiments to determine the optical conditions for the production and assay of staphylokinase

Optimal time of harvesting. Cultures were incubated at 37° and 1 ml. samples taken at intervals to determine the yield of staphylokinase at different times. Graphs of growth and yield of staphylokinase against time of incubation showed that production of staphylokinase at first lagged behind growth, but then proceeded rapidly, reaching a maximum yield per ml. at about 6½ hr. for all three strains under these conditions (Fig. 1). It was therefore decided to incubate all cultures for 6½ hr. after the 10 % inoculum had been made.

Optimal time of incubation with the test fibrin clots. Six parallel series of MLyD tests were prepared and the results read after 1, 2, 4, 8, 18 and 24 hr. The longer times were used as it is known (Gerheim & Ferguson, 1949) that staphylokinase requires long incubation to activate plasminogen. After 1 or 2 hr. of incubation no lysis had occurred and as the time was increased from 4 to 18 hr., the end-point was found in progressively higher dilutions of the supernatant fluid. Incubation beyond 18 hr. gave no further change in the end-point and all further tests were therefore incubated for this time.

Variability of results. Twenty samples of the same culture supernatant fluid were tested to see whether the staphylokinase end-point was always found in the same dilution. All the tests gave an end-point with the same dilution of antiserum or with

one different by only 20 %. A series of tests with different dilutions of supernatant fluid showed that a stoichiometric relationship existed between supernatant fluid dilution and antiserum dilution (Table 1).

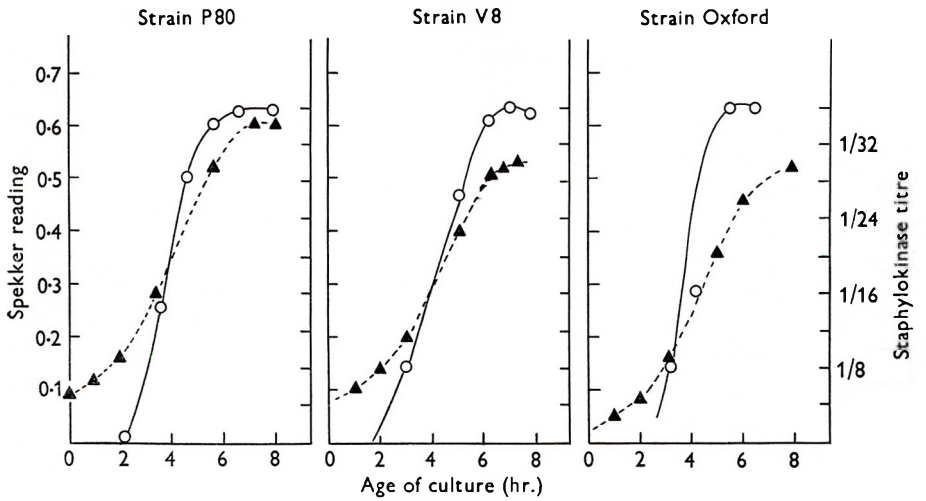

Fig. 1. Relationship between time of incubation of cultures and growth and yield of staphylokinase. ▲—▲, growth; ○—○, yield of staphylokinase.

Effect of temperature on the production of staphylokinase

Cultures were incubated at 35°, 37°, 39°, 41° and 43° and the yields of staphylokinase assayed. Occasionally growth at 43° was very low; in such cases, the cultures were discarded and new ones grown. In all other cases, the growth obtained was of the same order, i.e. about 5–6 mg. bacterial dry wt./ml. In every test, cultures at 37° were included as controls.

Table 1. *α-Haemolysin activity: relationship between dilution of supernatant fluids of staphylococcal cultures and dilutions of antiserum*

Reciprocals of dilutions of supernatant fluids	Reciprocals of dilutions of antiserum							
	32	64	128	256	512	1024	2048	4096
	lysis (+) or no lysis (−) of clot							
2	−	−	+	+	+	+	+	+
4	−	−	−	+	+	+	+	+
8	−	−	−	−	+	+	+	+
16	−	−	−	−	−	−	+	+
32	−	−	−	−	−	−	+	+

The amount of staphylokinase/mg. bacterial dry wt. detected at the different temperatures was greatest at 35° and smallest at 43° (Fig 2). When the logarithm of the amount of staphylokinase (y) was plotted against temperature (x) the regression of y on x was linear. All three strains showed the same slope, i.e. $-0{\cdot}0702$ ($\pm 0{\cdot}0064$) but the positions of the lines differed. The curves for strains V8 and

Oxford were identical, while that for strain P80 was significantly lower. The coefficient 0·0702 implies that the amount of staphylokinase produced decreased by 18 % for each degree rise in temperature.

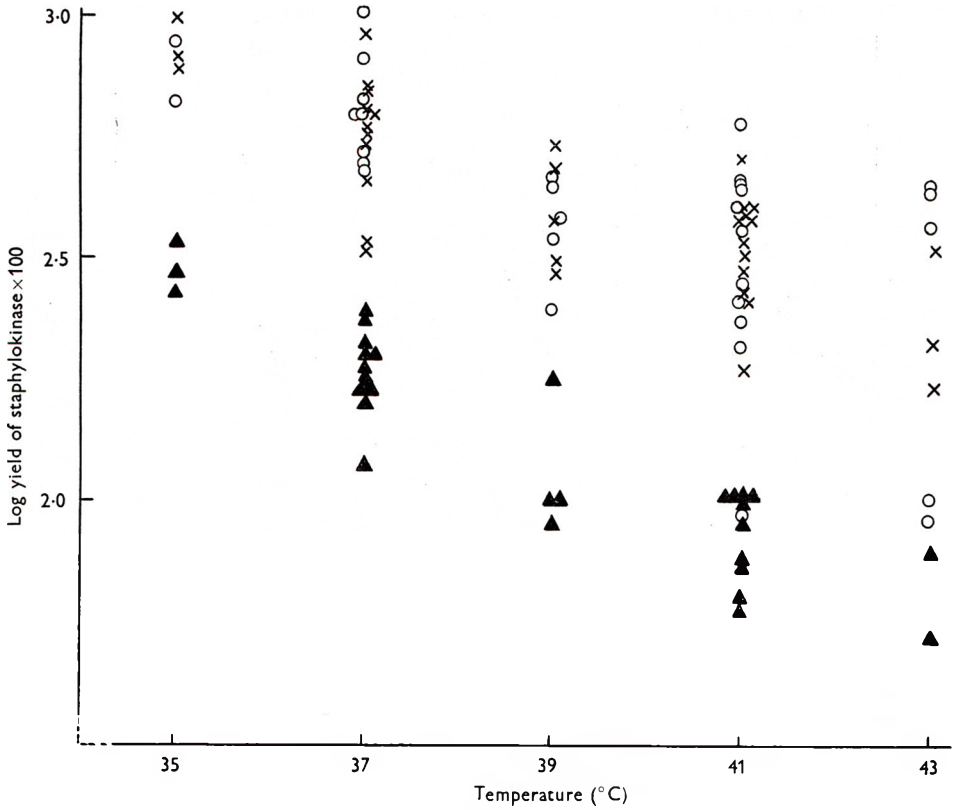

Fig. 2. Yields of staphylokinase produced by staphylococcal strains P80, V8 and Oxford at temperatures ranging from 35° to 43°. ▲, P80; ○, V8; ×, Oxford.

Several samples of supernatant fluid from cultures of all three strains at 37° containing a known amount of staphylokinase were kept at 43° for 6½ hr. and then re-tested. The amount of staphylokinase was unchanged and it was concluded that the lower yields obtained at the higher temperatures were not due to destruction of staphylokinase.

Effect of temperature on the production of α-haemolysin

Cultures of the staphylococci grown at temperatures ranging from 37° to 41° were tested for α-haemolysin content. The values obtained did not differ significantly at the different temperatures.

Effect of temperature on the production of F and S components of leucocidin

Supernatant fluids tested for F and S components of leucocidin showed no differences in the amounts produced at the different temperatures.

Effect of temperature on the production of hyaluronidase

Two temperatures, 37° and 41°, were selected for these tests; a comparison of the amounts of hyaluronidase produced at these temperatures is shown in Table 2. The supernatant fluids from cultures incubated at 37° contained about 5–8 times as much hyaluronidase as those incubated at 41°. When supernatant fluids from cultures incubated at 37° were kept at 41° for 14 hr., the results obtained were unchanged, showing the original difference to be due to decreased production of hyaluronidase.

Table 2. *Production of hyaluronidase at* 37° *and* 41°

Strain	t.r.u. per mg. at 37°	t.r.u. per mg. at 41°
P80	8·0	1·2
	6·0	< 1·0
	5·0	< 1·0
	5·5	1·2
	7·3	1·4
V8	10·7	2·4
	8·9	2·1
	7·2	1·6
	> 10·0	2·45
	6·7	1·5
Oxford	30·0	4·8
	27·2	3·4
	> 13·0	4·4
	19·0	3·1
	17·0	2·5

DISCUSSION

The experiments described show that for these three strains of coagulase-positive staphylococci, growth at a temperature higher than 35° resulted in a progressive and marked diminution in the yields of staphylokinase and hyaluronidase, while total mass of growth, α-haemolysin and PV leucocidin remained unaltered. If it be permissible to apply these results to conditions of staphylococcal sepsis *in vivo*, then a rise in body temperature of this order in fever might be held to exert a suppressive effect upon staphylokinase and hyaluronidase production in the animal tissues. Even so, the role of these and other extracellular substances produced by staphylococci in relation to invasiveness and virulence is obscure. Hyaluronidase production seems unrelated to virulence for any given strain (see Bøe, 1944). The use of staphylokinase and coagulase production as indicators of staphylococcal virulence has been challenged. Kapral & Isabel (1960) produced mutants which had lost the power to produce staphylokinase (both bound and soluble), yet these strains were as virulent for rabbits as was the parent strain. Another mutant, which retained its ability to produce coagulase, almost completely lost its invasiveness and virulence. Virulence, as many observers have suggested, is probably a complex phenomenon in which many products of the staphylococcus, some as yet unidentified, may be involved.

I wish to thank Dr G. P. Gladstone for guidance and help in this work; Mr J. Scott for the statistical analysis of the results; Drs R. A. Kekwick, D. Riding and

A. M. Woodin for generous samples of human fibrinogen and thrombin, hyaluronic acid, and purified F and S components of leucocidin, respectively; and Miss June Tyrell and Miss Eve Cooper for their able technical assistance.

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Printed in Great Britain

Experimental Conditions for Nitrate Reduction by Certain Strains of the Genus *Lactobacillus*

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(*Received* 14 *October* 1960)

SUMMARY

Costilow & Humphreys's (1955) observation that certain strains of *Lactobacillus plantarum* reduced nitrates under certain conditions was confirmed. Two strains of *L. fermenti* also reduced nitrates. In static culture, agar and anaerobiosis were not essential for nitrate reduction, contrary to speculations in the literature. Nitrate reduction was possible only in media with restricted carbohydrate and with the pH value maintained at a relatively high value within the activity range of nitrate reductases. For good growth, lactobacilli for the nitrate test have been customarily grown in media with high carbohydrate content, with consequent low final pH values. This seems to be the essential reason why the genus *Lactobacillus* had previously been defined as unexceptionally nitratase-negative.

INTRODUCTION

Until recent years the genus *Lactobacillus* has been described as unable to reduce nitrates (*Bergey's Manual*, 1948; Rogosa *et al.* 1953). However, Costilow & Humphreys (1955) reported that 18 of 38 strains of *Lactobacillus plantarum* reduced nitrates. This result was achieved through the use of the BBL indole-nitrite medium (Baltimore Biological Laboratory, Inc.) which has an initial pH value of 7·2 and a composition of 2 % (w/v) Trypticase, 0·2 % (w/v) Na_2HPO_4, and 0·1 % (w/v) each of glucose, KNO_3 and agar. Negative results, consistent with earlier general experience, were obtained with broth media, such as the BBL indole-nitrite medium minus agar, or Difco nitrate broth. To explain this, Costilow & Humphreys (1955) reasoned that agar would tend to decrease the oxygen tension of the medium and stated: 'It is obvious that the oxygen tension of the medium was the most important factor in nitrate reduction....' From inspection of the formula of the BBL indole-nitrite medium it is evident that it contains very little fermentable carbohydrate (0·1 %, w/v) and is very highly buffered. Also the initial pH, 7·2, is high. This suggests that nitrate reduction may be a function of pH value and that the nitrate reductase enzymes may have a relatively high and narrow pH activity range. For example, Woods (1938) found with *Clostridium welchii* that, when the reduction rate at pH 6·8 was taken as 100 %, it was 50 % at pH 6·4 and only 10 % at pH 6·1 after 25 min. After 35 min., the reduction rate decreased further at pH 6·5 to only 10 %. Similar results were obtained with a strain of

Escherichia coli. Nason & Evans (1955) studied a purified enzyme from *Neurospora crassa* which catalysed the reaction $TPNH + H^+ + NO_3^- \rightarrow TPN^+ + NO_2^- + H_2O$ and the enzyme exhibited a sharp optimum for activity at pH 7·0 in phosphate buffer. Zucker & Nason (1955) found maximum activity between pH 8·0 and 9·0 for the intermediate enzyme, hydroxylamine reductase, in denitrification by *N. crassa*. Najjar (1955) obtained similar high pH optima with *Pseudomonas stutzeri* and *Bacillus subtilis* for the enzymic conversion of NO_2^- and NO to N_2. Because of the importance of nitrate reduction as a taxonomic criterion, a wide range of *Lactobacillus* species, and certain streptococci and pediococci, were tested in experiments designed to discover the appropriate conditions for nitrate reduction.

METHODS

The strains of lactobacilli were representative of 13 species in the collection of Dr M. Elisabeth Sharpe (National Institute for Research in Dairying, NIRD, University of Reading), obtained through original isolations by Dr Sharpe or other workers, and from such culture colleetions as the American Type Culture Collection (ATCC), the (British) National Collection of Type Cultures (NCTC), the (British) National Collection of Industrial Bacteria (NCIB) and the (British) National Collection of Dairy Organisms (NCDO maintained at NIRD). Each strain had been carefully studied by numerous tests (Rogosa *et al.* 1953; Rogosa & Sharpe, 1959) including serological procedures (Sharpe, 1955) wherever specific group antisera could be prepared.

There were strains of *Lactobacillus plantarum*. The origins of the following 7 are of interest: *AR1* NIRD 17/5; *P4*, NCTC 6376 from ATCC 8014; *P16*, ATCC 8014; *AR5*, *L. arabinosus* V743 Tittsler; *P30*, V322 Tittsler; *P7*, NIRD 1–4; *P8*, NIRD 1–8. Strains *AR1*, *P4*, *P16* and *AR5* are identical and are really only a single strain designated *L. arabinosus* 17/5 by Fred, Peterson & Anderson (1921), but ascribed different numbers in different culture collections.

Five strains of *Lactobacillus fermenti* were tested. Strain *F1* is identical with ATCC 9338 and NCTC 6991, and strain *F4* is NCTC 7230.

The remaining strains were distributed numerically as follows: *Lactobacillus acidophilus*, 5; *L. brevis*, 10; *L. buchneri*, 1; *L. bulgaricus*, 9; *L. casei*, 14; *L. cellobiosus*, 2; *L. delbrueckii*, 2; *L. helveticus*, 1; *L. jugurti*, 3; *L. lactis*, 11; *L. leichmannii*, 2; *L. salivarius*, 4.

In addition, one strain each of the following group D streptococci was tested: *Streptococcus bovis*, *S. durans*, *S. faecalis* var. *liquefaciens*, *S. faecalis* and *S. faecium*. Among group N streptococci were two strains of *S. cremoris*, one of *S. lactis* and two of *S. lactis* var. *diacetilacticus*. Three strains of *Pediococcus cerevisiae*, including ATCC 8081, were also tested.

The media used were: (1) the BBL indole-nitrite medium; (2) Difco nitrate broth; (3) various modifications of these such as the addition of agar, yeast extract, increased buffering with Na_2HPO_4, and changes in the concentration of glucose. The conditions for each experiment will be described in the tabulation of results.

Cultures were inoculated with one drop from good growth in MRS broth (de Man, Rogosa & Sharpe, 1960) and incubated for 6–7 days at 37° except for *Lactobacil-*

lus plantarum, *L. brevis*, *L. casei*, the streptococci and the pediococci which were incubated at 30°.

When anaerobiosis was desired it was obtained by using McIntosh & Fildes jars filled with either H_2 or a 90 % H_2 + 10 % (v/v) CO_2 mixture, and combining residual traces of O_2 with H_2 by using the electrically heated catalyst.

The reagents for the detection of nitrite were: solution 1 containing 2 g. sulphanilic acid in 250 ml. 5N-acetic acid; solution 2 containing 1·5 ml. dimethyl-α-naphthylamine in 250 ml. 5N-acetic acid. The latter reagent was recommended by Wallace & Neave (1927) and Tittsler (1930) as superior to the α-naphthylamine reagent because the colour develops instantaneously, is more intense, and does not fade in a reasonable period of time. For the nitrite test, one drop of culture was deposited in a white porcelain spot plate followed by one drop each of solutions 1 and 2. The pink to red colour in positive tests developed at once and was unequivocal. The spot plate technique was often superior to tests made directly in the tubes. Some media contain substances (thioglycollate, for example) which inhibit colour development, but with the quantities of culture and reagents described for the spot plate test, colour development was uninhibited. Negative controls on the media and a positive control with *Micrococcus aureus*, phage type 80, were included routinely. All negative tests were confirmed by reducing residual nitrate to nitrite with Zn dust on the spot plate and testing for nitrite as described. Zn reductions in the tubes often gave ambiguous and false results.

RESULTS

The only species in which some strains reduced nitrates were *Lactobacillus plantarum* (7 of 12) and *L. fermenti* (2 of 5 tested). All other species of lactobacilli, and the streptococci and pediococci examined, were unable to reduce nitrates under any of the experimental conditions. Thus, Costilow & Humphreys's (1955) observation that certain strains of *L. plantarum* can reduce nitrates under appropriate conditions was confirmed.

Some typical data from experiments concerning the role of agar, yeast extract, initial pH and anaerobiosis in nitrate reduction by *Lactobacillus plantarum*, are presented in Table 1. When Difco nitrate broth (DNB) or DNB supplemented with agar and yeast extract was used at an initial pH 6·4 (a pH value favourable for growth) all strains repeatedly failed to reduce nitrates aerobically or anaerobically and the pH value decreased sharply to a final value of 3·4. At the relatively unfavourable value pH 7·4, however, the cultures reduced nitrates, the pH decrease was markedly less, and the final pH value was about 6·4, i.e. 3 units less than the decrease obtained when the initial value was pH 6·4.

In BBL indole-nitrite (IN) medium nitrate reduction was sometimes suppressed under anaerobic conditions, as illustrated by the data for strain P30. In IN medium supplemented with Difco yeast extract (YE) nitrate reduction was also occasionally absent or weak. From observation of growth it was obvious that these weak or negative responses were related to total growth and that growth in IN medium + YE was even poorer than in the relatively poor IN medium. Also, it is clear from Table 1 that nitrate reduction, both aerobically and anaerobically, took place in the absence of agar, which was irrelevent to the process. Yeast extract in broth media,

Table 1. *Effect of composition of culture medium, anaerobiosis, and* pH *value on nitrate reduction by* Lactobacillus plantarum

Experimental medium	Uninoculated control				Strain P30				Strain P4			
	Aerobic		Anaerobic†		Aerobic		Anaerobic		Aerobic		Anaerobic	
	NO_2*	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH
Difco nitrate broth (DNB)	–	6·4	–	5·9	–	3·4	–	3·4	–	3·4	–	3·4
DNB + 0·1 % agar	–	6·4	–	6·0	–	3·4	–	3·4	–	3·4	–	3·4
DNB + 0·1 % agar + 0·3 % Difco yeast extract (YE)	–	6·4	–	6·0	–	3·4	–	3·4	–	3·4	–	3·4
DNB + 0·1 % agar + 0·3 % Difco yeast extract (YE)	–	7·4	–	6·0	+	6·1	–	6·0	+	6·4	+	5·7
BBL indole-nitrite (IN) medium	–	7·0	–	6·9	+	6·4	–	6·4	+	6·4	+	6·4
IN medium + 0·3 % YE	–	7·1	–	6·9	–	6·3	–	6·4	+	6·4	sl	6·4
IN medium with agar omitted (IN broth)	–	7·1	–	6·7	+	5·7	–	5·7	+	5·8	tr	5·8
IN broth + 0·3 % YE	–	6·9	–	6·7	+	5·7	–	5·8	+	5·8	–	5·8
IN broth + 0·3 % YE + 0·9 % glucose	–	7·0	–	6·7	–	5·5	–	4·9	–	5·6	–	5·0

* NO_2 = nitrate reduction; sl and tr = slight and trace reduction, respectively; – = negative and + = positive.
† Anaerobic conditions 90 % (v/v) H_2 + 10 % CO_2 (v/v).

Table 2. *Relation between fermentable carbohydrate, final* pH *value, and nitrate reduction by* Lactobacillus plantarum, *under aerobic and anaerobic conditions*

Medium	AER*		ANH_2		$ANH + CO_2$		AER		ANH_2		$ANH_2 + CO_2$	
	NO_2†	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH
	Uninoculated control						Strain P30					
BBL indole-nitrite (IN) medium	–	7·3	–	7·3	–	6·6	+	6·6	+	6·6	–	6·3
IN medium + 0·1 % glucose	–	7·3	–	7·4	–	6·6	–	5·8	tr	6·0	–	5·7
IN medium + 0·3 % glucose	–	7·1	–	7·2	–	6·5	–	4·9	–	4·9	–	4·7
IN medium + 0·5 % glucose	–	7·1	–	7·2	–	6·6	–	4·7	–	4·6	–	4·2
IN medium without agar	–	7·3	–	7·4	–	6·6	+	6·8	+	6·6	–	6·4
Medium	AER		ANH_2		$ANH_2 + CO_2$		AER		ANH_2		$ANH_2 + CO_2$	
	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH
	Strain P4						Strain AR5					
BBL indole-nitrite (IN) medium	+	6·8	+	6·6	+	6·4	+	6·8	+	6·8	+	6·2
IN medium + 0·1 % glucose	+	5·9	+	5·9	+	5·8	+	6·0	–	6·0	sl	5·7
IN medium + 0·2 % glucose	–	4·8	–	4·9	–	4·8	+	5·0	–	5·0	–	4·9
IN medium + 0·5 % glucose	–	4·5	–	4·4	–	4·4	–	4·5	–	4·5	–	4·3
IN medium without agar	+	6·8	+	6·7	+	6·6	+	6·9	+	6·8	+	6·5

* AER = aerobic incubation; ANH_2 = anaerobically in 100 % H_2; $ANH_2 + CO_2$ = anaerobically in 90 % (v/v) H_2 + 10 % CO_2 (v/v).
† NO_2 = nitrate reduction; – = negative and + = positive; sl and tr = slight and trace reduction, respectively.

contrary to the experience with agar-containing media, did not inhibit nitrate reduction. What is most germane to this discussion is that cultures in media such as IN medium without agar + YE supplemented with conventional quantities of glucose (0·9 %, w/v), had relatively low terminal pH values and also did not then reduce nitrates.

The above results strongly indicate that induced anaerobic conditions, yeast extract, and agar are not essential for nitrate reduction by these strains. Rather, it appears that the pH value, fermentable carbohydrate, and buffer capacity are the principal factors which effect nitrate reduction. Table 2 presents typical data which confirm this hypothesis. Anaerobiosis previously was achieved with 90 % (v/v) H_2 + 10 % (v/v) CO_2. Since CO_2 may decrease the pH value of the medium, anaerobiosis in further experiments was also maintained by an atmosphere of 100 % H_2. As little as 0·1 % (w/v) added glucose inhibited nitrate reduction by *Lactobacillus plantarum* P30. Nitrate reduction by this strain seemed most sensitive to slight acidity from the fermentation of glucose, but all nitrate positive strains in IN medium were, with one exception, unable to reduce nitrates when 0·3 % (w/v) glucose was added and the pH value consequently decreased to 5·0 or less. With 0·5 % (w/v) glucose, nitrate reduction was never observed. Again, positive reactions were generally fewer or less intense in anaerobic as compared with aerobic cultures, and it is quite clear that agar had no enhancing effect. These repeated results with agar do not agree with those of Costilow & Humphreys (1955). In experiments which repeated their experimental protocol exactly as described, but in which the pH values of comparable broth and agar media were adjusted electrometrically exactly to the same initial value of 7·1, there was no difference in nitrate reduction in broth or agar containing media.

The strain of *Micrococcus aureus*, phage type 80, which was used as a positive control, reduced nitrate to nitrite at pH 7·0. But at pH 7·5 or above the organism consistently also reduced nitrite completely, with accumulation of N_2. In this case it would appear that the nitrite reductase enzyme had an even higher pH activity range than the nitrate reductase.

DISCUSSION

Clarke (1959) emphasized the nitrate reducing property of some anaerobic bifid organisms named *Lactobacillus bifidus*. In the judgement of the writer and of Orla-Jensen, Orla-Jensen & Winther (1936), Rogosa & Sharpe (1959), and Sundman, Bjorksten & Gyllenberg (1959) who described these organisms as 'organisms previously incorrectly designated *Lactobacillus bifidus*', these organisms should not be properly within the genus *Lactobacillus* but rather be allocated elsewhere. A more extensive discussion is reserved for the future.

The plausible argument that anaerobic technique is essential for nitrate reduction in static culture is not new. ZoBell (1932), in a study of nitrate reduction by *Brucella* spp., advocated the use of a medium made semisolid with 0·3 % (w/v) agar, which he thought would lower the O/R potential and thus encourage nitrate reduction. The basal medium was a poor one containing only 0·2 % (w/v) peptone, 0·1 % (w/v) beef extract, and 0·3 % NaCl (w/v) as nutrients. He observed markedly improved growth in the semisolid medium. In the absence of comparative aerobic and

anaerobic results in broth media it is just as reasonable to assume that the agar was exerting nutritional effects or was adsorbing toxic materials.

It has already been shown in the present experiments that agar and anaerobic cultivation are unessential for nitrate reduction by *Lactobacillus plantarum*. The statement by Costilow & Humphreys (1955) 'that oxygen tension is a very important factor in the ability of bacteria to reduce nitrates' and their remarks concerning Sacks & Barker (1949) can be misunderstood. Sacks & Barker (1949) said: 'Weissenberg (1897) tested the ability of three denitrifying bacteria to reduce nitrate and nitrite in shallow layers of medium exposed to air and in the complete lack of oxygen. Weissenberg found that complete denitrification occurred in the anaerobic cultures, *whereas aerobically nitrate was reduced only as far as nitrite*.' (Italics mine.) Sacks & Barker proceeded to test the effect of different partial pressures of oxygen on denitrification by *Pseudomonas denitrificans*, using special precautions to maintain equilibrium between the atmosphere and the culture medium. This was done in manometric experiments by shaking the flasks at the rapid rate of 150 oscillations/min. Under these conditions both nitrate and nitrite reduction were inhibited.... 'but even in a solution fully saturated with air the rate of nitrate reduction is 29 per cent of the anaerobic rate'. The influence of oxygen tension on the formation of nitrate- and nitrite-reducing enzyme systems during growth was determined in experiments in which the bacteria were grown anaerobically 'and aerobically while being vigorously aerated with 1·0, 5·0, or 20·6 per cent oxygen in nitrogen'. It was found that: 'The formation of *nitrite*-reducing enzymes is decreased 29 per cent by 1 per cent oxygen and is completely prevented by oxygen at a level of 5 per cent or higher. The lowest oxygen level capable of preventing the formation of *nitrite*-reducing enzymes may be considerably below 5 per cent; no data are available in the range between 1 and 5 per cent oxygen. The formation of *nitrate*-reducing enzymes is much less sensitive to oxygen during growth; 1 per cent oxygen causes no detectable inhibition and even saturation of the culture medium with air does not completely prevent the formation of such enzymes.' (Italics mine.) Furthermore, at an oxygen tension of 5% when nitrite reduction was nil, nitrate reduction was still 65% of the anaerobic reaction.

The usual conditions for the nitrate-reduction tests are static cultures, not 'aerobically while being vigorously aerated'. Also, negative oxidation-reduction potentials are reached by lactic acid bacteria in carbohydrate media during early 'aerobic' growth (Frazier & Whittier, 1931). It seems highly probable that the deleterious effect of oxygen on nitrate reduction has not been clearly differentiated from its effect on nitrite reduction and that an argument has been applied from the inapplicable case of denitrifying bacteria which reduce nitrates to nitrites and then reduce nitrites to other products. But in the routine test for nitrate-reduction the reagents test for nitrite accumulation. Even with denitrifying bacteria which also reduce nitrite, the disappearance of nitrite and residual nitrate indicates a positive reaction.

Some years ago the writer's laboratory tested hundreds of lactobacilli for nitrate reduction in anaerobic culture with completely negative results. Included were many strains of *Lactobacillus plantarum*, among which were some that were positive when tested later by Costilow & Humphreys (1955). These have also been found to reduce nitrate in the present work. Thus, the argument which involves decreased

oxygen tensions was not appealing in itself as an explanation for previous negative results. We have seen that the reaction of the medium is a critical factor in the reduction of nitrates. Generally, nitrate media for denitrifying bacteria contain extremely little added carbohydrate or more often none at all. It is not difficult to see now that with the acidogenic lactobacilli relatively small concentrations of fermentable carbohydrate may inhibit or prevent nitrate reduction because of pH changes. It has previously been customary to culture lactobacilli for the nitrate test in media containing enough carbohydrate for good growth. With the accumulation of acidic fermentation products the pH value, even in early growth, shifts beyond the activity range of nitrate reductases. This seems the highly probable reason why the genus *Lactobacillus* has previously been described as unexceptionally nitrate-negative.

It is not clear why only certain strains of *Lactobacillus plantarum* and *L. fermenti* were nitrate-positive. There were no obvious correlations between this and any other properties of the organisms. Most species grew poorly in the media tried. Attempts to improve growth by the use of massive inocula of washed organisms or to induce adaptation, and changes in the buffer content of media, have not yet changed the nitrate-negative character of any organisms.

I gratefully acknowledge the hospitality of the Director and staff of the National Institute for Research in Dairying, University of Reading, England, where most of this work was done. Particularly I acknowledge the kind help of Dr M. Elisabeth Sharpe who provided me with laboratory facilities, technical aid and many cultures.

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ADDENDUM

Clarke (1959) reported nitrate reduction by *Lactobacillus brevis* NCTC 2797. Dr M. Elisabeth Sharpe of the NIRD has confirmed that this strain reduces nitrates under appropriate conditions as described in this paper; but she has found that in fact this strain is not *L. brevis* but is *L. fermenti* as confirmed by nutritional and serological tests and growth temperatures.

J. gen. Microbiol. (1961), **24**, 409–421
With 1 plate
Printed in Great Britain

Purification and Properties of Neuraminidase from *Vibrio cholerae*

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(*Received* 21 *October* 1960)

SUMMARY

A method is described for the purification of neuraminidase from culture fluids of *Vibrio cholerae*. Five steps are involved: fractionation with methanol, adsorption to and elution from human red cells, fractionation with ammonium sulphate, chromatography on columns of hydroxyl apatite, crystallization. From 35 l. of culture filtrate, an average yield of 21 % of the original enzyme activity was obtained as crystals. The degree of purification was about 5000 fold. Purified neuraminidase possessed $12{\cdot}6 \times 10^6$ units of biological activity/mg. dry weight, and gave a value for $E^{1\%}_{280\,m\mu}$ of 8·96, measured at 0·025 % (w/v). Enzyme activity was stimulated by calcium ions and inhibited by ethylenediaminetetra-acetate. In the presence of 0·001 M-$CaCl_2$, neuraminidase showed maximum activity at pH 5·6. With sialyl lactose as substrate, a value of $1{\cdot}2 \times 10^{-3}$ M was found for the Michaelis constant. At an enzyme concentration of 0·16 μg./ml., $V_{max.}$ was 0·021 μM *N*-acetylneuraminic acid/min./ml. The enzyme was stable when dried from the frozen state and stored under vacuum at 0°. A suspension of crystals in water also retained activity when stored at 0°. Solutions of crystalline neuraminidase showed a small increase in activity when stored at 0° for several weeks. This effect was greatest at pH 6·7 and 8·5 but was barely detectable at pH 4·6. At pH 5·6 or 6·7, the enzyme lost about 20 % of its activity over a period of 2 hr. at 37° (concentration = 15 μg./ml.) No proteolytic activity nor *N*-acetylneuraminic acid aldolase activity was detected in the crystalline preparation.

INTRODUCTION

The ability of culture fluids of *Vibrio cholerae* to render human red cells inagglutinable by influenza virus was first noticed by Burnet, McCrea & Stone (1946) who described the active principle as the receptor-destroying enzyme (RDE) of *V. cholerae*. Gottschalk & Lind (1949) early provided evidence about the chemical activity of RDE but the enzyme was not characterized as a glycosidase until 1956 (Gottschalk, 1956; Heimer & Meyer, 1956). The enzyme has been termed sialidase (Heimer & Meyer, 1956) or neuraminidase (Gottschalk 1957) and its action defined as 'the hydrolytic cleavage of the glycosidic bond joining the keto group of *N*-acetylneuraminic acid to D-galactosamine' (Gottschalk 1957).

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Early work on the purification of the enzyme (Ada & French, 1950) was hampered by the complex nature of the culture medium and the low enzyme titre obtained. These difficulties were overcome when it was found that addition of *N*-acetylneuraminic acid or certain derivatives to a simple medium greatly stimulated enzyme production (Ada & French, 1957, 1959*a*; French & Ada, 1959). A dialysate of bovine colostrum enriched with glycerol and peptone was found to be a suitable medium for enzyme production (Ada & French, 1957). Following growth in this medium and subsequent heating to kill the organisms, filtration yielded a crude extract from which it was possible to purify the enzyme (Ada & French, 1959*b*). The present paper presents details of the purification procedure and some properties of the crystalline enzyme. The succeeding paper (Pye & Curtain, 1961) reports some physical properties of the enzyme.

METHODS

Peptone. Evans bacteriological peptone or Difco proteose peptone no. 1 was used.

Glycerol. A.R. quality.

Peptone water. A solution of glycerol (0·5 %, v/v) and peptone (1 %, w/v) adjusted to pH 7·4 with NaOH and sterilized by autoclaving (15 lb./sq.in.; 20 min.)

Methanol. A preparation labelled 'acetone-free' was found to be satisfactory.

Anti-frothing reagent. Undecanol (3 %, v/v) in peanut oil; sterilized by heating for 1 hr. at 180° on 3 successive days.

EDTA. Ethylenediaminetetra-acetic acid solution, adjusted to pH 7·4 with NaOH.

Hydroxyl apatite. Prepared according to Tiselius, Hjerten & Levin (1956). This material could be stored at 0–4° for periods of at least 1 month.

Sialyl lactose. The method of preparation was described by French & Ada (1959).

Saline. An aqueous solution of NaCl (0·15 M).

Bovine plasma albumin. Fractionation V prepared by the Armour Laboratories.

Phosphate buffers. Phosphate buffers (pH 6·8) were prepared by dilution of M stock solution (0·5 M-NaH_2PO_4; 0·5 M-Na_2HPO_4).

Tris-maleate buffers. These were prepared by mixing 0·1 M 2-amino-2-hydroxymethylpropane-1:3-diol (tris) and 0·1 M-maleic acid solutions.

Estimation of protein concentration. This was generally determined by the method of Lowry, Rosebrough, Farr & Randall (1951). On some occasions, it was assessed from optical density measurements at 280 mμ. In certain kinetic studies, a sample of the enzyme solution was dried (100°) and weighed.

Estimation of N-*acetylneuraminic acid* (N-*ANA*). Total *N*-ANA was estimated by the direct Erhlich reaction (Werner & Odin, 1952). Free *N*-ANA was estimated by the thiobarbituric acid method (Warren, 1959).

Drying of neuraminidase. Samples of the purified enzyme were dried from the frozen state *in vacuo* in an apparatus described elsewhere (Holden, 1958). We wish to thank Mr H. F. Holden for this courtesy.

Titration of neuraminidase activity

(*a*) *Biological method.* The method used and definition of a unit of enzyme activity has been described elsewhere (French & Ada, 1959).

(*b*) *Chemical method.* The substrate solution was constituted as follows: sialyl lactose solution (10 %, w/v), 0·8 ml.; $CaCl_2$ solution (0·01 M), 1·5 ml.; tris + maleate buffer (pH 5·6) containing 0·05 % (w/v) bovine plasma albumin, 12·7 ml. Samples (0·35 ml.) of this were brought to 37·1° and 0·05 ml. of enzyme solution added. At appropriate intervals (usually 10 min.), 0·1 ml. samples were pipetted into tubes containing 0·1 ml. water, 0·1 ml. of the periodate reagent (Warren, 1959) was added immediately and the amount of liberated *N*-ANA determined. In all experiments, the rate of release of *N*-ANA at zero time was estimated by extrapolation.

Titration of proteolytic activity

Casein substrate. Defatted casein (0·5 g.) was suspended in water and 0·1 N-NaOH added cautiously until the protein just dissolved (final volume = 100 ml.). This solution was dialysed for 16 hr. at 0° against veronal + acetate buffer.

Buffer. 25 ml. 0·04 M-sodium barbiturate, 25 ml. 0·04 M-sodium acetate, adjusted to pH 6·5 with N-HCl. Volume brought to 100 ml.

Trypsin. A crystalline preparation was dissolved in buffer, dilutions made in buffer and used immediately.

Test. Enzyme solution (0·1 ml.) was added to 1 ml. samples of the substrate solution, the solutions mixed and then incubated for 1 hr. at 37°. After cooling in ice water, 0·25 ml. of 40 % (w/v) trichloroacetic acid was added, the solutions mixed, kept at 0° for 15 min. and then centrifuged (2000 *g*; 15 min. 0°). The absorption at 280 mμ of the clear supernatant liquid was read in 0·5 cm. optical cells.

Titration of N-*ANA-aldolase activity*

N-*ANA.* A crystalline preparation derived from sialyl lactose (French & Ada, 1959).

N-Acetylmannosamine. A crystalline sample kindly provided by Dr S. Roseman.

Test. To 0·2 ml. of *N*-ANA solution (1·7 %, w/v) was added 0·3 ml. of phosphate buffer (0·01 M; pH 6·8) or neuraminidase dissolved in 0·3 ml. buffer. One drop of toluene was added, and after removal of 0·15 ml. as the zero time sample, the tubes were stoppered and incubated at 37° for 24 hr. The *N*-acetylhexosamine content of the zero time and 24 hr. samples was estimated by the method of Reissig, Strominger & Leloir (1955), with *N*-acetylmannosamine as standard.

Production of neuraminidase

Organism. The properties, cultivation and storage of the 4Z strain of *Vibrio cholerae* were described previously (French & Ada, 1959). Twenty-four hr. before bulk growth of the organisms, nutrient broth (100 ml.) was inoculated from an agar slope culture and incubated (6 hr., 37°). The whole of this culture was used as inoculum for 1 l. peptone water which was then incubated for 16 hr. at 37°. The peptone water culture was used as inoculum for the bulk medium (see below).

Preparation of medium. Bovine colostrum (about 6 l.; first milking after parturition) was dialysed (Visking 27/32 tubing) against 35 l. water previously saturated with chloroform. Dialysis was carried out at 0–4° for 8–14 days in a stainless steel can with a closely fitting lid, the can being briefly shaken each day. The colostrum was then discarded, the clear diffusate warmed to about 45° and the chloroform

removed by aeration with sterile air (2–3 hr.). Glycerol was added to a final concentration of 0·75 % (v/v). Peptone (360 g.) was dissolved in 1 l. of water, the pH adjusted to about 7 and insoluble material removed by centrifugation. The clear supernatant liquid was added to the colostrum dialysate and adjusted to pH 7·3 with 20 M-NaOH. The medium was filtered (0–4°) through sterilizing pads into sterile containers.

Growth of organism. The medium was transferred to a sterile stainless-steel can and warmed to and maintained at 37°. The can was fitted with a transparent lid through which the shaft of a stirrer and an aeration tube passed. The latter was placed so that sterile air emerged under the blades of the stirrer. The medium was inoculated with the peptone water culture of *Vibrio cholerae* (see above), the stirrer started and air passed through the medium at about 150 l./hr. Frothing was controlled by the addition of the anti-frothing preparation. Incubation was continued for about 10 hr.

Destruction of organisms and preparation of crude extract. Dry $CaCl_2$ was added to the culture to give a final concentration of 0·5 % (w/v). The organisms usually aggregated during this procedure. After adjustment to pH 6·0 with 10 N-HCl, the mixture was brought to 56°, maintained at this temperature for 30 min. to kill the organisms, and then cooled (16 hr., 0–4°). When the clumped organisms settled out, the clear supernatant liquid was obtained by decantation (the sedimented organisms amounted to about 1 l. in volume). Otherwise the organisms were removed by filtration through a pad of filter-aid. For ease of handling, the cleared liquid (supernatant liquid or filtrate) was divided into two portions for the next treatment; one part was stored at 0–4° in the presence of toluene for 2–3 days and the other part processed immediately.

Purification procedure

Methanol fractionation of crude extract. All procedures in this step were carried out at 0–2°. The cleared liquid (above) was adjusted to pH 5·0 with 10 N-HCl. Methanol, precooled to about −30°, was added during a period of about 5 hr. until the concentration reached 60 % (v/v). The resultant precipitate was allowed to settle during 2 days. Most of the clear supernatant liquid was decanted and discarded. The precipitate was recovered from the rest by centrifugation (2000 *g*; 30 min.). Cold water was added to the packed precipitate to give a slush which was then dialysed against several changes of distilled water to remove methanol (2–3 days). The suspension was centrifuged (2000 *g*; 30 min.) and the sediment re-extracted once with an equal volume of water. The supernatant liquids were pooled and called 'neuraminidase concentrate'.

Preparation of human red cell eluate. Human blood was collected into Rous and Turner fluid and the cells washed three times with saline. Unless cells were used on the day collected, a considerable degree of haemolysis occurred in this step and increased the difficulty of later purification. The washed red cells were distributed into two equal portions of 200 ml., each of which was distributed into centrifuge cups and held at 0–4°. To the 'neuraminidase concentrate' (0–4°) was added NaCl and $CaCl_2$ to give final concentrations of 0·85 % and 0·1 % (w/v), respectively. N-NaOH was added cautiously until the pH value reached 8·5. The slight precipitate which formed was immediately removed by centrifugation (2000 *g*; 10 min.;

0–4°). The clear supernatant liquid was then added to one portion of red cells, stirred for 1 min. and then centrifuged (2000 *g*; 3 min.; 0–4°). The supernatant liquid was removed and immediately added to the other portion of red cells. Mixing and centrifugation was again carried out as above. The extracted fluid (supernatant liquid) was discarded, the red cells pooled and suspended in 400 ml. saline containing 0·01 M-EDTA and kept at about 20° for 10 min., with occasional gentle stirring. The suspension was centrifuged (2000 *g*; 10 min.; 20°) and the supernatant liquid removed. The red cells were re-extracted as above with another 200 ml. of EDTA saline. The supernatant liquids were pooled and re-centrifuged (2000 *g*.; 20 min.; 0–4°), yielding a clear slightly pigmented supernatant liquid (red cell eluate).

Ammonium sulphate fractionation. An equal volume of aqueous saturated $(NH_4)_2SO_4$ (pH 6·5) was immediately added to the red cell eluate. After standing for 16 hr. at 0–4°, the fine precipitate was recovered by centrifugation (supernatant liquid discarded), resuspended in 5–10 ml. water and dialysed (48 hr., 0–4°) against 0·1 M-NaCl + 0·001 M-phosphate buffer (pH 6·8). The dialysed fluid was centrifuged (2000 *g*; 10 min.; 0–4°) and the residue discarded.

Chromatography on hydroxyl apatite. This procedure was carried out at 0–4°. A column (10 cm. × 1·6 cm.) of hydroxyl apatite was prepared and washed well with a solution containing 0·1 M-NaCl + 0·001 M-phosphate buffer (pH 6·8). The enzyme solution was passed through the column which was then washed with about 20 ml. of the above phosphate saline solution. The effluent was discarded and the column developed with a gradient from 0·1 M-NaCl + 0·001 M-phosphate buffer (pH 6·8) to 0·1 M-NaCl + 0·25 M-phosphate buffer (pH 6·8). The conditions of the gradient were described previously (see Drake, 1955, p. 18, case 4 with $k = 2$ and $V1 = 40$ ml.). Under these conditions, the neuraminidase began to elute when about 40 ml. had passed through the column. The eluate was collected in a fraction collector (1·3–2·0 ml. per tube) and the protein concentration in each tube estimated by measuring the absorption at 280 mμ. When haemolysis in the preceding stage had been slight, one sharp protein peak showing biological activity was obtained. The enzyme content of samples containing protein was estimated. Those samples which showed close agreement between enzyme activity and protein content (see Fig. 1*a*) were pooled. When more than one protein peak was present or when the neuraminidase peak trailed very badly, the samples containing most of the enzyme were pooled and again chromatographed as above. In either case, the appropriate tubes were pooled, dialysed against distilled water (48 hr.; 0–4°) and dried from the frozen state *in vacuo*.

Crystallization of neuraminidase. This procedure was carried out at 0–4°. The dried material was dissolved in 1–1·5 ml. water and dilute acetic acid added to pH 5·0. If the solution became opaque, it was clarified by centrifugation. (If too much acetic acid was added, the solution was neutralized with dilute NH_4OH, again dialysed and dried.) The solution was then cautiously brought to about pH 4·5 and rocked gently for 2–3 days. Crystallization was usually achieved by seeding; it was complete after 3 days. The crystals were then washed with water saturated with CO_2 and stored in water at 0–4°. They were dissolved by the addition of 0·01 N-NH_4OH.

RESULTS

Production of neuraminidase

Maximum production of neuraminidase in the culture medium under the conditions described usually occurred within 10 hr. In a typical experiment, the titre of enzyme (per 0·25 ml., estimated by the biological method) at different times after the inoculation of the medium was: 6 hr., 100; 7·5 hr., 350; 8·5 hr., 1300; 9·5 hr., 2400. After heating to kill the organisms, this fluid had a titre of 2200. In this experiment, most of the sialyl lactose present in the original medium (as estimated by the direct Ehrlich reaction) had been destroyed by 10 hr. (see French & Ada, 1959). Crude extracts from which crystalline neuraminidase has been obtained possessed titres (per 0·25 ml.) ranging from 1100 to 4000.

Table 1. *Purification of neuraminidase of* Vibrio cholerae

Procedure	Volume (ml.)	Activity* (units/ 0·25 ml.) ($\times 10^{-3}$)	Total units ($\times 10^{-6}$)	Protein† (mg/ml.)	Activity (units/ mg.)	Yield (%)	Purification
Crude extract	35,000	4	560	5·5	2·9	100	—
1. Methanol fractionation	810	160	520	5·5	116	93	40
2. Human red cell eluate	800	110	352	0·3	1,470	63	500
3. Ammonium sulphate fractionation	11·1	3,200	142	2·7	4,730	26	1,630
4. Chromatography on hydroxyl apatite	8·0	4,800	154	2·15	8,940	28	3,080
5. Crystallization	1·2	24,000	115	6·0	16,000	21	5,500

* Estimated by the biological method. At each stage during the purification procedure, samples were diluted 1/100 in calcium acetate broth and stored at −20°. All enzyme activities were determined on the same day.

† Determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Evaluation of purification procedure

The detailed analysis of the purification procedure as applied to one crude filtrate of a *Vibrio cholerae* culture is presented in Table 1. Measurements of the volume, enzyme activity and protein content of each fraction allowed an estimate to be made of the yield of pure enzyme and the degree of purification achieved. The major part of the overall purification achieved occurred in the first three steps (methanol fractionation, adsorption to and elution from red cells, ammonium sulphate fractionation). However, the recovery of enzyme was often poor at one or the other of these stages. In the experiment quoted, the major loss of activity occurred during the ammonium sulphate fractionation. Sometimes the greatest loss occurred at the preceding stage of adsorption to and elution from red cells. The low recovery often encountered here was not due to destruction of enzyme; the remaining portion could be accounted for in the fluid after extraction with red cells. In some experiments, a higher recovery of enzyme in the eluate was obtained by increasing greatly the number of red cells used as absorbent.

Chromatography of the ammonium sulphate precipitate on columns of hydroxyl apatite showed a single protein peak with a very steep leading edge, but the trailing edge varied in steepness from batch to batch. Figure 1 *a* shows the pattern of enzyme

activity and protein concentration given by the preparation quoted in Table 1. The close correspondence between enzymic activity (estimated by the biological method) and protein concentration seen at all stages of the peak except the tail end was fairly typical. A protein peak showing a more pronounced trailing edge and thus a greater disparity between protein concentration and enzymic activity was seen occasionally. For example, such a pattern was shown by a preparation in which substantial haemolysis had occurred during elution from red cells. In this case, the most active fractions were pooled and again chromatographed, then yielding a pattern like that shown in Fig. 1*b*.

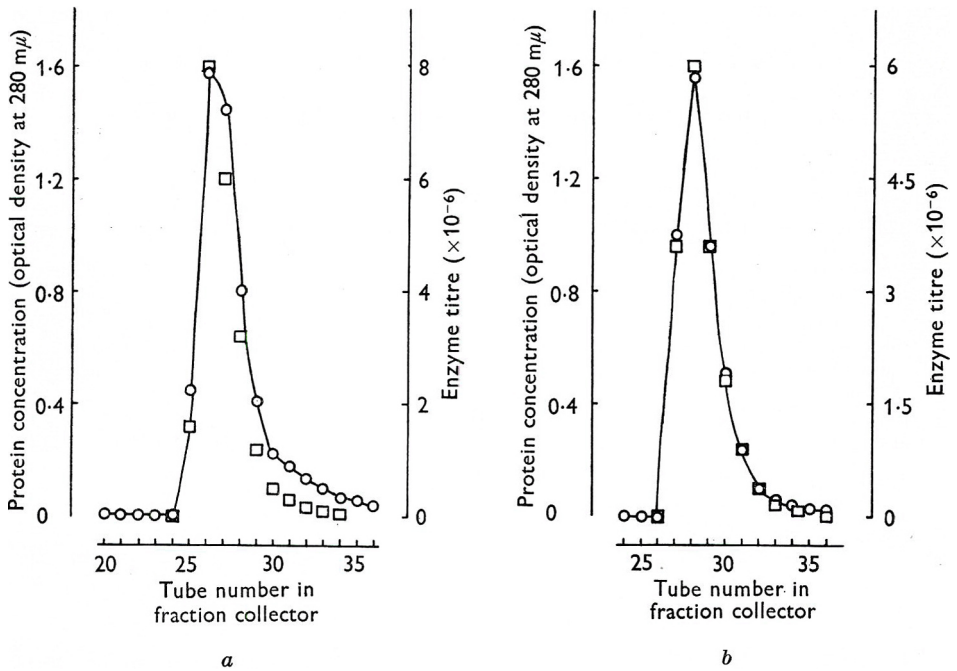


Fig. 1. Purification of *Vibrio cholerae* neuraminidase by chromatography on hydroxyl apatite. The elution pattern was obtaned by using a concentration gradient of phosphate buffer (pH 6·8). (*a*) First chromatographic run. (*b*) Re-chromatography of pooled selected fractions from first run. Protein concentration, ○—○; enzyme titre, □—□.

Coloured impurities were removed from the preparation at the chromatography stage so that crystallization was effected from a colourless solution. Crystallization completed the purification procedure. In the experiment quoted, the overall result was a decrease in volume from 35 l. to 1·2 ml., a yield of 20 % of the original activity as crystals and a 5500-fold purification. Nine batches of crystalline enzyme have been prepared. In six batches, the recovery was estimated and found to vary between 33 and 7 % with a mean value of 21 %.

One batch of enzyme behaved differently. The procedure applied differed in one respect from that described above in that the product of methanol fractionation was stored at −20° for 14 days. The crude extract had a very high titre (7500), but extensive losses were sustained at each stage of the purification procedure, the

overall recovery at the ammonium sulphate stage being only 7 %. Chromatography on hydroxyl apatite showed a very broad protein peak. No attempt was made to obtain crystals from this batch.

Properties of neuraminidase

Neuraminidase crystallizes as needles (Pl. 1). Fig. 2 shows the ultraviolet absorption spectrum of the pure enzyme. $E^{1\%}_{280\,m\mu}$ was 8·96, measured at 0·025 % (w/v). Application of the method of Lowry *et al.* (1951) for protein estimation to a solution of the enzyme (0·0152 %, w/v) yielded an optical density (0·5 cm. optical cell) of 0·194 at 750 mμ. The pure enzyme had a biological activity of $12{\cdot}6 \times 10^6$ units/mg. dry weight.

Failure to detect proteolytic activity. Crystalline enzyme (20 μg.) failed to show any sign of proteolytic activity against casein at pH 6·5; the test could have detected the presence of 0·02 μg. trypsin.

Failure to detect N-*ANA-aldolase activity.* Samples of *N*-acetylneuraminic acid (*N*-ANA; 3·4 mg.) were incubated with buffer or with buffer containing 20 μg. neuraminidase. After 24 hr., less than 15 μg. of *N*-acetylmannosamine was detected in either reaction mixture. This corresponds to less than 1 % conversion of *N*-ANA to *N*-acetylmannosamine.

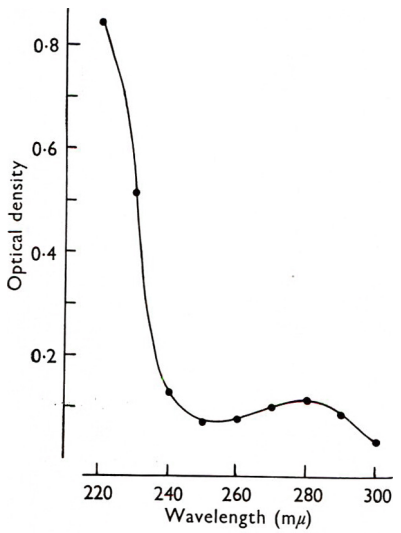


Fig. 2

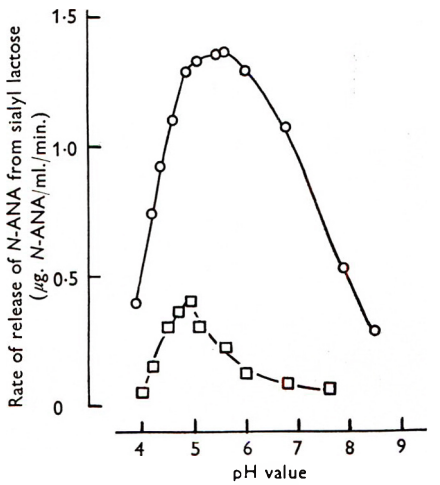


Fig. 3

Fig. 2. Ultraviolet absorption spectrum of *Vibrio cholerae* neuraminidase. Neuraminidase was dissolved in 0·08 M-phosphate buffer (pH 6·8). Concentration of enzyme, 0·025 % (w/v).

Fig. 3. The activity of *Vibrio cholerae* neuraminidase at different pH values. Composition of medium: sialyl lactose, 0·17 % (w/v); buffer 0·3 ml., prepared by mixing 0·1 M-tris + 0·1-M maleic acid; neuraminidase, 0·15 μg./ml.; total volume, 0·4 ml. □—□, no added $CaCl_2$; ○—○, added $CaCl_2$ (0·001 M).

Kinetic studies

Experiments were first carried out to determine the pH value at which optimum enzyme activity of purified enzyme preparations was shown. The suspension of

enzyme crystals (0·025 ml.) was dissolved in 0·225 ml. 0·01 N-NH_4OH and the volume brought to 1 ml. by addition of tris + maleate buffer (pH 4·9). A further dilution into buffers of different pH values was made shortly before the start of the experiment. Under these conditions, the enzyme showed a maximum activity at pH 4·9 (Fig. 3, lower curve).

Influence of cations on the activity of neuraminidase. Calcium ions were found to stimulate enzyme activity. The results of a typical experiment are shown in Fig. 4, in which the effect of addition of different concentrations of $CaCl_2$ to the enzyme + substrate solutions is illustrated. Maximum stimulation was found at 0·001 M-$CaCl_2$. Stimulation was not observed when the $CaCl_2$ concentration was 0·1 M.

Enzyme activity was also affected by certain other cations. The chlorides of manganese, magnesium, barium, zinc or sodium were added to the medium to a final concentration of 0·001 M. The rate of release of *N*-ANA from sialyl lactose at pH 4·9 (expressed as μg. *N*-ANA/ml./min.) was in the presence of $CaCl_2$, 2·0; $MnCl_2$, 1·0; $ZnCl_2$, 0·7; $MgCl_2$, 0·4; $BaCl_2$ and NaCl, 0·3.

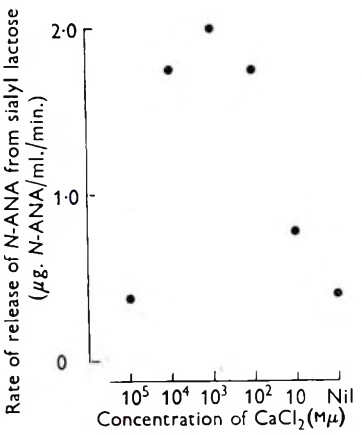


Fig. 4

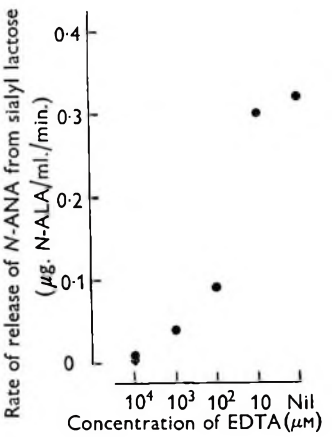


Fig. 5

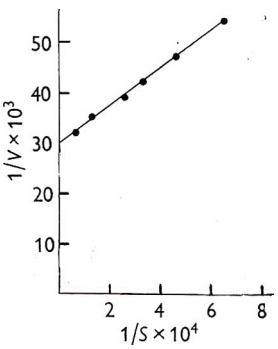


Fig. 6

Fig. 4. The activity of *Vibrio cholerae* neuraminidase in presence of added $CaCl_2$. Composition of medium: sialyl lactose, 0·17 % (w/v); tris + maleate buffer, 0·06 M (pH 5·6); neuraminidase, 0·27 μg./ml.

Fig. 5. The activity of *Vibrio cholerae* neuraminidase in the presence of added ethylenediaminetetra-acetate (EDTA). Composition of medium: sialyl lactose, 0·17 % (w/v); tris + maleate buffer, 0·06 M (pH 6·6); neuraminidase, 0·27 μg./ml.

Fig. 6. Activity of *Vibrio cholerae* neuraminidase as a function of substrate concentration. S is the concentration (M) of substrate; V is the velocity of action (plotted on an arbitrary scale).

Inhibition of enzyme activity by ethylenediamine tetra-acetate. In Fig. 5 are plotted the results of one of two experiments to investigate the effect of added EDTA on the activity of neuraminidase. Increasing inhibition of enzyme activity occurred as the concentration of EDTA was raised. In a separate experiment, enzyme (75 μg./ml.) was stored in 0·01 M-EDTA (pH 5·6) for 40 hr. at 0–4°. It was then diluted and tested in the presence of 0·001 M-$CaCl_2$ and showed full activity.

Effect of pH *value on neuraminidase activity.* In the presence of 0·001 M-$CaCl_2$ neuraminidase showed maximum activity at pH 5·6. Below pH 5 and above pH 6, the activity decreased rapidly (Fig. 3, upper curve).

Enzyme constants. Constants were determined at 37·1° in tris + maleate buffer (pH 5·6; 0·07 M) containing $CaCl_2$ (0·001 M), bovine plasma albumin (0·03 %) and sialyl lactose. A Lineweaver & Burk (1934) plot of the data yielded a value of $1·2 \times 10^{-3}$ M for the Michaelis constant K_m (Fig. 6). At an enzyme concentration of 0·16 μg./ml., $V_{max.}$ was 0·021 μM-*N*-ANA/min./ml.

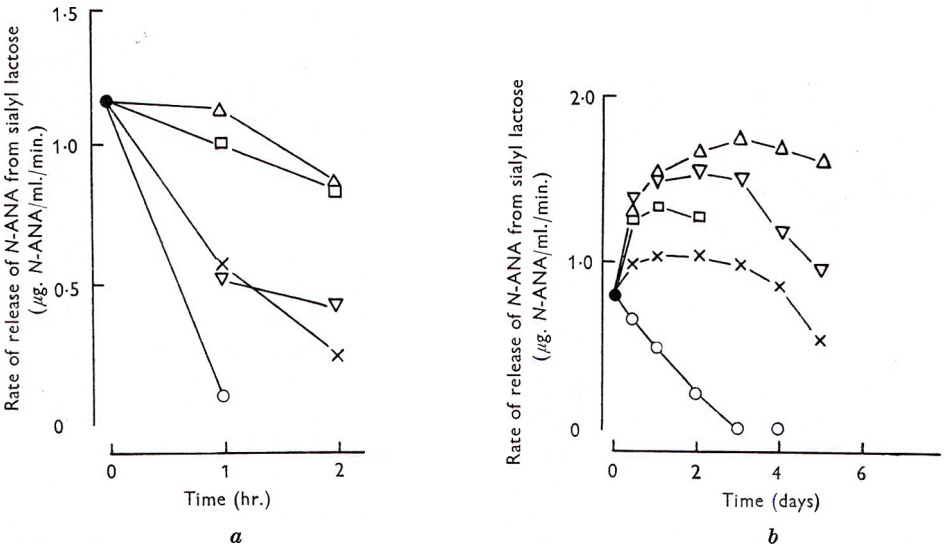


Fig. 7. Stability of *Vibrio cholerae* neuraminidase stored at (*a*) different pH values at 37° and (*b*) at 0–4°. The activity of the stored enzyme (about 15 μg./ml.) was determined in the standard way at pH 5·6 and in the presence of 0·001 M-$CaCl_2$. pH 8·5, ▽; pH 6·7, △; pH 5·6, □; pH 4·6, ×; pH 3·9, ○.

Stability of purified neuraminidase

The stability of neuraminidase in solution was tested at 37° and 0–4°. Some tests were also carried out on the dry enzyme.

Stability at 37°. Neuraminidase lost activity when incubated at 37°. This was first noticed when estimating the velocity in the chemical test for enzyme activity. For example, the decrease in velocity per min. at different pH values was as follows: pH 4·3, 0·45 %; pH 5·0, 0·4 %; pH 6·2, 0·3 %; pH 7·9, 0·2 %.

To find the pH value of optimum stability, some crystals were dissolved in NH_4OH and brought to about pH 5·6. Samples (0·1 ml.) were added to 0·9 ml. volumes of buffer at pH 3·9, 4·6, 5·6, 6·7 and 8·5 (final enzyme concentration about 15 μg./ml.). These mixtures were then incubated at 37° for 1 hr.; some of the pH 5·6 sample was kept at 0°: Samples from each mixture were then added to a solution of sialyl lactose and $CaCl_2$ buffered at pH 5·6 and the enzyme activities determined. The enzyme solutions, incubated at 37°, had the following activities expressed as a percentage of the control solution kept at 0°: pH 3·9, 9 %; pH 4·6, 50 %; pH 5·6, 112 %; pH 6·7, 130 %; pH 8·5, 42 %. In a second experiment, dissolved enzyme was stored at 0–4° (pH 5·6) for 18 hr. before incubation at 37°. The results of this

experiment are plotted in Fig. 7*a*. The enzyme was most stable in the range pH 5·6–6·7, about 24% of the activity being lost in 2 hr. The enzyme was particularly unstable at pH 3·9.

Stability at 0–4°. The experimental procedure followed here was similar to that described in the preceding paragraph except that the enzyme solutions (at pH 3·9, 4·6, 5·6, 6·7, 8·5) were held at 0–4° for several weeks. At intervals samples were removed and the enzymic activity determined at pH 5·6. The results are plotted in Fig. 7*b*. The most striking feature was the rise in activity shown by enzyme stored at the higher pH values. After this initial rise, the activity again slowly decreased.

Stability of dried enzyme. Purified enzyme to be sent to other laboratories was dried from the frozen state in glass ampoules which were then sealed under vacuum. When stored at 0–4°, such material retained nearly full activity (as tested by the biological method) for at least 6 months.

DISCUSSION

Purification procedure. Several factors have contributed to the successful outcome of this work. In the first place the presence in the medium of sialyl lactose, an inducer of neuraminidase (French & Ada, 1959), ensured the production of high titres of enzymes. The crude extract thus had a high specific activity (enzyme units/mg. protein). Secondly, the medium contained only simple ingredients which were not precipitated by 60% (v/v) methanol in water at 0° and this largely accounted for the 40-fold purification achieved in the first step. In addition, the enzyme was concentrated about 40-fold in this step and thus was contained in a volume small enough to allow rapid extraction with red cells, by using ordinary laboratory facilities.

The process of specific adsorption to and elution from red cells, first introduced by Burnet & Stone (1947), played an important part in the purification procedure. The recovery rate of this step was about 50%. Attempts to increase the yield of enzyme in the eluate were unsuccessful; increasing the number of red cells used as adsorbent did not always result in improved yields. The use of ethylenediaminetetra-acetic acid at room temperature to effect rapid elution of the enzyme, instead of prolonged incubation of enzyme and red cells at 37° (Ada & French, 1950), decreased the contamination of the eluate with red cell breakdown products.

Precipitation of the enzyme with ammonium sulphate was a convenient way of decreasing the volume to be handled and of restoring the protein concentration to a high value which was maintained in the later stages of the process. Although not formally demonstrated, our experience was consistent with the belief that removal of most of the impurities by chromatography on hydroxyl apatite was a prerequisite for a high yield of crystalline enzyme. While the yield of crystalline enzyme was satisfactory there were two stages in the procedure where substantial losses occurred. Experiments in progress may show ways of decreasing these losses.

Properties of the enzyme. No information about the chemical properties of the enzyme is yet available. A preliminary examination of the physical properties is reported in the succeeding paper (Pye & Curtain, 1961). The results suggested that the crystalline enzyme was homogeneous by the usual physical criteria.

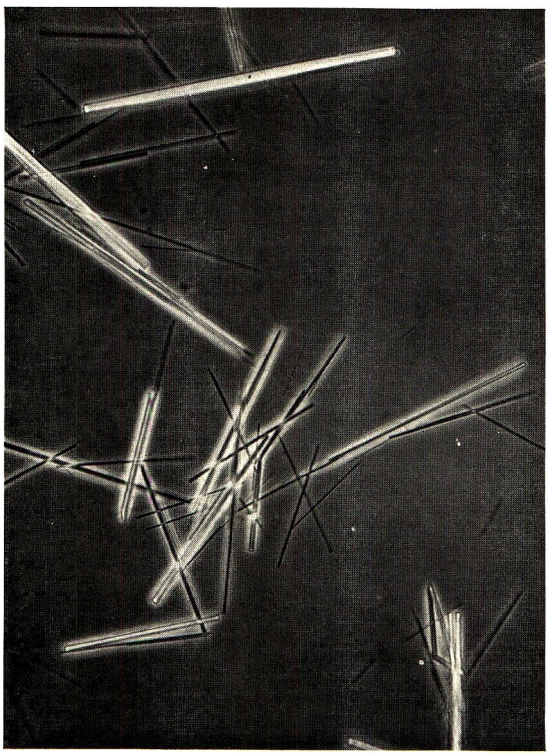
Sialyl lactose was used as the substrate in kinetic studies for several reasons. It is a well characterized compound, is readily prepared in large amounts, and since it is very soluble, high substrate concentrations can be achieved. However, kinetic constants may vary with the substrate used.

The addition of Ca (0·001 M) to the reaction medium stimulated neuraminidase activity (cf. Burnet & Stone, 1947). High concentrations (0·1 M) of Ca did not stimulate activity, a feature which has been noticed with other metal+enzyme systems. The addition of Ca ion to the medium changed the shape of the pH–activity curve; the value for optimum activity was displaced from pH 4·9 to pH 5·6. The mode of action of added Ca ion (whether it combined with enzyme or substrate or acted as a link beween enzyme or substrate) remains to be investigated. The activity shown by neuraminidase in the absence of added Ca ion was inhibited by ethylenediaminetetra-acetic acid which suggests that the enzyme as isolated contained a metal as an essential component.

A puzzling feature of the stability results was the initial increase in activity on storage of the enzyme, particularly at 0–4°. Here the rise was observed at all pH values except 3·9 and was greater as the pH value increased. At the higher values tested (pH 6·7, 8·5) this increase occurred over 3 weeks, reaching a value about double the original. This effect was less clear at 37°. It seems likely that two opposing factors were operating: one was a change of the enzyme to a more active form (disaggregation?), a process favoured by storage of dilute solutions at high pH values, and the other was thermal inactivation of the enzyme. This finding means that the calculated values for K_m and $V_{max.}$ should be regarded as approximate rather than absolute. It should also be pointed out that, despite the variation in thermal stability shown by enzyme stored at different pH values, the curve in Fig. 3 is essentially a true indication of the activity of the enzyme at each pH value, since velocity values were obtained by extrapolation to zero time.

Schramm & Mohr (1959) independently published a method for the purification of neuraminidase from *Vibrio cholerae* cultures. The properties of their preparation are different from those described here. In addition to the low molecular weight (10,000–20,000) they found their preparation was very unstable unless stored in the presence of chelating agents or cyanide. The K_m value of their preparation ($1–2 \times 10^{-4}$ M) is different from that found with our preparation ($1·2 \times 10^{-3}$ M) but this might be due to the smaller enzyme molecule and the different substrate used by Schramm & Mohr (sheep red cells). We cannot as yet offer any experimental evidence which would explain the many differences between the two preparations.

We wish to thank the different organizations, particularly the Victorian Department of Agriculture, who have supplied us with bovine colostrum. The work was supported by the National Health and Medical Research Council, Canberra, Australia. The authors are also indebted to Mr K. Clarke for excellent technical assistance.



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EXPLANATION OF PLATE

Crystalline neuraminidase (phase contrast; ×300). Photograph by Mr E. Matthaei.

J. gen. Microbiol. (1961), **24**, 423–425
With 1 *plate*
Printed in Great Britain

Electrophoretic, Sedimentation and Diffusion Behaviour of Crystalline Neuraminidase from *Vibrio cholerae*

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(*Received* 21 *October* 1960)

SUMMARY

Crystalline neuraminidase prepared from *Vibrio cholerae* culture fluids by Ada, French & Lind (1961) was examined by moving boundary electrophoresis, analytical ultracentrifugation and diffusion. The various corresponding physical constants were obtained. The results indicated that, within the limits of the methods used, the crystalline protein was enzyme and was homogeneous.

METHODS

Neuraminidase. Crystalline neuraminidase (a pool of three batches) was supplied by Dr G. L. Ada. Because of the scarcity of the purified enzyme, all physical measurements were carried out on this pool. The enzyme was submitted first to diffusion analysis, then to electrophoresis and finally to sedimentation in the ultracentrifuge. After each examination, the enzyme was dialysed against distilled water and concentrated by sublimation of water from the frozen state *in vacuo*. In some cases, a considerable time elapsed between experiments and during these periods the dissolved enzyme was stored at $-20°$.

Buffers. For electrophoresis: (1) sodium veronal (pH 8·5, 0·012 M) + sodium chloride (0·09 M); (2) sodium phosphate (pH 6·7, 0·0056 M) + sodium chloride (0·09 M); (3) sodium acetate (pH 5·1, 0·0145 M) + sodium chloride (0·09 M). The ionic strength of each of these buffers is 0·1.

For sedimentation: (4) sodium veronal (pH 8·5, 0·01 M) + sodium chloride (0·1 M); (5) sodium phosphate (pH 6·7, 0·01 M) + sodium chloride (0·1 M).

For diffusion: the sodium phosphate buffer (no. 5) used for sedimentation.

Moving boundary electrophoresis. This was carried out at 4° in a Tiselius type equipment with cylindrical lens schlieren optical system. A 2 ml. optical cell was used. The assembly was closed on the anode side. At the conclusion of each run the cell was closed and the contents of the two limbs and the base removed separately for biological assay of enzyme activity (French & Ada, 1959). We are indebted to Dr P. E. Lind for carrying out these estimations. Mobilities of the boundaries observed and of the enzyme activities were calculated for the descending limb by using the conductivities of the supernatant buffer solutions.

Ultracentrifugation. An air-driven Beams-Pickels type of centrifuge with a cylindrical lens schlieren optical system was used at a rotor speed of 800 rev./sec. and a temperature of about 20°.

Diffusion. The diffusion experiment was carried out at 1·5° in a 2 ml. cell in the Perkin–Elmer Model 38A electrophoresis apparatus with the phase-plate gradient optical system of Moore & Opperman (1956). The cell bath of this instrument was maintained to ±0·02° by circulating water from a refrigerated tank (built by Cordley & Hayes, New York, for the Perkin–Elmer Corporation).

RESULTS

Electrophoresis. The electrophoretic patterns observed at pH 6·7, 8·5 and 5·1 are shown in Pl. 1, fig. 1(*a*), (*b*), (*c*). In each case a single peak was observed which migrated towards the anode. In the first two analyses the peaks were asymmetrical. During dialysis against the pH 5·1 buffer, a slight precipitate formed and this was removed by centrifugation. The peak subsequently observed during electrophoresis at pH 5·1 was almost symmetrical. The mobilities of these boundaries and of the enzyme activities are given in Table 1.

Table 1. *Electrophoretic mobilities of* Vibrio cholerae *neuraminidase at various* pH *values*

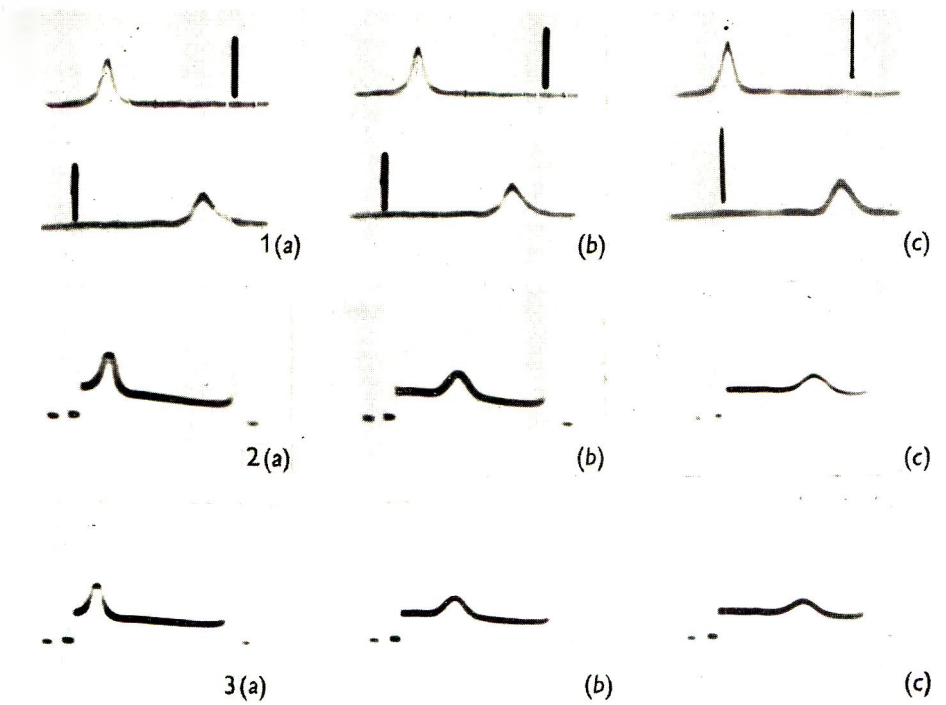
pH value:		6·7	8·5	5·1
Mobilities (10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1})	Protein boundary:	3·8	3·9	2·1
	Enzyme activity:	3·8	4·1	2·1

Sedimentation. The enzyme sedimented as a single symmetrical boundary at pH 6·7 and 8·5 (see Pl. 1, fig. 2 (*a*), (*b*), (*c*) and fig. 3 (*a*), (*b*), (*c*)). At pH 6·7, the sedimentation was observed at concentrations of 0·9%, 0·6% and 0·3% (w/v). There was no apparent dependence of sedimentation coefficient on protein concentration within the accuracy of the methods used. At pH 6·7, s_{20} was 5·5 S; at pH 8·5, 5·3 S.

Diffusion. At a concentration of 0·72% (w/v) and pH 6·7 the *Vibrio cholerae* neuraminidase gave a value for D_{20} of $5{\cdot}5 \times 10^{-7}$ cm.^{2}sec.$^{-1}$.

DISCUSSION

The results of the sedimentation and electrophoresis measurements left little doubt that we were dealing with a protein preparation which was substantially homogeneous, at least within the limits of the methods used. The small amount of material insoluble at pH 5·1 might have been denatured or aggregated neuraminidase and as such could have been responsible for the asymmetry of the electrophoretic boundaries at pH 6·7 and 8·5. The agreement between the electrophoretic mobilities of the enzymically active material and the protein boundaries at three pH values suggested that the crystalline protein was in fact neuraminidase. This was supported by the finding that a single peak with constant specific activity was obtained during chromatography of the material on hydroxy-apatite (Ada *et al.* 1961). No attempt was made to correlate the sedimenting boundaries with enzymic activity. The mobility values indicated that the isoelectric point of neuraminidase


1 (a)
(b)
(c)
2 (a)
(b)
(c)
3 (a)
(b)
(c)

was below pH 5·1. The instability of the enzyme at lower pH values (Ada *et al.* 1961) precluded any attempt to determine the actual value.

If a partial specific volume of 0·75 be assumed, calculation from the sedimentation and diffusion coefficients yields a molecular weight of 90,000 and a frictional ratio (f/f_0) of $1{\cdot}2_0$. These results were obtained with a phosphate buffer at pH 6·7; the sedimentation coefficient in veronal buffer at pH 8·5 was not significantly different. Schramm & Mohr (1959) obtained a sedimentation coefficient of 1·3S in veronal buffer at pH 8·5 in presence of potassium cyanide for a neuraminidase isolated from filtrates of *Vibrio cholerae* cultures; they found evidence of aggregation in other media (unspecified). There are at present insufficient data to account for the differences between the two enzyme preparations.

We are grateful to Mr H. F. Holden who carried out the sedimentation runs and to Mr I. J. O'Donnell for the use of a Cambridge Universal Measuring Machine. One of us (J.P.) was supported by the National Health and Medical Research Council, Canberra, Australia.

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EXPLANATION OF PLATE

Fig. 1. Moving boundary electrophoresis of *Vibrio cholerae* neuraminidase. (*a*) pH 6·7; 5·8 volt cm.$^{-1}$ for 189 min. (*b*) pH 8·5; 5·7 volt cm.$^{-1}$ for 177 min. (*c*) pH 5·1; 5·6 volt cm.$^{-1}$ for 327 min. In each case the upper and lower patterns represent the ascending and descending limbs, respectively. The vertical line indicates the initial position of the boundary.

Fig. 2. Sedimentation of *Vibrio cholerae* neuraminidase. 0·9 % (w/v) at pH 6·7. Speed of rotor, 800 rev./sec. From left to right, exposures at 43 (*a*), at 111 (*b*) and at 164 min. (*c*) after reaching full speed.

Fig. 3. Sedimentation of *Vibrio cholerae* neuraminidase. 0·8 % (w/v) at pH 8·5. Speed of rotor 800 rev./sec. From left to right, exposures at 37 (*a*), at 97 (*b*) and at 157 min. (*c*) after reaching full speed. In every case sedimentation occurs from left to right.

J. gen. Microbiol. (1961), 24, 427–431
With 3 plates
Printed in Great Britain

Morphological Variation in *Spirillum* spp., with Observations upon the Origin of the Hyphomicrobia

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(*Received* 2 *November* 1960)

SUMMARY

Pure cultures of *Spirillum serpens* grown on solid medium contained variants of very diverse morphological appearance; these were relatively straight rods, and branched coccobacillary and coccal forms. Flagella were in the normal lophotrichous arrangement, peritrichous, single or absent. Some forms possessed characters resembling those of the hyphomicrobia. The development of Rhodomicrobium in cultures of photosynthetic spirilla was also observed. It is suggested, not only that the spirilla possess potentialities consistent with a suggested role as the ancestral bacterial type, but also that Rhodomicrobium may be a growth form of a photosynthetic spirillum. Appearances found in the parasitic *Hyphomonas polymorpha* and in certain pleuropneumonia-like organisms were considered to support this concept.

INTRODUCTION

It was suggested by Bisset (1950) that the spirilla probably conform most closely to the ancestral bacterial type, and that many of the morphologically distinct types considered to have arisen from them may be regarded as adaptations to a terrestrial existence, a process paralleled in many natural groups. In support of this idea, the present paper describes a variety of morphological forms adopted by a single species of spirillum (*Spirillum serpens*) when cultivated on the surface of solid medium. These forms simulate many of the major characters of the hypothetically more advanced bacteria, and indicate a potential, in spirilla, for the production of such characters, in accordance with the evolutionary theory in question. The existence of a complex life-cycle in spirilla is well attested (e.g. Pease, 1956*a*; Watanabe, 1959, 1960; and others). Certain of the appearances here described are attributable to phases in such a cycle, others are probably not. At the same time, some light may be thrown by these observations upon the problem of the origin and nature of the hyphomicrobia; of these three genera are recorded, each of one species, variously classified by different authorities, possessing a similar and very unusual morphology, and being credited with great versatility in their modes of life. The first descriptions of *Hyphomicrobium vulgare* attributed to it the power of fixing nitrogen (Rullman, 1897; Stutzer & Hartleb, 1899), although it is now regarded as an oligotrophic saprophyte. *Rhodomicrobium vannielii* is considered to be a photosynthetic purple sulphur bacterium (Duchow & Douglas, 1949). *Hyphomonas polymorpha* was isolated in circumstances suggestive of pathogenicity towards man (Pongratz, 1957). All these micro-organisms are distinguished by their morphology

and mode of growth, which is quite unlike that of the recognizable forms of other bacteria, in that the cells are borne upon narrow, often ramifying filaments, and reproduce by the outgrowth of similar filaments, upon which small buds appear and grow up into new cells. The cytology of this process was described by Murray & Douglas (1950), but the mechanism cannot be regarded as well understood. Despite the stalk-like filaments, this mode of reproduction is unlike that of the true caulobacteria, which undergo fission in a relatively normal manner (Bisset, 1955, pp. 121–4). Although true motility is possible only in small detached elements, flagella are commonly borne upon various parts of the ramifying growth, in all recognized genera. The attachment is described as polar in *Hyphomicrobium vulgare* (Mevius, 1953) and *Hyphomonas polymorpha* (Pongratz, 1957), while Douglas & Wolfe (1959) considered the flagellation of *Rhodomicrobium vannielii* to be peritrichous; but the electron micrographs of Pease (1956*b*) show polar attachment in *R. vannielii*. Our own view is that the normal criteria of flagellar insertion must be applied with caution to micro-organisms of this morphology.

The circumstances in which Hyphomicrobium and Rhodomicrobium are usually observed are of importance in any consideration of their possible nature and relationships. They arise, in a manner for which no real explanation has hitherto been offered, in ageing and degenerate cultures of other bacteria, especially nitrifying bacteria in the first case, and photosynthetic spirilla in the second. Some strains, arising in this manner, can be isolated and subcultured, others appear to grow freely only in mixed culture.

METHODS

Observations were made upon a strain of *Spirillum serpens* (provided by Dr Phyllis Pease) which had been isolated from pond water and maintained in pure culture for 6 years; also upon four strains of *Rhodomicrobium vannielii*, observed in ageing cultures of photosynthetic spirilla, and two of *Hyphomonas polymorpha* provided by Dr E. Pongratz, by whom they were isolated from a nasal discharge.

The medium used was similar to that of Duchow & Douglas (1949); its composition (%, w/v) was: yeast extract (Oxoid), 0·2; sodium acetate, 0·2; sodium citrate, 0·2; ammonium sulphate, 0·1; magnesium sulphate, 0·01; di-potassium hydrogen phosphate, 0·05; sodium chloride, 0·2; at pH 7·2, solidified with 2% (w/v) agar; in Petri dishes for *Spirillum serpens*, and dispensed in small screw-capped bottles with caps tightly fastened, for the photosynthetic forms. The organisms used grew at room temperature; *Hyphomonas polymorpha* grew on meat infusion agar at 37°.

Electron micrographs were made upon material taken directly from fluid cultures and dried upon collodion films, or from suspensions in distilled water in the case of growth on solid medium.

RESULTS

The morphology of *Spirillum serpens* in fluid culture, or on initial subculture on solid medium in Petri dishes, was very characteristic, consisting of spiral rods of moderate length, with a tuft of ten to fifteen flagella at each pole (Pl. 1, fig. 1). On continued subculture on solid medium the colonies lost their original smooth appearance and showed a rough spreading form. When microscopically examined, these rough colonies were seen to consist mainly of typical spirilla, but with about 20–30% of other forms including relatively straight rods (Pl. 1, figs. 2, 4) and

branched forms (Pl. 1, figs. 4, 5, 6). Flagellation was mainly of the normal polar type, but peritrichous arrangements were also seen, both in straight rods (Pl. 1, fig. 2) and in spirilla (Pl. 1, fig. 3). In the later stages of culture, after 7–10 days, typical minute swarm-cells began to appear (Pl. 1, fig. 7; cf. Pease, 1956*a*), some of which possessed the characteristic single polar flagellum, whereas other small rod-shaped organisms were non-flagellate (Pl. 2, figs. 8, 12). In association especially with the latter were two forms of unusual appearance: (*a*) spherical cells somewhat resembling free protoplasts, but with coiled flagella (Pl. 2, figs. 8, 9); (*b*) stalked cells (Pl. 2, figs. 11–13). The special interest of these lay in their resemblance to hyphomicrobia (compare Pl. 2, figs. 14, 15; Pl. 3, figs. 16–18). Another type of cell arising at this stage in cultures of *S. serpens* was not exceptional in itself, being a short oval, without flagella, but once more its resemblance to certain stages in the life-cycle of hyphomicrobia was most striking (Pl. 2, fig. 10; compare Pl. 2, fig. 15).

Rhodomicrobium vannielii was observed in the later stages of crude cultures ot photosynthetic spirilla (Pl. 2, figs. 14, 15). It began to appear in cultures aged 2–3 weeks, and gradually increased in proportion until the entire culture was of this form, consisting of oval cells joined by narrow branched filaments. Flagella were seen on occasional cells in most fields; they were very frequently coiled (Pl. 3, figs. 16–18). As these cultures aged, after 5–6 weeks or longer, both stalks and flagella became less obvious, and the bacteria often aggregated in stellate clumps (Plate 3, figs. 18, 19). In this condition they quite closely resembled those eubacteria, including spirilla (Watanabe, 1959, 1960), which adopt a similar habit, except for the appearance of an occasional stalk or projecting filament, or more rarely a coiled flagellum. Subculture from the earlier stages of culture usually gave a mixture of hyphomicrobia and other bacteria; but after the stellate phase had commenced, the hyphomicrobia were seldom detectable in subcultures until these had again aged.

The hyphomonas strains were distinguishable from Rhodomicrobium in that they branched much less freely, and produced mainly short irregular flagella (Pl. 3, fig. 20). The characteristic form was a globular cell attached by a slender filament to a smaller cell (presumably in course of development) at the other end. For comparison, observations were made upon growth forms of pleuropneumonia-like organisms, maintained in culture in this laboratory, some of which bore a distinct resemblance to hyphomicrobia (Pl. 3, fig. 21).

DISCUSSION

The theory of bacterial evolution proposed by Bisset (1950) and subsequently elaborated (Bisset, 1955), depends for its validity upon the potentialities of spirilla as forebears of all or most of the main groups of bacteria. The postulated development of different flagellar types from polar-flagellate aquatic ancestors appears to be acceptable to certain workers in this field (Leifson, 1960, p. 18) by whom the theory has been adopted. The observations reported in the present paper provide evidence that spirilla, when constrained to adopt an unnatural mode of existence on solid medium, may produce morphological forms which simulate, in certain respects, those bacteria whose morphology is believed to be an adaptation of the original spirillar form to a terrestrial environment. The appearance of straight or

branched rods with typically spirillar flagella, or of peritrichous flagella upon spiral rods, may be regarded as indicating that all these variant characters are separately associable with the problem of adaptation to terrestrial environments. At the same time, the mixture of normal and variant characters in these organisms serves to confirm their identity with the original strain. This obviation of any suggestion of contamination is important also in consideration of the stalked forms, and others resembling hyphomicrobia, which occur in the latter stages of culture. The accepted theory of origin of hyphomicrobia is that they exist as contaminants in cultures of autotrophic bacteria, which they eventually overgrow. However, the existence of comparable forms in pure cultures of spirilla, and the peculiarities of the life-cycle of strains of Rhodomicrobium derived in the normal way from cultures of photosynthetic spirilla, render it at least equally probable that Rhodomicrobium also is a variant or growth form of a Rhodospirillum. These observations are not unique, in that Watanabe (1959) not only illustrates spirilla in stellate clusters of the appearance of those adopted by Rhodomicrobium, but also forms derived from the growth-cycle of saprophytic spirilla that bear a strong resemblance to hyphomicrobia. It is significant that hyphomicrobial morphology can be adopted by the parasitic *Hyphomonas polymorpha*, which also has flagella of degenerate appearance. The coiled flagella found in Rhodomicrobium and in the hyphomicrobial forms of *Spirillum serpens* occur in other bacterial groups (Leifson, 1960), but are especially distinctive of Rhodomicrobium. The resemblance already noted between hyphomicrobia and certain stages of pleuropneumonia-like organisms is demonstrated with even greater clarity in the figures of Ledingham (1933) and Turner (1935). It may perhaps be stated, without further elaborating the argument here, that these organisms are now accepted by some workers to be growth forms of true bacteria. Thus it would appear that the adoption of a morphology of this type is quite widespread among bacteria of different groups.

In view of the suggestion already made, in support of the evolutionary concept quoted at the beginning of this discussion, that the variant forms described here are adaptations to growth on solid medium, it is interesting to consider that the hyphomicrobial morphology is adapted to growth in attachment to solid surfaces, usually the sides of vessels containing fluid medium. Even the very small swarmer cells, with their single polar flagella, are capable of motility in the thin film of moisture on an agar surface, where normal spirilla are usually completely immobilized. In appearance, these swarmers closely resembled vibrios, and although not strictly variants as in the case of the bacillary branched peritrichously-flagellate or hyphomicrobial forms, they afford further evidence of the ability of spirilla to produce or simulate most of the characters of the bacterial groups to which, on the hypothesis of Bisset (1950, 1955), they may be considered as ancestral.

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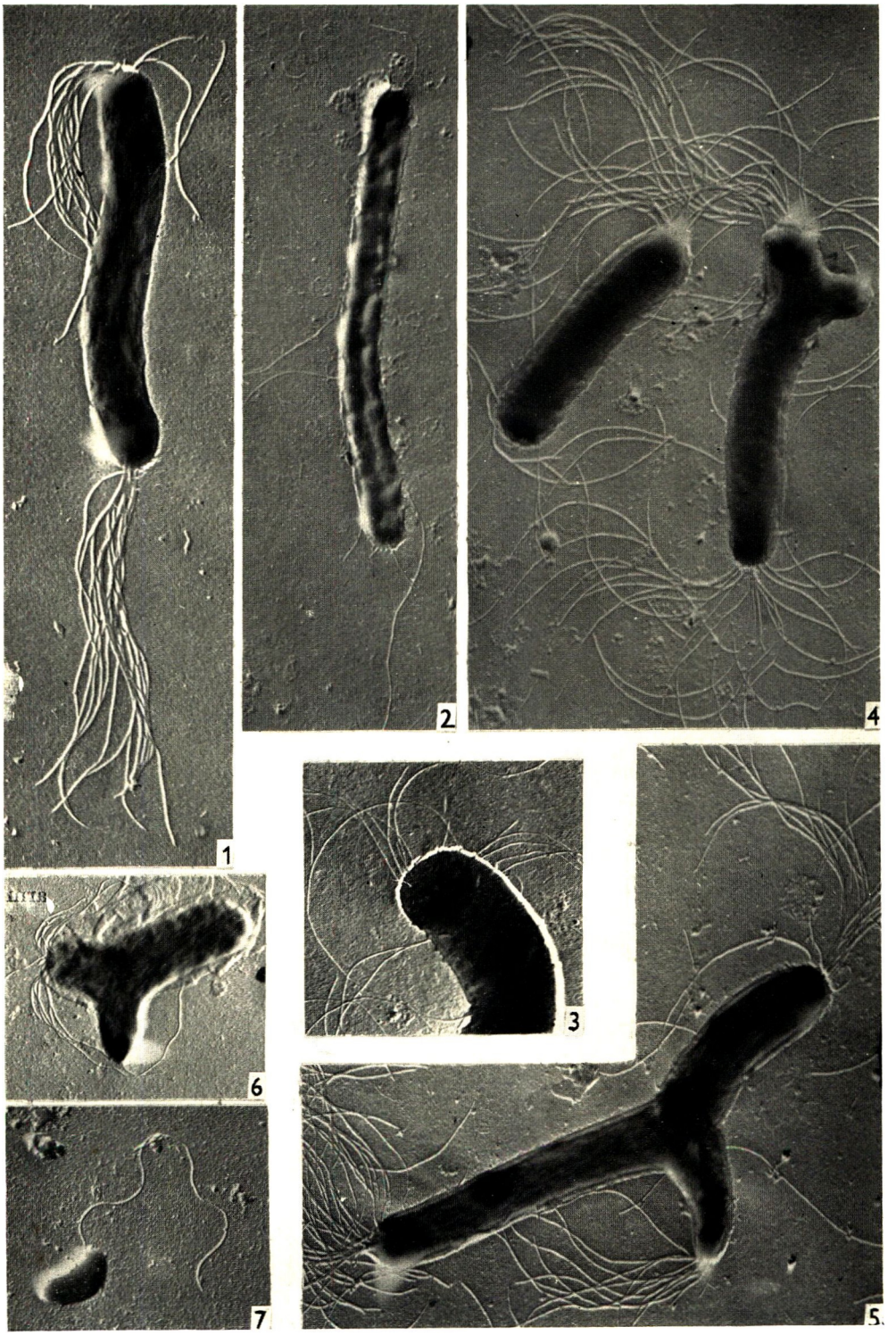

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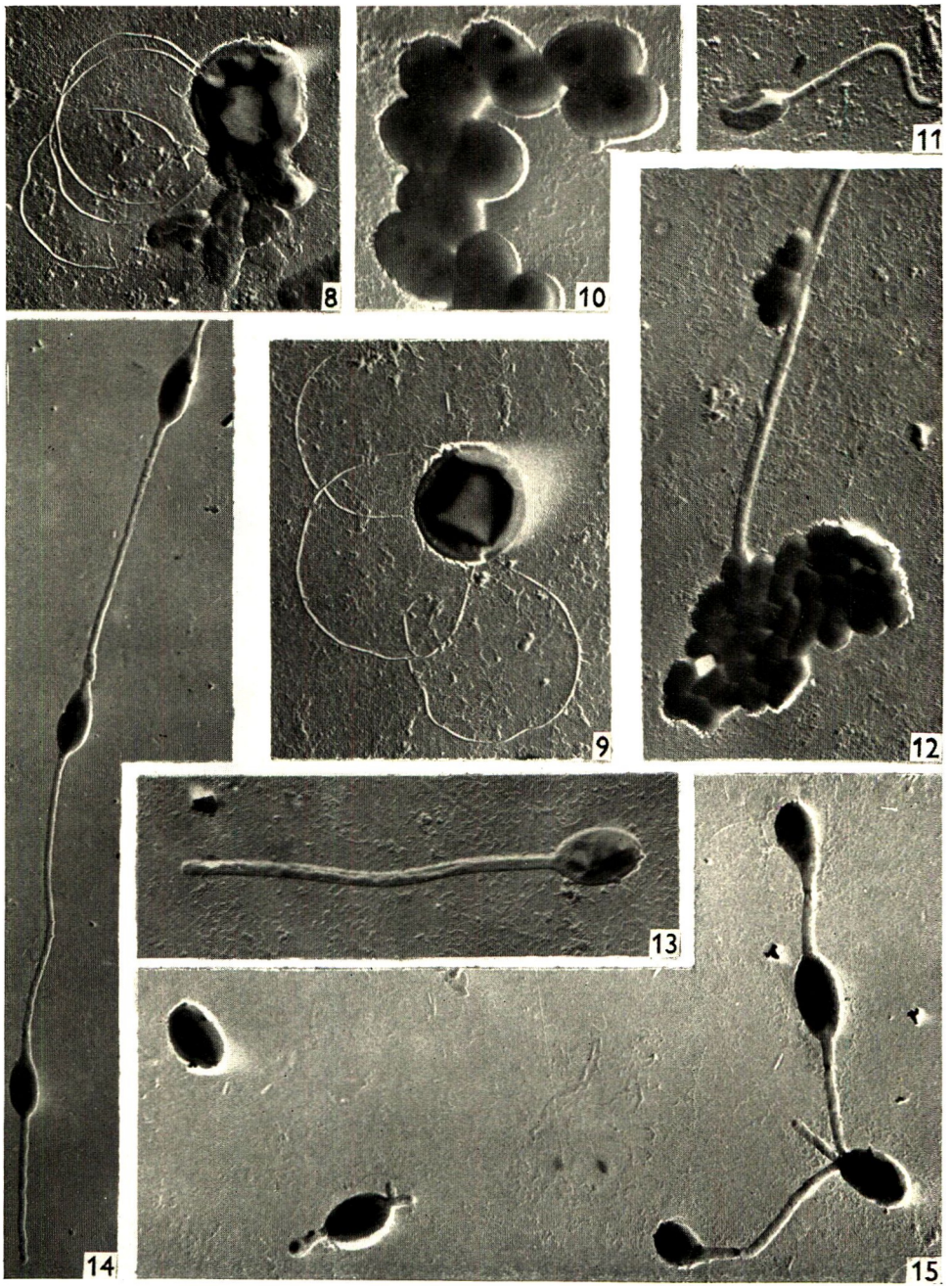
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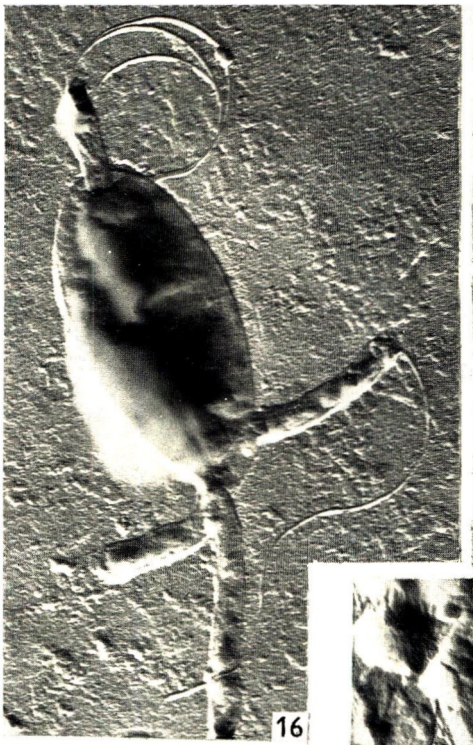
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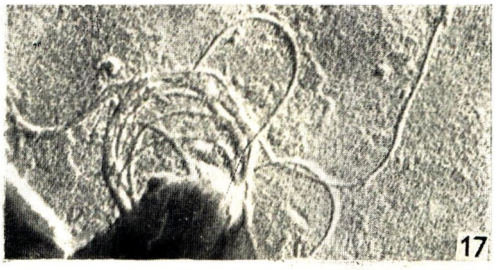
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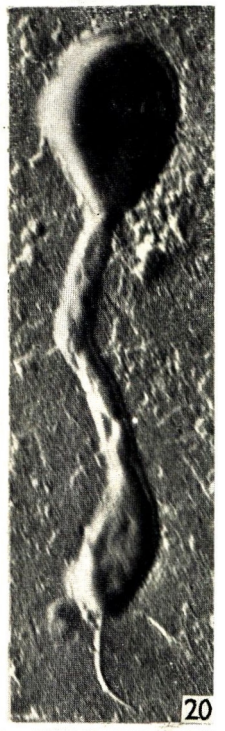
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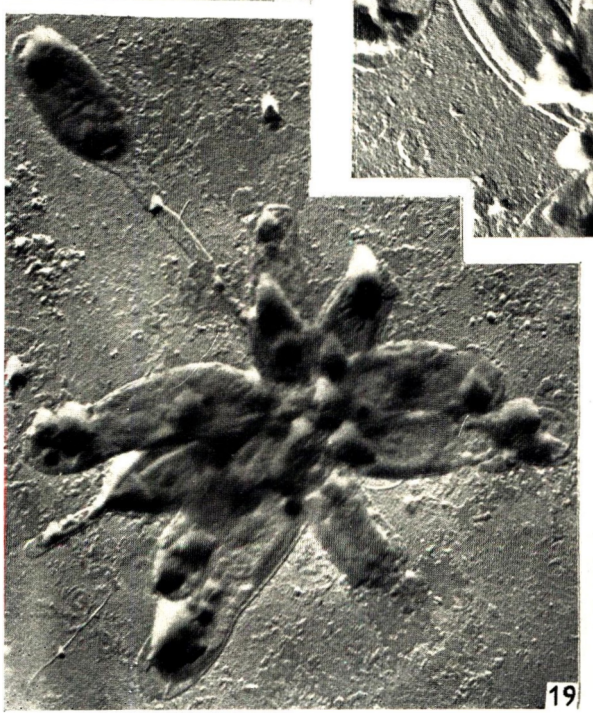
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EXPLANATION OF PLATES

(All figures are electron micrographs, gold-palladium shadowed.)

PLATE 1

Fig. 1. *Spirillum serpens:* normal appearance, spiral with polar flagella. ×10,000.

Fig. 2. *S. serpens:* rod form with irregular peritrichous flagella. ×10,000.

Fig. 3. *S. serpens:* spiral form with peritrichous flagella. ×10,000.

Fig. 4. *S. serpens:* straight and branched rods with polar flagella. ×10,000.

Figs. 5, 6. *S. serpens:* branched rods with polar flagella. ×10,000.

Fig. 7. *S. serpens:* swarmer cell, with single flagellum. ×10,000.

PLATE 2

Figs. 8, 9. *Spirillum serpens:* spherical cells (protoplasts?) associated with swarmers, showing coiled flagella. ×10,000.

Fig. 10. *S. serpens:* non-flagellate oval cells (compare fig. 15). ×10,000.

Figs. 11–13. *S. serpens:* stalked forms associated with swarmers (compare figs. 14, 15). ×10,000.

Figs. 14, 15. *Rhodomicrobium vannielii:* branched filaments and individual cells (compare figs. 10–13). ×7,500.

PLATE 3

Figs. 16, 17. *R. vannielii:* showing coiled flagella. ×20,000.

Figs. 18, 19. *R. vannielii:* stellate clumps, mainly bacillary forms, with occasional stalks and coiled flagella. ×10,000.

Fig. 20. *Hyphomonas polymorpha:* cell with short filament and bud bearing single, short flagellum. ×20,000.

Fig. 21. Organism of contagious agalactia (goat) showing hyphomicrobial forms. ×15,000.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its thirty-first General Meeting at the Microbiological Research Establishment, Porton, near Salisbury, Wilts, on Thursday, Friday and Saturday, 29 *and* 30 *September and* 1 *October,* 1960. *The following communications were made:*

THURSDAY, 29 SEPTEMBER 1960

ORIGINAL PAPERS

Breakage of Bacteria in a Modified Extrusion Press. By J. W. T. WIMPENNY (*Department of Bacteriology, Guy's Hospital Medical School, London*)

Method of Dissolving Triacetyl-Oleandomycin for the Assay of Sensitivity of Micro-Organisms. By J. W. CZEKALOWSKI (*Department of Bacteriology, The School of Medicine, Leeds*)

Natural and Experimental Infections with PPLO in Rats and Mice. By RUTH M. LEMCKE (*Lister Institute, Chelsea Bridge Road*)

Experiments Relating to the Survival of Bacteria Introduced into the Sheep Rumen. By P. N. HOBSON and S. O. MANN (*The Rowett Research Institute, Bucksburn, Aberdeen*)

Reflections on Microbial Survival Induced by some New Penicillins. By R. KNOX (*Department of Bacteriology, Guy's Hospital Medical School, London*)

Cation requirements of HeLa Cells in Tissue Culture. By H. V. WYATT (*Department of Bacteriology, The School of Medicine, Leeds*)

Quantitative Determination of the D Antigen Content of Living and Dead Polio-virus Preparations. By A. J. BEALE (*Glaxo Laboratories Ltd., Sefton Park, Bucks.*)

Conjugal Transfer of Immunity to Bacteriophage Multiplication. By K. W. FISHER (*M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, London*)

Dilution as a Method of Investigating the Establishment of Lysogeny in *Salmonella typhimurium*. By B. E. B. MOSELEY and R. H. GORRILL (*Department of Bacteriology, Guy's Hospital Medical School, London*)

FRIDAY, 30 SEPTEMBER 1960

PAPERS ON ASPECTS OF MICROBIAL SURVIVAL

The Preservation of Bacteria on Drying and Reconstitution. By B. R. RECORD (*M.R.E. Porton*)

Survival of Organisms in Favourable Environments. By E. O. POWELL and K. P. NORRIS (*M.R.E. Porton*)

The Growth and Survival of *Zymosarcina ventriculi*. By T. BAUCHOP and E. A. DAWES (*Department of Biochemistry, University of Glasgow*)

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