J. gen. Microbiol. (1961), **24**, 165–175
Printed in Great Britain

Toxicity of the Extracellular Phase of *Prymnesium parvum* Cultures

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(*Received* 7 *July* 1960)

SUMMARY

Cytotoxic material ('prymnesin') excreted into the growth medium by the phytoflagellate *Prymnesium parvum* was partially purified and crudely characterized. It proved to be a highly potent haemolysin; its solubility properties and some other characteristics are those of a lipid, perhaps a saponin. The high haemolytic activity which characterized the toxin was often also accompanied by a high ichthyotoxic activity. A method of locating haemolytic activity in a paper chromatogram is described. Fish were killed not by prymnesin alone, but were rendered highly susceptible to prymnesin poisoning by a range of cofactors: streptomycin sulphate, calcium chloride, magnesium chloride. Ichthyotoxicity in cofactor + prymnesin systems conformed within a wide concentration range to the relationship: $a \times b = Q$, where a is a prymnesin concentration, b a minimum concentration of cofactor which elicits a lethal effect in presence of a, and Q is a cofactor-specific constant. The recognition of this relationship has enabled a sensitive assay to be developed. Practical implications are discussed and the hypothesis proposed that prymnesin acts on fish by inducing a state of abnormal permeability of the gill.

INTRODUCTION

Outbreaks of mass mortality among gill-breathing inhabitants of pond waters have been associated with blooms of a toxigenic phytoflagellate *Prymnesium parvum* Carter (Otterström & Steeman-Nielsen, 1940; Reich & Aschner, 1947; Shilo & Aschner, 1953; Yariv, 1955, 1958; Shilo & Rosenberger, 1960). Severe losses are inflicted on the carp industry of Israel by this agent. The present communication describes a procedure for the recovery of stable and partially purified toxic material 'prymnesin' from the culture fluid of *P. parvum* cultures, describes some general properties of the preparation and discusses methods for its assay.

METHODS

Cultivation of Prymnesium parvum. Pure cultures were kindly made available to us by Mrs Mira Shilo. Strain no. 1 was originally isolated in Israel from fish ponds by Reich & Kahn (1954) and strain no. 2 by Droop (1954) in Scotland from supralittoral pools.

Pure cultures were made in a medium composed of 0·02 % (w/v) liver extract (Difco), 0·02 % (w/v) Bacto-tryptone (Difco) and 10 % (w/v) sea water in distilled water in 500 ml. conical glass flasks maintained under fluorescent lamps at 20°.

A 2-week-old culture was used in the proportion of 1:10 for inoculum. Large-scale preparations were made from samples of pond water (batch size about 50 l.) which had been transferred during prymnesium bloom to a tank which was maintained at ambient temperature (10–25°) under fluorescent lighting for 1 to 2 weeks, in which time the toxic activity reached its peak.

Detection and measurement of haemolytic activity

Reference standard. Digitonin (Merck, C. P.) served as a standard for reference; the stock solution was at a concentration of 0·026 % (w/v) in water. At a dilution of 1/200 in isotonic sodium chloride, this effected 50 % lysis of bovine erythrocytes within 45 min. in conditions of a standard assay described below.

Preparation of samples for assay. Samples to be assayed were brought to pH 5·0 and to isotonic salt concentrations. Extracts of dry prymnesin preparations were prepared by either of the following methods. (*a*) Sample (about 2 mg.) was moistened with a drop of 0·1 M-sodium hydroxide and suspended in 10 ml. water. The suspension was cleared by centrifugation; the supernatant fluid was immediately brought to pH 3–4 with dilute hydrochloric acid, whilst the sediment was extracted again as before. The extracts were pooled, the pH adjusted and brought to a known total volume and to appropriate salt concentration. (*b*) Sample was stirred with successive portions of methanol until all the haemolytic activity had been taken into solution. Extracts in methanol were diluted at least 1/100 with buffered saline before assay.

Standard assay system. Since prymnesin often lost activity rapidly at pH > 5 (see Results), assays were performed in acidified blood. As bovine erythrocytes withstand acid conditions better than do sheep erythrocytes, they were used in preference to the latter. The blood was taken into an equal volume of isotonic salt solution (0·073 M-sodium chloride, 0·042 M-sodium citrate, 0·114 M-glucose; pH 6·1) and stored at 4°. Before assay, stored red cells were washed 3 times in the cold with 10 vol. of buffered saline (0·130 M-sodium chloride adjusted to pH 5·0 with 0·020 M-citrate). The assay system consisting of toxin with approximately 0·4 % (v/v) bovine erythrocytes in buffered saline at pH 5·0 was incubated for 45 min. at 35 ± 0·5°.

The degree (%) of haemolysis was determined colorimetrically, essentially as described by Collier (1951). The values of optical density were respectively 340 and 190 (galvanometer units measured in a Klett galvanometer with filter 54) for 0 and 100 % haemolysis. The decrease of optical density was closely proportional to the % haemolysis at all values of the latter.

A unit of haemolytic activity was defined as that amount of prymnesin which effected 50 % lysis of bovine erythrocytes in one ml. of standard assay system. This unit is equivalent to 1·3 μg. digitonin. Figure 1 shows that in the range 0·8–1·2 units there was an almost linear relationship between concentration of haemolysin and % haemolysis. Titration within an accuracy of ± 7 % generally required 30 units toxin in a solution at a concentration of at least 3 units/ml.

Test of haemolytic activity on paper. Loci of haemolytic activity were detected in paper chromatograms by means of an erythrocyte spray (2 %, v/v, bovine erythrocytes in 0·137 M-sodium chloride + 0·020 M-sodium phosphate; pH 6·8). This was applied to the vertically suspended paper from a horizontal direction, starting from the top and working downwards in such a way as to leave a dry area at the

top of the paper strip while excess of fluid accumulated at the bottom. While the strip dried in air (10–20 min.), fluid flowed through it in an upward direction. Whilst such a flow did not cause migration of erythrocytes, any haemoglobin released by haemolysis was moved by the current and formed a dark spot in the region above the haemolytic locus; a bleached area remained below the dark spot. Certain non-haemolytic hydrophobic substances caused the erythrocytes to aggregate with attendant whitening or darkening of the locus (Table 1). However, as such changes were not accompanied by migration of haemoglobin, they were readily distinguished from a true haemolytic action. A reaction observed by Heftman & Hayden (1952) at sapogenin loci on paper is believed by us to have been of this non-specific kind and to have been interpreted erroneously as a sign of haemolytic activity.

Table 1. *Specificity of the haemolysis reaction on paper*

Substance*	Solvent	Reaction†	Minimum reactive dose (μg.)
Digitonin	Methanol	*h*	0·8
Prymnesin‡	*n*-Propanol + water (1 + 1, v/v)	*h*	1·5
Lysolecithin	Water	*h*	5
Saponin	Methanol	*h*	40
Digitogenin	Methanol	*nh*	8
Cholesterol	Methanol	*nh*	8
Kryptogenin acetate	Ethanol + chloroform (1 + 1, v/v)	*nh*	16
Tristearin	Ethyl ether	*nh*	100

* Lysolecithin was kindly supplied by Mr G. Rimon. Digitogenin and kryptogenin acetate were made available through the courtesy of Professor C. Djerassi.

† A haemolytic reaction is designated *h* and a non-specific colour change by *nh*.

‡ Fraction Xb (see text) was used for this experiment.

Measurement of ichthyotoxic activity

Fish. Gambusia minnows (25–40 mm. long) collected from pools in Beisan Valley, Israel, served as the test organism. The animals were maintained in the laboratory in tanks of tap water under a gentle stream of air.

Standard assay system. Ichthyotoxicity was measured on the basis of the ability of prymnesin to enhance the susceptibility of fish to the lethal action of streptomycin. Groups consisting of five fish were immersed in beakers at 20° into 20 ml. of solution containing prymnesin, sodium bicarbonate at 5 mM and streptomycin sulphate at 2·0 mM (0·1 %) in distilled de-ionized water. The solution was adjusted to pH 8·0 with NaOH and held within $\pm 0{\cdot}2$ pH units throughout the assay. The 'fish unit' of toxin was defined as that minimum amount of prymnesin/ml. which killed all five minnows present in 20 ml. of standard solution containing streptomycin sulphate at pH 8·0 during 3 hr. at 20°. In the conditions of this assay, streptomycin sulphate alone or prymnesin alone did not kill any of the fish. The titrations of toxicity were reproducible within ± 10 %. A total of about 100 fish was generally used per titration.

Ichthyotoxin preparation. Except as otherwise indicated, prymnesin at stage Z of purification (see under Results) was used in experiments on ichthyotoxicity. Stock solutions (pH 4·0) in water were stored at 6°.

RESULTS

Supernatant fluids from pure cultures of *Prymnesium parvum* increased in toxicity during the first 2 weeks. Haemolytic titres reached 6–30 units/ml. and ichthyotoxic titres about 6 units/ml.

Purification of toxic principles

A pure culture (strain no. 1) of *Prymnesium parvum* with an activity of 16 haemolytic units/ml. at 16 days was cleared by centrifugation (12,000 rev./min. in a Sharples centrifuge) and the culture fluid brought to pH 10–11 with conc. sodium hydroxide, added with vigorous stirring. A flocculent precipitate, consisting mainly of magnesium hydroxide, was allowed to sediment (1 hr.), packed down by centrifugation and dissolved in N-hydrochloric acid (100 ml.). The solution (pH 2–3) was dialysed against 0·1 mM-hydrochloric acid through cellophan at 6° for 3 days. On freeze-drying the material remaining in the dialysis sac (dialysand) 140 mg. of a fluffy, somewhat hygroscopic powder (Z) was obtained. About 50 % of the original haemolytic activity was recovered in this fraction. Its haemolytic and ichthyotoxic activity remained unchanged during prolonged storage (1 year at −15° in an evacuated ampoule). Product Z was converted into a granular product (Y) by grinding under cold absolute acetone. Solution of this material in 50 ml. methanol gave on evaporation under reduced pressure 12·5 mg. of a slightly yellow powder (X) having an activity of 4800 haemolytic units/mg. A large-scale operation conducted on a monoalgal rather than pure culture is summarized in Table 2.

Table 2. *Purification and resolution of active principle in supernatant fluid of monoalgal* Prymnesium parvum *culture*

Purification procedure was essentially as that described in the experiment with a pure culture (see text).

Fraction*	Recovery of haemolytic activity (% of starting material)	Dry weight (mg.)	Haemolytic activity (units/mg.)	Ichthyotoxic activity (units/mg.)
Culture fluid	100	10^6	0·5	0·15
Z	78	299	1240	400
Y	78	204	1640	530
X	78	52	6700	1900
Xa	6	4	6400	—
Xb	68	44	6700	1700

* Letter symbols are used to designate successive steps in purification as described in the text.

Resolution of toxin in a propanol+water system. Product X was washed in absolute *n*-propanol (20 ml.) without loss of activity and then dispersed in *n*-propanol + water (90 + 10, v/v). About 11 % (fraction Xa) of the total activity was taken into solution by this solvent. The residue was dispersed in 30 ml. of *n*-propanol + water system (50 + 50, v/v), to afford a solution which was cleared by centrifugation and then concentrated under reduced pressure with successive additions of absolute *n*-propanol. On evaporation of the solvent, 7·4 mg. of a light yellow powder (Xb) were recovered. This contained most of the original activity at

5600 haemolytic units/mg., a value nearly eight times that found with digitonin in the standard assay conditions.

Chromatographic resolution of toxin on cellulose. Suitable solvent systems applied on a paper sheet (Table 3) or on a cellulose column (Fig. 2) resolved the activity of product Z into at least two components. An all-glass column (600 mm. long and 35 mm. in diameter) contained Whatman's Ashless Cellulose Powder pretreated as recommended by Stoll & Kreis (1951). The developing system was ethyl acetate + *n*-propanol + water (8 + 2 + 3, v/v). Fraction Z of prymnesin (50,000 units contained

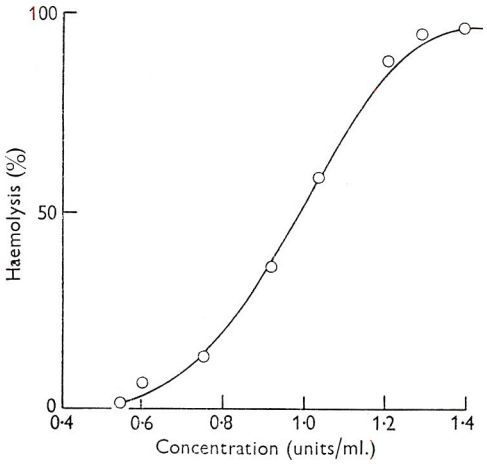

Fig. 1. Concentration effect function of prymnesin.

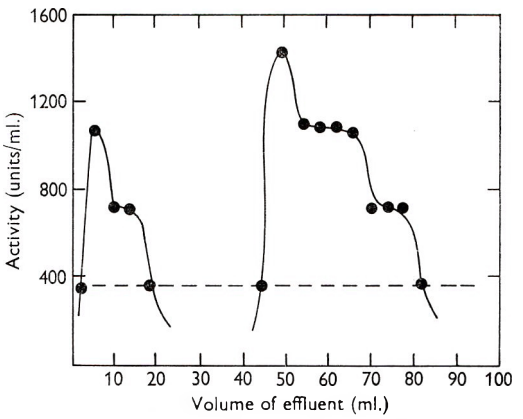

Fig. 2. Chromatographic resolution of prymnesin on a cellulose column.

Table 3. *Mobility of various haemolysins on paper*

Whatman no. 1 paper was used with a development time of 15 hr. at room temperature.

Solvent system (v/v)	Substance	Appropriate R_f*
n-Amyl alcohol + acetic acid + water (7 + 3 + 10)	Prymnesin	**0·06**; 0·17
n-Butanol + water (1 + 1)	Prymnesin	0·18
Ethyl acetate + *n*-propanol + water (8 + 2 + 3)	Prymnesin	**0·21**; 0·34†
n-Amyl alcohol + *n*-butanol + water (1 + 4 + 5)	Prymnesin	0·45
Ethyl acetate + *n*-propanol + water (7 + 5 + 10)	Prymnesin	0·90
n-Butanol saturated with water	Lysolecithin	0·68
	Digitonin	0·05
	Prymnesin	0·05

* Where toxin was resolved into components, the R_f value of the major component is given in bold figures.

† Ninhydrin-positive substances (test performed as by Berry & Cain, 1949) in the sample were resolved by this system and gave R_f values of 0·00, 0·06, 0·15. At the two haemolytic loci, however, no substance which gave a positive reaction with ninhydrin was detected.

in 60 mg.) was applied in the aqueous phase of the solvent and developed in the organic phase. Successive 4·0 ml. fractions of effluent were examined for activity > 360 units/ml. by paper test. A fast-moving fraction (corresponding to Xa in Table 2) represented 20 % of the total activity put in the column; a slow-moving fraction (corresponding to Xb in Table 2) represented ⩾ 60 % of the total activity

applied to the column. The furfural test (Feigl, 1954) for carbohydrate in both these fractions was positive. The minimum haemolytic activity which was detectable by the analytical procedure used is indicated in the figure by the broken horizontal line. A major (slow) component in product Z had the same R_f value as the active compound found in the Xb fraction described above. The R_f value of the minor (fast) component of product Z corresponded to the active compound in the Xa fraction.

General properties of toxin

Some solubility properties of the prymnesin are given in Table 4. Information about keeping qualities of this preparation, and on behaviour with precipitating agents and adsorbents as well as miscellaneous other features, is collected in Table 5.

Table 4. *Solubility of prymnesium haemolysin in organic liquids*

A sample (2 mg.) of prymnesin purification Z was stirred with 0·2 ml. solvent, centrifuged, and the supernatant fluid then tested for haemolytic activity.

Haemolytic activity	
1000 units or more/ml.	200 units or less/ml.
Methanol, pyridine*, *n*-butanol (sat. with water), *n*-propanol + water (1 + 1, v/v), *e*-systems†.	*n*-Propanol, *n*-butanol, acetone, diethyl ether, light petroleum, ethyl acetate, ethyl acetate (sat. as to water), benzene, chloroform, carbon tetrachloride.

* Activity in pyridine declined rapidly.
† *e*-systems = aqueous and organic phase of ethyl acetate + *n*-propanol + water (8 + 2 + 3, v/v).

Prymnesin solutions (10–100 units/ml.) in water showed a marked decrease in titre when kept for 60 min. at 35°. When solutions which had been partially inactivated by such treatment were subsequently brought to pH 4·0, their original activity was rapidly restored.

Relation of ichthyotoxic and haemolytic activities. Though the ratio of haemolytic activity to ichthyotoxic activity in a given preparation of prymnesin remained roughly constant throughout purification (Tables 2, 5), wide variations in ratio were encountered between different batches of toxin. Several preparations which exhibited high haemolytic activity indeed had no apparent ichthyotoxicity.

Observations on ichthyotoxicity of water samples from pure cultures and from ponds during prymnesium bloom. As reported by Liebert & Deerns (1920), Otterstrøm & Steeman-Nielsen (1940) and Reich & Aschner (1947), samples of water taken from ponds during prymnesium bloom were frequently but not invariably ichthyotoxic. When toxic samples were dialysed through cellophan (72 hr.; 6°; pH 4·0), the separated dialysate and dialysand had no lethal effect on fish, but were again toxic when pooled.

Passage through a cation-exchange column (H^+ form of Amberlite IRC50) abolished the ichthyotoxicity of pond-water samples. On elution of the column with N-HCl, material was recovered which conferred toxicity on dialysand prepared from a toxic water sample. The active factor in the eluate was not destroyed by ashing. Dialysand of toxic water samples formed a toxic system

Table 5. *Influence of various treatments on cytotoxic activities of prymnesin*

Aqueous solutions contained 10–100 units prymnesin/ml.; solutions in organic liquids represented extracts of product Z at a concentration of about 1000 units/ml. The pH values of the aqueous solutions were adjusted with HCl to pH 0·0, 4·0 and 6·0 and with $NaHCO_3$ to 8·0. Except as otherwise stated, all treatments tabulated below were performed at 25° and lasted for 5 min.

Treatment	Tested property or reagent	Solvent	Particulars of treatment	Recovery of original activity (%) in Liquid phase	Recovery of original activity (%) in Solid phase[2]
1	Storage	Water	700 hr.; 6°; pH 4	**100**[1]	*
2	Storage	Water	24 hr.; pH 4	**100**	*
3	Storage	Water	24 hr.; pH 8	0	*
4	Thermostability	Water	15 min.; 98°; sealed vessel; pH 4	**100**	*
5	Thermostability	Water	15 min.; 98° (boiling); pH 4	0	*
6	Thermostability	*n*-Propanol	15 min.; 94° (boiling)	100	*
7	Thermostability	Methanol	15 min.; 62° (boiling)	100	*
8	Hot acid	Water	15 min.; 98°, pH 0	0	0[3]
9	Water/gas interphase	Water	24 hr.; stream of N_2; pH 4	60	*
10	Diffusibility	Water	72 hr.; 6°; cellophan membrane; pH 4	**100**[4]	*
11	Cellulose	Water	Paper (Whatman no. 1); pH 4	30	70[5]
12	Ca phosphate	Water	15 min.; Na-acetate 5 mM; pH 4	44[6]	—
13	$Mg(OH)_2$	Water	0·1 M-$MgCl_2$; initial pH 4 brought to pH 11	0	57[7]
14	$(NH_4)_2SO_4$	Water	6°; 0·24 of sat.; pH 6	0	34[8]
15	Chloroform	Water	6°; shaking; pH 4	0[9]	32[10]
16	Cholesterol, acetone or *n*-amyl alcohol	Methanol	Reagent at 1 %	60 to 80	—[11]
17	Diethyl ether	Methanol	Reagent at 1 + 1 (v/v)	36	42[12]
18	Petroleum ether	*e*-System[13]	Reagent at 1 + 1 (v/v)	30	56[12]

[1] Values denoted by bold figures designate solutions that were tested also as to ichthyotoxic activity. In all these cases, ratio of haemolytic to ichthyotoxic activity was found not to have been altered significantly by the treatment in question.

[2] Absence of precipitate formation is indicated by asterisk.

[3] The precipitate was soluble in benzene.

[4] The dialysate contained no activity.

[5] Methanol served as eluent.

[6] One ml. gel suspension (prepared according to Singer & Kearney, 1950) was added to 10 ml. prymnesin solution. At pH 7–10, little or no activity was adsorbed by this reagent.

[7] Precipitate, consisting mostly of $Mg(OH)_2$, was dissolved at pH 2 (adjusted with HCl) and dialysed before titration.

[8] The solid, being of low specific gravity, floated when the suspension was centrifuged; it was dissolved in water.

[9] Material in solution in the aqueous and in the organic phase of the solvent system was inactive.

[10] The precipitate was collected at the chloroform–water boundary and dissolved in water after alkali treatment.

[11] Precipitate formed with 1 % reagent was not visibly dissolved and failed to release any activity into either methanol or 0·01 M-NaOH in water. Increase of the concentration of reagent by several fold failed to elicit any further precipitation.

[12] After removal of this precipitate, increase of the ether concentration to 2 vol. gave no further precipitate, but at 3 vol. a second precipitate formed with attendant disappearance of all activity from solution. The activity recovered on solution of the second precipitate into methanol represented 40–70 % that in the original supernantant fluid.

[13] e-system = organic phase of ethyl acetate + *n*-propanol + water (8 + 2 + 3, v/v).

when it was mixed with 10 mM-calcium chloride, 10 mM-magnesium chloride or 2 mM-streptomycin sulphate. In absence of dialysand the same solutions were innocuous to fish. Chlorides of monovalent cations (Na, K) at 20 mM were not toxic to the fish either in the presence or absence of dialysand. Similar results were also found when dialysed centrifugates from pure cultures of *Prymnesium parvum* were used for these experiments in the place of the pond-water dialysands.

Effect of prymnesin on susceptibility of fish to poisoning by various compounds

Toxicities of various inorganic salts and of streptomycin sulphate in the presence and absence of prymnesin are compared in Table 6.

The manner in which LD100 of selected compounds varied with the prymnesin concentration is shown by Fig. 3. These data fit a simple relationship: $a \times b = Q$, where a is prymnesin concentration, b is the minimum concentration of cofactor which elicits a lethal effect in the presence of a, and Q is a cofactor-specific constant whose value defines the LD100 of the cofactor for systems in which prymnesin is at unit concentration.

Cofactors all had practically the same LD100 values when measured in absence of prymnesin on prymnesin-pretreated minnows as when measured in the presence of prymnesin (see Table 7). The time lapse between the beginning of exposure to cofactor and the onset of death greatly exceeded the duration of the previous immersion of the fish in the prymnesin solution. This implies that the survival times of fish in the cofactor + prymnesin systems probably depended on the time required for the action of cofactor on the prymnesin-sensitized fish, rather than on the time during which the sensitization by prymnesin was accomplished.

Table 6. *Influence of prymnesin on ichthyotoxicity of various compounds*

Compound	Buffer (5 mM; pH 8·0)	LD100 (mM) of compound in absence of prymnesin	LD100 (mM) of compound in presence of prymnesin (1·0 units/ml.)
$CaCl_2$	$NaHCO_3$	60	1
Streptomycin SO_4	$NaHCO_3$	20	2
$CaSO_4$	$NaHCO_3$	> 20	5
$MgCl_2$	$NaHCO_3$	80	5
$MgCl_2$	Na_2HPO_4	> 40	20
KCl	Na_2HPO_4	> 20	—
NaCl	Na_2HPO_4	> 50	—

The LD100 value of prymnesin in the standard assay system was doubled when a solution of prymnesin (20 units/ml.) was held for 6 hr. at room temperature at about pH 10 before the test was carried out. The solution, thus partially inactivated, was rapidly restored to its original titre by subsequent incubation at pH 4·0. Thus there appeared to be at least a qualitative resemblance between the effects of successive exposure to alkali and acid on ichthyotoxicity and haemolytic activity, respectively.

To learn whether plant saponins require cofactor for the manifestation of the lethal effect on fish, methanol-soluble components of a commercial saponin pre-

paration (British Drug Houses Ltd.) were taken into water and dialysed against dilute acid (10^{-4} N-hydrochloric acid for 3 days). The LD100 values of the dialysand at pH 6·0, 7·0 and 8·0, respectively (pH adjusted with 0·01 M-sodium phosphate), were as follows (in μg. original crude preparation/ml. final system): 250, 167, 125. Addition of cofactor (20 mM-$MgCl_2$) to such dialysands did not alter their LD100 values.

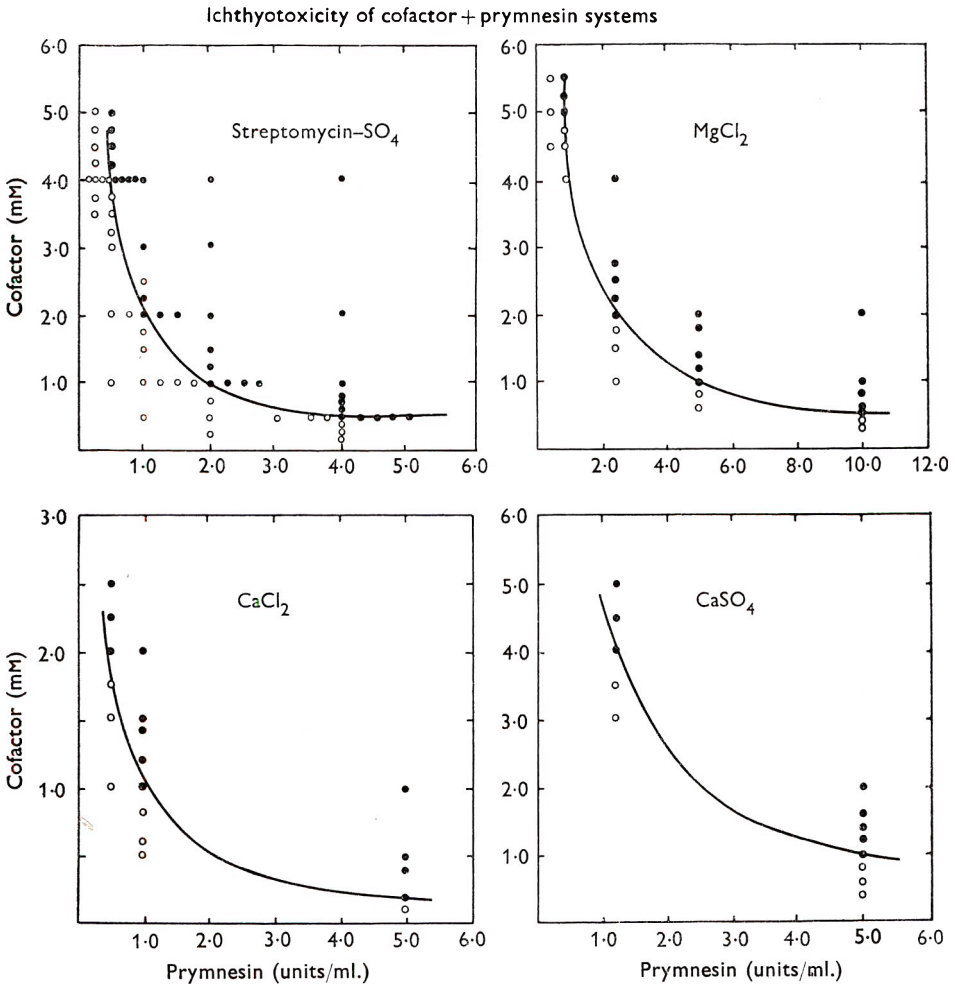

Fig. 3. Ichthyotoxicity of cofactor + prymnesin systems. Test mixtures contained in each case prymnesin and a single added salt under the standard conditions. Fish were exposed to prymnesin at graded dilutions in the presence of a fixed cofactor concentration and to cofactor at graded dilutions in presence of a fixed concentration of prymnesin. Solution mixtures in which one or more of the animals survived for 3 hr. are recorded by ○ and those in which no animal survived by ●. Drawn curves are calculated from equation (1) on the basis of appropriate values assigned to Q (streptomycin sulphate, 2·0; magnesium chloride, 5·0; calcium chloride, 1·0; calcium sulphate, 5·0), concentration of cofactor being expressed in mM and that of prymnesin in units/ml. of final system.

Table 7. *Effect of prymnesin on the susceptibility of fish to streptomycin sulphate*

Minnows were immersed in prymnesin solution and then transferred to solution containing streptomycin sulphate but no prymnesin.

Treatment with prymnesin		Concentration of streptomycin sulphate (mM)			
		1	2	3	4
	Duration	Max. survival time (min.)			
Units/ml.	(min.)				
1·5	5	180	110	82	67
	15	180	90	65	59
3·0	5	.	57	.	.
	15	.	55	.	.
5·0	5	60	38	.	.
	15	64	35	.	.

DISCUSSION

A simple diagnostic procedure to indicate whether prymnesin was the causal agent in any particular outbreak of mass mortality among fish has been sought. Some properties of prymnesin which are disclosed in this paper might be useful in this connexion. They are in particular the non-dialysability of this agent, its rather unusual solubility pattern and its dependence for action after dialysis on an added cofactor. Solubility in methanol and in *n*-propanol water solvent systems distinguishes the toxic material in prymnesin from simple proteins and polysaccharides. The observed properties are consistent with the view that the toxic material is lipid and in which there are present both non-polar and polar moieties, both of which contribute significantly to the over-all behaviour. It has also been noted that the ratio of haemolytic to ichthyotoxic titre in a preparation made from a given starting material tends to maintain nearly a constant value throughout successive stages in purification, but that a wide variation in the activity ratio can be observed as between preparations made from different batches of culture fluid. The results are consistent with the view that a family of compounds possessing a similar basal structure are concerned in the cytotoxic activities of *Prymnesium parvum*. The homogeneity of even the most potent of our preparations has not been established. Several of the observed properties of the prymnesins (ichthyotoxicity and haemolytic activity, non-dialysability against water, general solubility features, formation of insoluble inactive complexes with certain alcohols, the capacity to undergo precipitation by magnesium hydroxide and ammonium sulphate) are also exhibited by saponins (see Kofler & Wolkenberg, 1925; Windaus, 1910; Windaus & Weinhold, 1932). But saponin does not require a cofactor.

Though prymnesin in aqueous solution exhibits the behaviour of a colloid, fish immersed in such a solution are promptly sensitized. The rapidity of the action suggests that the immediate target is an exposed organ, probably the gill. Attention has been directed to the fact that the preparations which manifest ichthyotoxicity also have haemolytic activity. Both cation and anion in a salt + prymnesin system affect the toxicity. Moreover, prymnesin causes fish to become susceptible to poisoning by such widely different compounds as calcium chloride and streptomycin sulphate. To account for these observations the hypothesis can be considered that

the mode of the action of prymnesin on fish resides in an impairment of the permeability mechanism of the gill.

We are indebted to Dr M. Shilo for advice and criticism and thank Mr S. Sarig for many courtesies. Financial assistance from the Fish Breeders' Association of Israel and a grant-in-aid from the Ford Foundation are gratefully acknowledged. The work presented in this communication was carried out by one of us (J.Y.) in partial fulfilment of requirements for a Ph.D. degree.

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แผนกห้องสมุด กรมวิทยาศาสตร์
กระทรวงอุตสาหกรรม

J. gen. Microbiol. (1961), **24**, 177–186
With 1 *plate*
Printed in Great Britain

Purification of Murray Valley Encephalitis Virus

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(*Received* 27 *July* 1960)

SUMMARY

Methods are described for the purification of Murray Valley encephalitis virus from baby mouse brain extract. The method finally chosen involved treatment with protamine, ultracentrifugation, adsorption to and elution from hydroxyl apatite and a second ultracentrifugation. The degrees of recovery of virus infectivity and haemagglutinin were about 15% and 50%, respectively. Measurement of the absorption in the ultraviolet region and examination in the electron microscope suggested that a substantial degree of purification had been achieved. The stability of the infectivity of purified virus was less than that of crude virus. Purified virus yielded infective 'ribonucleic acid' on treatment with phenol. The yield was consistent with the possibility that the potential infective 'ribonucleic acid' in crude virus was contained in infective virus particles.

INTRODUCTION

In 1943 Taylor, Sharp, Beard & Beard showed that purified preparations of eastern equine encephalomyelitis virus contained protein, lipid and ribonucleic acid. This work provided the first indication that the nucleic acid associated with an animal virus might be entirely of the ribose type. Interest in the composition of these viruses has been stimulated by reports that crude preparations of some viruses in this group, upon treatment with phenol, yielded fractions which appeared to consist mainly of ribonucleic acid (RNA) and were infective (Wecker & Schäfer, 1957; Cheng, 1958; Anderson & Ada, 1959). These findings suggest that RNA may carry the genetic information necessary for the production of virus particles which contain lipid as well as nucleoprotein in their structure. The present paper describes a method for purifying Murray Valley encephalitis (MVE) virus from crude extracts of infected baby mouse brains. There was a high recovery rate but the purified virus lost infectivity very rapidly. Nevertheless, it yielded high titre infective 'ribonucleic acid' on treatment with phenol.

METHODS

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

Bicarbonate cystine saline (*BCS*). A solution of $NaHCO_3$ (0·025 M), cystine (0·85 mM) and NaCl (0·15 M). The cystine was dissolved in saline by boiling, and after cooling the $NaHCO_3$ was added. The solution was filtered through a Seitz sterilizing pad.

Veronal cystine saline (*VCS*). An aqueous solution of sodium barbiturate (0·018 M), HCl (0·009 N), cystine (0·85 mM), NaCl (0·15 M); pH 7·9.

Phosphate buffer. An aqueous solution of K_2HPO_4 (1·8 M) and KH_2PO_4 (0·2 M).

Phosphate saline. An aqueous solution containing NaH_2PO_4 (0·05 M), NaOH (0·044 M), NaCl (0·13 M); pH 7·7.

Phenol. Phenol (A.R.) was distilled *in vacuo*, the distillate saturated with glass-distilled water and stored at 0–4°.

Diethyl ether. This ether was prepared as described previously (Anderson & Ada, 1959).

Normal rabbit serum (*NRS*). Animals in a laboratory colony were bled from the ear vein and serum separated from the clot at 37°. Sera were pooled and filtered through a Seitz sterilizing pad.

Eggs. White Leghorn/Black Orpington cross eggs were incubated at 39° until used and thereafter at 36°.

Source of virus. A strain of Murray Valley encephalitis (MVE) virus, isolated in 1951, was used as 8th mouse passage material. It was inoculated intraperitoneally into mice 5 days old and the brains were harvested when the mice were moribund after a further 4 days.

Preparation of virus extract. Infected baby mouse brains were ground in the appropriate solvent by using a glass mortar fitted with a Teflon pestle which rotated at about 4000 rev./min.

Preparation of infective 'RNA'. The standard procedure described previously (Anderson & Ada, 1959) was slightly modified in that the virus solution was treated twice with phenol, each time for a period of 6 min.

Preparation of normal mouse brain extract. Brains were harvested under chloroform anaesthesia from mice 8 or 9 days old and ground in the desired solvent with the mortar and pestle described above. Grinding was carried out for 3 min. and the fluid clarified by centrifugation (2000 *g*, 5 min., 0–4°).

Titration of virus and infective 'RNA'. Titration in 12-day chick embryos was done on the chorioallantoic membrane, the membranes being 'dropped' with an inoculum of 0·05 ml. Titration in mice 5–8 days old was by intraperitoneal inoculation of 0·05 ml., or intracerebral injection of 0·01 ml. Infectivity endpoints were determined as 50 % lethal end-points at 4 days in chick embryos; mice were observed for deaths over a period of 12 days.

Haemagglutination. Adult goose red cells were prepared in Alsever's solution and stored for periods up to 4 weeks at 4°. Just before use they were washed three times with saline and made up to 1 % (v/v) suspension in phosphate saline. In the haemagglutination test, haemagglutinin was serially diluted in tenfold falling dilutions in 0·5 ml. volumes of phosphate saline. To each tube was added 0·25 ml. of 1 % goose red cells and the contents mixed. Titrations were read after standing at 22° for 45 min.

'Fluorocarbon' treatment. A proprietory preparation, labelled Freon 113, was used. Equal volumes of infective solution and fluorocarbon were ground at 0–4° in the mortar described earlier. After the treatment the aqueous phase was recovered by centrifugation.

Protamine sulphate. A preparation obtained through the courtesy of the Commonwealth Serum Laboratories, Melbourne.

Hydroxyl apatite. This was prepared according to the technique of Tiselius, Hjertén & Levin (1956). It was stored under phosphate buffer (0·001 M K_2HPO_4, 0·001 M KH_2PO_4; pH 6·8) at 0–4° for periods up to 4 weeks.

Column chromatography. Columns of hydroxyl apatite were prepared about 3 hr. before use. They were pre-washed with about 20 ml. BCS.

Ultracentrifugation. All ultracentrifugation was carried out in a model L Spinco ultracentrifuge fitted with a 40 rotor.

Ultraviolet spectrophotometry. A Uvispek apparatus was used, all readings being made in 0·5 cm. optical cells.

Electron microscopy. A Siemens Type E4 electron microscope was used. The infective sample was treated for 16 hr. at 4° with one half its volume of 3 % (w/v) formaldehyde solution. The sample was then dialysed against distilled water (24 hr.; 4°) and mounted from a high-pressure spray gun on formvar covered grids. The droplets were shadowed with gold-manganin and examined at magnifications of about ×15,000 and at 60 kV with a 50 μ objective aperture.

RESULTS

Preliminary attempts at purification

In early experiments several techniques were tried for their value in the purification of MVE virus. The criteria of usefulness included the degree of purification achieved, the yield of virus and the subsequent stability of the virus. The techniques were applied to solutions of either crude or partially purified virus, and comprised both those which precipitated non-viral material (treatment with protamine, fluorocarbon or ammonium sulphate) and those which achieved a selective concentration of virus particles (sedimentation or column chromatography).

Treatment with protamine. Warren, Weil, Russ & Jeffries (1949) found that protamine precipitated non-viral material from crude preparations of virus but did not decrease the infectivity of the solution. They removed excess protamine with heparin. Protamine behaved similarly with crude preparations of MVE virus. In 13 experiments the change in titre varied from a gain of 1·0 log unit to a loss of 1·0 log unit of infectivity, the mean change being a loss of 0·1 log unit which was not significant. Protamine treatment decreased the u.v. absorption at 260 mμ and 300 mμ by an average of 80 % and 90 %, respectively. The method of purification finally chosen included treatment with protamine but not heparin.

Treatment with fluorocarbon. Gessler, Bender & Parkinson (1956) found that some fluorocarbons when mixed vigorously with crude preparations of certain viruses preferentially removed non-viral material from solution. In the present work, some purification was achieved when MVE virus preparations were briefly treated with fluorocarbon. The best results were obtained when infected fluids, previously treated with protamine, were exposed to fluorocarbon for 5 min. No loss of infectivity occurred, though the % decrease of absorption at 260 mμ and 300 mμ was 26 ± 14 (S.D.) and 45 ± 15 (S.D.), respectively. When virus, which had been purified either by sedimentation in the ultracentrifuge or by ammonium sulphate fractionation as described below, was treated with fluorocarbon, the recovery of infectivity was very erratic.

Ammonium sulphate fractionation. Crude preparations of virus were first treated

with protamine, then with fluorocarbon, and finally with ammonium sulphate. In six experiments the mean loss of infectivity due to the ammonium sulphate step was 0·7 log ± 1·2 log unit. In a seventh experiment, dialysis against buffered saline (pH 7·7) at 0–4° for 16 hr. to remove ammonium sulphate, resulted in a further loss of 1·3 log unit. This dialysed material lost another 1·4 log unit of infectivity after one fluorocarbon treatment.

Adsorption to and elution from hydroxyl apatite. The virus present after treatment of crude preparations with protamine could be adsorbed to columns of hydroxyl apatite. In preliminary experiments, 30 ml. protamine-treated extract were adsorbed to a column (3 cm. × 1·5 cm.) of hydroxyl apatite which was then developed with a gradient of phosphate concentration (0·02 M–2·0 M). The virus eluted under these conditions and the infectivity were quantitatively recovered. However the elution profile was very broad and, though marked purification occurred, little concentration was achieved. Later work showed that by using a deposit obtained by centrifugation of the protamine-treated extract, a smaller capacity column could be used and a sharper elution pattern obtained.

Ultracentrifugation. Virus in eluates from hydroxyl apatite columns could be sedimented by ultracentrifugation. In four experiments, high titre eluates were pooled and centrifuged at 90,000*g* for 30 min. and the rotor then stopped with the brake on. The virus formed a firm pellet at the bottom of the tube. About 95 % of the supernatant fluid was removed by Pasteur pipette and the remainder used for resuspending the virus pellet. In four experiments the average recovery of virus was 100 %, 90 % being in the sediment. When such virus was again centrifuged under the same condition the pellet was not adequately dispersed and the recovery of infectivity was appreciably lower. In the technique finally adopted the conditions of centrifugation were changed in order to overcome this difficulty.

Adopted procedure for purification of MVE virus

The choice of a standard procedure was governed not only by the yield of virus and the degree of purification achieved but also by the lability of the purified virus. Because of this it was desirable that concentration should be accompanied by purification; therefore after protamine treatment the virus was concentrated by sedimentation in the ultracentrifuge. This had the advantage of removing much of the soluble protein so that a smaller column of hydroxyl apatite could be used. The complete procedure took about 6 hr., and all steps were carried out between 0 and 4°. Sterile reagents and containers were used.

Sixty freshly-harvested infected mouse brains were ground in 90 ml. BCS and the solution clarified by centrifugation at 2000*g* for 5 min. Ten ml. of a warm saline solution containing 360 mg. protamine sulphate was added with stirring to the supernatant fluid (termed 'original extract'). The mixture was shaken mechanically (oscillation/min. = 100; amplitude = 5 cm.) for 30 min. after which a clear supernatant fluid (protamine-treated extract) was obtained by centrifugation (2000*g*; 10 min.). The protamine-treated extract was centrifuged in a 40-rotor at 93,000*g* for 15 min., the brake being left off to slow deceleration. The virus was seen as a loose milky concentrate at the bottom of the tube with a small clear pellet to one side. The supernatant fluid was carefully removed, leaving about

0·5 ml. in which the virus was readily resuspended with a Pasteur pipette (termed 'deposit 1').

The pooled deposits (about 5 ml.) were applied to a column (2 cm. × 1 cm.) of hydroxyl apatite and the column washed with 10 ml. BCS. The column was then developed with about 5 ml. portions of BCS to which 2M phosphate buffer (pH 7·7) had been added to yield final concentrations of 0·025M, 0·05M, 0·1M, 0·2M, 0·3M, 0·4M and 0·5M phosphate. The flow rate was about 15 ml./hr.

The 0·1M and 0·2M eluates were pooled and centrifuged (93,000*g*, 12 min.). The sedimented virus was then seen as before and was resuspended with a Pasteur pipette in about 0·7 ml. of the supernatant fluid. The products thus obtained were termed 'supernatant 2' and 'deposit 2'.

Evaluation of standard procedure

The standard procedure for purification was applied to nine crude preparations of virus. The various fractions were examined for their % recovery of virus, as estimated by haemagglutination, infectivity, and u.v. absorption. The latter examination also gave some indication of the chemical nature of each fraction.

Recovery of viral haemagglutinin. The original extracts showed haemagglutination in only five of the nine samples; in these five the titres ranged from 2500 to 4000. All this haemagglutinin was recovered between the 0·1, 0·2 and 0·3M phosphate eluates, and in four of the five cases the haemagglutinin was present almost entirely in the 0·1 and 0·2M fractions. There was again a quantitative recovery of haemagglutinin in supernatant fluid 2 and in deposit 2, the distribution between these two fractions being in the ratio 1:3·4. In four out of five experiments, the haemagglutinin titre of deposit 2 was between 2×10^5 and 4×10^5.

In those experiments where it was sought, an inhibitor of haemagglutination was found in some fractions. The inhibitor was not titrated in the classical fashion, but some estimate of its amount was obtained by noting the extent of the prozone in a standard titration of haemagglutinin. This prozone extended to the dilutions 1/200, 1/200 and 1/300 in titrations of three original extracts, with haemagglutinin titres of 3000, 3000 and 4000, respectively. A prozone occurred irregularly in the 0·1M to 0·5M eluates; in those 0·1M and 0·2M eluates where the prozone occurred, it extended to dilutions of 100 to 3000; in the 0·3M to 0·5M eluates, to dilutions of 5 to 30. In five experiments the 'titre' of prozone ranged from 10 to 300 in supernatant 2, and in three experiments from 20 to 80 in deposit 2.

It was felt that if sufficient inhibitor were present it might completely obscure haemagglutinin. This was accepted as the explanation of the absence of detectable haemagglutinin in four of the nine original extracts. Four of the nine 0·025M eluates and five of the 0·05M eluates also lacked haemagglutinin although the infectivity titres were such that considerable haemagglutinin titres would have been expected. Here too the effect was attributed to an excess of inhibitor.

These experiments throw little light on the nature of the inhibitor(s) of haemagglutinin. In one experiment, normal baby mouse brains were extracted and subjected to the standard fractionation procedure. The ability of the fractions to inhibit viral haemagglutination was tested, the haemagglutinin used being a 0·3M phosphate

eluate prepared on the same day. This had a titre of 4000 and was used at a dilution containing 12 A.D. The original extract was found to have an inhibitor titre of 60; none of the other fractions had any activity.

Recovery of infectivity

In nine experiments the ELD50 of the original extract from infected brain had a mean value of 8·2 log. (range 7·6–8·7). In five experiments the average recovery of infectivity in different fractions was as follows: deposit 1, 71 %; effluent, usually less than 0·001 %; eluate 0·025M, 2 %; eluates 0·1M + 0·2M, 70 %; eluates, 0·3M + 0·4M + 0·5M, 6 %; supernatant 2, 5 %; deposit 2, 15 %. Most of the infectivity was recovered in the 0·1M and 0·2M eluates which were therefore chosen for final centrifugation. The greatest loss was during the final centrifugation.

The ratio of ELD50 to haemagglutinin titre was calculated for certain fractions in three of the experiments where haemagglutinin was present in the original extract. Mean values were: the original extract, $3{\cdot}4 \times 10^4$; deposit 1, $1{\cdot}1 \times 10^4$; the 0·025M eluate, $> 20 \times 10^4$; the 0·1M + 0·2M eluates, $1{\cdot}5 \times 10^4$; the 0·3M + 0·4M eluates, $0{\cdot}2 \times 10^4$; deposit 2, $0{\cdot}6 \times 10^4$. The ratio for original extract and for combined 0·1M and 0·2M eluates were similar. The high value for the earlier eluate was attributed to the suppression of haemagglutinin by inhibitor; the low value for the later eluates was attributed to dead haemagglutinating virus, a hypothesis which is supported by the low ratios of ELD50 to u.v. absorption values of these eluates. It was first thought that concentrations of phosphate higher than 0·2M might destroy infectivity but the addition of phosphate to 0·2M eluates was found not to decrease the ELD50 value.

U.v. absorption. In Table 1 are recorded the absorption of fractions from normal and from infected brain extracts at 260 mμ and values of the ratio, absorption at 260 mμ: absorption at 280 mμ. In both cases the values quoted were from single experiments. The results from the experiment with infected brain were typical of 5 experiments. As seen in columns 1 and 2 of Table 1, the normal brain absorbed more than the infected brain extract. The protamine extracts of each preparation absorbed to about the same degree. Deposit 1 derived from the infected extract absorbed more than did deposit 1 from the normal extract, because of the presence of virus in the former. The effluent from each column showed similar degrees of absorption. A substantial difference in the elution profile from the infected column compared with the normal column was first apparent at the 0·1M eluate stage. This and later eluates from the normal column contained negligible amounts of material absorbing at 260 mμ, whereas in the infected column these were the very eluates where there was an accumulation of virus. The peak was in the 0·2M eluate consistently in nine experiments. This effect was seen visually in eluates from the infected column, the 0·2M eluate having a greater opacity than the 0·1M and 0·3M eluates. The other fractions were clear, as were all eluates from the normal column.

Of the substances likely to be present in appreciable amounts in the fractions, only three absorbed well at 260 mμ and 270 mμ: namely, proteins and nucleoproteins and small molecular weight purine and pyrimidine derivatives. For each of these, the ratio 260 mμ:280 mμ lay in a typical range; for proteins, it was less than 1; for nucleoproteins, 1 to 2, and for small molecular weight purine and pyrimidine

Table 1. *Ultraviolet absorption values of fractions from normal and from MVE-infected brains, and the infectivity titres of fractions from infected brains*

Values were from one experiment.

Fraction		Volume (ml.)	OD 260 mμ* Normal	OD 260 mμ* Infected	OD 260 mμ : OD 280 mμ Normal	OD 260 mμ : OD 280 mμ Infected	Infectivity (log_{10})
Original extract		90	23·4	14·4	1·26	1·22	8·3
Protamine extract		—	1·80	1·92	1·83	1·84	8·2
Deposit 1		—	2·44	3·24	1·98	1·58	9·4
Column effluent		—	0·74	0·61	2·15	2·4	4·4
Column eluates (M)	0·025	5	0·097	0·109	1·12	1·07	7·0
	0·05	5	0·091	0·113	0·86	0·83	7·5
	0·1	5	0·037	0·238	0·68	1·34	8·2
	0·2	5	0·037	0·579	0·78	1·38	9·2
	0·3	5	0·01	0·212	—	1·37	8·6
	0·4	5	0·01	0·031	—	—	8·2
	0·5	5	0·01	0·01	—	—	—
Supernatant 2		9·3	—	0·132	—	1·18	8·2
Deposit 2		0·7	—	4·42	—	1·56	9·6

* OD = optical density.

derivatives, it was greater than 2. The latter compounds were not deposited under the conditions of the present experiments. As about 70 % of the absorbing material in the 0·1 M and 0·2 M eluates was sedimented and as the remaining 25 % (supernatant 2) had a ratio of 1·18, it seemed that small molecular weight purine and pyrimidine derivatives were virtually absent; the sedimented material with a ratio of 1·56 was therefore largely nucleoprotein. The value of the 260 mμ:280 mμ ratio of the 0·1 M and 0·2 M eluates was higher in the 'infected' column (average 1·36) than in the control column (average 0·73); this difference was mostly due to nucleoprotein.

Properties of purified virus

The properties of deposit 2 were investigated in four ways: u.v. absorption and studies in the electron microscope indicated substantial purity; the purified virus yielded infective 'RNA', but was less stable to heat than was crude virus.

Ultraviolet absorption spectrum. An average absorption curve was calculated from the values for eight preparations of deposit 2. The resulting curve was typical of nucleoproteins. The value of the ratio, 260 mμ:280 mμ, was $1{\cdot}48 \pm 0{\cdot}06$.

Examination in the electron microscope. Plate 1, a micrograph of deposit 2, shows little else in the preparation but virus particles.

Liberation of infective 'RNA' from purified virus preparations. In three experiments infective 'RNA' was made by phenol treatment of original extract and of deposit 2. For this purpose original extract was diluted 1/10 and the final deposit was diluted 1/1000 in normal baby mouse brain extract before phenol treatment; these dilutions were allowed for in the final calculation. The ratio of virus titre to titre of 'RNA' derived from that virus (V:R ratio) was 2·3, 2·4 and 3·6 log (average 2·8) for the three original extracts and 2·0, 3·0 and 3·4 log (average 2·8) for the three corresponding preparations of deposit 2.

Stability studies. The best diluents for crude virus were earlier found to be NRS (10 %, v/v) and mouse brain extract (Anderson & Ada, 1959) in which the virus was comparatively stable. Both crude virus in the form of original extract and purified virus in the form of deposit 2 were diluted 1/100 in NRS (10 %, v/v) and held at 37°. Infective titres were, for crude virus, at 0 min., 8·1 log; at 15 min., 8·1 log; at 60 min., 8·0 log. For purified virus, at 0 min., 9·0 log; at 15 min., 9·8 log; at 60 min., 8·8 log. This increase in titre after 15 min. was found regularly. In one experiment of this type where infectivity titrations were done in triplicate, the average values for purified virus were at 0 min., 9·2 log; at 7½ min., 9·4 log and at 15 min. 9·6 log. End-points in the titration of samples incubated 30 or 60 min. were very close but appeared to indicate a decrease in titre.

Purified virus was less stable in the absence of NRS. It lost infectivity when held at 37° either undiluted or diluted 1/1 in VCS. Typical figures in the latter diluent were, at 0 min., 8·5 log; 30 min., 7·2 log and at 60 min., less than 6·2 log. However, in this diluent, at 20°, virus lost only 0·4 log of infectivity over a period of 60 min.

DISCUSSION

The outstanding impression from the present work was the ease with which semi-purified or purified MVE virus lost infectivity. It was not clear whether this was a reflexion of an intrinsic instability of pure virus or whether it was due to damage caused by the method of purification. The diluent in which pure virus was least unstable was a 1/10 dilution in normal rabbit serum. When diluted in this medium and held at 37° pure virus showed an initial increase in titre and then a slow decay. The early increase of about 0·5 log may have indicated the resurrection of individual virus particles, but was probably due to the disaggregation of clumped virus. The later decay was attributed to instability of virus.

The instability of purified virus made it difficult to assess virus recovery rates during purification. Through the first part of the purification procedure (from original extract to the combined 0·1 M and 0·2 M eluates) there was an average recovery of 70 % of infective virus. Through the second part of the purification procedure (from eluates to final deposit) infectivity was decreased by 73 % but haemagglutinin was not lost at all, and the u.v. absorption results also pointed to a satisfactory recovery of viral material during this phase. It thus seemed that most of the original virus was recovered in deposit 2, and that the final yield was vitiated only by the instability of the infectivity of the purified virus.

The purity of deposit 2 has been estimated both by electron microscopy and by examination in the ultraviolet spectrophotometer. The results of electron microscopy were more easily interpreted, and the pictures left little doubt that virus-like particles comprised most of the material in deposit 2.

From the u.v. absorption pattern it was not possible to determine accurately the degree of purity, but the results were consistent with the belief that deposit 2 was relatively pure virus. First, a comparison of the u.v. absorption profiles of eluates from infected columns and from normal columns suggested that nearly all of the absorbing material in the 0·1 M and 0·2 M phosphate eluates from the infected column was virus, and the small amounts of soluble protein present in these fractions would have been largely eliminated during the final centrifugation. Secondly, the value

of the ratio, O.D. 260 mμ:O.D. 280 mμ for 8 preparations of deposit 2 was relatively constant (1·48 ± 0·06). These are the wavelengths of maximum absorption of nucleic acid and protein, respectively, and an alteration in the relative concentrations of these two components between one preparation of deposit 2 and another would have resulted in a substantial change in this ratio. Such a change was not observed and the constancy was taken to indicate that deposit 2 contained mainly one type of particle. Thirdly, the absolute value of this ratio (1·48) was consistent with the major component being nucleoprotein in character.

Some estimate of the degree of purification achieved was obtained from the value of the ratio of haemagglutinin to u.v. absorption. This was 700- to 1100-fold higher for deposit 2 than for the crude brain extract. Although the estimate of the u.v. absorption of the crude extract was unduly high because of light scattering effects, it was concluded that the above ratio meant that a better than 500-fold purification had been achieved.

An obvious next step would have been to determine the number of virus particles per infective unit of purified virus preparation. Many such attempts were made in this laboratory, but all were unsuccessful because of the failure to obtain suspensions consisting mostly of single particles for electron microscopy.

It was found earlier that treatment of crude MVE virus with phenol gave rise to infective 'RNA' (Anderson & Ada, 1959). A prime reason for developing a method of purifying MVE virus was to see whether phenol treatment of pure virus liberated 'RNA' and whether the potential 'RNA' in crude preparations was present only in mature virus particles. The average value of the ratio, infectivity of virus: infectivity of 'RNA' (V:R) was 2·8 for both crude and purified preparations. However, the constancy of this ratio may be misleading. It was indicated above that the infectivity of deposit 2 was lower than was expected from its haemagglutinin titre and optical density. As it was not known whether this loss of infectivity was due to damage to protein, lipid or nucleic acid (see Ada & Anderson, 1959) it was difficult to know the meaning of a value of 2·8 for the V:R ratio. For this reason and because of the batch variation in V:R ratios, it is not possible to state whether all the potential 'RNA' in crude virus preparations is contained in mature virus particles. Nevertheless, it is clear beyond reasonable doubt that purified MVE virus yields infective 'RNA'; the figures were not inconsistent with the idea that the potential infective 'RNA' in crude preparations derived from mature virus.

This work was supported by a grant from the National Health and Medical Research Council of Australia.

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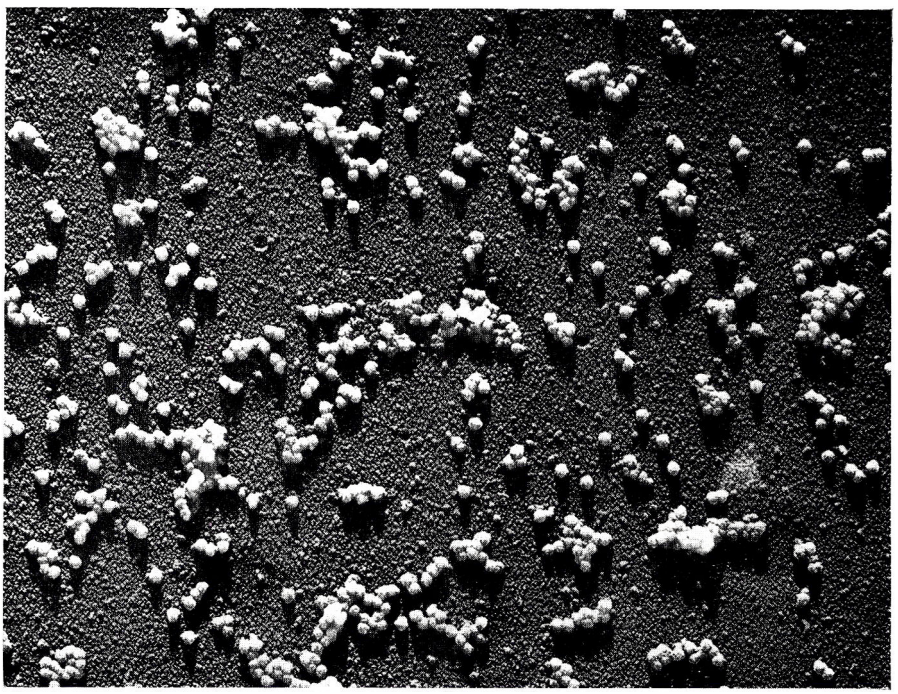
EXPLANATION OF PLATE

Electron micrograph of purified Murray Valley encephalitis virus. Shadowed with gold manganin. Magnification ×40,000.



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(*Facing p.* 186)

J. gen. Microbiol. (1961), **24**, 187–190
Printed in Great Britain

A Strain of *Bacillus circulans* Capable of Growing under Highly Alkaline Conditions

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(*Received* 23 *July* 1960)

SUMMARY

An organism capable of growing at pH values up to 11·0 appeared, presumably as a contaminant, in the course of 'training' experiments designed to produce alkali-resistant strains of *Bacillus cereus* Frankland & Frankland; this organism has now been characterized as *B. circulans* Jordan. Of 26 other strains of *B. circulans* studied, none grew at pH 10·7. The alkali-resistant *B. circulans* strain showed little loss of resistance after many transfers on neutral medium. When it grew in alkaline media it lowered the pH value of these media.

INTRODUCTION

It was reported previously (Kushner & Lisson, 1959) that strains of *Bacillus cereus* Frankland & Frankland, an organism that will not normally grow above pH 9·5, could be 'trained' by successive subcultures in media of gradually increasing pH value to grow at pH 10·3. The training process was a long one, requiring more than 50 transfers during more than a month. In four experiments it was not possible to make *B. cereus* grow at pH values higher than 10·3, even when it was transferred daily in media of this pH value for an additional month. In one experiment, however, the culture, which had been slowly trained to grow at pH 10·0 took on, in five transfers, the ability to grow at pH 11·0. Bacteria removed when the culture had acquired the ability to grow at pH 10·0 appeared, microscopically and in colonial form, like the alkali-resistant *B. cereus* described by Kushner & Lisson (1959). Those removed after the culture grew at pH 10·7 and subcultured on neutral or alkaline buffered agar, however, were quite distinct from *B. cereus* in that the rods were thinner than those of *B. cereus* and, on agar, formed small translucent colonies. This organism has now been kept for more than 2 years by transfer on neutral or alkaline buffered nutrient agar. It is not considered likely that it was derived from *B. cereus*, but it is thought to be a contaminant which entered during the training procedure. Because of its ability to grow under highly alkaline conditions, it was further investigated.

METHODS

The conditions of growth for the experiments reported in Fig. 1 were those described earlier (Kushner & Lisson, 1959). Phosphate-buffered nutrient broth (BNB) and phosphate-buffered nutrient agar (BNA), final pH 7·4, were prepared

by dissolving dried nutrient broth and dried nutrient agar (Difco) in 0·1 M phosphate buffer (pH 7·5) to give a medium of final pH 7·4. Alkaline media were prepared by the addition of sterile N-NaOH, according to previously determined titration curves. For examining this organism, the methods and media used were similar to those of Smith, Gordon & Clark (1952) and Knight & Proom (1950). Tests were made on BNA (pH 7·4 and 10·7); BNB (pH 7·4 and 10·7); in routine laboratory media (pH 7·0); in media made up in 0·1 M-phosphate buffer (pH 7·5). Incubation was at 37°, except where otherwise stated.

Spore sculpture. The surface configuration of spores was kindly examined for us by Mr D. E. Bradley by the electron microscope and the carbon replica method of Bradley & Williams (1957).

RESULTS

Morphological characters of the highly alkali-resistant organism

The organism conforms in all respects to the description of *Bacillus circulans* given by Smith *et al.* (1952). No difference was found in the morphology on BNA (pH 7·4) and nutrient agar (pH 7·0). On BNA (pH 10·7) at 24 hr. the rods were slightly thinner and longer than on BNA (pH 7·4). The swollen sporangia and oval spores were similar to those formed on BNA (pH 7·4).

Spore sculpture. The sculpture of the spore surface was found by Mr D. E. Bradley by using the electron microscope and carbon replica method of Bradley & Williams (1957) to be smooth.

Growth characters

No difference was obtained from the description of *Bacillus circulans* by Smith *et al.* (1952) apart from the inability to grow anaerobically. No growth was obtained on the surface of nutrient agar and BNA + glucose (1 %, w/v) slopes or streak plates in McIntosh and Fildes jar (aerobic controls positive), and surface growth only in BNA and nutrient agar + glucose (1 %, w/v) shake tubes. The organism appears therefore to be an obligate aerobe. Colonial form and amount of growth appeared similar on BNA (pH 7·4) and BNA (pH 10·7).

Physiological characters

The organism conforms to the description of *Bacillus circulans* by Smith *et al.* (1952) apart from the inability to produce acid from glucose. No growth was obtained on inorganic salts basal medium + sugar up to 7 days at 30° or 37°. Growth but no acid was obtained on nutrient agar slopes with bromcresol purple indicator + glucose, xylose, arabinose, sucrose, mannitol, maltose or raffinose after 7–14 days at 30° or 37°.

Nutrition. The nutritional requirements of this organism were satisfied by a medium containing salts, acid-hydrolysed casein (Knight & Proom, 1950) thiamine and biotin. An ammonia basal medium (Knight & Proom, 1950) did not replace the casein basal medium.

Examination of strains of Bacillus circulans *for alkali resistance*

The above evidence indicated that this organism could be classified as a strain of *Bacillus circulans* Jordan. A collection of 26 strains of *B. circulans* from different sources was therefore examined to assess the occurrence of alkali resistance in this

species. Fourteen strains from the Wellcome Research Laboratory collection (Knight & Proom, 1950) and 12 isolated from soil (by M.E.C.) were tested for ability to grow in alkaline media. The cultures were maintained routinely on nutrient agar (pH 7·0) and were subcultured several times in BNB (pH 7·4) at 30° and 37° before testing.

Cultures (24 hr.) in BNB (pH 7·4) at 30° and 37° were inoculated by adding two 4 mm. loopfuls to BNB at pH 7·4 and 10·7. Alkali resistance was indicated by growth in BNB at pH 10·7; results were recorded after growth for 24 hr. at 30° and 37°. None of the 26 strains tested grew in BNB at pH 10·7 in 24 hr.; with the exception of one strain (CN3378), all grew within 24 hr. in BNB at pH 7·4.

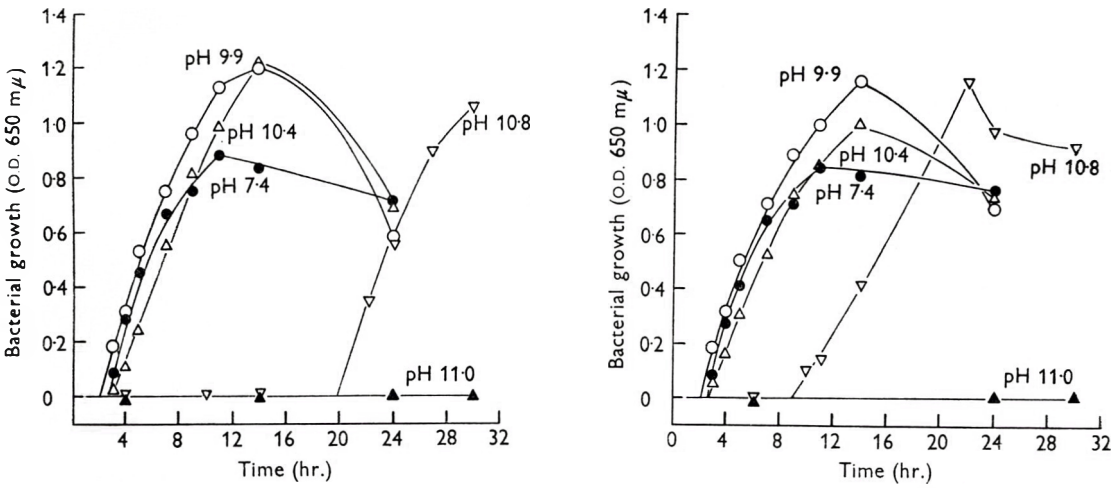

Fig. 1 *a*, *b*. The effect of pH value on growth of alkali-tolerant *Bacillus circulans*. *a*, Bacteria maintained for 1 year at pH 7·4; *b*, bacteria maintained for 1 year at pH 10·7. Bacteria were grown for 20 hr. on BNA of pH 7·4 (*a*) or 10·7 (*b*), resuspended in BNB (pH 7·4) and an inoculum equiv. 0·025 mg. dry wt. bacteria in 0·3–0·5 ml. broth added to tubes containing 10 ml. BNB of the pH values shown. Incubation, shaking, and measurement of optical density were as described by Kushner & Lisson (1959).

Growth at different pH *values and the retention of alkali tolerance*

The effect of the pH value of the medium on the growth of this organism in BNB is shown in Fig. 1. Comparison was made between bacteria which had been maintained for 1 year on BNA (pH 7·4) with transfers at 2-week intervals (Fig. 1 *a*) and bacteria which had been similarly maintained at pH 10·7 (Fig. 1 *b*). Both cultures grew after a slightly shorter lag period at pH 9·9 than at pH 7·4. In the highly alkaline media in which growth occurred, cultures reached a higher density than they did at pH 7·4. A 1-year long transfer on BNA of pH 7·4 had caused little loss of alkali resistance, the only difference between the two cultures appearing at pH 10·8, where the bacteria maintained at pH 7·4 grew after a lag period 11 hr. longer than did those maintained at pH 10·7. When first isolated, these bacteria grew in shaken cultures of pH 11·0. Two years later, at the time of the experiment shown in Fig. 1, they still grew on BNA at pH 11·0 but did not grow in shaken BNB of this pH value. In this, they resemble alkali-resistant *B. cereus* (Kushner & Lisson, 1959), which grew at a slightly higher pH value on agar than in liquid medium.

In the late stages of incubation with shaking, there was a decrease in the optical density of all cultures. No clumping of the bacteria was observed, and the culture became viscous, indicating that lysis was probably taking place. As with alkali-resistant *Bacillus cereus*, the growth of the present organism in alkaline media lowered the pH value. After incubation for 30 hr. the pH value of the culture which was originally at 7·4 had increased to 7·8, and the pH values of all other cultures had decreased to 8·8–9·0.

DISCUSSION

The swollen sporangia and oval spores produced by this alkali-resistant organism indicate that it is a *Bacillus* sp. of morphological group 2 (Smith *et al.* 1952). The results of the physiological tests classify the organism as a strain of *Bacillus circulans*. Inability to produce acid from glucose and to grow anaerobically is, however, not typical of *B. circulans*, although atypical reactions of this kind are not uncommon with strains of this species-complex. The smooth surface sculpture of the spores is similar to that found in some strains of *B. circulans*, although other strains of this species may show longitudinal parallel ribs or reticulation (Bradley & Franklin, 1958). The nutritional requirements of this strain are also consistent with those found by Knight & Proom (1950) for some strains of *B. circulans* and for *Bacillus* species in general. Since alkali resistance was not found in 25 other strains of *B. circulans* the high resistance shown by this particular strain is, thus far, unique.

The alkali-resistant *Bacillus circulans* strain has been sent to the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.

We are indebted to Mr D. E. Bradley (A.E.I. Research Laboratories, Aldermaston Court, Berkshire) for the examination of the spore sculpture by the carbon replica method, and to Professor B. C. J. G. Knight for advice during part of this investigation. Mr T. A. Lisson provided valuable technical assistance during the early stages of this work. This paper is contribution no. 653, Forest Biology Division, Research Branch, Department of Agriculture, Ottawa, Canada.

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J. gen. Microbiol. (1961), **24**, 191–200
Printed in Great Britain

The Effect of Oxygen on Freeze-dried *Escherichia coli*

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SUMMARY

When *Escherichia coli* organisms were suspended in distilled water and freeze-dried the maximum loss of viability did not occur during the drying process proper, but during the time of contact of the dried organisms with air between the primary and the secondary drying periods. By substituting other gases for air at this stage, it was proven that oxygen was the active agent involved. The dried organisms which were exposed to different pressures of air and oxygen at different temperatures proved to be extremely sensitive to traces of oxygen, even at very low temperatures. The implications of this oxygen effect in connexion with existing freeze-drying procedures, as well as some preliminary kinetic experiments concerning the shape of the survival curve, are discussed.

INTRODUCTION

High viabilities of bacterial suspensions after freeze-drying can usually be obtained only when the organisms are suspended in quite complex media during lyophilization. A survey of the literature (see, for example, Harris, 1954) shows that the discovery of these media has usually beeen a 'hit and miss' affair, and only in exceptional cases was an attempt made to rationalize the success of a certain medium (Fry & Greaves, 1951; Naylor & Smith, 1946). The lethal effect of freezing and drying on bacteria is indeed only very poorly understood, if at all. Studies of the nature of the damage caused by freezing different micro-organisms (Mazur, 1959; this article gives references to most of the current work) have failed to prove that freezing causes the principal or even any important damage during the process of lyophilization. Organisms which withstand freezing well may be killed by lyophilization when not protected by a suitable medium. In addition to freezing, there appeared therefore to exist one or more lethal stages in the drying process itself. An attempt to determine the viability of the bacteria, not only after the completion of the freeze-drying process, but also during its different stages, has apparently been made only once (Fry & Greaves, 1951) and then, as will be shown further below, not under the most suitable conditions.

Most of the work dealing with lyophilization so far has been concerned with finding a medium in which the highest viability of the freeze-dried bacteria could be obtained. Under these circumstances it would be difficult to detect a phase of maximum killing, even if it existed, since when the overall viability of the freeze-dried bacteria remained comparatively high during the whole process, the statistical fluctuations in the viability determinations would be large enough to obscure a

decrease in the viability during any one of the stages of freeze-drying. If one carries out the lyophilization of bacteria, which are resistant to freezing, under conditions under which high mortality is to be expected in the drying process, it should be possible to find out whether the mortality in this process is gradual, or whether it is confined to a single defined stage of the drying. As it has long been known (Fry, 1954; and confirmed by us) that freeze-drying of *Escherichia coli* in distilled water yields very poor results, we used this method to get high mortality in the lyophilization process. Moreover, freeze-drying from distilled water would leave the dried bacteria free from the components of the medium, and the results would be likely to be more generally valid. As the test organism has to be washed with, and suspended in, distilled water, the bacterium should be resistant to osmotic shock: *E. coli* has this property.

METHODS

Organism. A locally isolated strain of *Escherichia coli*, E/65, was used, unless otherwise indicated.

Culture methods. Organisms grown on freshly prepared nutrient agar slopes for 24 hr. at 37°, were washed off with, and re-suspended in, sterile nutrient broth to a concentration of approximately 10^7 organisms/ml. This suspension was used to inoculate Roux bottles containing Difco nutrient broth solidified with about 2·5% (w/v) agar. After incubation for 20 hr. at 37°, the organisms were washed off into distilled water, sedimented at 20,000 *g*, washed well with distilled water and then re-suspended in the same medium. This procedure was repeated. Finally, the organisms were resuspended in distilled water to a concentration of $1{\cdot}5–2{\cdot}0 \times 10^{11}$ organisms/ml. This suspension, from a sample of which a viable count was made, was then used for the lyophilization experiments.

Viable counts. Counts were performed by diluting a sample in gelatin buffer (0·5%, w/v, gelatine in M/15 phosphate buffer, pH 6·8). Portions (0·5 ml.) of the appropriate dilutions were mixed with melted nutrient agar at 44° in Petri dishes. At least three dishes were used at each dilution. After the agar had solidified, the dishes were inverted and opened on wire screens in a forced-air incubator for 15 min. This procedure completely prevented spreading of colonies on the agar surface as well as between the agar and the bottom of the dish. The dishes were then closed and incubated for 48 hr. at 37°, and the colonies counted. Dilutions were selected so as to obtain between 50 and 500 colonies/dish.

Lyophilization

Apparatus. The apparatus used was of the simple manifold type. The ampoules were connected through high-vacuum rubber tubing to the manifold, the diameter of which was large enough to offer only negligible resistance to vapour flow. The condenser consisted of a stainless steel Dewar-type flask and was cooled by a solid CO_2+ethanol mixture. By a single stage rotary oil pump, a pressure of less than 10μ Hg was produced; the pump was protected from water vapour by a phosphorus pentoxide trap. The pressure in the system was measured by a Pirani gauge connected to the system through a water vapour trap. Air which was bled into the system after lyophilization, was dried by passing it through sulphuric acid,

soda lime and through a small-bore long U-tube immersed in a solid CO_2 + ethanol mixture.

The secondary drying system, which was used in part of our experiments, consisted of a long large-bore horizontal cylinder which contained phosphorus pentoxide on stainless steel trays. Ampoules were connected to this system through manifolds. This system was evacuated by a double stage rotary oil pump to a pressure less than $0{\cdot}5\mu$ Hg.

Lyophilization procedure. The bacterial suspensions were distributed in 1 ml. amounts into (neutral) glass ampoules (outside diameter 15 mm.; length 50 mm.). The contents of the ampoules were quick-frozen in a solid CO_2 + ethanol mixture and stored overnight at a temperature not exceeding $-70°$. This storage did not affect the viability of the suspensions. The ampoules to be lyophilized were connected to the manifold while still immersed in the freezing mixture until a vacuum of at least 50μ Hg was reached in the drying system. When this pressure had been obtained, the freezing mixture was removed and drying continued, the heat of sublimation being supplied by the surrounding atmosphere. Primary drying, as judged by the disappearance of moisture from the outside of the ampoule, was completed after 20–30 min. By that time the contents of the ampoule had been converted into a fluffy powder. The disappearance of moisture from the outside of the ampoule as criterion of dryness is only a very crude indication and depends among many other factors on the outside temperature and dew point. The drying was, therefore, continued for at least another 60–90 min. When secondary drying was required, dry air was bled into the system, then the ampoules were disconnected from the manifold, their necks drawn out in a small flame, and the ampoules were connected as soon as possible to the secondary drying system. After completion of the secondary drying the ampoules were sealed under vacuum.

For viable counts, the contents of the ampoules were reconstituted by adding gelatin + buffer immediately after opening the sealed ampoules, and the counts performed as soon as possible (never later than 20–30 min. after reconstitution, the material being kept meanwhile in the refrigerator).

RESULTS

To ascertain the point at which the bacteria were killed in the freeze-drying, viability curves were taken at every stage of the freeze-drying of a suspension of *Escherichia coli* in distilled water (freezing, primary drying, admitting dry air into the ampoules, drawing out and constricting the necks of the ampoules, secondary drying, sealing in vacuum). The counts were performed at intervals as short as technically feasible. The contents of three ampoules were reconstituted and their volume adjusted to that of the original sample. Table 1 represents the protocol of a typical experiment; the results are summarized in Fig. 1.

No significant change in viability occurred after freezing and storing in the frozen state, and only a small and gradual drop was found during the primary drying; at the end of that period 50% of the original population was still viable, although lyophilization was carried out in distilled water. On the other hand, a sudden drop in viability occurred between the end of the primary and the beginning of the secondary drying. During the secondary drying itself, there occurred only a small and gradual loss of viability.

Table 1. *Viability of* Escherichia coli *during lyophilization from distilled water*

Time from start of drying (min.)	Manipulation	Viable organisms/ml. Sample 1	Sample 2	Sample 3	Average	Relative viability (%)
—	Before freezing	$1{\cdot}2 \times 10^{11}$	$1{\cdot}2 \times 10^{11}$	$1{\cdot}3 \times 10^{11}$	$1{\cdot}3 \times 10^{11}$	100
0	After 20 hr. at −70° drying starts	$1{\cdot}2 \times 10^{11}$	$1{\cdot}1 \times 10^{11}$	$1{\cdot}2 \times 10^{11}$	$1{\cdot}2 \times 10^{11}$	92
15	—	$1{\cdot}2 \times 10^{11}$	$1{\cdot}2 \times 10^{11}$	$1{\cdot}1 \times 10^{11}$	$1{\cdot}2 \times 10^{11}$	91
30	—	$1{\cdot}1 \times 10^{11}$	$1{\cdot}0 \times 10^{11}$	$9{\cdot}8 \times 10^{10}$	$1{\cdot}0 \times 10^{11}$	78
45	—	$9{\cdot}8 \times 10^{10}$	$9{\cdot}4 \times 10^{10}$	$1{\cdot}1 \times 10^{11}$	$9{\cdot}8 \times 10^{10}$	77
75	—	$7{\cdot}9 \times 10^{10}$	$9{\cdot}0 \times 10^{10}$	$8{\cdot}7 \times 10^{10}$	$8{\cdot}5 \times 10^{10}$	67
110	End of drying; air admitted	$7{\cdot}8 \times 10^{10}$	$8{\cdot}1 \times 10^{10}$	$5{\cdot}0 \times 10^{10}$	$6{\cdot}3 \times 10^{10}$	50
160	Secondary drying starts	$9{\cdot}7 \times 10^{9}$	$6{\cdot}4 \times 10^{9}$	$6{\cdot}4 \times 10^{9}$	$7{\cdot}8 \times 10^{9}$	6·1
215	—	$5{\cdot}8 \times 10^{9}$	$4{\cdot}2 \times 10^{9}$	$3{\cdot}8 \times 10^{9}$	$4{\cdot}6 \times 10^{9}$	3·6
355	End of secondary drying	$5{\cdot}8 \times 10^{9}$	$6{\cdot}1 \times 10^{9}$	$3{\cdot}7 \times 10^{9}$	$5{\cdot}2 \times 10^{9}$	4·1

The high mortality between primary and secondary drying may be ascribed to either of the following causes: (1) the change of pressure experienced by the organisms, when they were brought from vacuum to atmospheric pressure; (2) the effect of air *per se*. The rise in temperature of the dry powder during the constriction of the neck of the ampoule had no influence on the process.

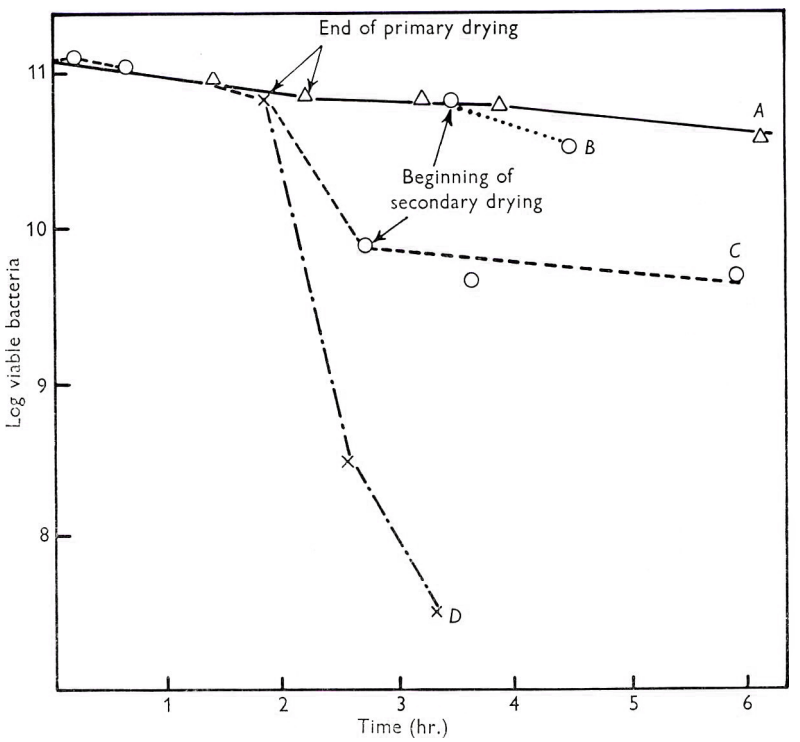

Fig. 1. Viability of *Escherichia coli* suspended in distilled water during the lyophilization procedure. The organisms were exposed to different atmospheres between the primary and the secondary drying: *A*, in vacuum; *B* in hydrogen; *C* in air; *D* in oxygen.

Organisms dried from distilled water were exposed to different gases at atmospheric pressure and at 28°. As controls, organisms were kept under vacuum for the same time and temperature. In view of the above results, the secondary drying was omitted. The results for hydrogen, air, oxygen, and vacuum for *Escherichia coli* E/65 are given in Fig. 1, and for oxygen, air and vacuum for *E. coli* B/r in Fig. 2. The results for these two organisms were similar, except that the B/r strain appeared to be somewhat less sensitive. The viability counts for nitrogen were puzzling at first: the mortality was quite high as compared to hydrogen, although much lower than for air. Experiments showed that the purification of commercial nitrogen by

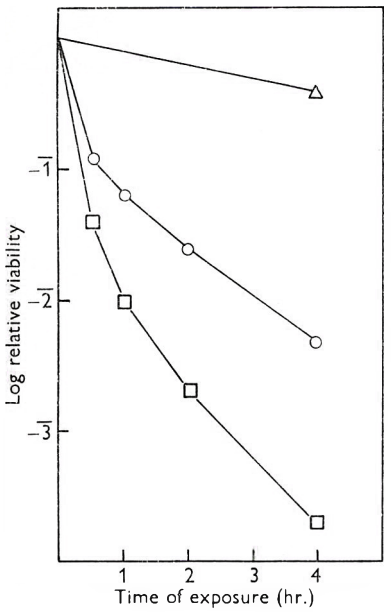

Fig. 2

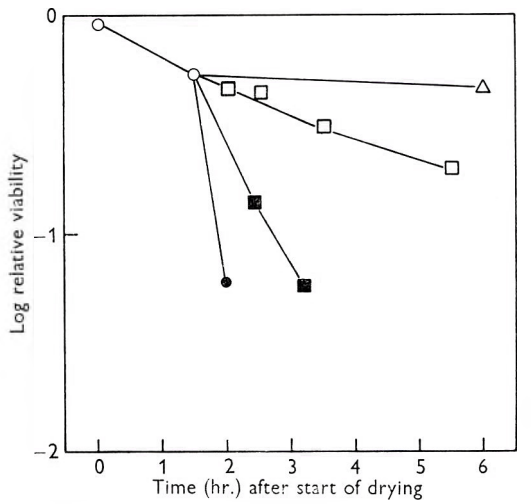

Fig. 3

Fig. 2. Exposure of *Escherichia coli* B/r, dried from distilled water to vacuum, air or oxygen at 28°. △, vacuum; ○, air; □, oxygen.

Fig. 3. Viability of *Escherichia coli* dried from distilled water and exposed to nitrogen 'purified' from oxygen by different methods. △, vacuum; ●, air; □, nitrogen purified by Fieser's solution; ■, nitrogen purified by alkaline pyrogallol.

alkaline pyrogallol (5%, w/v, pyrogallol in a 50%, w/v, aqueous solution of potassium hydroxide) was not efficient. When Fieser's solution (Fieser, 1924; 16%, w/v, $Na_2S_2O_4$, 4%, w/v, sodium β-anthraquinone-sulphonate in a 14%, w/v, aqueous solution of sodium hydroxide) was used, better results were obtained. They are summarized in Fig. 3.

As the efficiency of the removal of oxygen from the nitrogen increased the viability of the dried suspensions exposed to nitrogen increased. On the other hand, even Fieser's solution did not seem to absorb all traces of oxygen under the conditions used by us. As will be shown presently, the suspensions dried from distilled water are extremely sensitive even to minute traces of oxygen.

Effect of changes in the pressure of oxygen and in the temperature of exposure

Figure 4 shows that the viability of the dried suspension exposed to oxygen for 3 hr. at room temperature depended on the oxygen pressure. The extreme sensitivity of the suspensions to quite low oxygen pressures was pronounced; about 95% of these organisms were killed in 3 hr. by oxygen at a pressure as low as 10 mm. Hg. (In these experiments part of the pressure range was covered by exposure to air, part by exposure to oxygen. Contrary to expectations, in the overlapping region of these two curves the results were not identical; it is not clear at present what caused the discrepancy.)

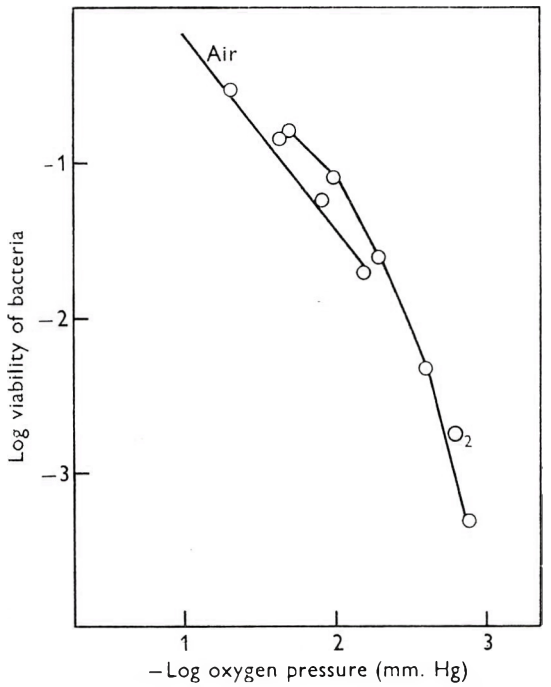

Fig. 4. Viability of *Escherichia coli* dried from distilled water and exposed to air and oxygen at different pressures for three hours at room temperature.

Figure 5 describes the viability of the dried suspensions when exposed to air at atmospheric pressure for 3 hr., but at different temperatures. Although the reaction between oxygen and the dried suspension was highly temperature-dependent, the mortality of the dried suspensions exposed to oxygen was easily measurable even at temperature as low as −30°. When the exposure was prolonged beyond 3 hr., killing was evident at even lower temperatures.

Shape of the survival curves

As seen in Fig. 2, the plot of the logarithm of the survival in dried suspensions exposed to air or oxygen against time of exposure yielded a curve which is concave upwards. This may have been due to a consumption of oxygen and corresponding

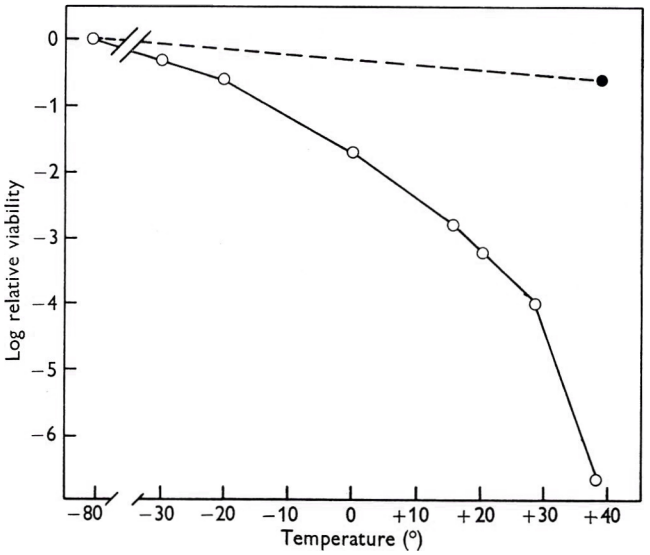

Fig. 5. Viability of *Escherichia coli* dried from distilled water and exposed to air at atmospheric pressure for three hours at different temperatures. ●, control in vacuum; ○, exposed to air.

fall in its pressure, which in turn would cause a slowing down of the reaction rate. That this is not the case is shown in Table 2, in which two conditions are compared: in one case the organisms were exposed to the normal volume of oxygen, while in the other a volume about twenty times as large was used. The results were not significantly different. In another experiment described in Table 3 different amounts of organisms were exposed to the same volume of oxygen. The results show that in

Table 2. *Viability of* Escherichia coli *freeze-dried from distilled water and exposed to different volumes of air at atmospheric pressure at* 28°.

Original density of suspension $1{\cdot}2 \times 10^{11}$/ml. Each figure represents the average of three samples.

Volume of air (ml.)	Period of exposure to air (min.) 30	120	240
	Viability (%)		
10	8·0	0·7	0·05
200	5·8	0·4	0·06

Table 3. *Viability of* Escherichia coli *freeze-dried from distilled water at different concentrations of suspension, and exposed to air at atmospheric pressure at* 28°.

Original concentration of organisms	Period of exposure to air (min.) 60	120	240
	Viability (%)		
$1{\cdot}5 \times 10^{11}$/ml.	1·1	0·3	0·03
$1{\cdot}5 \times 10^{10}$/ml.	1·4	0·5	0·03

this case the relative inactivation of the suspensions was independent of the original concentration of organisms. We see, therefore, that on the one hand the organisms were inactivated independently of each other, and on the other that the concentration of the inactivating agent was not rate-limiting under the conditions of our experiment. One would, therefore, expect an exponential survival curve. This point is discussed below.

DISCUSSION

The fact that oxygen is deleterious to lyophilized bacteria has been recognized for a long time (Rogers, 1914); for this reason freeze-dried substances are generally stored under vacuum or in inert gases. In some instances authors have tried to decrease the mortality in lyophilized material by adding anti-oxidants to the suspending medium (Naylor & Smith, 1946); however, it is difficult to see how anti-oxidants are able to counteract the oxidation by traces of air in the dry state.

Experiments carried out to determine the effect of different gases on freeze-dried bacteria (Naylor & Smith, 1946) demonstrated the lethal effect of air. This effect was a slow one and could be measured only after extended periods of storage; a 90% decrease in viable count was found for *Serratia marcescens* after storage in air for 48 days, as compared with an insignificant change when stored under vacuum. Interestingly enough, a decrease in viable count of 70–75% was found when these organisms were stored under tank nitrogen, or under nitrogen 'purified' (from oxygen) by alkaline pyrogallol. Similarly, unsatisfactory results with dry nitrogen were reported by Collier (1954). These results are not surprising in the light of the work described here.

Much more attention has usually been paid to the exclusion of moisture from the freeze-dried material than to the prevention of contact with air. Fry & Greaves (1951) sealed their ampoules under vacuum, rather to exclude unknown amounts of moisture than to exclude traces of air. Swift (1937), Stillman (1941), and Morton & Pulaski (1938) used dry air as storage atmosphere for their material, but this practice is no longer accepted (Fry, 1954). Meryman (1959) even suggested drying frozen material by passing dry air over it, instead of using the conventional vacuum. It seems, therefore, that the effect of oxygen on dried cells has been generally underestimated. In our work two facts have come to light. (1) It is possible to obtain a high survival in suspensions freeze-dried from distilled water, i.e. without any protective medium, when these suspensions are carefully protected from traces of oxygen. This is true at least in the case of *Escherichia coli*, which is resistant to hypotonic shock and to freezing in distilled water. (2) The failure reported by other workers in the lyophilization of suspensions from distilled water is due to the extreme sensitivity of the organisms to air. In the process of primary drying the neck of the ampoule should have a bore as large as possible. Since it is very difficult to seal such a wide neck under vacuum, the usual procedure is to let dry air into the system after the primary drying and to constrict the neck of the ampoule before the secondary drying. It is seen now that under these conditions drying of organisms from distilled water would not be successful. Substituting commercial nitrogen (even when 'purified' by conventional methods) for air between the primary and secondary drying, would not help very much, unless it is freed from oxygen by highly efficient reagents. Naylor's experiments (Naylor & Smith, 1946), mentioned

above, which demonstrated only a much smaller effect of air on freeze-dried suspensions, were performed on bacteria which were embedded in the drying medium devised by these workers. This would suggest that their medium contained a substance protecting the dried organisms from oxygen. This has indeed been proven to be the case (Lion & Bergmann, unpublished).

Although careful exclusion of air prevented the death of most of the *Escherichia coli* organisms freeze-dried from distilled water, yet 50% were killed during the primary drying. This might be due to the traces of air diffusing through imperfect connexions of a vacuum system. Some air is also liberated during evacuation from the inner walls of the system, and furthermore, the small bubbles of air originally dissolved in water become trapped in the ice when the suspension is frozen and are liberated during freeze-drying when the ice evaporates. Although these traces of air may account fully for the loss of viability during the drying process, other causes of mortality are by no means excluded and, furthermore, the death of organisms during storage of freeze-dried material may occur even in the complete absence of oxygen. Complete absence of even traces of oxygen is extremely difficult to achieve, and these traces may have a long time in which to react with the organisms.

Some remarks about the shape of the survival curve depicted in Fig. 2 may be in order; its form is rather difficult to interpret kinetically. In our experiments oxygen was in excess. On the other hand, the relative inactivation rate seemed to be independent of the initial density of the suspension of organisms; thus no interaction between the organisms seemed to occur. Under these conditions, a first-order inactivation curve would be expected for a homogeneous population. As this was not the case, some heterogeneity must be present. The hypothesis that part of the population is genetically more resistant to oxygen is highly unlikely, since no mutant more resistant to freeze-drying (which nearly always includes such an oxygen effect) than the original population has ever been isolated. A metabolic or physiological heterogeneity cannot be excluded, although in our case it was not very probable, since the organisms were harvested long after the completion of the log phase and very carefully washed with distilled water before use. In general, our curve resembles very much the inactivation curve of poliomyelitis virus by formaldehyde. An analogous model to that proposed by Gard (1957) for this virus might be formulated with some justification. We believe, however, that more detailed kinetic studies should first be performed. The similarity between the inactivation curves of dried bacteria by oxygen and of virus by formaldehyde leads us to expect that the lethal effect of oxygen on unprotected freeze-dried bacteria may be utilized to prepare vaccines from killed bacteria. This method is now under investigation.

This paper forms part of a thesis submitted to the Hebrew University (by M.B.L.) in partial fulfilment of the requirements for the degree of Ph.D.

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J. gen. Microbiol. (1961), **24**, 201–206
Printed in Great Britain

Inter-species Change in Thiobacilli

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(*Received* 2 *August* 1960)

SUMMARY

Inter-species conversion has been observed in thiobacilli, starting from isolates of single bacilli.

INTRODUCTION

Since the early studies on the thiobacilli by Nathansohn (1902) and Beijerinck (1904) work has been directed mainly to the elucidation of their metabolic pathways and their classification. During an investigation into the organisms responsible for the oxidation of sulphur compounds in gas works effluents, an attempt was made to classify the bacteria concerned. It soon became apparent that the classification given by Parker (1957) was inadequate, largely because most of the species had been studied by different investigators using different media and criteria. Several species were therefore obtained from the National Collection of Industrial Bacteria (NCIB) for comparative purposes. A note on the anomalous behaviour of *Thiobacillus thioparus* NCIB 8370 was published by Townshend & White (1960); this paper is concerned with the reason for this discrepancy.

METHODS

Organisms. Two of the organisms used were very similar, and as they closely resemble *Thiobacillus neapolitanus.* they will be referred to as 'neapolitanus type'. These organisms were: 'G' which was isolated from a laboratory activated sludge unit treating thiocyanate, and no. 5 which was given by Miss Adams of the National Chemical Laboratory. The other culture used, NCIB 8370, will be referred to in the paper as 8370.

Culture media. Oxoid Nutrient Agar was used for the nutrient media, and Oxoid Ion Agar no. 2 for the inorganic media, both obtained from Oxo Ltd. London. The various inorganic media used are given in Table 1. Medium S8 was used for anaerobic culture in a McIntosh and Fildes jar.

Stock cultures. These were maintained on S2 agar slopes at 10° and subcultured every 3 weeks. All incubations were at 30°.

Single organism isolations. These were carried out on the surface of an agar block, using a modification of the technique described by one of us (Johnstone, 1953) with phase contrast illumination. Portions of the block, each carrying a single organism, were transferred to 10 ml. amounts of the S2 medium in 50 ml. conical flasks.

Table 1. *Composition of inorganic culture media (g./l. distilled water)*

Compound	Media				
	S1	S2	S5	S7	S8*
$Na_2S_2O_35H_2O$	10·0	10·0	10·0	—	10·0
NH_4CNS	—	—	—	0·2	—
$Na_2HPO_412H_2O$	2·0	4·0	—	3·0	3·0
KH_2PO_4	—	4·0	2·0	1·8	1·8
$MgSO_27H_2O$	0·1	0·1	0·1	0·1	0·1
$(NH_4)_2SO_4$	0·1	0·1	0·1	0·1	0·1
$CaCl_2H_2O$	0·1	0·1	0·1	0·1	0·1
KNO_3	—	—	—	1·0	5·0
Na_2CO_3	0·2	0·2	0·2	0·2	0·2
$FeCl_36H_2O$	0·02	0·02	0·02	0·02	0·02
$MnSO_44H_2O$	0·02	0·02	0·02	0·02	0·02
pH	7·8	6·7	5·2	6·7	6·7

* Anaerobic.

RESULTS

Experiments with organism 'G'

In the preliminary isolation of organisms from the activated sludge unit, a pure culture was obtained by repeated single colony isolation; it closely resembled *Thiobacillus neapolitanus* but differed in showing a variable ability to decrease the pH value. Organism G was a strictly aerobic autotroph, which decomposed only thiosulphate media, and gave rise to a 'w' colony type (Table 2). Stock cultures showed colony variants within 2 months. These consisted of two colony types, one a round colourless colony not readily distinguishable from 'G' except that no sulphur was deposited, and a very thin flat colony with a crenate edge. One or both of these colonies appeared each time 'G' was re-isolated, but no other colony types were observed. Both these organisms grew on nutrient agar, the round colony type gave rise to a round greyish white colony, and the crenate type to a round opaque grey colony, around which the medium became brown after 7 days. Pure cultures of these organisms grew in thiosulphate liquid medium, but whereas the round colony type used the thiosulphate slowly, the breakdown of thiosulphate by the crenate organism was negligible. Attempts to obtain these variants from the laboratory atmosphere by exposing both liquid and solid media were unsuccessful. Although a mould grew in the liquid medium, no thiobacilli or the colony types described above were ever found.

At this time a culture of *Thiobacillus thioparus* NCIB 8370, was obtained, which gave unexpected results (Table 2), and an attempt was made to obtain a pure culture of *T. thioparus* from it, by means of single cell technique.

First series of isolation experiments

In the first series of isolations 8370 was grown on S2 liquid medium and the bacteria isolated during the log phase. The isolates were transferred to the S2 medium, and subcultured thence to various test media (Table 2). As may be seen from Table 2, seven of the twelve isolates grew, five of those within 5 days and a further two (I.7 and I.12) after 2 weeks. Four types of bacteria were dis-

Table 2. *Character of the isolates from NCIB* 8370 (*series* 1)

Isolate no.	Loss of sulphur compound in liquid media				Growth on agar plates (2% agar)					Colony* type
	S1	S2	S5	S7†	S1	S2	S5	S8‡	Nutrient	
I.4	+	+	+	+	+	−	+	−	−	w
I.6	−	−	−	−	+	+	+	+	+	r
I.7	+	+	+	+	+	−	+	+	−	w
I.8	−	−	−	−	+	+	+	+	+	r+c
I.9	+	+	+	+	+	+	+	+	+	r+c+w
I.10	+	+	+	+	+	+	+	+	+	r+w
I.12	+	+	+	+	+	−	+	+	−	w
Original 8370	+	+	−	−	+	+	−	+	+	w

* Colony types: w = round colony which deposited sulphur; r = round colony which did not deposit sulphur; c = crenate-edged colony which did not deposit sulphur.
† Thiocyanate. ‡ Anaerobic.

tinguishable by the test media used, I.4 ('w' colony type, strict aerobe) I.6 ('r' colony type, facultative anaerobe) 1.7 ('w' colony type facultative anaerobe), crenate organism ('c' colony type, strict aerobe). The distribution of the three colony types on various solid media (Table 3) shows clear differences between the four organisms. I.4 and I.7 were strictly autotrophic thiobacilli with similar colonial appearance, which decomposed thiosulphate and thiocyanate, but which differed in I.7's ability to grow anaerobically. I.6 was a heterotroph and a facultative anaerobe, which decomposed thiosulphate slowly. The crenate colony type was strictly aerobic and a heterotroph.

Table 3. *Colony types appearing on the agar plates* (*Series* 1)

Isolate no.	Media: Colony type				
	S1	S2	S5	S8‡	Nutrient
I.4	w	—	w	—	—
I.6	r	r	r	r	r
I.7	w	—	w	w	—
I.8	r+c	r+c	r+c	r	r+c
I.9	r+c+w	r+c	r+c+w	r	r+c
I.10	r+w	r	r+w	r	r
I.12	w	—	w	w	—

Colony types: As in Table 2 above. ‡ Anaerobic.

The most interesting aspect of this work was that in three cases a single bacterium had given rise to more than one type of organism. In addition, the two organisms which did not deposit sulphur on the plates were very similar to the variants found previously with 'G'. At this time the stock cultures of 8370 and 'G' were again tested, and 8370 had acquired the ability to decompose thiocyanate, thus paralleling the changes found in the isolates. 'G' also utilized thiocyanate, and the two stock cultures now appeared to be identical, each growing on all the test media and exhibiting the three colony types.

Table 4. *Characteristics of the isolates from* 8370 *and organism* no. 5 (*series* 2)

Isolate no. 8370	Loss of sulphur compound in liquid media				Growth on agar plates					Colony* type
	S1	S2	S5	S7†	S1	S2	S5	S8‡	Nutrient	
II.1	−	−	−	−	+	+	−	+	+	r
II.3	−	−	−	−	+	+	−	+	+	r
II.4	−	−	−	−	+	+	−	−	+	c
II.5	+	+	+	+	+	+	−	−	−	w
II.6	+§	−	−	−	−	−	−	−	−	w§
II.7	+	+	+	+	+	+	−	−	−	w
II.8	+§	−	−	−	+	+	−	+	+	r+w§
II.9	−	−	−	+§	+	+	−	+	+	r+w§
Original culture 8370	+	+	−	+	+	+	−	+	+	c+r+w
No. 5										
II.12	+	+	+	−	+	+	+	−	−	w
II.13	+	+	+	−	+	+	+	−	−	w
II.14	+	+	+	−	+	+	+	−	−	w
II.16	+	+	+	−	+	+	+	−	−	w
II.17	+	+	+	−	+	+	+	−	−	w
II.19	+	+	+	−	+	+	+	−	−	w
Original culture no. 5	+	+	+	−	+	+	+	−	−	w

* Colony types: w = round colony which deposited sulphur; r = round colony which did not deposit sulphur; c = crenate-edged colony which did not deposit sulphur.

† Thiocyanate.

‡ Anaerobic.

§ Grew after 16 days in the liquid media only.

Second series of isolation experiments

In this series 8370 and no. 5 (a neopolitanus type organism which had been maintained in these laboratories for 5 months without showing any colony variation) were used. The characteristics of the stock cultures were tested immediately before isolation and ten isolates were made from each culture (Table 4). Of the ten isolates from 8370, seven grew within 1 week and each was a pure culture. After 2 weeks a further organism II.6 was found to be growing, and at this time II.8 and II.9 were found to contain two types of organisms giving no subculture r and w colonies. In each case the later occurring w colonies were shown to use thiocyanate and were I.4 of the previous experiment. Six of the isolated bacteria from no. 5 grew; each was a pure culture with the characteristics of the parent strain.

DISCUSSION

The conclusive evidence that these organisms may change comes from the first series of isolations in which four changes were observed; in two instances a single bacterium gave rise to a mixture of two organisms, and in one case a mixture of three occurred.

The second series confirmed these results with two more examples; but additional evidence is also available from the behaviour of the stock cultures of 'G' and 8370. The original culture of 8370 did not decompose thiocyanate, despite the many experiments which were made in attempts to confirm the findings of De Kruyff, van der Walt & Schwartz (1957) on this point. Four months later, when the isolations were made, two distinct organisms were found, each of which decomposed thiocyanate, neither of which could have been present in the original culture. In view of this evidence the authors believe that single organisms of some strains of thiobacilli can give rise to the range of organisms described in this paper.

The evidence against contamination is strong. The variants were never found in liquid or solid media exposed in the laboratory and the fact that culture no. 5 has not changed over the same period of time suggests that contamination is not the cause. Of the four stock cultures maintained in these laboratories two have changed and two have not, which suggests that the cause is not environmental, but is inherent in the organisms. This evidence suggests that the change is genetic rather than adaptive, especially when it is recalled that organisms which have arisen can utilize thiocyanate, to which they have not been exposed in the laboratory.

The media chosen for these experiments clearly distinguish four organisms. Two of these, I.4 and I.7, are strictly autotrophic thiobacilli, and I.6 corresponds to *Thiobacillus trautweinii*, which uses thiosulphate slowly under aerobic and anaerobic conditions. As the crenate organism did not use thiosulphate, it would not be classed as a thiobacillus, despite its close relationship to this group. Using the criteria given by Parker (1957), the organisms described may be regarded as distinct species; on these grounds inter-species change may be said to have occurred.

From the stock culture 'G', which was originally a strictly autotrophic strain, both heterotrophs and autotrophs have arisen. These results support the suggestion of Vishniac & Santer (1957) that there is little fundamental difference in this group between autotrophic and heterotrophic metabolism, and we have shown that these thiobacilli can change relatively easily from one mode of life to the other. This interconversion of species of *Thiobacillus* explains the results which we originally found with NCIB 8370. For the same reason, it is possible that De Kruyff, van der Walt & Schwartz (1957), using NCIB 8370, were working with I.7 organisms.

The authors wish to thank Professor F. C. Happold of this University, and the late Miss M. E. Adams of the National Chemical Laboratory, for helpful discussion, and the Joint Research Committee of the Gas Council for permission to publish this paper.

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J. gen. Microbiol. (1961), **24**, 207–224
Printed in Great Britain

Decomposition of Pyrimidines by *Nocardia corallina*

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(*Received* 27 *July* 1960)

SUMMARY

A bacterial species which degrades the pyrimidines, uracil, thymine and cytosine by induced enzymes has been characterized as *Nocardia corallina* (strain S). All other strains of *N. corallina* investigated oxidized thymine, but varied in their abilities to oxidize uracil and cytosine.

Organisms adapted to pyrimidines converted uracil to barbituric acid and thymine to 5-methylbarbituric acid. Oxidation of uracil by thymine-grown organisms was almost entirely by a pathway in which barbituric acid was an intermediate. Oxidation of thymine by uracil-grown organisms was similarly almost entirely via 5-methylbarbituric acid.

Oxidation of uracil by uracil-grown organisms and of thymine by thymine-grown organisms occurred, at least in part, through the respective barbituric acids. Discrepancies between the theoretical and observed values for O_2 uptake suggested however that other pathways may also occur in these cases.

Pyrimidine-grown organisms oxidized 2-thiouracil to 2-thiobarbituric acid and 2-thiothymine to a compound which was probably 5-methyl-2-thiobarbituric acid. These products were not further degraded by the organism.

Barbituric acid was oxidized by uracil-grown organisms to CO_2, NH_3 and urea with concurrent oxidative assimilation. The oxidation of barbituric acid was inhibited by isobarbituric acid and sodium azide although barbiturase activity in cell-free extracts was not affected by these substances. Barbiturase preparations converted barbituric acid anaerobically to malonic acid, CO_2 and NH_3, but barbituric acid was not degraded by whole organisms under anaerobic conditions. Whole organisms, grown on uracil, degraded urea but did not oxidize malonic acid. Acetic and propionic but not malonic or barbituric acids were activated by cell-free extracts as judged by hydroxamate formation. From the evidence presented, it is unlikely that free malonic acid is an intermediate in the breakdown of barbituric acid.

INTRODUCTION

Pyrimidine catabolism may be initiated either by a reduction yielding a dihydropyrimidine or by an oxidation giving a barbituric acid. The degradation of pyrimidines through dihydropyrimidines has been described in (*a*) animal tissues (Fink, Fink & Henderson, 1953; Fink, Cline, Henderson & Fink, 1956; Canellakis, 1956; Fritzson, 1957; Fritzson & Pihl, 1957); (*b*) *Pseudomonas aeruginosa* for uracil and thymine (Fink, Cline & Koch, 1954); (*c*) *Clostridium uracilicum* for uracil

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(Campbell, 1957*a–c*); (*d*) *Zymobacterium oroticum* and some species of *Corynebacterium* for orotic acid (Lieberman & Kornberg, 1953, 1954, 1955; Reynolds, Lieberman & Kornberg, 1955). A reductive pathway for uracil catabolism by *Torula utilis* was suggested from growth studies (Di Carlo, Schultz & Kent, 1952), but it is now known that dihydro-orotic acid is not attacked by this organism (Batt, Martin & Ploeser, 1953).

Pyrimidine catabolism with barbituric acids as intermediates has been demonstrated with certainty only in bacteria; species active in this respect have been isolated by the elective culture technique with pyrimidines as main source of carbon and nitrogen. The organisms obtained include a *Mycobacterium* sp. and a *Corynebacterium* (strain 161) (Hayaishi & Kornberg, 1952), a *Bacterium* sp. (Wang & Lampen, 1952*a*) and two strains of *Nocardia corallina* (Lara, 1952*a*; Batt & Woods, 1951). Results obtained, with both whole organisms and partially purified

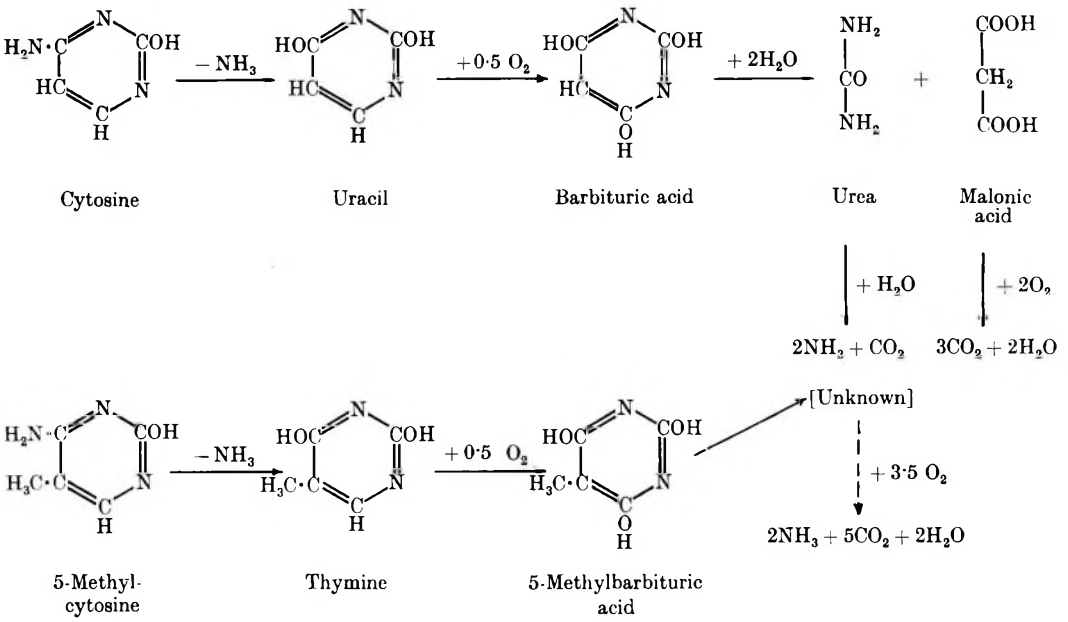

Fig. 1. Pathways of oxidative catabolism of pyrimidines (after Hayaishi & Kornberg, 1952).

enzyme preparations, have led to the schemes for oxidative pyrimidine catabolism shown in Fig. 1. Barbituric acid was isolated as a product of uracil oxidation by whole organisms (Hayaishi & Kornberg, 1952), and this reaction has also been demonstrated with enzyme preparations from several different bacteria (Hayaishi & Kornberg, 1952; Wang & Lampen, 1952*b*; Lara, 1952*b*). The conversion of thymine to 5-methylbarbituric acid has been shown with enzyme preparations (Hayaishi & Kornberg, 1952), but the acid has not been clearly demonstrated to be an intermediate with intact organisms.

Barbituric acid was hydrolysed to malonic acid and urea by extracts of uracil-adapted organisms of a strain of *Mycobacterium* (Hayaishi, 1952) and a similar enzyme (barbiturase) was described in cell-free extracts of *Nocardia corallina*

grown on thymine (Lara, 1952*b*). Crude extracts of both these organisms showed urease activity and the products of the breakdown of barbituric acid were malonic acid, NH_3 and CO_2. Intact organisms of both species oxidized barbituric acid.

Barbiturase had no action on 5-methylbarbituric acid, 2-thiobarbituric acid or isobarbituric acid (Hayaishi & Kornberg, 1952). Intact organisms of the *Mycobacterium* sp. were tested for barbiturase activity by Hayaishi & Kornberg (1952) who stated that 'the removal of barbituric acid, which is carried out by cell-free enzymes at the same rate in the presence or absence of oxygen, does not occur under anaerobic conditions when intact cells are used'. The possibility was considered that energy may be required for the entry of barbituric acid into the organisms. Malonic acid was poorly utilized by uracil-adapted *Mycobacterium* sp. and although the organism may be impermeable to malonic acid, the possibility was suggested by Hayaishi & Kornberg (1952) that barbiturase produced from barbituric acid a labile derivative of malonic acid which was degraded to malonic acid by the isolation procedures.

The present paper describes the isolation and characterization of a bacterium able to utilize pyrimidines as the main source of carbon and nitrogen for growth. The main object of the work done with it was to investigate the possible pathways of degradation of a given pyrimidine when the organism was grown on the same or a different pyrimidine.

METHODS

Organisms. The bacterium with which most of the work was done was isolated from a sample of medium *A* (0·5 % KH_2PO_4, 0·2 % $MgSO_4,7H_2O$ and 0·2 % $CaCl_2$; pH 7·2) containing uracil (0·5 %) which had been exposed to the atmosphere for 12 hr. at room temperature (Batt & Woods, 1951). Following successive single colony isolations on tryptic meat digest agar, the organism was identified by Professor H. L. Jensen as a strain of *Nocardia corallina*; it will be referred to subsequently as strain S of this species.

The organism was aerobic, Gram-positive, non-motile, rod-shaped, non-sporulating, not acid-fast and it exhibited considerable pleomorphism when grown on different media. Neither acid nor gas was produced from glucose, sucrose, lactose, maltose, dulcitol or salicin, whereas acid but no gas was formed from sorbitol and mannitol. Gelatin was not liquefied and milk was not clotted; H_2S was produced by the organism growing in tryptic meat digest broth. By direct microscopy of young cultures (12–24 hr.) on agar small mycelia with granules were observed which were, possibly, the rudiments of aerial hyphae.

Four other strains of *Nocardia corallina* were made available by Professor Jensen and were designated strains P.F.M., 117, K_3 and Th_3. The growth conditions established for strain S were used for these organisms.

Growth tests. Medium *A* (5 ml.) supplemented with the test substance (20 mM) and Difco yeast extract (0·01 %) was distributed in 150 × 19 mm. tubes and autoclaved at 121° for 20 min. After inoculation with a dilute suspension of organisms (0·1 ml.; 0·05 mg. dry wt./ml.) harvested from a tryptic meat agar slope (30°; 24 hr.) the tubes were incubated at 30° in racks sloped at 5° above horizontal. Growth was estimated with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral density filter. The relationship between dry wt. of

organisms and instrument reading was linear up to a reading of 40; a reading of 20 was equivalent to 0·2 mg. dry wt. of organisms/ml.

Preparation of suspensions of organisms. For most experiments medium *A* (150 ml.) supplemented with a pyrimidine (1 g./l.) and yeast extract (Difco) (0·1 g./l.), was distributed in Roux bottles and autoclaved at 121° for 20 min. The inoculum was 0·75 mg. dry wt. of organisms harvested from a tryptic meat agar slope (24 hr.) The bottles were incubated horizontally in air at 30° for 65 hr. The organisms were harvested by centrifuging (2000 *g* for 20 min.), washed in the culture volume of 67 mM-phosphate buffer, pH 7·2, and resuspended in the same buffer at 10 mg. dry wt./ml.

For a few experiments the organisms were grown on tryptic digest of meat broth under the same conditions and with the same inoculum.

Preparation of cell-free enzyme preparations. A suspension of washed organisms (60 mg. dry wt. in 6 ml. of 67 mM-phosphate buffer, pH 7·2) was mixed with 4 g. ballotini beads (No. 13; Chance Bros., Smethwick, Staffs.), and 0·1 ml. of tributylcitrate, cooled in ice-water and shaken on a vibratory tissue disintegrator (H. Mickle, Gomshall, Surrey; Mickle, 1948) at maximum amplitude for 30 min. After centrifuging at 2500 *g* the straw-coloured supernatant fluid was stored at 0°.

Acetone powders were prepared by suspending 100 mg. dry wt. of organisms in 5 ml. of distilled water, chilling, and adding with vigorous stirring to acetone (50 ml.) previously cooled to $-10°$. The deposit after centrifuging was resuspended in acetone (20 ml.) and again centrifuged. After three further washings with acetone (10 ml.) the residue was freed from acetone by evacuating for 3 hr.; the yield of powder was 73 mg.

Estimations. Manometric estimations of O_2 consumption and CO_2 production were carried out by conventional methods (Umbreit, Burris & Stauffer, 1949). The manometer vessels contained 0·1 M-phosphate buffer (pH 7·2; 1·0 ml.), suspension of organisms (0·5 ml.) equivalent to 5 mg. dry wt. and distilled water (0·5 ml.) in the main compartment and 20 mM substrate solution (0·5 ml.) or distilled water (for the controls) in the sidebulb. The temperature was 30°.

For the estimation of non-gaseous products, the manometer vessels were chilled in ice-water, the contents poured into chilled tubes and centrifuged. The clear supernatant fluids were decanted and stored at 0°.

Pyrimidines were estimated spectrophotometrically. The samples were diluted 1 in 50 with either 0·1 N-HCl or 0·1 N-NaOH. The estimation of pyrimidines in binary mixtures followed the method described by Hotchkiss (1948) for determining purines in mixtures. The method of Markham & Smith (1949) was used for the separation and identification of the pyrimidines on paper chromatograms.

Ammonia was estimated in the presence of urea by distillation in the apparatus described by Markham (1942) after the addition of 0·35 M-borate buffer (pH 8·5). Urea was estimated by the difference in ammonia content of the solution before and after incubation with urease (Nimmo-Smith & Appleyard, 1956).

The method of Rose (1955) was used for the detection and estimation of hydroxamates formed from hydroxylamine. The enzyme preparation (0·5 ml.) was incubated in a total volume of 0·85 ml. with the substrate (340 μmole), adenosine triphosphate (3 μmole), Tris(2-amino-2-hydroxymethylpropane-1:3-diol; 100 μmole), $MgCl_2$ (10 mole) and hydroxylamine (930 mole); pH 7·7. After 30 min. at 30°, 10 % (w/v)

trichloroacetic acid (1 ml.) was added and the mixture centrifuged. Ferric chloride solution (4 ml.; 1·25 % (w/v) in N-HCl) was added to the supernatant fluid and the colour intensity estimated in a photoelectric colorimeter using a 540 mμ filter.

Chemicals. 5-Methylbarbituric acid was synthesized by the method of Holmberg (1945) from diethylmethylmalonate and urea, the product obtained having a m.p. of 197° (uncorrected). 5-Hydroxymethyluracil was prepared by reacting formaldehyde with uracil in an alkaline solution (R. E. Cline, personal communication). Thymine glycol was synthesized by the method of Baudisch & Davidson (1925), and melted with decomposition at 210°. All other compounds used were obtained commercially.

RESULTS

Growth of the organism on pyrimidines

Nocardia corallina (strain S) grew in medium *A* containing either uracil, thymine, cytosine, 5-hydroxymethyluracil, barbituric acid or 5-methylbarbituric acid as the main source of carbon and nitrogen. In each case the lag phase was shortened by the addition to the medium of a small amount of yeast extract (Difco; 0·01 %) which also supplied a growth requirement of the organism for thiamine (Martin & Batt, 1957). The growth response to various pyrimidines of the five available strains of *N. corallina* were compared (Table 1); all grew well on thymine but they differed in their ability to utilize uracil and cytosine.

Table 1. *Growth of strains of* Nocardia corallina *on pyrimidines*

The medium (see Methods section) contained the pyrimidine stated (20 mM); incubated for 72 hr.

Strain of *N. corallina*	Growth (EEL reading) with		
	Uracil	Thymine	Cytosine
S	10	10	7
P.F.M.	7	11	3
227	3	11	4
K_3	1	15	2
Th_3	0	10	0

The substances thought by Cerecedo (1927, 1931) to be intermediates in the catabolism of uracil and thymine in dogs (dialuric acid, isodialuric acid, isobarbituric acid and thymine glycol) did not support the growth of strain S. The following substances also failed to support the growth of this organism on medium *A* supplemented with 0·01 % yeast extract (Difco): 5:6-dihydrouracil, 5:6-dihydrothymine, orotic acid, 2-thiothymine, 2-thiouracil, 2-thiobarbituric acid, 6-methyluracil, isocystosine, 2-aminopyrimidine, 2-amino-5-methyl-4-hydroxyprimidine, 5-amino-2:4-dihydroxypyrimidine, 4:6-dihydroxypyrimidine.

General action of suspensions of strain S on pyrimidines

Suspensions of organisms harvested after growth on either uracil or thymine rapidly oxidized uracil, thymine, barbituric acid and 5-methylbarbituric acid (Figs. 2, 3). Organisms grown on tryptic digest of meat broth oxidized all four pyrimidines

only after a lag of at least 4 hr.; the enzyme systems are therefore induced by the presence of the substrate.

Organisms grown on the pyrimidines and tested on uracil, thymine and the barbituric acids completely removed the substrates, i.e. the rate of O_2 uptake decreased to values equal to those for the endogenous respiration and no pyrimidine remained.

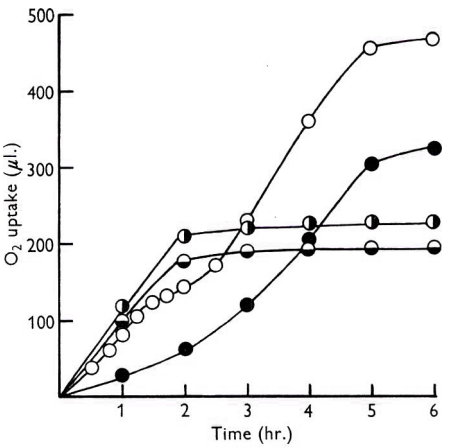

Fig. 2

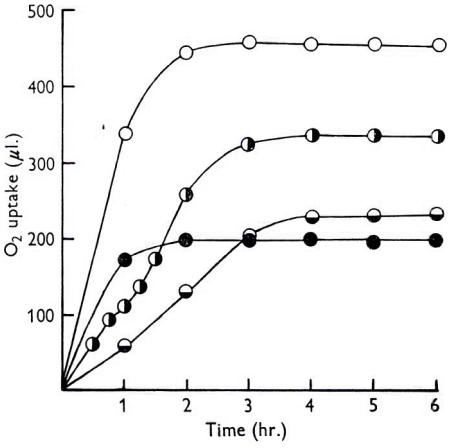

Fig. 3

Fig. 2. Uptake of oxygen by uracil-grown organisms (strain S) acting on thymine (○), 5-methylbarbituric acid (●), uracil (◑) and barbituric acid (◒). Conditions as described in Methods section with 10 μmole of substrate. All values corrected for endogenous O_2 uptake.

Fig. 3. Uptake of oxygen by thymine-grown organisms (strain S) acting on thymine (○), 5-methylbarbituric acid (●), uracil (◑) and barbituric acid (◒). Conditions as for Fig. 2.

The endogenous respiration of suspensions was high and, under the test conditions, amounted to about one-third of the O_2 uptake resulting from the degradation of the pyrimidine. Justification for subtracting the endogenous O_2 uptake from the experimental values in the presence of pyrimidines was obtained by determining the O_2 uptake with a constant amount of organism and different amounts of uracil; the corrected O_2 uptake values were directly proportional to the amount of substrate (Table 2).

Table 2. *Total oxygen uptake with varying amounts of uracil*

Uracil-grown organisms (strain S) incubated (as in Methods section) with the amount of uracil stated. The reaction was continued until the rate of O_2 uptake with uracil decreased to that with the organism alone. Corrected values are those in which the endogenous O_2 uptake value has been deducted.

Amount of uracil (μmole)	O_2 uptake: Corrected μmole	Corrected μmole/μmole uracil	Uncorrected μmole	Uncorrected μmole/μmole uracil
17·90	18·6	1·04	24·2	1·35
8·95	9·3	1·04	13·1	1·46
4·47	4·5	1·01	7·3	1·63

Urea, NH_3 and CO_2 were produced from uracil, thymine, barbituric acid and 5-methylbarbituric acid by organisms grown on either uracil or thymine. However, the values for the final O_2 uptake were much lower than the theoretical values calculated for the complete degradation of the pyrimidines to CO_2 and NH_3 (Table 3). No products other than urea, NH_3 and CO_2 were detected at the completion of the experiments of Table 3. The possibility that some of the substrate was undergoing oxidative assimilation was tested by carrying out the oxidations in the presence of sodium azide. With organisms grown on a given pyrimidine and

Table 3. *Total oxygen consumption during the oxidation of various pyrimidines*

Suspensions of organisms (strain S) grown on either uracil or thymine were incubated as described in the Methods section with 10 μmole of the stated pyrimidine. The O_2 uptake values have been corrected for the endogenous uptake. The theoretical O_2 uptake values were calculated for the complete conversion of the pyrimidines to CO_2 and NH_3.

	O_2 uptake (μmole/μmole pyrimidine) for organisms grown on		Theoretical O_2 uptake (μmole/μmole pyrimidine)
Substrate	Uracil	Thymine	
Uracil	1·01	1·48	2·5
Barbituric acid	0·87	1·03	2·0
Thymine	2·07	2·05	4·0
5-Methylbarbituric acid	1·47	0·88	3·5

tested on the same pyrimidine there was a marked increase in O_2 consumption although the theoretical value for complete oxidation was not reached (Table 4). With organisms grown on uracil and tested on thymine (and vice versa) even the lower concentration of azide used inhibited O_2 uptake (Table 4).

5:6-Dihydrouracil and 5:6-dihydrothymine were not oxidized by suspensions of organisms grown on uracil and thymine respectively.

Table 4. *Effect of sodium azide on the oxygen uptake with uracil and thymine*

Organisms grown as stated on either uracil or thymine were incubated as described in the Methods section with either uracil or thymine in the presence of the stated concentration of sodium azide. The reaction was normally continued until the O_2 uptake decreased to the endogenous rate; * however indicates that the results were calculated after 5 hr. incubation even though the rate was still above the endogenous value.

	O_2 uptake (μmole/μmole pyrimidine) with			
	Uracil-grown organisms oxidizing		Thymine grown organisms oxidizing	
Conc. of azide (mM)	Uracil	Thymine	Uracil	Thymine
0	1·07	2·12	1·43	1·90
5	1·92	0·62	1·06	2·82
20	1·5*	0·49	0·47	2·5*
Theoretical value for complete oxidation	2·5	4·0	2·5	4·0

Oxidation of uracil and thymine to the corresponding barbituric acids

Thymine oxidation by organisms grown on uracil. The rate of uptake of O_2 showed a sudden change at a value of approximately 0·5 mole O_2/mole thymine (Figs. 2, 4). After this time no thymine remained but a compound was present which absorbed

ultraviolet radiation. At the end of the first phase of thymine oxidation (about 60 min.) 0·12 mole CO_2 and 0·09 mole NH_3/mole thymine were present but only traces of urea were detected (Fig. 4). The oxidation product of thymine had identical $\lambda_{max.}$ values in 0·1 N-HCl (262 mμ) and 0·1 N-NaOH (268 mμ) to those for synthetic 5-methylbarbituric acid. On the assumption that the oxidation product is 5-methylbarbituric acid, the amount present at the point of inflexion of the O_2 uptake curve is equivalent to 95–100 % conversion of the added thymine to the acid. Both disappearance of thymine and production of 5-methylbarbituric acid were complete at the time O_2 consumption reached 0·5 mole/mole thymine originally present (Fig. 4).

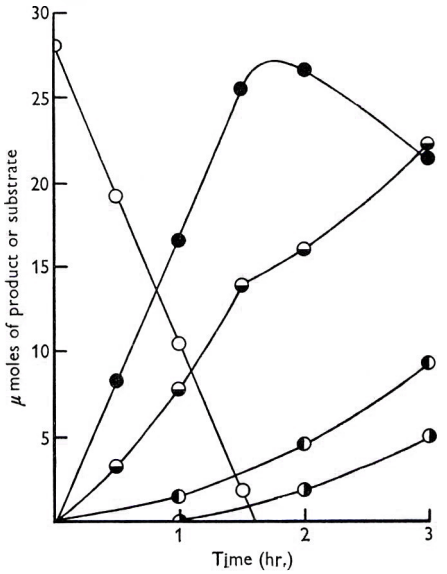

Fig. 4

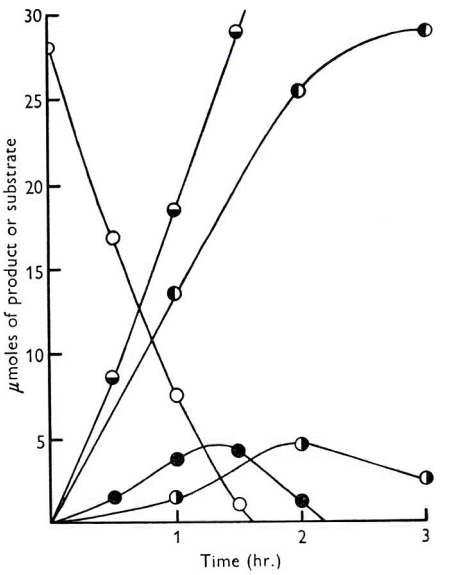

Fig. 5

Fig. 4. Metabolism of thymine (28 μmole) by organisms grown on uracil. Thymine (○), 5-methylbarbituric acid (●), O_2 uptake (◒), NH_3 (◐) and urea (◑). All values corrected for control values without substrate.

Fig. 5. Metabolism of thymine (28 μmole) by organisms grown on thymine. Symbols as for Fig. 4.

The primary oxidation product of thymine was isolated and characterized as 5-methylbarbituric acid as follows. Uracil-grown organisms were incubated with thymine in a number of manometer vessels until the change in rate of O_2 uptake occurred. After removing the organisms by centrifuging, the supernatant fluid was evaporated to dryness under vacuum. The residue was dissolved in a minimum volume of water and 30 % (w/v) $CaCl_2$ added until no further precipitation occurred. The filtrate obtained after removal of the calcium phosphate was evaporated to dryness and the residue extracted with β-ethoxyethanol; the extract was again evaporated. Crystallization from ethanol yielded a white solid (30 mg., m.p. 193°) which gave a mixed melting-point with authentic 5-methylbarbituric acid (m.p. 197°) of 195°. The absorption maximum (262 mμ in 0·1 N-HCl and 268 mμ in 0·1 N-NaOH) and R_F values (Table 5) were identical with the values for 5-methylbarbituric acid.

The curve for O_2 uptake with 5-methylbarbituric acid was almost identical to the curve for the second phase of thymine oxidation (Fig. 2). 5-Methylbarbituric acid is spontaneously and rapidly oxidized in air, under both acid and neutral conditions, to 5-hydroxy-5-methylbarbituric acid (Nishikawa, 1931; Stuckey, 1942). When 5-methylbarbituric acid (10 μmole) in phosphate buffer (0·08 M; pH 7·2) was shaken aerobically in a manometer vessel, the theoretical O_2 uptake for conversion to 5-hydroxy-5-methylbarbituric acid was reached in 30 min. However, when 5-methylbarbituric acid was incubated with a suspension of organisms (Fig. 1) in a solution of the same phosphate concentration and pH, the rapid conversion to 5-hydroxy-5-methylbarbituric acid did not occur; the products of the oxidation in the presence of the organism were CO_2, NH_3 and urea. 5-Methylbarbituric acid was detected in the reaction mixture throughout the period of oxidation (5 hr.). The presence of the actively metabolizing suspension of organisms effectively suppressed the formation of 5-hydroxy-5-methylbarbituric acid; the latter compound was not itself oxidized by the organism.

Table 5. *Chromatography of pyrimidines and products formed from them by strain S*

Solvent systems: (A) *n*-butanol saturated with water, (B) isopropanol + water (100:30), (C) isopropanol + water ammonia; the same solvent as (B), a beaker containing ammonia (sp.gr., 0·88) being placed in the chromatography tank, (D) isopropanol + water + HCl (10 N) (680:156:164).

All of the oxidation products showed as yellow spots on chromatograms sprayed with 0·1% methyl red in borate buffer pH 8·5 indicating that they were all acidic compounds.

	R_F with (solvent system)			
	(A) Butanol + water	(B) Isopropanol + water	(C) Isopropanol + water + ammonia	(D) Isopropanol + water + HCl
Pyrimidine				
Uracil	0·35	0·60	0·54	0·67
Thymine	0·51	0·69	0·64	0·76
2-Thiouracil	0·57	0·66	0·58	0·70
2-Thiothymine	0·69	0·75	0·67	0·79
Barbituric acid	0·05	0·20	0·43	0·49†
5-Methylbarbituric acid	0·05	0·23	0·49	—*
2-Thiobarbituric acid	0·05	0·26	0·48	0·66†
Oxidation product formed from				
Uracil	0·05	0·21	0·44	0·49†
Thymine	0·05	0·24	0·50	—*
2-Thiouracil	0·05	0·25	0·47	0·66†
2-Thiothymine	0·08	0·30	0·58	—*

* 5-Methylbarbituric acid spots were obtained with acid solvents only when relatively large quantities were chromatographed. The compound formed from thiothymine resembled 5-methylbarbituric acid in being unstable in acid solvents.

† The barbituric acids showed as yellow spots on chromatograms run with isopropanol + water + HCl. The oxidation product from thiouracil also showed as a yellow spot on the chromatograms.

Thymine oxidation by organisms grown on thymine. In this case O_2 consumption was more rapid and no sudden change in rate occurred (Figs. 3, 5). Only a small amount of 5-methylbarbituric acid accumulated transiently during the incubation (Fig. 5); the same was true of urea, but NH_3 was formed rapidly from the outset.

Uracil oxidation by organisms grown on thymine. A change in the rate of O_2 uptake was observed at a point corresponding to 0·5 mole O_2/mole uracil (Fig. 3). After this point, no uracil remained but a compound had accumulated which absorbed ultraviolet radiation. This compound disappeared slowly on further incubation (Fig. 6). The absorption characteristics of the compound were identical in 0·1 N-HCl ($\lambda_{max.}$ 257 mμ) and 0·1 N-NaOH ($\lambda_{max.}$ 259 mμ) to the values for barbituric acid; the R_F values for the oxidation product determined on paper chromatograms with several solvent systems were the same as the values for barbituric acid (Table 5).

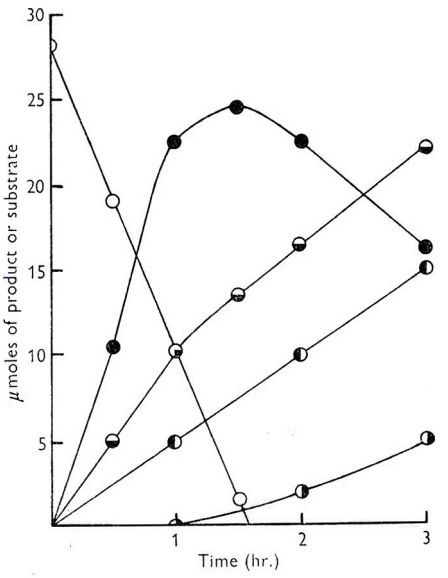

Fig. 6

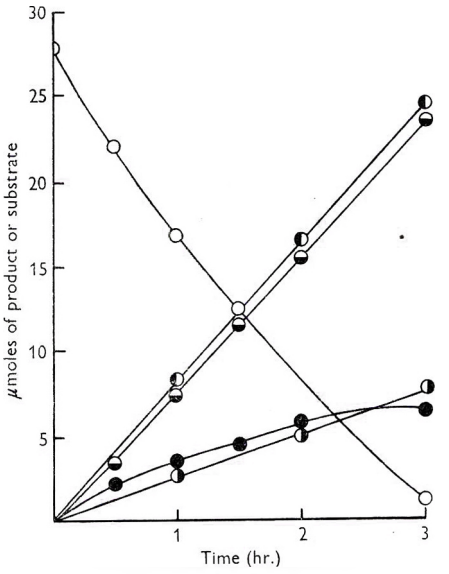

Fig. 7

Fig. 6. Metabolism of uracil (28 μmole) by organisms grown on thymine. Uracil (○), barbituric acid (●), O_2 uptake (◒), NH_3 (◐) and urea (◑). All values corrected for control values without substrate.

Fig. 7. Metabolism of uracil (28 μmole) by organisms grown on uracil. Symbols as for Fig. 6.

At its maximum the amount of barbituric acid formed was equivalent to about 90 % of the uracil added (Fig. 6). Formation of NH_3 was linear from the outset, but urea was only detected after 2–3 hr. The rate of O_2 uptake in Fig. 6 is for 28 μmole of uracil added initially; when the ratio of amount of organisms to pyrimidine concentration was reduced, as in this experiment, the change in rate of O_2 uptake was usually less pronounced than that shown in Fig. 3.

Uracil oxidation by organisms grown on uracil. As with the oxidation of thymine by thymine-grown organisms no abrupt changes in the rate of O_2 consumption were observed during the incubation (Figs. 2, 7). Accumulation of barbituric acid was small and transitory (Fig. 7). Urea and NH_3 production were both linear but the latter was the more rapid.

Oxidation of 2-thiouracil and 2-thiothymine

During a search for compounds capable of inhibiting pyrimidine catabolism it was found that strain S, grown in the presence of either uracil or thymine, oxidized 2-thiouracil and 2-thiothymine with an overall O_2 uptake of 0·5 mole O_2/mole of 2-thiopyrimidine removed. The products of thiopyrimidine oxidation absorbed ultraviolet radiation. During the oxidation of 2-thiothymine there was a change in the $\lambda_{max.}$ value (pH 7·2) from 275 to 272 mμ, while with 2-thiouracil as substrate the change was from 270 to 265 mμ; the $\lambda_{max.}$ for 2-thiobarbituric acid at pH 7·2 is 265 mμ. The rates at which 2-thiouracil and 2-thiothymine were oxidized were approximately equal to the rates of oxidation of uracil or thymine by the same bacterial suspension.

The oxidation products of 2-thiouracil and 2-thiothymine were chromatographed in various solvents and the R_F values compared (Table 5) with different reference compounds including the products formed when the organism oxidized uracil and thymine. The acidic nature of the compounds formed from 2-thiouracil and 2-thiothymine was demonstrated by spraying the chromatograms with a buffered solution of methyl red. When the oxidation of 2-thiouracil or 2-thiothymine by thymine-grown organisms was carried out in unbuffered medium the pH fell rapidly (for 2-thiothymine from 6·8 to 4·0) and only a small O_2 uptake was observed. The results indicate that the product formed from 2-thiothymine was 2-thiobarbituric acid and that 2-thiothymine, by analogy, was probably converted to 2-thio-5-methyl-barbituric acid; an authentic sample of the last-named substance was not available. As these reactions were only observed with pyrimidine-adapted organisms, it was concluded that the oxidations were catalysed by the enzyme systems induced by uracil and thymine. 2-Thiobarbituric acid (and presumably 2-thio-5-methylbarbituric acid) was not attacked by pyrimidine-adapted suspensions of the organism.

Catabolism of barbituric acid

Effect of growth medium. Barbituric acid was oxidized immediately and rapidly to CO_2, NH_3 and urea by suspensions of strain S which had been grown on uracil, thymine or barbituric acid, whereas when grown in media containing no added pyrimidines no oxidation occurred until after a lag of about 8 hr. It appears that barbituric acid, uracil and thymine can all induce the enzymes required for the degradation of barbituric acid. The O_2 uptake curves obtained when organisms grown on barbituric acid were tested with uracil, thymine, barbituric acid and 5-methyl-barbituric acid (Fig. 8) were similar to those obtained when uracil-grown organisms oxidized these four pyrimidines (fig. 2) suggesting that barbituric acid can also induce enzymes for the initial attack on uracil and thymine.

Requirement for oxygen. Suspensions of the organism (grown on uracil) had no action on barbituric acid in an atmosphere of N_2.

Products of barbituric acid catabolism. The equations for the oxidation of barbituric acid by suspensions of strain S (1) and for the complete oxidation of barbituric acid to CO_2 and NH_3(2) are:

$$C_4H_4N_2O_3 + 0{\cdot}88O_2 \rightarrow 2{\cdot}52CO_2 + 0{\cdot}72NH_3 + 0{\cdot}36CO(NH_2)_2, \quad (1)$$

$$C_4H_4N_2O_3 + 2O_2 + H_2O = 4CO_2 + 2NH_3. \quad (2)$$

The rates of production of ammonia and urea from barbituric acid by uracil-grown organisms were compared with the rate of removal of the acid and the rate of O_2 uptake (Fig. 9). The total O_2 uptake values were reproducible to within 10%; for six experiments the values (corrected for the endogenous O_2 uptake) were 0·89, 0·85, 0·89, 0·81, 0·90 and 0·92 μmole O_2/μmole barbituric acid removed. The incomplete recovery of carbon and nitrogen (as CO_2, NH_3 and urea) suggested the possibility that concurrent oxidative assimilation was occurring during the oxidation of barbituric acid.

Effect of sodium azide. An increase of 54% in the total O_2 uptake was obtained when barbituric acid was oxidized by suspensions of the organism in the presence of 2 mM-sodium azide; this was the largest increase observed with the range of concentrations (0·1 mM–0·2 M) of azide tested.

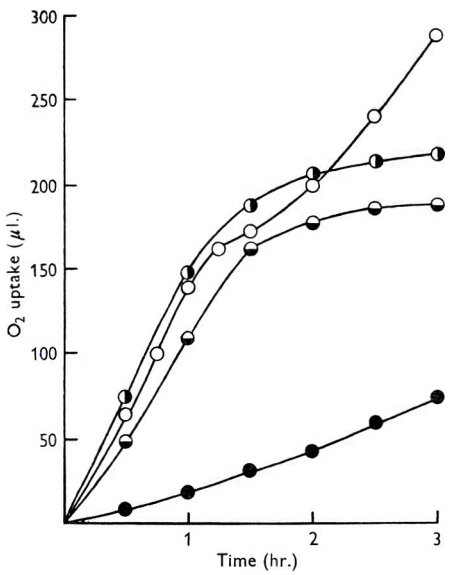

Fig. 8

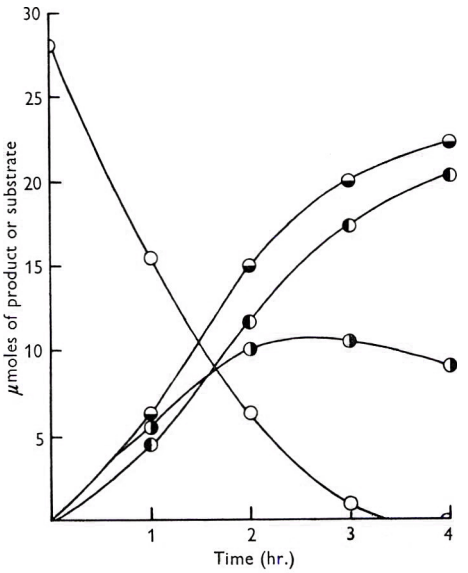

Fig. 9

Fig. 8. Uptake of oxygen by barbituric acid-grown organisms acting on thymine (○), 5-methylbarbituric acid (●), uracil (◑) and barbituric acid (◒). The amount of each substrate was 10 μmole. All values corrected for endogenous O_2 uptake.

Fig. 9. Metabolism of barbituric acid by organisms grown on uracil. Barbituric acid (○), O_2 uptake (◒), NH_3 (◐) and urea (◑). All values corrected for control values without substrate.

High concentrations of azide (0·2 M) completely inhibited the oxidation of barbituric acid by uracil-grown organisms and also caused barbituric acid to accumulate in the reaction mixture when similar organisms were oxidizing uracil. With thymine-grown organisms acting on uracil in the presence of 0·2 M-azide, the accumulation of barbituric acid was equivalent to the amount of uracil which had disappeared. The activity of the enzymes catalysing the initial attack on uracil and thymine was not affected by azide at this concentration.

Effect of isobarbituric acid. During a search for compounds which might be either intermediates in barbituric acid catabolism or inhibitors of its utilization, iso-

barbituric acid was found almost completely to suppress the oxidation of an equimolar concentration of barbituric acid (Table 6).

Degradation by cell-free extracts. Extracts made from uracil-grown organisms with the Mickle tissue disintegrator decomposed barbituric acid anaerobically to NH_3, CO_2 and malonic acid; 9 μmole of barbituric acid was removed by 1 ml. of enzymic extract in 1 hr. A similar extract from thymine-grown organisms had

Table 6. *Effect of isobarbituric acid on pyrimidine catabolism*

Organisms grown on either uracil or thymine as indicated were incubated for 3 hr. with uracil or barbituric acid (each 10 μmole) in the absence or presence of isobarbituric acid (10 μmole). After removing the organisms by centrifuging, the supernatant fluids were analysed for pyrimidines.

	Pyrmidines (μmole) formed or removed by			
	Uracil-grown organisms with isobarbituric acid		Thymine-grown organisms with isobarbituric acid	
	Absent	Present	Absent	Present
Uracil removed	10	6·6	10	10
Barbituric acid removed	10	1	10	1
Barbituric acid formed from uracil	0	1	0	5

only one-quarter of the activity. Malonic acid (50 mg.) was isolated by the method of Hayaishi (1952) from the products of a large-scale experiment in which enzymic extract (8 ml.) was incubated with barbituric acid (200 mg.) in 12 ml. of 0·2 M-phosphate buffer (pH 7·2) in the presence of toluene (0·5 ml.) at 30° until the barbituric acid had been decomposed (28 hr.). The enzyme catalysing the anaerobic breakdown of barbituric acid will be referred to as barbiturase; the crude preparation used above also contained urease.

Sodium azide and isobarbituric acid, which both inhibited the oxidation of barbituric acid by whole organisms, were without action on the activity of barbiturase in the cell-free extracts.

Urea was degraded by intact organisms, acetone powders and extracts made with the Mickle tissue disintegrator. It is likely that the primary products of barbiturase action are malonic acid and urea, and that the latter is then decomposed to NH_3 and CO_2 by the urease present in the preparations.

Metabolism of dicarboxylic acids

Several dicarboxylic acids were tested as possible sources of carbon for aerobic growth in medium *A* supplemented with phosphate buffer (0·1 M; pH 7·2), thiamine (3 mM) and $(NH_4)_2SO_4$ (25 mM); the carbon sources were added, as the sodium salts, at a final concentration of 0·1 mM. Succinic, malic, fumaric and methylmalonic acids supported the growth of the organism but no growth was obtained with malonic, ketomalonic and tartronic acids.

Suspensions of uracil-grown organisms did not oxidize malonic acid. In addition malonic acid had no effect on the oxidation of uracil or barbituric acid by such organisms.

Formation of hydroxamic acids by cell-free extracts. Hayaishi (1955) showed that

malonic acid was degraded to acetic acid by cell-free enzyme preparations of *Pseudomonas fluorescens* with the intermediate formation of coenzyme A derivatives of malonic acid and acetic acid. If these two acids are intermediates in the aerobic catabolism of barbituric acid by strain S it is possible that the organism would contain enzyme systems for their activation and conversion to coenzyme A derivatives. Activation was tested by the method of Rose (1955) by studying the formation of hydroxamates by cell-free extracts in the presence of hydroxylamine and adenosine triphosphate; a number of other carboxylic acids were also tested (Table 7). The enzymic extract contained activation systems for acetic and propionic acids but not for malonic acid or the other substances tested.

Table 7. *Formation of hydroxamates by cell-free extracts of strain S*

Cell-free extracts were prepared with the Mickle tissue disintegrator and incubated with the test substrates as described in the Methods section.

Substrate	Colorimeter reading
None	0
Acetate	12
Propionate	22
Malonate	0
Barbiturate	0
Uracil	0
Lactate	1
Succinate	2
β-Hydroxypropionate	0

Oxidation of acetic acid. Since acetic acid or a derivative (acetyl coenzyme A) was considered as a possible intermediate in barbituric acid catabolism its oxidation by suspensions of strain S was studied in the presence and absence of sodium azide. It was oxidized rapidly and without lag, irrespective of the nature of the growth medium from which the organism had been harvested. With organisms grown on uracil, the final O_2 uptake was 1·04 mole O_2/mole acetate and concurrently, 1·1 mole CO_2/mole acetate was produced. The oxidation of acetic acid was partially uncoupled by azide (2 mM) which gave an increase from 1·04 to 1·66 mole O_2/mole acetate removed; the theoretical value for complete oxidation is 2 mole O_2/mole acetate.

Analysis of medium after growth

It was considered possible that strain S metabolized barbituric acid by two pathways, and that one of these, catalysed by barbiturase, yielded malonic acid which was not further utilized by the organism. The possibility was tested by determining whether malonic acid accumulated during growth on uracil. Organisms were grown on uracil as described for the preparation of washed suspensions except that a sample of sterile medium was removed before inoculation for pyrmidine analysis. After culture for 65 hr. the organisms were removed by centrifugation and the supernatant fluid analysed for pyrimidines, corrections being made for evaporation of water during the incubation period. The amount of uracil removed was 54·5% of that initially present and barbituric acid was formed in amounts equivalent to 5·5% of the uracil added.

The supernatant fluid was evaporated to a small volume, acidified with 3N-H_2SO_4 and continuously extracted with ether for 48 hr. The very small amount of material extracted by the ether contained no malonic acid as indicated by chromatography on paper. No substances could be sublimed from the ether extract.

Pyrimidine oxidation by strain K_3

Nocardia corallina strain K_3 grew much better on thymine than on uracil or cytosine (Table 1). Suspensions of thymine-grown organisms rapidly oxidized both thymine and 5-methylbarbituric acid and uracil was converted to barbituric acid (Table 8). Barbituric acid was metabolized only slowly.

Table 8. *Pyrimidine catabolism by suspensions of strain K_3*

Organisms grown on thymine and incubated aerobically in manometer vessels (as described in Methods section) with 10 μmole of the substrate indicated.

	Substrate			
	Uracil	Thymine	Barbituric acid	5-Methylbarbituric acid
Substrates removed (μmole)	8·0	8·8	1·5	10·0
Barbituric acid formed (μmole)	7·0	—	—	—
5-Methylbarbituric acid formed (μmole)	—	3·0	—	—

DISCUSSION

The long lag period which elapsed before pyrimidines were attacked by suspensions of *Nocardia corallina* from medium containing no added pyrimidine makes it clear that the enzyme systems required for the overall oxidative metabolism of pyrimidines by strain S were induced by the presence of the substrate. Either uracil or thymine can induce the enzyme(s) for an initial attack on both pyrimidines, yielding the corresponding barbituric acids, and this enzyme will be referred to as the uracil-thymine oxidase (Hayaishi & Kornberg, 1952). Growth on either uracil or thymine also induced in the organism ability to oxidize (probably to the corresponding barbituric acids) both 2-thiouracil and 2-thiothymine and it is probable that uracil-thymine oxidase also catalyses these reactions. A cell-free preparation of a similar enzyme from a *Mycobacterium* sp. was found by Hayaishi & Kornberg (1952) not to oxidize 2-thiouracil.

Since neither 5:6-dihydrouracil nor 5:6-dihydrothymine supported growth of strain S and were not oxidized by washed suspensions it is unlikely, unless these substances do not enter the organism, that a preliminary reduction (see Introduction) is an initial step in the overall oxidation of uracil and thymine.

The O_2 uptake with thymine and uracil by organisms grown with either pyrimidine was considerably less than the values required for complete oxidation to CO_2 and NH_3, but no other product apart from urea (whose conversion to CO_2 and NH_3 requires no O_2) was detected. It is probable therefore that extensive oxidative assimilation of the substrate into cell material was taking place. However, O_2 uptake was increased by an uncoupling agent (sodium azide) only when organisms were oxidizing the pyrimidine on which they had been grown and not,

for example, when thymine-grown organisms were oxidizing uracil (Table 4). The failure to demonstrate oxidative assimilation by the use of azide in the latter case may be explained as follows. It may be assumed that organisms grown on uracil contain fully developed all the enzymes required for the complete oxidation of uracil. On the other hand, organisms grown on thymine are adapted for the conversion of uracil to barbituric acid, but the enzymes for the degradation of the latter are not fully induced and barbituric acid at first accumulates almost quantitatively (Fig. 6). It is suggested that azide inhibits the formation of the enzymes which metabolize barbituric acid and give rise to the intermediates used in oxidative assimilation. This view is supported by the observation that barbituric acid itself was oxidized by uracil-grown organisms and that the oxidation was partly uncoupled from assimilation by azide; barbituric acid was not however oxidized by thymine-grown organisms in the presence of azide.

Similar arguments apply to the converse case in which oxidative assimilation could not be demonstrated with uracil-grown organisms metabolizing thymine. It is concluded that failure to demonstrate oxidative assimilation with azide in these two cases does not mean that such assimilation does not occur.

When uracil was oxidized by organisms grown on thymine there was an initial and almost theoretical accumulation of barbituric acid (Fig. 6) which was later further oxidized (Fig. 3); the bulk of uracil metabolism therefore occurred via barbituric acid. With uracil-grown organisms, however, barbituric acid was present in only small amounts at any stage of the reaction. The results might be explained on the assumption that the enzymes for further metabolism of barbituric acid are not fully developed in thymine-grown organisms; the initial rate of O_2 uptake with barbituric acid was only one-third that with 5-methylbarbituric acid (Fig. 3). However, if the whole of the uracil was also metabolized by uracil-grown organisms through the intermediate formation of barbituric acid it would be expected that the O_2 consumption with barbituric acid should be 0·5 mole less than that with uracil, i.e. by the amount of O_2 required for the conversion of uracil to barbituric acid. Experimentally the difference was only 0·15 mole O_2 (Table 3).

The position with regard to oxidation of thymine by organisms grown on uracil and thymine, respectively, was analogous. 5-Methylbarbituric acid was established as an intermediate with certainty only with uracil-grown organisms. With thymine-grown organisms a little of the acid accumulated, but the O_2 uptake values for thymine and 5-methylbarbituric acid differed by almost 1·2 mole instead of the expected 0·5 mole. It is concluded therefore that in the case of the oxidation of uracil and thymine by organisms grown on uracil and thymine, respectively, there is a possibility that a second pathway exists in which the corresponding barbituric acids are not intermediates.

There is evidence from work with cell-free extracts of various bacteria (Hayaishi & Kornberg, 1952; Lara, 1952*b*) that barbituric acid is initially attacked by an enzyme, barbiturase, which degrades it anaerobically to malonic acid and urea. The same enzyme is present in cell-free extracts of the present organism but intact organisms attacked barbituric acid only when O_2 was present. Furthermore isobarbituric acid and sodium azide inhibited completely the metabolism of barbituric acid by whole organisms, but did not affect the barbiturase activity of cell-free extracts. The possible role of barbiturase in the aerobic metabolism of barbituric acid

by whole organisms is therefore not clear; some of the difficulties have been considered by Hayaishi & Kornberg (1952). Suspensions of strain S harvested from growth on either barbituric acid or uracil rapidly oxidized barbituric acid to CO_2, NH_3 or urea with considerable concurrent oxidative assimilation.

Hayaishi (1955) showed the presence in *Pseudomonas fluorescens* of enzymes for the conversion of malonic acid to a coenzyme A derivative and for the conversion of the latter to acetic acid through the intermediate formation of acetyl coenzyme A. Evidence for the activation of malonic acid by cell-free extracts of strain S was sought by a study of hydroxamate formation but none was found; acetate was activated under the same conditions. Malonate was not oxidized by pyrimidine-adapted suspensions of strain S nor could it be detected in the medium after growth of the organism on uracil. The possibility exists that an active derivative of malonate is formed directly from barbituric acid and that this is immediately metabolized without intermediate conversion to free malonate. It is also possible that malonate does not permeate the organism, though this would not explain the lack of activation by cell-free extracts.

One of the authors (R. D. B.) is deeply indebted to the Wellcome Foundation for a personal grant during part of the time of this work. He was a Nuffield Dominions Demonstrator in the University of Oxford for the remainder of the period. The research was aided by a grant to the Department from the Rockefeller Foundation.

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J. gen. Microbiol. (1961), **24**, 225–237
With 3 plates
Printed in Great Britain

Morphogenesis of Mycoplasma and Bacterial L-form Colonies

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(*Received* 8 *August* 1960)

SUMMARY

The development and mechanism of formation of Mycoplasma and bacterial L-form colonies were studied. The micro-organisms penetrate into the agar within a few hours after inoculation. It is suggested that penetration is caused by capillary forces which draw the minute plastic organisms into the dried agar gel, together with the water surrounding them. Penetration does not take place when the agar surface is very moist. The organisms appear to develop in the interstices of the fibrillar network of the agar gel and form a firm and elastic ball-like colony growing in all directions. When reaching the agar surface the growth spreads into the thin free water film which covers the agar, forming the peripheral zone typical of mature colonies. Factors which decrease the thickness of the free water film, like drying the medium or increasing the agar concentration, cause a decrease of the peripheral zone dimensions. Factors which retard growth, such as high concentrations of inorganic salts or hydrogen ions, inhibit the formation of the peripheral zone by limiting the initial ball-like growth inside the agar, rendering it incapable of reaching the agar surface. Typical colonies composed of both central and peripheral zones appear also on other fibrillar media such as the cellulose pellicle of *Acetobacter xylinum* and gelatin.

INTRODUCTION

Mycoplasma organisms (PPLO) and the L-forms of bacteria share a typical colony morphology, the 'fried-egg' colony. This colony is made up of a central zone, embedded in the agar and a peripheral zone on the agar surface (Klieneberger-Nobel, 1960; Dienes, 1960). The morphology of the colony, unique for these organisms, serves as one of the most important criteria in distinguishing them from other bacteria. Although much work has been done on the morphology of Mycoplasma and L-forms, the information concerning the mode of formation of the colonies is scanty (Klieneberger, 1934; Dienes, 1945). Our aim was, therefore, to study the development of these peculiar colonies and the mechanisms involved in their formation.

Two features shared by the Mycoplasma and L-forms seem to be responsible for the formation of the 'fried-egg' colonies: the very small dimensions (100–300 mμ) of the minimal reproductive units (Klieneberger-Nobel, 1956) and their plasticity due to the absence of a rigid cell wall (Klieneberger-Nobel, 1960).

METHODS

Organisms. *Mycoplasma laidlawii* strain A (PG8), *M. laidlawii* strain B (PG9), *M. bovigenitalium* (PG11), *M. gallisepticum* (PG31), *M. mycoides* var. *mycoides* (PG1), *M. neurolyticum* (PG 28) and the stable L-form of *Proteus* were kindly given by Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). *M. mycoides* var. *capri* and the stable L-form of *Streptobacillus moniliformis* were the gift of Dr E. Klieneberger-Nobel (The Lister Institute for Preventive Medicine, London). *M. hominis* was isolated in our laboratory from a case of vaginitis.

Media. The medium used for stock cultures and most experiments was a modified Edward medium (Butler & Knight, 1960*a*) where pooled inactivated human serum replaced horse serum. Beef heart for infusion, peptone and yeast extract used for compounding Edward medium were products of Difco Laboratories Inc. (Detroit 1, Michigan, U.S.A.). Following the suggestion of Klieneberger-Nobel (1956) the medium was enriched by addition of a nutrient broth culture filtrate of *Staphylococcus aureus* in a final concentration of 12·5% (v/v). Edward medium was solidified with 1·25% (w/v) Bacto-agar (Difco, certified) except for some experiments where 12% (w/v) Bacto-gelatin (Difco) was used.

Coagulated serum medium (Löffler's medium) was prepared by mixing three parts of bovine serum with one part of Difco nutrient broth containing 1% (w/v) glucose. The medium was poured into Petri dishes and sterilized by inspissation at 85° for 2 hr.

Cellulose medium. *Acetobacter xylinum* was grown on the medium of Hestrin & Schramm (1954) for 2 days at 30°. The thick pellicle formed was removed, washed thoroughly with sterile distilled water and immersed in sterile distilled water at 6° for 3 days. The water was changed daily. The white cellulose pellicle was then soaked in liquid Edward medium, transferred to a filter paper disk to remove excess liquid and finally placed on the surface of solid Edward medium.

Conditions of growth. Test tubes ($6 \times \frac{5}{8}$ in.) containing 5 ml. of liquid Edward medium were inoculated with 0·1 ml. of the stock cultures and incubated statically in air at 37°. Optimal growth of all strains occurred usually after 48 hr. incubation. Decimal dilutions of these broth cultures were made in a solution of 1% (v/v) Edward medium in 0·01 M-K_2HPO_4 buffer (pH 8·0; Butler & Knight, 1960*b*). Standard drops (0·02 ml. each) of these dilutions were placed on solid medium plates which had previously been dried by leaving them open in the 37° incubator for 1–1·5 hr. The dilutions used for inoculation were chosen so as to give no more than 500 colonies per drop area. The plates were placed in closed tins containing moist cotton wool and incubated at 37° for 3 days.

Measurement of colony and central zone diameters. The growth which appeared on plates was inspected by means of an ordinary light microscope, using slightly oblique transmitted light. Measurement of colony and central zone diameters was carried out with a ×10 objective and a ×5 ocular fitted with a micrometer disk. The drop area was divided by a line through its centre cut by means of a razor blade, and 50 colonies were measured along this line, assuring the representation of colonies growing in the periphery and centre of the drop area.

Vertical sections of colonies. Large colonies were selected for this purpose. The section was cut through the centre of the colony by means of a razor blade. For good

results the thickness of the section had to be not greater than 0·2–0·3 mm. The sections were transferred to slides and examined by the light microscope.

Technique for demonstrating penetration of micro-organisms into the agar. Standard drops of diluted suspensions of the organisms tested were placed on the surface of dried Edward medium plates. Immediately after the drops had dried and at various time intervals later, small agar squares (larger than a drop area) were cut from the Edward medium and placed on the drop areas, care being taken not to form air bubbles. The agar slices were removed, turned over and placed on slides. The agar plates and slices were then incubated at 37° for 3 days in a moist atmosphere. The number of colonies appearing in the drop areas on the plates and on the corresponding agar slices was determined by using the light microscope (magnification ×25).

RESULTS

The development of Mycoplasma and bacterial L-form colonies

Edward medium plates were inoculated with suspensions of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis*. Visible colonies appeared after 24-hr. incubation at 37° (Pl. 1, figs. 1, 4). At this phase of growth almost all colonies in both organisms appeared to be small spheres embedded in agar. This was proved by the circular shape of vertical sections through the colonies and by the inability to remove the colonies on scraping the agar surface with a razor blade.

Further incubation for another 24 hr. caused enlargement of *Mycoplasma laidlawii* strain A colonies, some of which had already developed the peripheral zone (Pl. 1, fig. 2). The L-form grew faster than the Mycoplasma and almost all colonies developed into the 'fried egg' type after 48 hr. incubation (Pl. 1, fig. 5). The peripheral zone of the colonies, located on the agar surface, was easily removed by scraping. Mature colonies of both organisms may be seen in Pl. 1, figs. 3, 6. These colonies had big peripheral zones.

The development of colonies was also followed quantitatively by measuring the diameter of the colonies and their central zones (Fig. 1). The central zone was the first to develop, followed by the formation of the peripheral zone later on. The central zone grew only a little after 48-hr. incubation, whereas the peripheral zone continued to grow, until it occupied the greater part of the colony after incubation for 96 hr.

Vertical sections of mature colonies

All Mycoplasma and L-form strains used in this investigation were examined. The colony sections showed the same basic structure in all organisms: a hemispherical central zone which is embedded in the agar and a thin peripheral zone which spreads over the agar surface (Pl. 2, figs. 7–10). The central zone was a firm and elastic structure in comparison with the very soft peripheral zone.

Penetration of Mycoplasma and L-forms into the agar and formation of the central zone

In order to understand the mechanism of formation of Mycoplasma and L-form colonies we thought it worth while to investigate why the growth of these organisms begins in the agar. Experiments to this purpose showed that the organisms penetrated into the agar medium before multiplication. As may be seen from

Table 1, immediately after the drops had dried about 50% of the Mycoplasma organisms could be transferred to the agar slices. Further incubation gradually decreased the number of organisms that could be transferred and at 6–8 hr. after inoculation almost all organisms remained on the agar plate.

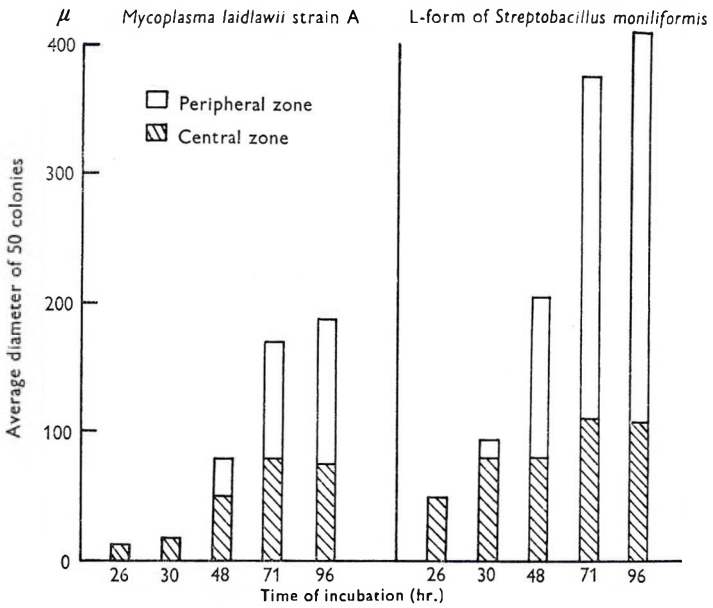

Fig. 1. The development of colonies of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* on solid Edward medium.

The sum of the number of colonies which developed on the drop area on the plate and the corresponding agar slice remained constant within the first 8 hr. of experiment, indicating that no mortality or multiplication took place during this period. After 24-hr. incubation the number of colonies which appeared on the agar slices increased and became equal to the number of colonies on the corresponding drop areas. However, in most experiments the growth was confluent. This was due to the smear of the surface peripheral zones which began to appear at this time (Pl. 1, figs. 2, 4).

The four Mycoplasma strains tested behaved similarly; almost all organisms penetrated into the agar within 6–8 hr. after inoculation. The L-forms of *Proteus* sp. and *Streptobacillus moniliformis* differed in this respect since the number of organisms which could be transferred to the agar slice did not change significantly during the first 8 hr. of the experiment.

For comparison the same experiment was repeated with *Staphylococcus aureus* and *Streptococcus pyogenes*. More than 50% of the organisms inoculated could be transferred to the agar slices after 3-hr. incubation, indicating that these organisms did not penetrate into the agar.

Mycoplasma penetration into the agar did not occur when the surface of the agar was moist. Moist Edward agar plates were prepared by pouring different amounts of sterile distilled water on the agar surface. With *Mycoplasma laidlawii* strain A as

Table 1. *The penetration of Mycoplasma and L-forms of bacteria into agar*

All experiments were done in duplicate and some in triplicate. The table gives typical results of some of the experiments. For technical details see text.

a = number of colonies found on the drop area on the plate.
b = number of colonies found on the corresponding agar slice.

Hr. after inoculation	*Mycoplasma laidlawii* strain A		*Mycoplasma laidlawii* strain B		*Mycoplasma mycoides* var. *capri*		*Mycoplasma bovigenitalium*		L-form of *Proteus*		L-form of *Streptobacillus moniliformis*		*Staphylococcus aureus**		*Streptococcus pyogenes**	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0	110	80	55	44	167	160	55	45	152	215	83	78	6	16	29	63
1	136	70	45	48	166	102	68	26	201	152	70	71	11	16	25	68
3	140	45	80	32	210	55	59	30	244	154	125	105	10	14	120	260
6	170	33	103	12	250	45	58	25	216	162	129	128	—	—	**	**
8	200	30	90	8	270	18	70	35	190	138	144	79	**	**	**	**
24	235	225	**	**	**	**	**	**	**	**	**	**	—	—	—	—

*, in this experiment thallium acetate and penicillin were omitted from the Edward medium.
**, confluent growth.

inoculum, growth was inspected after incubation at 37° for 3 days in a moist atmosphere. The colonies which appeared on the surface of the moist plates were large, flat, and had either minute central zones or none at all (Pl. 2, figs. 11, 12).

Suspensions of Mycoplasma or L-forms were mixed with melted Edward agar at 45° and poured into plates. Most colonies developed deep inside the agar, forming ball-like structures, in contrast to the lens-like deep agar colonies of Eubacteria such as *Staphylococcus aureus* (Pl. 3, figs. 13–15).

Factors influencing the form and dimensions of the peripheral zone

It was assumed that the peripheral zone of Mycoplasma and L-form colonies develop in the thin free water film which is present on the agar surface (Knaysi, 1951). This view was supported by the demonstration of the spreading of peripheral zones along cotton-wool fibres placed on the agar (Pl. 3, figs. 16, 17).

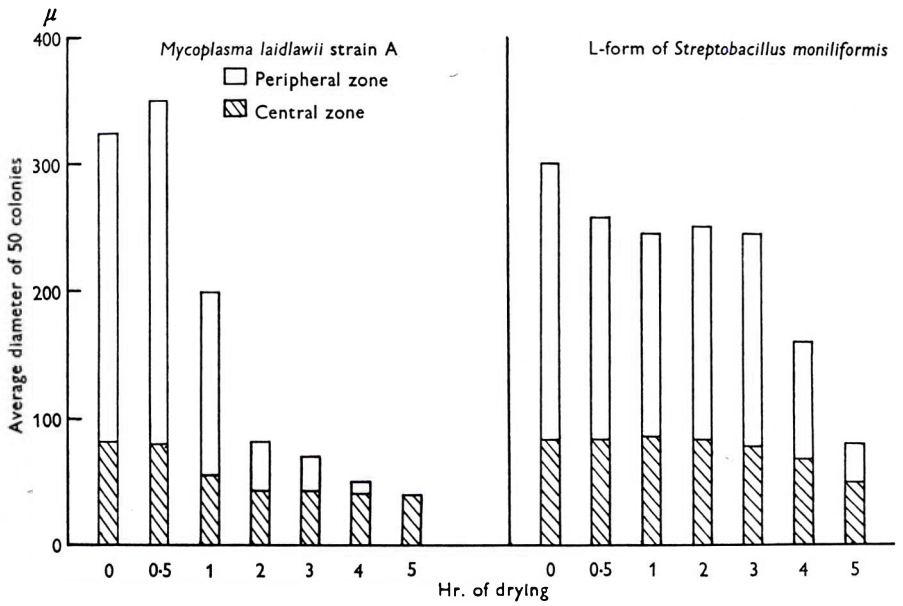

Fig. 2. The effect of pre-drying the Edward medium on the morphology of colonies of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis*.

The need of a humid atmosphere for optimal growth of Mycoplasma and L-forms has been stressed by many authors (Edward, 1954; Klieneberger-Nobel, 1954). Drying of the plates and subsequent incubation in a dry atmosphere decreases the free water film on the agar surface, possibly affecting the formation of the peripheral zones. To test this assumption, solid Edward medium in Petri dishes was dried for different periods of time by opening the plates in a 37° incubator. After the inoculation with suspensions of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* the plates were placed in tins without moist cotton wool and incubated at 37° for 3 days. The dimensions of the peripheral zones were found to be markedly decreased in the case of the dried plates (Fig. 2). Drying for 5 hr. caused complete disappearance of the peripheral zones in all colonies of *M. laidlawii* strain A

and in almost all colonies of the L-form of *S. moniliformis*. The rate of reduction of the central zone diameter due to drying of the medium was much smaller than that of the peripheral zone diameter.

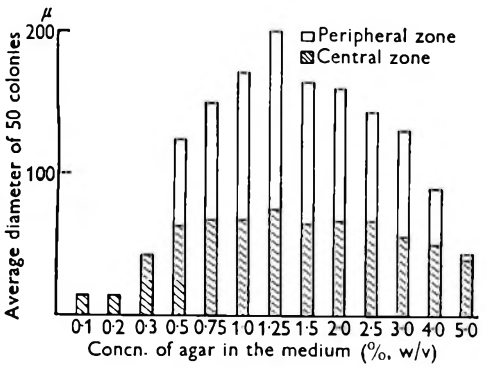

Fig. 3

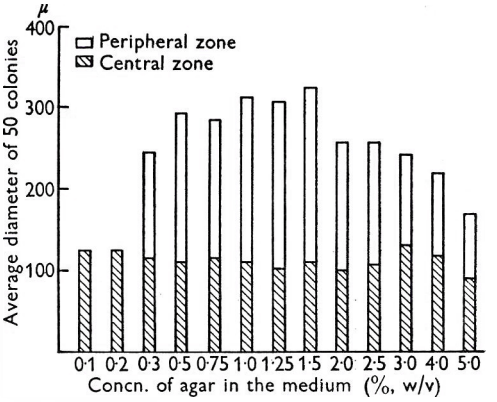

Fig. 4

Fig. 3. The effect of agar concentration in Edward medium on the morphology of *Mycoplasma laidlawii* strain A colonies.

Fig. 4. The effect of agar concentration in Edward medium on the morphology of colonies of the L-form of *Streptobacillus moniliformis*.

The effect of agar concentration on the morphology of the colonies

The effect of changes in the agar concentration in Edward medium on the morphology of colonies of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* is illustrated in Figs. 3 and 4. With low agar concentrations (0·1–0·3 %, w/v), the medium was semisolid and *M. laidlawii* strain A grew in small irregular ball-like colonies at various depths of the medium. A hazy surface growth was also present. Typical colonies, consisting of both central and peripheral zones, appeared at a concentration of 0·5 % (w/v). Increasing the concentration of the agar to 1·25 % (w/v) increased the diameter of the colonies, mainly that of the peripheral zones. Further increase in agar concentration caused a gradual decrease of the colony diameter, affecting primarily the peripheral zone. At a concentration of 5 % (w/v) agar, almost all colonies were without peripheral zones. Similar results were obtained with the L-form of *S. moniliformis* (Fig. 4). The diameter of the central zone of the L-form colonies did not change significantly by changing the agar concentration of the medium. The optimal agar concentration for the development of the peripheral zone was about 1·5 % (w/v); higher concentrations inhibited its formation. A hazy surface growth was observed with low agar concentrations (0·1–0·2 %, w/v).

Morphology of Mycoplasma colonies on solid media other than agar

Mycoplasma laidlawii strains A and B and *M. mycoides* var. *capri* were grown on Edward medium solidified with 12 % (w/v) gelatin instead of agar. The plates were incubated for 3 days at 25°. Growth appeared mostly in the shape of ball-like colonies at various depths of the medium. Some colonies near the surface also

developed peripheral zones. Liquefaction of the gelatin by the organisms after further incubation masked this typical colony morphology.

Mycoplasma laidlawii strain A gave rise to colonies composed of central and peripheral zones when grown on the cellulose pellicle of *Acetobacter xylinum*. Many colonies, however, had no peripheral zones since they had developed deep inside the pellicle, resembling the deep agar colonies of Mycoplasma (Pl. 3, fig. 13).

Mycoplasma laidlawii strain A gave flat surface colonies without central zones when grown on coagulated serum medium. The same type of surface colonies, not penetrating into the medium, appeared when this organism was grown on the surface of a cellophan dialysis membrane placed on solid Edward medium.

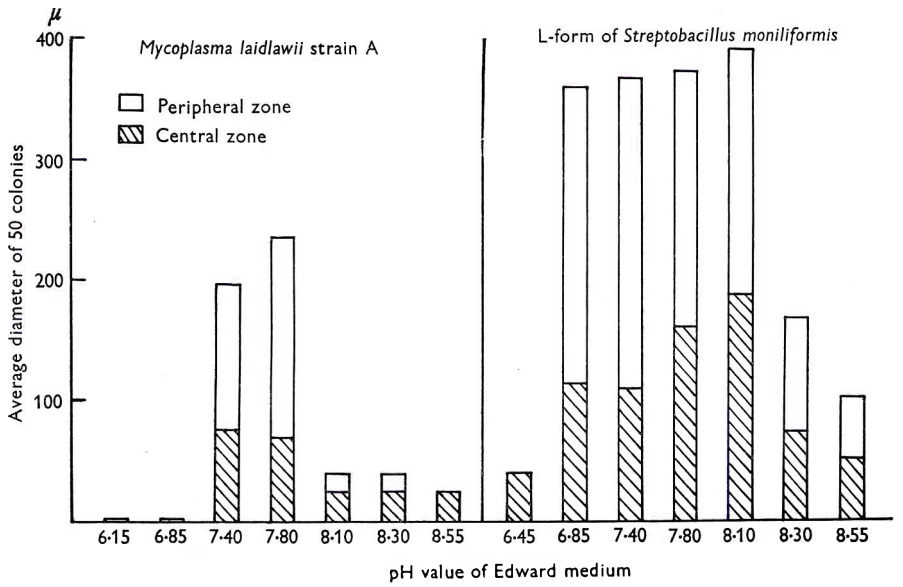

Fig. 5. The effect of pH value on the growth of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* in Edward medium.

The effect of inorganic salts and hydrogen ion concentration on the morphology of Mycoplasma and L-form colonies

Sodium chloride or $Li_2SO_4.H_2O$ were incorporated into solid Edward medium in concentrations of 0·5, 1·0, 2·0 and 3% (w/v). The plates were inoculated with *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* and the growth inspected after 3 days of incubation at 37°. Normal growth of *M. laidlawii* strain A occurred at concentrations 0·5 and 1·0% (w/v) with both salts. At a salt concentration of 2·0% (w/v) a partial inhibition of growth was noted; the colonies were much smaller and many lacking in peripheral zones. With 3·0% (w/v) of $Li_2SO_4.H_2O$ none of the colonies had peripheral zones, while NaCl at a similar concentration inhibited growth completely. The growth of the L-form of *S. moniliformis* was completely inhibited with 2·0% and 3·0% concentration of both salts. At a salt concentration of 1% (w/v) most colonies appeared with central zones only, while 0·5% (w/v) of NaCl or $Li_2SO_4.H_2O$ gave normal colonies.

The pH value of the medium has a marked influence on the growth of Mycoplasma organisms (Edward, 1954; Peoples, Morton & Feo, 1957). The growth and colony morphology of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* were tested at various pH values of Edward medium (Fig. 5). *M. laidlawii* strain A did not grow at pH values below 6·85; optimal growth occurred at pH 7·8. Increasing the pH above 8·0 decreased growth, causing most colonies to appear without peripheral zones. At pH 8·55 peripheral zones were absent from all colonies. The L-form of *S. moniliformis* was less sensitive to pH changes than the Mycoplasma, but inhibition of growth also occurred at low or high pH values, accompanied by the appearance of small colonies without peripheral zones.

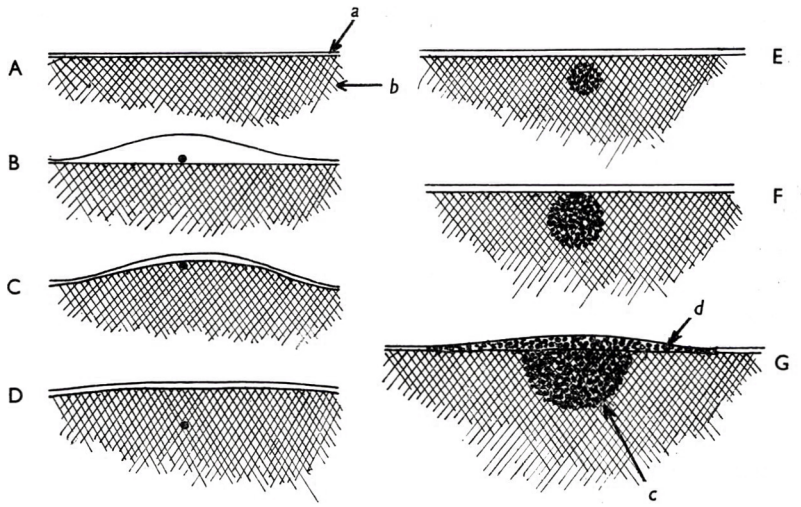

Fig. 6. Diagram to illustrate the development of Mycoplasma and L-form colonies. (A) Vertical section through the agar before inoculation: *a*, free water film; *b*, network of agar fibrils. (B) A drop containing a viable particle of Mycoplasma or L-form is placed on the agar. (C) C. 15 min. after inoculation: the drop is absorbed by the agar forming a slight swelling. (D) C. 3–6 hr. after inoculation: the viable particle has penetrated into agar. (E) C. 18 hr. after inoculation: a small ball-like colony has been formed inside the agar. (F) C. 24 hr. after inoculation: the ball-like colony approaches the agar surface. (G) C. 24–48 hr. after inoculation: the growth spreads into the free water film forming the peripheral zone: *c*, central zone; *d*, peripheral zone.

DISCUSSION

On the basis of our findings and those already reported by others, we are able to propose a possible mechanism for the formation of Mycoplasma and L-form colonies (shown schematically in Fig. 6). Under normal conditions the multiplication of these organisms begins inside the agar. The inoculated organisms are drawn into the agar gel together with the water surrounding them. The dried agar surface has strong imbibition properties, and water is absorbed quickly by capillary forces (Porter, 1946). This can be demonstrated by placing a drop of water on the surface of a dry agar plate; the drop is absorbed within a few minutes, resulting in a slight swelling of the agar at the drop area. When the agar surface is moist the absorption of additional water is decreased or even abolished. Therefore penetration of the organisms is very slight, or absent altogether, resulting in the formation of surface

colonies with small or no central zones (Pl. 2, figs. 11, 13). Hence, the forces responsible for drawing the micro-organisms into the agar are the capillary forces of the agar gel. The determinant factors of the actual penetration, however, are the very small dimensions and the plastic nature of the Mycoplasma and L-forms, allowing these organisms to enter between the agar fibrils and to move in the interstices of the fibrillar network of the agar gel.

Klieneberger-Nobel (1956) showed by filtration experiments and electron microscopy that L-form cultures contain viable particles of various sizes, the smallest of which have a diameter of about 300 mμ and occur only in small numbers. In contrast, cultures of Mycoplasma are composed mainly of very small viable particles, which have an average diameter of about 100–150 mμ. These differences between L-forms and Mycoplasma might explain the results shown in Table 1. Most viable particles of Mycoplasma penetrated into the agar, whereas only a part of the viable particles of the L-forms showed this ability.

The absence of motility in Mycoplasma and L-forms (Klieneberger-Nobel, 1954, 1960) excludes the possibility of active penetration of the organisms into the agar. Gravity forces do not appear to play any role in the penetration since this process is not affected by incubating the inoculated plates upside down. Actinomycetes are known to penetrate into the agar by their filamentous growth (Knaysi, 1951). Some Mycoplasma species also produce filaments and Freundt (1960) has expressed the opinion that this is a common feature of all Mycoplasma species. The possibility that the Mycoplasma penetrate into the agar through the formation of filaments is, however, disproved by the fact that penetration is accomplished within a few hours after inoculation, before the occurrence of any growth and multiplication (Table 1).

It is further shown by our results that the multiplication of the Mycoplasma and L-forms begins inside the agar at various distances from the surface, depending on the initial depth of penetration of the viable particle giving rise to the colony. During the process of multiplication the organisms do not push aside the agar, because they can penetrate and occupy the interstices between the agar fibrils. This is demonstrated by the finding that the growth of Mycoplasma and L-forms in the agar results in the formation of firm ball-like colonies instead of the lens-shaped colonies formed by the usual Eubacteria inside the agar (Pl. 3, figs. 13–15). The formation of lens-shaped deep agar colonies is explained by the inability of the usual bacteria to penetrate into the interfibrillar spaces, therefore causing the agar to split during the multiplication; the colony then spreads along this split (Knaysi, 1951). Staphylococci and streptococci do not penetrate into the agar, as shown in Table 1. It may be concluded that the central zone with Mycoplasma is a ball composed of a network of agar fibrils, with the soft micro-organisms occupying the interstices. The agar fibrils are responsible for the firmness of the central zone.

The ball-like growth inside the agar continues to grow and expand in all directions until it reaches the agar surface which is usually covered by a thin film of free water (Knaysi, 1951). At this stage the organisms spill into this free water film, forming the peripheral zone. The surface tension of the water is responsible for the circular shape of the peripheral zone. The latter is very soft because it does not contain agar, in contrast to the firm central zone. Cotton-wool fibres on the agar cause the peripheral zones of adjacent colonies to lose their round form and to spread along the fibres due to changes in the surface tension of the water surrounding the

fibres. This is further evidence for the location of the peripheral zone in the free water film of the agar.

The dimensions of the peripheral zone are determined by the thickness of the free water film. The thinner the film, the smaller will be the peripheral zone, and the colony as a whole. This explains, at least partly, the importance of a fresh medium and a humid atmosphere for optimal growth of Mycoplasma (Edward, 1954).

It is interesting to note that the large bodies, typical of L-forms and Mycoplasma, appear only at the peripheral zone of the colony (Liebermeister, 1960). This phenomenon might be explained by remembering that inside the agar the organisms are pressed together by the agar fibrils, whereas in the peripheral zone they are free to expand either by growth of the cytoplasm, or by swelling due to low osmotic pressure of the free water film.

The necessity of a fibrillar structure of the medium for the formation of the central zone was demonstrated with the cellulose pellicle of *Acetobacter xylinum*, known to be composed of a network of cellulose fibrils (Mühlethaler, 1949). The typical morphology of Mycoplasma and L-form colonies on gelatin medium apparently depends also on the fibrillar nature of this gel. Hayflick & Stinebring (1960) reported the formation of typical Mycoplasma colonies on the fibrillar plasma clot. On the other hand coagulated serum, which is not fibrillar, does not allow penetration of the organisms and the typical 'fried egg' colony does not appear with this medium (Dienes, 1960).

Apparently the wider interstices between the cellulose and gelatin fibrils, compared to agar at a concentration of 1·25 % (w/v), enable the organisms to penetrate deeper into the cellulose and gelatin media. Thus, the inside growth frequently cannot reach the surface and grows into a ball-like colony inside the medium. The same may happen when the concentration of the agar in Edward medium is decreased. The wider interstices between the fibrils at low agar concentrations explain also the smallness and the irregular boundaries of the central zones observed at these concentrations. The larger viable particles of the L-forms (Klieneberger-Nobel, 1956) apparently do not penetrate quite so deeply into the agar (Table 1), thus explaining the development of large central zones and the appearance of peripheral zones at lower agar concentrations than with the Mycoplasma. The decrease of the peripheral zone dimensions observed at high agar concentrations might be explained by the diminution of the free water film under these conditions (Knaysi, 1951). Increasing the agar concentration also causes decrease of the central zone diameter, apparently due to the inhibition of penetration of the organisms into the medium.

It is noteworthy that when the medium becomes unfavourable for growth, as in the presence of high concentrations of inorganic salts or unsuitable hydrogen ion concentrations, the peripheral zone is the first to disappear, presumably because the initial ball-like growth inside the agar is not big enough to reach the surface and form the peripheral zone. This observation might explain the formation of the T-colonies of Mycoplasma (Shepard, 1960), which are composed apparently only of central zones embedded in the agar (Hayflick & Stinebring, 1960). This is due to the unsuitability of the medium to support optimal growth of these exacting Mycoplasma strains. This was also our experience while testing defined media for Mycoplasma. Poor media only gave rise to this type of agar-embedded colonies without peripheral zones.

Our thanks are due to Dr M. Benziman for supplying us with the cellulose pellicle of *Acetobacter xylinum*.

This work was supported in part by a grant from the Hebrew University—Hadassah Medical School Research Fund.

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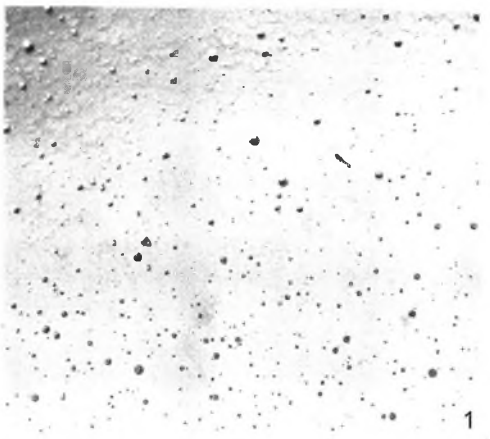
EXPLANATION OF PLATES

PLATE 1

Figs. 1–3. The development of *Mycoplasma laidlawii* strain A colonies on Edward medium after 24, 48 and 96 hr. incubation at 37°. × 50.

Figs. 4–6. The development of the L-form of *Streptobacillus moniliformis* colonies. Medium and incubation periods as above. × 50.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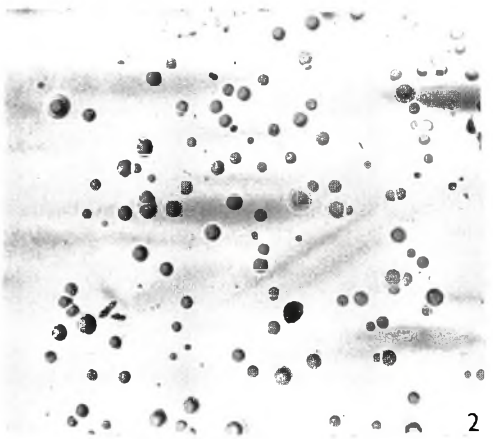
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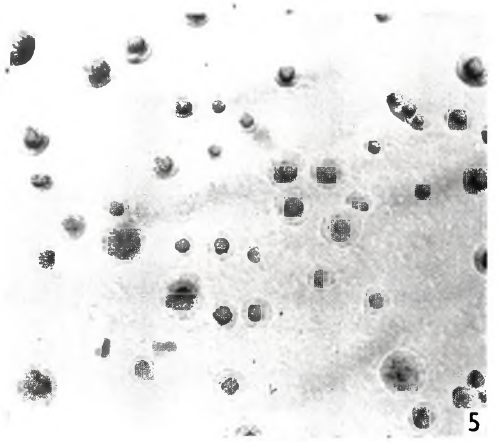
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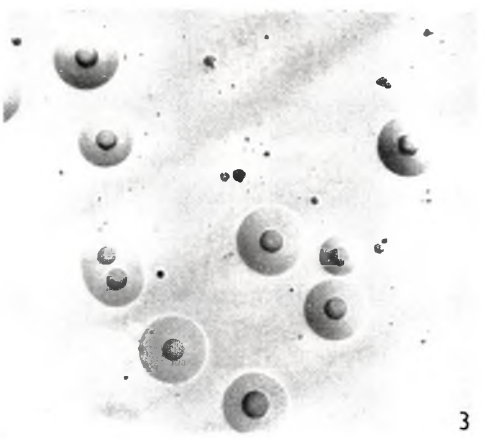
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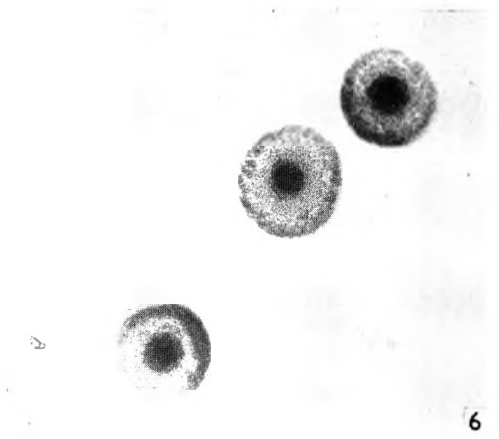
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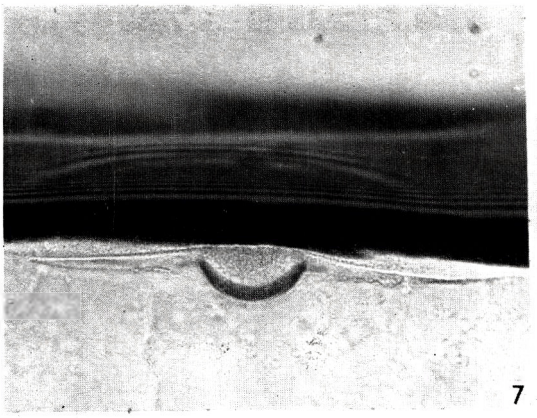
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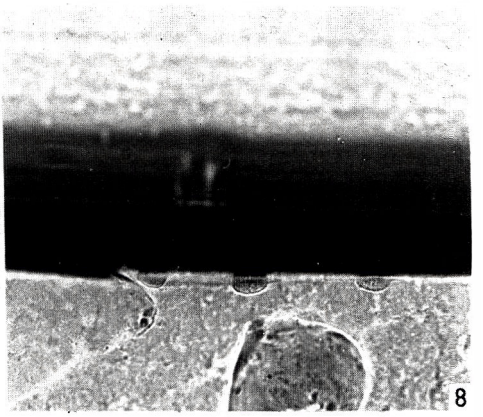
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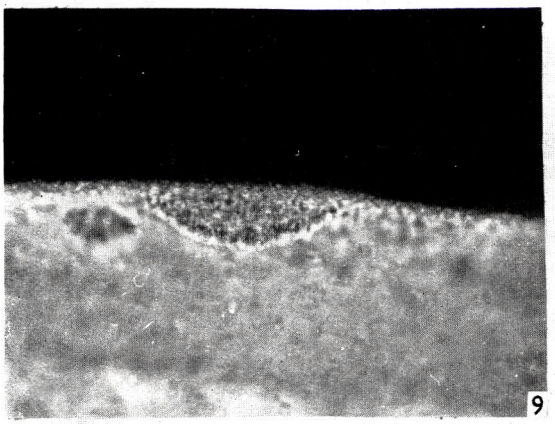
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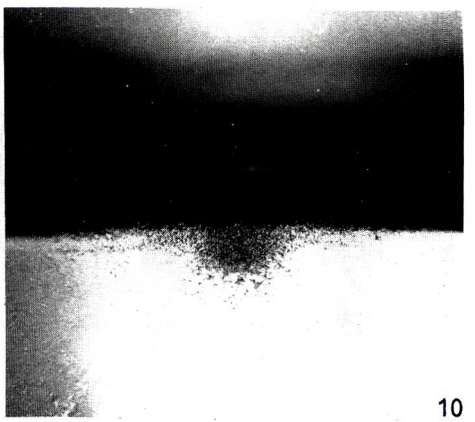
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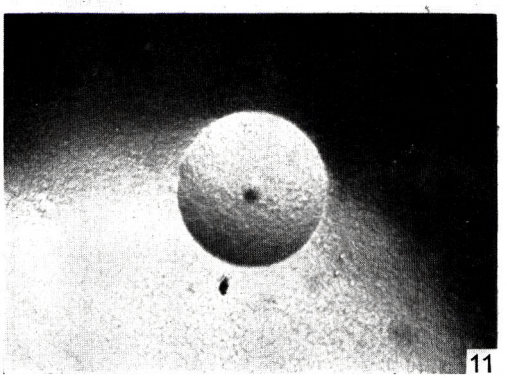
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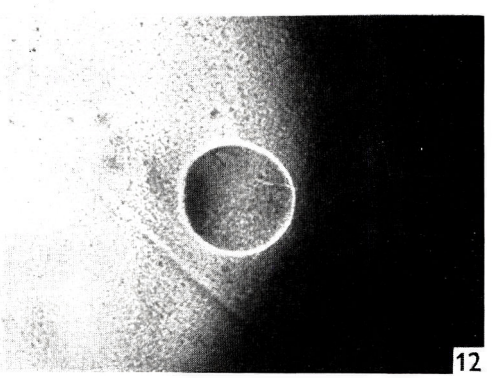
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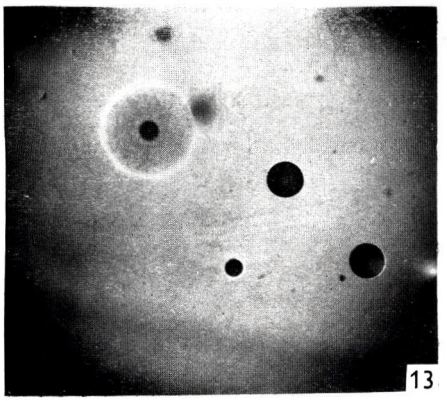
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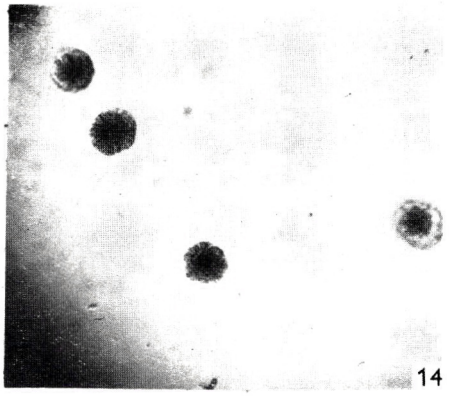
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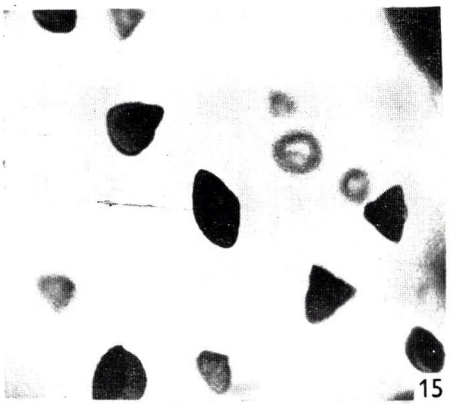
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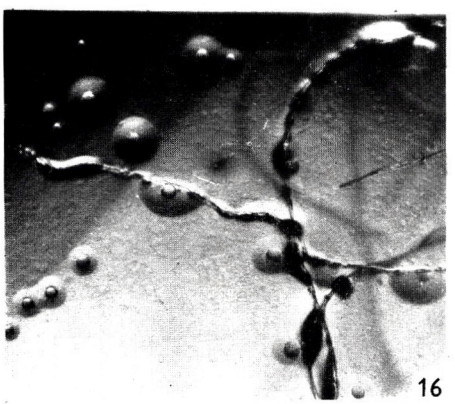
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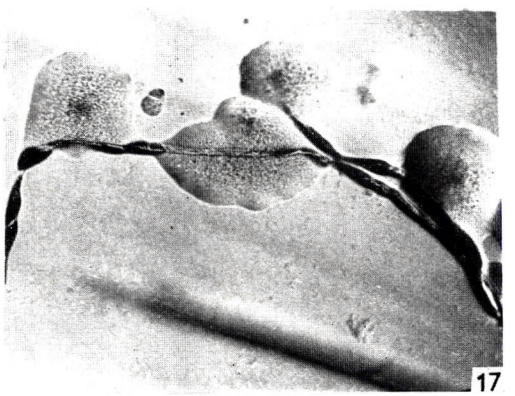
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PLATE 2

Figs. 7–10. Vertical sections of colonies.

Fig. 7. *Mycoplasma laidlawii* strain A. ×150.

Fig. 8. *Mycoplasma mycoides* var. *capri*. ×150

Fig. 9. *Mycoplasma gallisepticum*. ×600.

Fig. 10. L-form of *Proteus*. ×150.

Figs. 11, 12. Colonies of *Mycoplasma laidlawii* strain A grown on moist Edward agar. ×50.

PLATE 3

Fig. 13. Deep Edward agar colonies of *Mycoplasma laidlawii* strain A. Including one surface colony with a peripheral zone. ×50.

Fig. 14. Deep Edward agar colonies of the L-form of *Streptobacillus moniliformis*. One small surface colony is seen at the right end of the photograph. ×50.

Fig. 15. Deep Edward agar colonies of *Staphylococcus aureus*. ×50.

Fig. 16. *Mycoplasma laidlawii* strain A colonies touching cotton-wool fibres on the surface of Edward medium. ×50.

Fig. 17. L-form of *Streptobacillus moniliformis* colonies touching cotton-wool fibres on the surface of Edward medium. ×50.

J. gen. Microbiol. (1961), **24**, 239–246
Printed in Great Britain

The Influence of Hydrogen Ion Concentration and 2:4-Dinitrophenol on Orthophosphate Accumulation in *Tetrahymena pyriformis*

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(*Received* 9 *August* 1960)

SUMMARY

The relationship of the pH value of the suspension medium to inorganic phosphate accumulation by *Tetrahymena pyriformis*, strain W, in the presence and absence of 2:4-dinitrophenol was studied. Investigations were conducted to ascertain whether variations of the conditions of culture influenced phosphate accumulation. The pH value of the suspension medium had an influence on the amount of radiophosphate accumulation. The pH optimum, under the conditions used, was 6·5. The pH value of the culture medium, in the range tested, had little or no influence on the pH optimum observed in the suspensions, although differences in total phosphate accumulation were noted. Approximately 70% of the orthophosphate accumulated by the organisms was in the form of inorganic phosphate, which leads to the postulate of an active transport mechanism for phosphate accumulation in Tetrahymena. 2:4-Dinitrophenol was most effective in lowering radiophosphate accumulation in an acidic medium, and became less effective with increasing alkalinity. The results can be explained on the basis of the penetration of only the undissociated molecule into the organisms. It is suggested that 2:4-dinitrophenol interferes with the transport mechanism involved in phosphate entry.

INTRODUCTION

The growth of *Tetrahymena pyriformis* has been reported to be attenuated in the presence of 2:4-dinitrophenol (DNP). This growth inhibition is annulled by the addition of stigmasterol or related sterols to the medium (Conner, 1957). Numerous investigations concerning the effects of DNP on a variety of biological systems indicate that metabolic activities which require a constant supply of energy are adversely affected. DNP retards or attenuates active transport of cations in yeast (Conway, 1955), protein synthesis in normal and malignant rat livers (Frantz, Zamecnek, Reese & Stephenson, 1948), cleavage in Arbacia eggs (Clowes & Krahl, 1936), photosynthesis and photo-reduction in *Scenedesmus* (Gaffron, 1942), and sodium ion extrusion from the squid axon (Caldwell, 1957). The widespread effects of DNP on cellular activities can be explained in terms of an adverse effect on the

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enzymic reactions which lead to the production of energy-rich phosphate groupings such as adenosine triphosphate (ATP; Loomis & Lipmann, 1948). The formation of such groupings involves the esterification of orthophosphate (Hunter, 1951), and if such reactions were blocked, it might be expected that less orthophosphate would tend to enter the cell, either because of a lowered orthophosphate demand or a lowered availablity of energy necessary for accumulation. In some cases it has been shown that the extent to which DNP influences metabolic processes is related to the pH value of the solution used for suspending the biological material (Caldwell, 1957; Simon, 1953; Hamburger & Zeuthen, 1957). This indicates the necessity of controlling the pH value when evaluating the effect of a given concentration of DNP on a biological system. The extent to which DNP affects phosphate metabolism may depend on the intracellular concentration of the substituted phenol, which in turn might depend on the pH value of the suspension medium. Also, the influence of the pH value on the amount of phosphate accumulated is an important consideration.

This paper deals with the influence of the pH value of the medium on DNP inhibition of growth of Tetrahymena, on orthophosphate accumulation in the presence and in the absence of DNP, and with the relationship of orthophosphate accumulation to the pH of the medium in which the organisms were cultured. The distribution of the accumulated phosphate between organic and inorganic forms was also determined.

METHODS

The organisms, *Tetrahymena pyriformis*, strain W, were cultured under axenic conditions in a medium composed of (%, w/v): 2, proteose peptone (Difco); 0·1, yeast extract (Difco); 1, glucose; 0·2, sodium acetate; 0·15 tris buffer (2-amino-2-hydroxymethyl propane-1:3 diol methane; Sigma 121). In all cases, glucose was autoclaved separately and then added to the medium. For the various experiments the media were adjusted at the time of inoculation to pH values of 5·5, 6·5, 7·0 and 7·5 as determined by the Beckman pH meter. The organisms were incubated for 72 hr. in the dark at 25° ± 2°, harvested by centrifugation (200 *g*), washed three times with distilled water, and suspended in water to give an optical density reading of 0·5 on a Lumetron Colorimeter (Model 401 A) equipped with a 650 mμ filter. The packed organisms of 1 ml. of this suspension had a volume of 0·015 ml.

The phosphate accumulation experiments were carried out in flasks containing the following: 0·012 M-tris buffer, 0·01 M-sodium acetate, $1{\cdot}5 \times 10^{-8}$ M-radiophosphate (0·008 μc./ml. final concentration), 1×10^{-4} M-DNP when appropriate, and 1 ml. of Tetrahymena suspension. The final volume in all flasks was 10 ml. In the experiments involving fractionation of the phosphates, twice the number of organisms was added and a final volume of 20 ml. used.

The organisms were allowed to stand for 1 hr. in the presence of tris buffer; then sodium acetate and DNP were added to the appropriate flasks. Fifteen minutes later all flasks were charged with radiophosphate. After incubation for 2 hr. with radiophosphate the organisms were removed by filtration or centrifugation.

In the experiments involving the fractionation of the accumulated phosphate into acid-insoluble, acid-soluble organic and inorganic portions the following procedure was used. After the 2 hr. incubation period with the radiophosphate, 5 ml. of

suspension were filtered to remove the organisms. A 10 ml. portion of the suspension was added to an equal volume of 10 % (w/v) trichloroacetic acid (TCA), the mixture allowed to stand for 1 hr., followed by centrifugation. To a 10 ml. portion of the TCA supernatant fluid the following reagents were added in the order given: 1·0 ml. KH_2PO_4 (0·2 %, w/v); 2·0 ml. $MgSO_4.7H_2O$ (15 %, w/v); 1·5 ml. ammonia (sp.gr. 0·880). The contents were mixed thoroughly, covered, and allowed to stand overnight. The following morning the precipitate of magnesium ammonium phosphate was sedimented by centrifugation, washed with dilute ammonia (sp.gr. 0·880, diluted 1/10) and resedimented. The supernatant fluid was discarded, the tube drained by inversion and the precipitate dissolved in 1 ml. concentrated HCl, and the volume adjusted to 5 ml. with water. The temperature of all solutions was maintained at 5° during the fractionation procedure.

The radioactivity of three 1 ml. samples was determined by using a thin window Geiger-Muller tube and scaler. Constant geometry was maintained and all counts were corrected for background. The solutions on which counts were taken to assess radioactivity are designated as follows: measure of initial radioactivity in the suspension medium, T_0; suspension medium after removal of organisms, T_2; TCA supernatant fluid, TCA; inorganic phosphate, P_i. The P_i counts were taken at 103 % to compensate for the decay which occurred over the period of about 18 hr.

Direct counts were not made of the TCA-soluble organic or the TCA-insoluble fractions because quantitative recovery could not be achieved. To determine the values of these fractions and to correct for dilution the following calculations were made: total radiophosphate removed from the suspension medium (accumulated phosphate), $T_0 - T_2$; TCA insoluble, $T_0 - 2\text{TCA}$; TCA soluble organic, $2(\text{TCA} - P_i)$; inorganic phosphate, $2P_i - T_2$. Variance was computed for each set of original counts, corrected for background:

$$\frac{\Sigma(X-\bar{X})^2}{N-1},$$

where X is count value, $\bar{X}$ is mean value, N is number of samples counted.

Standard deviation of the calculated count values was determined as:

$$\sqrt{\frac{s_x^2+s_y^2}{N-1}},$$

where s_x^2 is variance of the 1st term, s_y^2 is variance of the 2nd term. (When x or y was increased by a factor of two to correct for dilution, s^2 was doubled for determination of standard deviation.)

Percentage deviation was found by taking the extreme values as given by standard deviations:

$$\frac{X+s_x}{Y-s_y}-\frac{X}{Y},$$

where X is the calculated count value for a given fraction, Y is either T_2 counts or accumulated phosphate in the fractionation experiments.

RESULTS

The influence of 2:4-*dinitrophenol and* pH *value of the culture medium on growth*

The results of the growth experiment (see Fig. 1) indicate that the rate of multiplication of the organisms decreased with increasing DNP concentration. At a

given concentration of DNP, the rate of growth decreased with increasing acidity. In the absence of DNP, no pH effect was seen in the final concentrations of organisms, confirming the findings of Kidder (1941).

Tetrahymena pyriformis used for the phosphate accumulation studies was grown in proteose-peptone medium adjusted to the desired pH value. The pH values of the media after growth for 72 hr. are given in Table 1. In all cases the pH value of the media moved towards neutrality. Orthophosphate accumulation was similar in organisms which had been grown in media initially at pH 5·5 and 6·5, but was somewhat lower in the case of organisms grown in media initially at pH 7·5. In fifteen experiments there was no exception to the pattern of radiophosphate accumulation, although differences in the absolute amounts of orthophosphate accumulated were noted from experiment to experiment. In general, a shift in the pH value of the suspension medium towards neutrality took place during the experimental period. However, in spite of this tendency to approach neutrality, the initial pH value of the suspension medium had an influence on the degree of orthophosphate accumulation. A pH optimum of 6·5 was found regardless of the conditions of culture used.

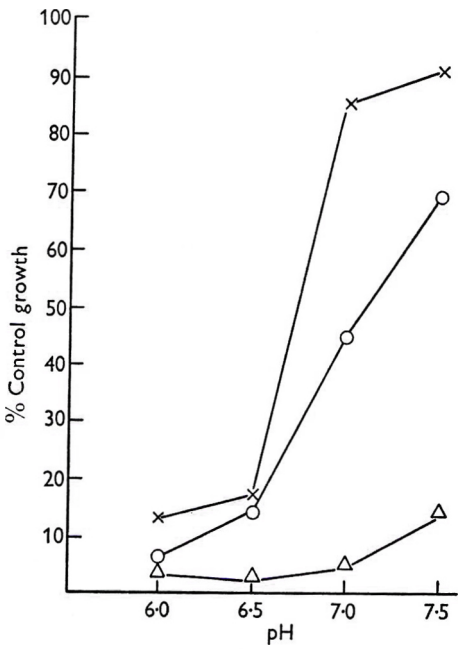

Fig. 1. Effect of 2:4-dinitrophenol (DNP) in media at different pH values on the growth of Tetrahymena. (×) 5×10^{-5}M, (○) 1×10^{-4}M, (△) 2×10^{-4}M-DNP. The media and conditions were as reported previously (Conner, 1959).

Orthophosphate accumulation by *Tetrahymena pyriformis* was inhibited by DNP. The maximal inhibition for a given concentration of DNP occurred when the suspension medium was acidic (see Fig. 3). In the presence of DNP, the pH optimum at 6·5 was abolished.

Since the pH value of the suspension medium had an effect on phosphate accumulation in absence of DNP, the data require clarification. When the amount of radio-

Table 1. pH *Value of culture media before and after growth of Tetrahymena*

The protozoans were maintained at 25° ± 2 for a period of 72 hr.

Initial pH of culture media	pH after growth for 72 hr.
5·5	6·3–6·4
6·5	6·7–6·8
7·5	7·2–7·3

phosphate accumulated at each pH value in the presence of DNP is compared with a control at that pH value, i.e. % DNP accumulation/% control accumulation, a better description of the influence of the pH of the suspension medium with regard to DNP inhibition is obtained (see Table 2). The distribution of accumulated phosphate (taken as 100%) was TCA-insoluble 19·8% ± 3·8, organic TCA-soluble 11·3% ± 3·7, inorganic phosphate 68·9% ± 3·5. In these experiments the organisms were grown at pH 6·5 and the pH value of the suspension medium was 7·0. The phosphate removed from the suspension medium by the Tetrahymena was 75% ± 5 (seven experiments). In a few experiments the organisms were incubated in a suspension medium at pH of 6·5 or 7·5. There was no significant variation in the distribution of phosphate under these conditions.

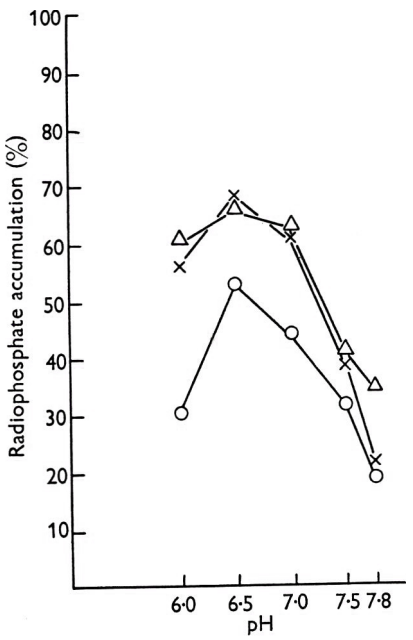

Fig. 2

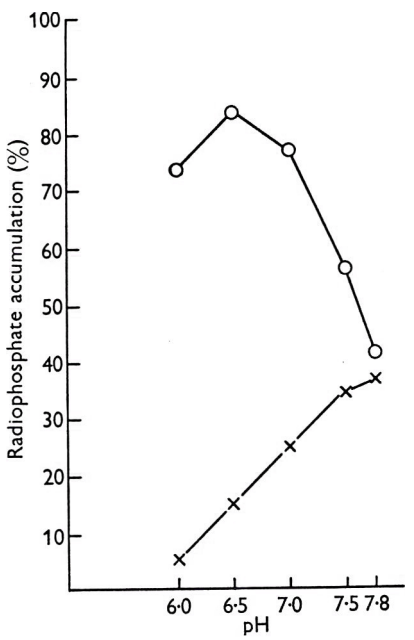

Fig. 3

Fig. 2. Influence of pH value of the suspension medium on radiophosphate accumulation by Tetrahymena grown in proteose peptone media of pH 5·5 (×), 6·5 (Δ) and 7·5 (○). Each point represents the mean % accumulation for five experiments.

Fig. 3. The influence of the pH value of the suspension medium on radiophosphate accumulation by Tetrahymena in the presence (×) or absence (○) of 1×10^{-4} M-DNP. The organisms were grown in the proteose peptone medium (pH 6·5).

Table 2. *Data from representative experiments on accumulation of radiophosphate by* Tetrahymena *in the presence and absence of* 10^{-4}M DNP *under various conditions of* pH *of culture medium and suspension medium*

Organisms grown in medium at pH 5·5 (see Table 1)					
Initial pH value of suspension medium (T_0)	6·0	6·5	7·0	7·5	7·8
Terminal pH value of control (T_2)	6·4	6·75	6·95	7·4	7·7
Terminal pH value in presence of DNP	6·55	6·8	6·95	7·4	7·7
% Accumulation control (no DNP)*	47·4	70·8	61·6	34·8	30·6
% Accumulation in presence of DNP*	1·4	11·2	35·0	39·1	27·9
% DNP/% Control	3	16	57	112	91
Organisms grown in medium at pH 6·5					
Initial pH value of suspension medium (T_0)	6·0	6·5	7·0	7·5	7·8
Terminal pH value of control (T_2)	6·6	6·8	6·9	7·3	7·6
Terminal pH value in presence of DNP	6·4	6·7	6·9	7·2	7·5
% Accumulation control (no DNP)*	74·7	85·7	78·7	56·7	41·6
% Accumulation in presence of DNP*	6·0	15·4	25·9	35·3	38·6
% DNP/% Control	8	18	33	62	93
Organisms grown in medium at pH 7·5					
Initial pH value of suspension medium (T_0)	6·0	6·5	7·0	7·5	7·8
Terminal pH value of control (T_2)	6·4	6·6	7·0	7·5	7·6
Terminal pH value in presence of DNP	5·7	6·75	7·0	7·35	7·65
% Accumulation control (no DNP)*	33·2	60·0	35·9	29·3	21·7
% Accumulation in presence of DNP*	0·0	11·3	23·8	20·4	18·4
% DNP/% Control	0	19	66	70	85

* In all cases the standard deviations were less than 2%.

DISCUSSION

The data presented indicate that the accumulation of orthophosphate varied with the pH value of the suspension medium, with a maximum at pH 6·5. Van den Honert (1933) found that phosphate accumulation in sugar cane varied with the pH value of the medium in a manner suggesting that the rate of accumulation was dependent on the concentration of the monobasic phosphate ion. He offered an explanation based on the assumption that the dibasic ions could not be absorbed. In support of such a hypothesis, van den Honert found that accumulation at high phosphate concentrations was independent of changes in the acidity of the medium. The data concerning phosphate accumulation by *Tetrahymena pyriformis* cannot be explained solely on the basis of the ionization state of the phosphate ion. Such an explanation would not account for the sharp maximum in accumulation at pH 6·5. Even though monobasic phosphate ions are in greater concentration at pH 6·0, accumulation is lower. The pH optimum could be explained if the mechanism concerned with orthophosphate entry involves specific sites in the cellular membrane which are sensitive to hydrogen ions. Thus, it is possible that two pH-dependent factors might be involved, the state of the phosphate ion and that of a carrier, the combined effect of these producing an optimum at pH 6·5. Goodman & Rothstein (1957) studied the effect of extracellular pH value on radiophosphate accumulation in yeast and reported that accumulation was maximal when the pH value of the suspension medium was 6·5.

The amount of radiophosphate accumulated by Tetrahymena cannot be accounted for on the basis of free diffusion or catalysed permeability. The total volume of organisms used in these experiments was about 0·030 ml., while the volume of the suspension medium was 20 ml. At the end of the 2 hr. incubation period about 30 % of the radiophosphate remained in the medium; thus phosphate had been concentrated by the organisms by a factor greater than 900. It was shown by Slater (1957) and confirmed in this laboratory (Conner, 1958) that no detectable efflux of radiophosphate occurred from Tetrahymena under the conditions used. This removal of radioactivity from the medium probably represents true accumulation and not phosphate ion exchange. This is an accumulation against a concentration gradient and can be explained on the basis of an active transport mechanism.

In Tetrahymena the most pronounced influence of a given concentration of DNP in inhibiting growth or in lowering radiophosphate accumulation occurred in the acid range. Since DNP is a weak acid and is less dissociated in an acidic medium, it seems possible to correlate the degree to which DNP lowers phosphate accumulation with the concentration of free acid. The experiments of DeDeken (1955) and Cross, Taggart, Covo & Green (1949) indicate that the ability of substituted phenols to influence biological processes is a function of the phenolic configuration, the mobility of the phenolic proton, and an inverse function of pK.

Hamburger & Zeuthen (1957) reported DNP to have a greater influence on respiration of *Tetrahymena pyriformis* in an acidic medium and suggested that the free acid is the physiologically active form. It seems, however, that an equally plausible explanation might be to assume that undissociated DNP has the greater ability to penetrate. If only the free acid were active or if both forms penetrated the cellular membrane to an equal extent, it would be necessary to postulate that intracellular pH varies with the pH value of the suspension medium in order to explain the observed results on respiration, growth and phosphate accumulation. Caldwell (1957) found that the extent to which DNP affected metabolic activity in the giant axons of *Loligo forbesi* depended on the concentration of the substituted phenol in the axoplasm, which in turn was dependent on the pH value of the solution bathing the axon. A similar situation might exist with Tetrahymena.

The pH optimum for phosphate accumulation was not seen when DNP was added. Thus in the presence of DNP some other factor becomes rate limiting. A lowered utilization or an intracellular increase of orthoposphate could account for the lowered phosphate accumulation and for the disappearance of the optimum in accumulation at pH 6·5. Such an explanation would be compatible with the current concept of the action of DNP as an uncoupling agent of oxidative phosphorylation and as a stimulant of ATPase activity (Lardy & Wellman, 1953). However, there are other reasonable explanations that could account for the results obtained. It is possible that DNP interferes with an active transport mechanism involved in phosphate entry: directly by combining with a carrier or indirectly by decreasing the supply of energy required for active transport (Conway, 1955). Work is in progress to test the validity of the above hypotheses.

The authors wish to thank Dr R. S. Davidon for the development of the statistics used, Miss Eileen Skromak for the growth studies and Mrs Jeanne Crosson for technical assistance. This work was supported in part by Bryn Mawr College, the

National Science Foundation Grant no. G7642, and the Public Health Service Grant no. 1065. Two of us (R.G. and M.S.K.) were participants in the Undergraduate Research Participation Programme of the National Science Foundation.

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J. gen. Microbiol. (1961), **24**, 247–252
Printed in Great Britain

A Disease of Finnock Due to *Vibrio anguillarum*

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(*Received* 10 *August* 1960)

SUMMARY

A disease of finnock (immature *Salmo trutta*) is described. The causal organism was found to be a Gram-negative, motile, curved rod which was also pathogenic to eels, perch, plaice and saithe. On comparison with other pathogenic vibrios from fish it was apparent that the organism was a variant of *Vibrio anguillarum*; the name *V. anguillarum* type C is suggested for it.

INTRODUCTION

Vibrio anguillarum has long been known to be responsible for red disease of eels, but the descriptions of the organism are various and incomplete, and it has been omitted from *Bergey's Manual of Determinative Bacteriology*, 7th ed. Nybelin (1935) collected and classified all the strains available at that time, and it is his description which will be used here for comparative purposes.

A disease of young Pacific salmon held in sea-water ponds was reported by Rucker, Earp & Ordal (1954) as due to a marine species of Vibrio. The fish develop extensive haemorrhages in the muscle and internal organs, and the disease which begins in April or May when the temperature reaches about 50° F. continues throughout the summer. Hoshina (1956) described a disease of rainbow trout which occurred from late autumn till spring in Japan. It affected both young fish and adults, and was characterized by the production of boil-like lesions or haemorrhages in the muscle. In 1957 Hoshina gave a fuller description of the causal organism which he named *V. piscium* var. *japonicus*. Rucker (1959) cited various outbreaks of disease due to *Vibrio* spp. in Pacific salmon, rainbow trout and steel head trout. The organism was found to be different from *V. comma* and was not pathogenic to warm-blooded animals.

Smith (1959) reported an outbreak of disease in finnock in Scotland and stated that it was due to a *Vibrio* species. The purpose of this paper is to describe the organism isolated from these finnock and by comparison with other similar organisms to show that it is *V. anguillarum*.

SPECIMENS EXAMINED

During the 6-year period (1954–59) 15 finnock and one sea trout were examined (Table 1).

The dead fish were usually found in late summer or early autumn and always in the tidal part of the river. It was also evident that no one sign of disease was common to all the specimens, but the same organism was isolated in every case. Very

often this organism grew in pure culture from the spleen, liver, kidney, heart blood and, sometimes, muscle. Sections were also examined but no consistent pathological change was revealed.

The same organism was isolated from a salmon found dead in the River Foyle in August 1959 (Edwards, O'Kelly, Napier & Fletcher, 1960) and the disease was confirmed in finnock and sea trout of the River Ythan as well as in finnock from the River Dee in the summer of 1960.

Table 1. *Specimens examined*

Date	Source	No. of specimens seen	No. of specimens examined	External appearance
September 1954	River Dee, George VI Bridge	3–4	1	Right pectoral fin congested; vent haemorrhagic
September 1955	Cowie Burn	1*	1	Vent distended; small abrasion on left operculum
August 1957	River Dee, Pots and Fords Fishings	*c.* 30	1	Vent haemorrhagic; haemorrhagic area on left flank
August 1958	River Dee, Victoria Bridge	*c.* 20	11	Varied from no sign of disease to congestion of fins, haemorrhage of vent and haemorrhagic areas on flanks
July 1959	River Dee, Torry Beach	2	2	Haemorrhagic swelling on right flank of one and right shoulder of the other

* Sea trout.

DESCRIPTION OF THE ORGANISM

(All tests carried out at 22° unless otherwise stated.)

Morphology. The organism is a small Gram-negative rod ($2–2{\cdot}5\mu \times 1\mu$) with a tendency to be curved, especially when direct smears or sections from fish are examined. It is motile by a polar flagellum, non-sporing, non-acid-fast and non-capsulate. Cultures on sea-water agar for 6 days or more show a variable number of oval bodies. These when examined in the phase-contrast microscope appear to have polar granulations. They are similar to the bodies mentioned by Liston (1955) in his description of gut group vibrios.

Growth on nutrient agar plate. Colonies are round, raised, mucoid, translucent and greyish yellow in colour. This organism is non-luminous.

Growth on sea-water agar plate. Colonies are round, raised, translucent but whiter than on nutrient agar. They are also of a more watery consistency.

Growth on nutrient agar slope. Raised mucoid, translucent, greyish yellow, tending to coalesce if growth is heavy.

Growth on nutrient agar stab. On the surface there was a spreading shiny mucoid growth and down the line of the stab there was a sharply defined area of growth. This showed the organism was a facultative anaerobe.

Growth on blood agar. The organism does not produce haemolysis on horse blood agar.

Growth in nutrient broth. A turbidity develops overnight and this is followed by the production of a pellicle and formation of a deposit. This deposit becomes viscid later.

Growth on Loeffler's serum slope. The organism grows as a smooth, yellow, shiny layer. The serum is liquefied.

Growth on potato slope. On alkaline potato slope the growth is smooth, shiny and yellow.

Biochemical reactions. The organism produces acid but no gas from glucose, fructose, sucrose, maltose, trehalose, starch, dextrin, glycogen, mannitol, sorbitol; slight acid but no gas from galactose and no acid or gas from arabinose, rhamnose, xylose, lactose, cellobiose, raffinose, inulin, glycerol, dulcitol, inositol, amygdalin and salicin. When tested by Hugh & Leifson's (1953) method the organism was found to belong to Group III*a*, i.e. anaerogenic fermenter. It is methyl red negative, produces acetylmethylcarbinol; does not produce indole; does not grow on Koser's citrate medium; does not produce hydrogen sulphide on ZoBell's medium; produces catalase on nutrient agar; reduces nitrate to nitrite; liquefies gelatin but has no action on filter-paper. In litmus milk a clot is produced and this is followed by proteolysis and reduction.

Table 2. *Pathogenicity experiments*

Fish	No.	Route of injection	Time to die (days)	External symptoms
Finnock	3	intraperitoneal	1–6	Two showed hamorrhage of the vent; one showed no external symptoms
Finnock	1	intramuscular	2	Inflammation of muscle at site of injection
Eels	4	intramuscular	2–7	Haemorrhagic swelling at site of injection and congestion of muscle
Brown trout	2	intraperitoneal	4–6	None
Perch	2	intraperitoneal	2	None
Plaice	1	intramuscular	22	Swollen haemorrhagic area about the size of a penny with a bluish black centre. Haemorrhage in peripheral muscle
Saithe	2	intraperitoneal	2–6	One showed no symptoms but the other developed a greyish area on its dorsal surface.

In all cases the organism was re-isolated after death but in no case was it found in an inoculated or uninoculated control.

Reaction to antibiotics. Evans's 'Senstests' were used. The organism was resistant to 2·5 i.u. penicillin but sensitive to 10 μg. terramycin. It was also sensitive to the vibriostatic agent 0–129 (2-4-diamino-6-7-di-isopropyl pteridine).

Metabolic properties. When the tap water used to make up peptone water was replaced completely or partially by sea water, the resulting growth was increased and appeared optimal when the concentration of salt in the medium was approximately 2%. The organism grew slowly at 5° and at 34° but not at 37°. On salt containing media it grew better at 22° than at 15°, but on ordinary

media the final growth was more or less equal at 15° and 22°. It grows from pH 6·0 to 9·0 with an optimum at 6·8. All these results are based on nephelometer readings. The organism will withstand heating to 40° for 10 min. but not to 45° for the same time.

Pathogenicity. Table 2 shows that the organism kills five species of fish including freshwater anadromous, catadromous and marine species.

DISCUSSION

When this organism is classified according to Skerman (1957) it is found to belong to the family *Vibrio* if it is treated as a curved rod or to the *Pseudomonas* family if a straight one. Haynes & Burkeholder (1957) state that the distinction of the straight rods of the *Pseudomonas* from the curved rods found in *Vibrio* is difficult as the curvature of the organism sometimes depends on the medium employed. It has, however, been found that the pseudomonads are oxidative in their action on carbohydrates while the vibrios are fermentative (Hugh & Leifson, 1953). This organism is fermentative, i.e. it can produce acid from carbohydrates under anaerobic conditions so that on this criterion it should belong to the *Vibrio* genus. In addition, it is sensitive to the 0–129 which Shewan, Hodgkiss & Liston (1954) state is a vibriostatic agent. This is further evidence that the organism is a *Vibrio* species.

For comparison the following cultures which had been isolated from diseased fish were obtained and examined.

(1) *Vibrio anguillarum*, National Collection of Marine Bacteria No. 6 (Torry Research Station, Aberdeen), isolated by Bagge & Bagge (1956) from diseased codling in Denmark.

(2) *V. piscium* var. *japonicus* isolated by Hoshina (1956) from rainbow trout in Japan.

(3) *V. ichthyodermis* isolated by Hodgkiss & Shewan (1950) from a plaice. This was initially called *Pseudomonas ichthyodermis* but the authors now consider it to be a *Vibrio*.

Vibrio anguillarum when injected intramuscularly into eels caused death in 2–6 days. It produced haemorrhage at the site of inoculation in two, swelling and congestion in the third and no symptoms in the fourth. The organism was re-isolated however from them all, and in every case was found in sections of the spleen, liver or muscle. Intramuscular injection of perch gave rise to swellings and the perch died in 6–10 days. The organism was re-isolated from one and found in liver and muscle sections. Trout also succumbed to intraperitoneal injection in 16–21 days. The peritoneum of one was distended and contained much reddish brown fluid while the vent of the second was slightly distended and haemorrhagic. The organism was re-isolated after death and observed in sections of muscle of both trout.

Neither *Vibrio piscium* var. *japonicus* nor *V. ichthyodermis* was pathogenic for eels but both killed one of four trout inoculated intraperitoneally. In both these cases the organism was re-isolated. This low pathogenicity may well be due to the fact that these organisms have been *in vitro* culture for some considerable time.

The above three cultures were also studied morphologically and biochemically, and were found to be very similar to the organism isolated from the finnock.

Vibrio anguillarum differed from the finnock culture only in its ability to ferment arabinose, glycerol and cellobiose and its inability to ferment starch. It produced indole and grew on Koser's citrate medium. *V. piscium* var. *japonicus* differed in its action on arabinose, starch inositol and cellobiose and *V. ichthyodermis* by its action on sorbitol, glycerol, starch and cellobiose, its inability to produce acetylmethylcarbinol and its ability to produce indole.

The descriptions of *Vibrio anguillarum* in the literature vary to some extent, but Nybelin (1935) described *V. anguillarum* as a Gram-negative curved bacillus which was actively motile, non-sporing and non-capsulate. It liquefied gelatin in a similar manner to the cholera bacillus, produced acid but no gas from glucose or maltose, did not produce hydrogen sulphide, had no reducing properties, was haemolytic (only a few strains examined); and was a halophile. He subdivided this species into two subtypes A and B. A was able to produce acid but no gas from sucrose and mannitol and to produce indole, whereas B had no action on these sugars and did not produce indole. Bagge & Bagge (1956) describe their isolate as *V. anguillarum* type A on this basis.

Hoshina (1957) ascribed the name *Vibrio piscium* var. *japonicus* to the organism he isolated from rainbow trout as he thought it resembled the organism *V. piscium* which David (1927) had isolated from carp. It seems, however, that it is more likely to be a variant of *V. anguillarum* as its growth characteristics are almost identical with those of *V. anguillarum*, and it does not produce indole or hydrogen sulphide to any great extent (I could find neither), whereas *V. piscium* produces them both; lastly Hoshina's culture and *V. anguillarum* both produce acid but no gas from sugars and reduce nitrates to nitrites, while *V. piscium* can neither ferment carbohydrates nor reduce nitrates. It would therefore seem that this organism would be more appropriately named *V. anguillarum*.

Likewise the organism described here falls into Nybelin's general classification except for the haemolysis of rabbit blood which was not studied and the reducing properties which this organism has. It appears, however, that this organism is intermediate between A and B. Nybelin (1935) did study such a strain. He obtained it from Schäperclaus who isolated it at Lübeck in 1932. This strain was able to produce acid from both sucrose and mannitol but could not produce indole. Nybelin considered it unnecessary to postulate a third type to cover this strain, as it might have been a weak indole producer, but the strain from finnock and that of Hoshina seem to be identical with the Lübeck strain and so should be called *V. anguillarum* type C.

No attempt has been made to study the antigenic structure of the strains considered here, as previous workers have shown that the structure is complex, and Nybelin (1935) found that cross-reactions rarely occurred in organisms isolated from different epizootics.

Strains of this organism have been lodged with the National Collection of Marine Bacteria at Torry Research Station, Aberdeen.

The author wishes to express her thanks to Mr K. A. Pyefinch for his interest and encouragement, to Miss E. F. M. Greig for her valuable technical assistance, and to Messrs F. G. T. Holliday, W. D. Irvine, G. G. J. Smart, R. Steven and interested anglers for the supply of specimens.

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J. gen. Microbiol. (1961), **24**, 253–260
Printed in Great Britain

The Sugar Composition of Streptococcal Cell Walls and its Relation to Haemagglutination Pattern

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SUMMARY

The sugar composition of cell walls of various streptococci and pneumococci was investigated by paper chromatography. Rhamnose was found in the walls of most streptococci bearing Lancefield group haptens but was not identified in the walls of pneumococci or most 'viridans' streptococci. In the group as a whole galactose and glucose were more regularly present than rhamnose. A significant correlation was found between the distribution of rhamnose and haemagglutination pattern, as determined by the presence of a widely distributed red cell sensitizing antigen and the production of an agent capable, like the receptor-destroying enzyme of the influenza virus, of modifying the antigenic properties of the red-cell surface.

INTRODUCTION

In recent years considerable advances have been made in our knowledge of the composition and structure of the bacterial cell wall. These advances were made possible by the development by Salton and others (Salton & Horne, 1951; Salton, 1952; Cummins & Harris, 1956) of satisfactory techniques for the preparation of purified cell-wall homogenates. On *a priori* grounds it seems likely that cell-wall composition would have considerable taxonomic value. This possibility is borne out particularly by the finding that the cell walls of Gram-negative bacteria show, as far as they have been examined, a wider range of amino acids and a higher lipid content than do those of Gram-positive organisms (Salton, 1952, 1953) and of generic differences amongst various Gram-positive bacteria in cell-wall sugar composition (Cummins & Harris, 1956, 1958).

An analysis of the cell-wall composition of various streptococci was reported by Cummins & Harris (1956). These workers found rhamnose to be a constituent of the cell walls of strains of streptococci of Lancefield groups A to G; from these results it seemed possible that rhamnose, which they found to be absent from the cell walls of staphylococci and certain other species, might be characteristic of the streptococci as a whole. They noted, however, differences between different strains of streptococci in their content of other sugars, and were in general of the opinion that the sugar and amino sugar composition of the cell wall was of taxonomic significance at species level. A quite different approach to the taxonomy of the streptococci was described by Stewart, Steele & Martin (1959). This proposed that these organisms might be classified by the haemagglutination technique on the

basis of the distribution of various red cell sensitizing antigens and of an agent—cell-modifying agent (CMA)—possessing properties similar to those of the receptor-destroying enzyme of the influenza virus.

The present communication reports the results of an investigation by paper chromatography into the sugar composition of the cell walls of various streptococci and pneumococci and considers in particular the relationship between cell-wall sugar content and haemagglutination pattern.

METHODS

Organisms. At least one member of each Lancefield group A to Q examined was from the National Collection of Type Cultures (N.C.T.C., Colindale, London, N.W.9). Two of the group K strains produced mucoid colonies on 5% (w/v) sucrose agar and may therefore be classified as *Streptococcus salivarius*. The strains of groups R and S examined were made available to us by the courtesy of Dr C. E. de Moor (Utrecht). The majority of the 'viridans' and group K strains were laboratory isolates from upper respiratory sources (throat swabs, sputa, saliva). They were selected in the first instance on the basis of the production of a viridans change on human blood agar incubated aerobically and were insensitive to 1 in 5000 ethyl hydrocuprein, were deoxycholate insoluble and did not produce mucoid colonies on sucrose agar. All strains classified as 'viridans' streptococci in addition gave negative tube precipitin reactions with antisera for Lancefield groups A to N. The Lancefield groups were determined on 48 hr. cultures in 0·5% (w/v) glucose digest broth extracted at 100° with 0·05 N-HCl. The precipitating sera used were those issued commercially by Burroughs Wellcome and Co.

Group O serum was also used routinely in our tests, but doubts about its specificity were raised by the finding of what appeared to be an excessively high proportion of reactions with 'viridans' strains. On further investigation the serum was found to react with a number of NCTC strains belonging to various other Lancefield groups. On examination by the Ouchterlony plate diffusion technique against the NCTC group O strain—8029—the serum was found to give a number of distinct precipitation lines. Pending fuller investigations the designation group O has therefore been withheld from all strains except NCTC 8029.

The pneumococci examined included one strain of each of types 2 (NCTC 7466), 14 and 16 and two strains of type 3. The remaining two strains were not typed.

Cell-wall analysis. Cultures for cell-wall analysis were obtained by overnight growth at 37° in Hartley's digest broth containing 0·2% (w/v) $NaHCO_3$ and 0·5% (w/v) glucose. Five hundred ml. of culture were usually sufficient for examination except in the case of the pneumococci when twice this volume was found necessary to give a satisfactory bacterial mass. The organisms were harvested by centrifugation at 3000 *g* and washed twice in 0·9% (w/v) saline.

The method used for the preparation of cell walls was essentially that of Cummins & Harris (1956), involving disintegration of the organisms by shaking in a Mickle disintegrator with no. 12 ballotini glass beads. Disintegration was considered complete when no intact organisms could be seen in Gram-stained films. The glass beads were removed by passage through a coarse sintered glass filter. The cell walls were separated by differential centrifugation and purified by washing with distilled

water, followed by treatment with trypsin and ribonuclease. The cell walls were then sedimented by centrifugation, washed in water and resuspended in water. Peptic digestion was omitted since control experiments with a number of strains showed no difference between pepsin-treated cell walls and walls which had not been treated with pepsin.

Random samples were examined from time to time in a Beckmann spectrophotometer to detect the presence of contaminating nucleotide material from the cytoplasm (Salton & Horne, 1951; Barkulis & Jones, 1957). In no case was any peak observed in the 260 mμ region.

Conditions and treatment. The method of hydrolysis employed followed that used by Cummins & Harris (1956) viz. treatment with 2N-H_2SO_4 at 100° for 2 hr. in sealed tubes. The hydrolysate was neutralized with $Ba(OH)_2$ and then dried over P_2O_5 in an Edwards centrifugal freeze-drying apparatus. Approximately 30 mg. dry weight of cell wall was used in each test; where this amount was not available the final volume of the hydrolysate was adjusted accordingly—a constant volume of this being applied to each chromatogram.

One-dimensional descending chromatograms on Whatman no. 4 paper with the solvent ethyl acetate + pyridine + water of Jermyn & Isherwood (1949) were found to resolve all the sugars present provided that the solvent front was allowed to run well off the paper. The sugars were revealed with aniline hydrogen phthalate, and hexosamines with the Elson and Morgan reagent. No attempt was made to separate individual hexosamines.

Haemagglutination techniques. Cultures for haemagglutination tests were obtained by growth for *c.* 18 hr. in Hartley's digest broth. The cultures were centrifuged until visibly clear and the supernatant fluids thus obtained used for cell treatment. For the preparation of absorbing suspensions 48 hr. cultures in digest broth containing 0·5 % (w/v) glucose were used. Human group O red cells, washed three times in saline before use and resuspended in saline to give a *c.* 5 % suspension were used for cell treatment. Treatment was carried out by incubating mixtures of equal volumes of cell suspension and of supernatant fluid for 2 hr. at 37°. At the end of this time the cells were sedimented by centrifugation, washed in saline and resuspended to give a 5 % suspension. For the haemagglutination tests proper one drop of cell suspension and one drop of serum dilution were mixed and the mixtures allowed to stand for 30 min. at room temperature; they were then examined for agglutination by inspection of the deposit with a × 10 microscope ocular.

The serum used for the detection of the Hickey antigen was obtained by immunization of a rabbit with the deposit from a culture of *Streptococcus pyogenes* in 0·5 % (w/v) glucose digest broth. The serum was heated to 56° for 30 min. and, in order to remove naturally occurring T agglutinins, was absorbed with red cells treated with the supernatant fluid of a digest broth culture of a 'viridans' streptococcus which produced the cell-modifying (receptor-destroying) enzyme. For test purposes the serum was used in a dilution of 1/20.

The serum used for the detection of cell-modifying activity was obtained by immunization of a rabbit with cells treated with allantoic fluid from chick embryos infected with the FMI strain (type A I) of influenza virus. The serum was heated to 56° for 30 min. and was heavily absorbed with normal red cells to remove species agglutinins. For test purposes it was used in a dilution of 1/40.

RESULTS

Cell-wall composition

The results of the cell-wall analyses are shown in Table 1. It will be seen that though in agreement with the findings of Cummins & Harris (1956) rhamnose was a component of the cell walls of the streptococci of groups A to G examined; its presence was not a general property of the streptococci since it was absent from the group O strain (NCTC 8029), from a number of group K strains, from the majority of the 'viridans' streptococci and from all the pneumococci. The two strains of *Streptococcus salivarius* classified as group K were rhamnose positive. The absence of rhamnose from the cell walls of pneumococci is consistent with the findings of Smith, Mills, Harper & Galloway (1957) who failed to detect this sugar in the cellular polysaccharide obtained by enzymic digestion of whole pneumococci.

Table 1. *Cell-wall sugar composition of various streptococci*

	Sugars			
	Gal + Gl +	Gal − Gl +	Gal + Gl −	Gal − Gl −
	Strains			
Rhamnose-positive	A (1), D (2), F (3), H (2), K (10), L (1), M (1), P (1), Q (1), R (2), S (2), 'Viridans' (11)	E (1), H (1), N (1), 'Viridans' (1)	B (1), D (1), G (1)	A (4), C (1)
Rhamnose-negative	K (3), O (1), 'Viridans' (21), Pneumococci (7)	K (1)	'Viridans' (3)	

Gal = galactose; Gl = glucose. Hexosamine present in all strains. Figures in parentheses indicate number of strains examined.

Surprisingly, considerably less variation was found in the group as a whole in the distribution of galactose and glucose than in that of rhamnose. With the exception of four strains of *Streptococcus pyogenes* and one group C strain, all strains possessed one or other of these sugars and most possessed both. From a taxonomic point of view it is significant, however, that of the seven groups of which more than one strain was examined, intra-group variation in either galactose or glucose content was encountered in four groups, namely A, D, H and K. The discovery of a group A strain which possessed both galactose and glucose is of interest in view of the fact that previous strains of this species which have been reported were found to be deficient in both these sugars (Barkulis & Jones, 1957; Cummins & Harris, 1956; McCarty, 1952; Salton, 1953; Schmidt, 1952). Both galactose and glucose were regularly present in the pneumococci and appeared to be somewhat more consistently present, though not significantly so, in the 'viridans' streptococci than in the streptococci identified as possessing Lancefield group haptens. Unlike Cummins & Harris (1956), who detected mannose in the cell walls of one group E and two group D strains, we have failed to identify mannose in any of our strains.

Relationship of cell-wall sugar content to haemagglutination pattern

The relationship between cell-wall sugar content and haemagglutination pattern as determined by the reactions with the CMA and Hickey sera is shown in Table 4. Tests for the Hickey antigen were carried out in the first place with the supernatant fluids of digest broth cultures. Strains which gave negative results in these tests were then retested by an absorption technique. For this purpose the sediments from 48 hr. cultures in digest broth containing 0·5 % (w/v) glucose were used, the sediment from 20 ml. culture being used to absorb 1 ml. unabsorbed Hickey serum diluted 1/20. Absorption was for 30 min. at room temperature. In each case two consecutive absorptions of the serum were carried out, the same amount of culture being used for the second absorption. The unabsorbed serum and samples of serum after each absorption were then titrated against red cells treated with supernatant fluid of the Hickey (*Streptococcus pyogenes*) strain. The results obtained with this method were quite clear cut, most Hickey-positive strains removing the antibody completely after the first absorption, and Hickey-negative strains causing at most a twofold decrease in titre after two absorptions. A typical absorption result is shown in Table 2.

Table 2. *Absorption of Hickey serum with representative strains of streptococci*

Figures given are reciprocals of serum dilution causing agglutination.

	Test cocci		
	Treated with Hickey supernatant fluid		Untreated
	Reciprocals of serum dilution		
	1st absorption	2nd absorption	
BI (CMA-positive viridans)	640	320	< 40
MM (CMA-positive group K)	320	320	< 40
NCTC 8029 CMA-positive group O	80	< 40	< 40
44 CMA-negative group K	< 40	.	< 40
Unabsorbed	640		< 40

CMA = cell-modifying agent.

In the earlier work, in which the distribution of the Hickey antigen was studied only in the supernatant fluids of digest broth cultures (Stewart *et al.* 1959), a very high negative correlation was found between the distribution of the antigen and that of the cell-modifying enzyme. With the more rigorous technique used in the present work it was found, however, that strains which give negative results for the Hickey antigen in digest broth might yet be shown by the absorption technique to possess it. One such culture was the group O strain (NCTC 8029). On repeated testing of this strain, however, occasional positive haemagglutination reactions were obtained with the supernatant fluids of glucose digest broth cultures. (For tests with glucose digest broth the red cells were suspended in phosphate buffered saline at pH 7).

The distribution of the four haemagglutination patterns determined as described in the preceding paragraph amongst the various streptococci examined is shown in Table 3. It will be seen that, excluding certain group K strains the Hickey antigen was present in all the streptococci bearing Lancefield group haptens examined, and that all except two of these were CMA negative. This distribution is in sharp contrast to that found in the pneumococci, all of which were Hickey-negative CMA-positive. In contrast, the 'viridans' streptococci showed a much greater diversity of haemagglutination type.

Table 3. *Haemagglutination patterns of strains examined*

	Haemagglutination type			
	Hickey + CMA −	Hickey − CMA +	Hickey + CMA +	Hickey − CMA −
	Number of strains			
Streptococci of groups A to S (excluding K)	26	0	2	0
Group K streptococci	9	5	0	0
'Viridans' streptococci	12	14	6	4
Pneumococci	0	7	0	0

CMA = cell-modifying agent.

From the results shown in Table 4 it will be seen that there was a considerable correlation, amongst the streptococci, between the presence of rhamnose as a cell-wall component and the haemagglutination type. This is best seen in the relative frequencies of the Hickey-positive CMA-negative and Hickey-negative CMA-positive types in rhamnose-positive and rhamnose-negative strains. Thus of 49 rhamnose-positive strains 41 were Hickey-positive CMA-negative and 6 were Hickey-negative CMA-positive. The rhamnose-negative strains, on the other hand, showed a quite different haemagglutination pattern; of 29 of such strains examined 6 were Hickey-positive CMA-negative and 13 were Hickey-negative CMA-positive ($\chi^2 = 20{\cdot}4$. $P < 0{\cdot}001$). This correlation was even greater when the results with digest broth cultures alone were considered. In this case 40

Table 4. *Relationship between streptococcal cell-wall sugar content and haemagglutination (HA) type*

		Cell-wall sugars					
		Rhamnose		Galactose		Glucose	
		+	−	+	−	+	−
HA type		Number of strains					
Hickey	CMA						
+	−	41	6	38	9	37	10
−	+	6	13	18	1	19	0
+	+	2	6	8	0	7	1
−	−	0	4	4	0	4	0

CMA = cell-modifying agent.
Results obtained with pneumococci excluded from this table.

of the rhamnose-positive strains were Hickey-positive CMA-negative and 6 were Hickey-negative CMA-positive, while of the rhamnose-negative strains 2 were Hickey-positive CMA-negative and 17 were Hickey-negative CMA-positive.

Of the 14 group K strains examined 9 were rhamnose-positive Hickey-positive CMA-negative, 4 were rhamnose-negative Hickey-negative CMA-positive, and one was rhamnose-positive Hickey-negative CMA-positive.

DISCUSSION

Our results have shown that rhamnose, though present in the cell walls of most streptococci possessing Lancefield group haptens, is not characteristic of the streptococci as a whole. In particular, it was found to be absent from the walls of the definitive group O strain examined, from those of certain group K streptococci and from those of the majority of viridans streptococci. The question therefore arises as to whether, amongst the streptococci, the presence of rhamnose as a cell-wall component is of importance as a criterion of phylogenetic relationship. Its presence in most streptococci bearing Lancefield group haptens and its absence from the pneumococci would suggest that it is. This view is reinforced by the finding that the distribution of rhamnose has a significant relation to haemagglutination pattern as determined by the presence of the Hickey antigen and the production of the cell-modifying enzyme. One is in fact tempted to see in this group of organisms two basic types from which others might have arisen by a process of hybridization namely: (1) a rhamnose-positive Hickey-positive CMA-negative type typical of the majority of the Lancefield group streptococci; (2) a rhamnose-negative Hickey-negative CMA-positive type characteristic of the pneumococci. These two types have in fact accounted for just over two-thirds of the strains of streptococci examined.

The status of group K strains as defined by these reactions is of interest. Group K streptococci have already been shown to be heterogeneous in respect of their capacity to produce mucoid colonies on sucrose agar—a property which is accepted as the definitive criterion of the species *Streptococcus salivarius* (Williams, 1956). In fact from the work of Williams the production of mucoid colonies on sucrose agar appears to be better correlated with various other physiological properties than is the possession of the K hapten. Group K strains are also heterogeneous in their haemagglutination patterns. The introduction of rhamnose as an additional parameter introduces still further heterogeneity into the group.

We wish to thank the Medical Research Council of Ireland for its support and to express our indebtedness to Miss P. Reddy, Miss K. O'Keefe and Miss P. McGown for skilled technical assistance.

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J. gen. Microbiol. (1961), **24**, 261–272
Printed in Great Britain

Morphological and Biochemical Features of 'Atypical' Mycobacteria

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(*Received* 22 *August* 1960)

SUMMARY

Morphological and biochemical features of 42 strains of 'atypical' mycobacteria and one strain of *Mycobacterium tuberculosis* were studied. Of the 42 atypical mycobacteria, 16 were originally classified in Runyon's group I, 4 in group II, 19 in group III, and 3 in group IV. The characteristics studied were bacillary morphology and staining properties on Kirschner and Löwenstein–Jensen media; colonial morphology on 7H-10 agar medium; pigmentation in the dark and after exposure to light; rate of growth and temperature requirements, with different methods of inoculation; growth on blood and nutrient agar plates, and in gelatin stabs; catalase activity on drug-free and on isoniazid-containing media; nicotinic acid (niacin) production. The sensitivity of the majority of the strains to 6 chemotherapeutic drugs was tested. The niacin test proved to be the most useful method for distinguishing the atypical mycobacteria from *M. tuberculosis*. In identifying strains of group I, their ability to produce yellow pigment after exposure to light was of most value, and their colonial morphology and their periodic acid-Schiff staining were also helpful. Strains of group II were identified by their ability to form yellow pigment in the dark, by their periodic acid-Schiff staining and by their colonial morphology. Strains of group III were identified by their rate of growth and buff pigmentation. Exceptionally a yellow pigment was formed, and such strains were identified principally by their colonial morphology and periodic acid-Schiff staining. Among the 19 strains classified originally as group III, 3 were reclassified into group IV. Strains of group IV were identified by their ability to grow on blood and nutrient agar plates within 3 days and in gelatin stab cultures within 2 weeks.

INTRODUCTION

In the last few years several publications have appeared dealing with human diseases caused by 'atypical' mycobacteria (Wood, Buhler & Pollak, 1956; Nassau & Hamilton, 1957; Beck, 1959; Lewis *et al.* 1959; Engbaek, Magnusson & Nielsen 1959). These authors, and others mentioned by Nassau & Hamilton (1957), stated that the diseases caused by atypical mycobacteria and by *Mycobacterium tuberculosis* are closely similar. Thus the correct diagnosis and treatment depends upon the precise identification of the causative agent. The identification of an infection by *M. tuberculosis* in routine diagnostic work often depends on the morphology and the staining characteristics of the bacilli as seen in smears made from clinical specimens and sometimes from cultures, together with the colonial morphology on Löwen-

stein-Jensen medium. These procedures are liable to considerable misinterpretation. The bacillary morphology and the degree of acid-fastness of *M. tuberculosis* and atypical mycobacteria may be similar. Colonies of *M. tuberculosis* on Löwenstein–Jensen medium may closely resemble those of some other strains of mycobacteria. Several morphological and biochemical tests (reviewed by Freerksen, 1960) have been suggested as additional aids for differentiating atypical mycobacteria from *M. tuberculosis*, such as microcolonial structure (Engbaek, 1952), cord formation (Middlebrook, Dubos & Pierce, 1947; Yegian & Budd, 1953), pigmentation of colonies (Tarshis & Frisch, 1952; Timpe & Runyon, 1954), the neutral red test (Dubos & Middlebrook, 1948; Hauduroy & Posternak, 1949), catalase activity (Middlebrook, 1954) and nicotinic acid (niacin) production (Konno, 1956). No single test has been found to be completely satisfactory for this purpose. Runyon (1959*a*, *b*) proposed a classification of the atypical mycobacteria based mainly on pigmentation of colonies and speed of growth. The purpose of the present investigation was to examine the morphological characteristics and some of the biochemical properties of a series of strains of atypical mycobacteria, classified into Runyon's four groups, and to see whether these characteristics are of value in differentiating these strains from *M. tuberculosis*. All of the strains used were of human origin.

METHODS

Strains

Mycobacterium tuberculosis, 1 strain, H37Rv. Atypical mycobacteria classified according to Runyon (1959*a*): group I, photochromogens (16 strains); group II, scotochromogens (4 strains); group III, Battey type (19 strains); group IV, rapidly growing (3 strains). The origin of the strains is set out in Table 1. All were obtained from human sputum or resected lung. The 13 British strains were obtained from 13 patients who had been examined in various British chest clinics and hospitals. Of these, 4 were described by Selkon & Mitchison (1959). All 29 strains from the United States of America were kindly sent by Dr E. H. Runyon, Veterans Administration Hospital, Salt Lake City, Utah, U.S.A.

Table 1. *The mycobacterial strains examined*

Group	No. of strains tested	Great Britain		United States of America	
		No. of strains	Strain no.	No. of strains	Strain no.
I. Photochromogen	16	10	0523, 0735, 1438, 1526S, 1526R, M1159, M1212, M1246, M1247, A725	6	15, 260, 265, 266, 353, 440
II. Scotochromogen	4	2	0112, M1177	2	50, 251
III. Battey type	19	1	M1208	18	160, 170, 171C, 223, 225, 277, 248, 470, 477, 487, 509, 513, 514, 520, 585, P7, P23, P25
IV. Rapidly growing	3	.	.	3	380, 481, 518

Examination of cultures in Kirschner medium. Kirschner medium (Mackie & McCartney, 1956) was inoculated from Löwenstein–Jensen cultures in screw-capped bottles, which were 3 weeks or less old, and which had not been opened since inoculation. Care was taken not to transfer lumps of bacteria to the Kirschner medium. After incubation for 14 days at 37°, films were made and they were then dried under an ultraviolet lamp for 30 min., placed in methanol for 5 min., and rinsed in water. Ziehl–Neelsen staining was performed in the usual way, with 5 min. decolorization with acid-alcohol (NaCl 20 g., HCl conc. 20 ml., 74° O.P. Spirit 1500 ml., distilled water 400 ml.) and counterstaining with Löffler's methylene blue for 5–15 min. Periodic acid-Schiff staining was performed as described by Csillag (1960).

Examination of cultures on Löwenstein-Jensen medium. Cultures were grown on Löwenstein–Jensen medium for 3 weeks at 37°. Smears, prepared and fixed as described above, were stained by the Ziehl–Neelsen and the Ziehl–Heidenhain (Uyeda, 1955) methods.

Colonial morphology. The morphology of colonies was examined on plates of oleic acid + albumin agar medium (7H-10 medium; Cohn, Middlebrook & Russell, 1959). The plates were inoculated from a 4-week growth on the same medium and incubated for 3–4 weeks at 37° in a hot room where they were occasionally exposed to artificial illumination. The colonies were formolized, and observed with a plate microscope ($\times 10$ magnification).

Pigmentation following incubation in the dark or after exposure to light. Two Löwenstein–Jensen medium slopes were inoculated with each strain. One of these was placed in a sealed box and incubated at 37° in the dark. The other slope was also incubated at 37° and, when growth first appeared, it was exposed to diffuse daylight for 1 hr., re-incubated for 24 hr. and compared for its pigmentation with the slope incubated in the dark. When only minor differences in pigmentation were found between the two slopes, the procedure was repeated on another batch of medium. Those strains which produced yellow or orange pigment when grown in the dark were examined in the same manner, but when growing on 7H-10 medium slopes.

Temperature requirements and rate of growth. The rate of growth at 37° and at 22° was studied on Löwenstein–Jensen medium slopes which had been inoculated with a loop containing a small amount of growth from a 3- to 4-week culture on the same medium ('clumped inoculum' method). With the photochromogens, the rate of growth at the same temperatures was also studied on the same medium which had been inoculated with a 3 mm. loopful of a suspension prepared by shaking about 2 mg. (moist weight) of bacillary mass with 0·4 ml. distilled water in a screw-capped bottle containing glass beads ('dispersed inoculum' method). Cultures were examined at daily intervals (except on Sundays) for 6 weeks.

Growth on blood and nutrient agar. Nutrient agar plates with or without 5% (v/v) horse blood were inoculated from a 3- to 4-week growth on 7H-10 agar plates. The cultures were incubated at 37° for 3 days.

Growth in gelatin stabs. Nutrient gelatin stab cultures were inoculated with a straight wire from a 3- to 4-week growth on 7H-10 medium plates. The cultures were incubated at 22° for 6 weeks.

The nicotinic acid (niacin) test. Tests for nicotinic acid were carried out as described by Gilani & Selkon (1958), after incubation for 6 weeks.

Catalase activity. Catalase activity (Middlebrook, 1954; Selkon & Mitchison, 1959) was studied on growth on drug-free Löwenstein–Jensen slopes and on slopes containing isoniazid in the sensitivity tests. In studying the catalase activity of isoniazid-resistant variants, Löwenstein–Jensen slopes containing 50 μg. isoniazid/ml. were inoculated with a large amount of bacillary growth, incubated for 4 weeks at 37° and the resulting growth tested.

Sensitivity tests. Sensitivity tests to isoniazid, *p*-aminosalicylic acid, streptomycin and *p*-acetamidobenzaldehyde thiosemicarbazone (thiacetazone) were performed as described by Selkon & Mitchison (1959). The sensitivity to 2-ethylisothionicotinamide (thioamide) and cycloserine was tested in a similar manner, with twofold serial drug dilutions in Löwenstein–Jensen medium. The results of a single test on each strain are reported; unexpected results were not repeated.

RESULTS

Bacillary morphology and staining

The strains were divided into types on the basis of their bacillary morphology ('BM') type and staining properties, as follows.

Group I

BM type Ia (15 strains, all except no. 1526R). Majority of organisms longer than those of *Mycobacterium tuberculosis* H37Rv, and strongly beaded. Tendency to formation of loose cords present, especially in Kirschner medium. Not acid-fast organisms only exceptionally seen with both staining methods, with 5 min. or 15 min. counterstaining. Periodic acid-Schiff staining positive.

BM type Ib (1 strain, no. 1526R). As above, but no tendency to cord formation, either in Kirschner or on Löwenstein–Jensen medium.

Group II

BM type II (4 strains). Size of organisms variable. Majority of organisms beaded. Cord-formation not observed in either medium. Not acid-fast organisms only exceptionally seen with both staining methods. Periodic acid-Schiff staining positive.

Group III

BM type IIIa (10 strains; all nos. except those listed as types IIIb–IIId below). Size of organisms nearly uniform and shorter than those of *Mycobacterium tuberculosis* H37Rv. Beaded or uniform staining. Tendency to cord formation not observed. Not acid-fast organisms rarely seen in growth on Kirschner medium. More not acid-fast organisms seen in growth on Löwenstein–Jensen medium, especially when stained either by the Ziehl-Neelsen method, with 15 min. counterstaining, or by the Ziehl–Heidenhain method. Periodic acid-Schiff staining negative.

BM type IIIb (7 strains, nos. P7, P23, 170, 223, 277, 487, M1208). As for type IIIa, but organisms not uniform in size; filaments, rods and coccoid forms being seen in the same smear. Nearly half of the organisms not acid-fast in growth on Löwenstein–Jensen medium, with all staining methods. Majority of organisms not acid-fast in Kirschner medium. Periodic acid-Schiff staining negative.

BM type IIIc (1 strain: no 171). As for type IIIb, but, after 8 preliminary serial subcultures at monthly intervals on Löwenstein–Jensen medium, exclusively not

acid-fast organisms seen in both media. Organisms of types IIIa, IIIb and IIId retained acid-fastness during similar passages. Periodic acid-Schiff staining negative.

BM type IIId (1 strain: no. 160). As for type IIIa, but tight cords present in growth from Kirschner medium. Periodic acid-Schiff staining negative.

Group IV

BM type IV (3 strains). Size of organisms similar to those of *Mycobacterium tuberculosis* H37Rv. Staining uniform. Tight cords present. Not acid-fast organisms only exceptionally seen in both media, with both staining methods. Periodic acid-Schiff staining negative.

Mycobacterium tuberculosis (H37Rv). Size of organisms typical for *M. tuberculosis*. Staining uniform or beaded. Tight cords present. Not acid-fast elements only exceptionally seen in both media, with both staining methods. Periodic acid-Schiff staining negative.

Colonial morphology

The strains were divided into types on the basis of their colonial morphology ('CM' types) on 7H-10 medium, as follows.

Group I

CM type Ia (13 strains, all nos., except those listed as type Ib). Circular, differences in size slight, low convex, finely granular surface, slightly crenated edge, central area of opacity, pigmented buff, butyrous.

CM type Ib (3 strains, nos. 440, 0735, 1526R). Circular, differences in size slight, raised, coarsely granular surface, crenated edge, some colonies umbonate, heaping between adjacent colonies sometimes seen, pigmented buff, friable, difficult to emulsify.

Group II

CM type II (4 strains). Circular, nearly uniform in size, high, convex, smooth surface, uniform structure, entire edge, pigmented yellow, butyrous.

Group III

CM type IIIa (11 strains: all nos. except those listed as types IIIb–IIIe below). Circular, size variable, domed, smooth surface, uniform structure, entire or slightly crenated edge, pigmented buff, butyrous.

CM type IIIb (2 strains, nos, 514, 520). As for type IIIa, but pigmented yellow.

CM type IIIc (1 strain, no. 513). As for type IIIa, but colonies irregular in shape.

CM type IIId (1 strain; no. 470). As for type IIIa, but size uniform and central area of opacity.

CM type IIIe (4 strains: nos. 160, 170, 225, 509). Circular, size very variable, effuse, umbonate, finely granular surface, entire or crenated edge, pigmented buff, friable.

Group IV

CM type IV (3 strains). Circular, size variable, low convex, coarsely granular surface, crenated edge, some colonies in each strain with centre umbonate, heaping between adjacent colonies seen, pigmented buff, friable.

Mycobacterium tuberculosis. Circular, size slightly variable, low convex, coarsely granular surface, crenated edge, centre umbonate, heaping between adjacent colonies seen, pigmented buff, friable.

Pigmentation

Pigmentation was observed on colonies grown systematically in the dark or exposed to light, with the following results.

Group I. All strains buff, when grown on Löwenstein–Jensen medium in the dark, yellow after 1 hr. exposure to light. One strain (no. 0735) pigmented yellow only after two exposures of 8 hr. to light.

Group II. Pigmented deep yellow in the dark and in the light on Löwenstein–Jensen and on 7H-10 medium.

Group III. Strains 514, 520, M1208 pigmented deep yellow in the dark and in the light on Löwenstein–Jensen and 7H-10 media. All remaining strains pigmented buff in the dark and in the light.

Group IV and *Mycobacterium tuberculosis*. Pigmented buff in the dark and in the light.

Temperature requirements and rate of growth

All of the atypical mycobacteria grew at 22° within 6 weeks when the cultures were inoculated by the clumped inoculum method. Among strains of *Mycobacterium tuberculosis*, H37Rv did not grow at this temperature; in further experiments with other strains growth occurred in a small proportion of them. Among the 16 group I strains whose cultures were inoculated by the dispersed inoculum method, three did not grow at 22° (nos. A725, M1247, 0523). Considerable differences were found in the rate of growth at 37°, depending upon the inoculum used. Growth was more rapid with a clumped inoculum than with a dispersed inoculum and, with the group III strains, growth occurred 3–4 days earlier when the slopes were inoculated from 6-week cultures than from 2- to 3-week cultures. Whichever inoculum was used, group IV strains grew more rapidly than the remaining strains of atypical mycobacteria, and these grew slightly more rapidly (1–4 days quicker) than *M. tuberculosis* H37Rv.

Growth on blood and nutrient agar

Strains of group I and group II did not grow within 5 days on blood agar and nutrient agar plates. Of the 19 group III strains, 16 did not grow on these media in this period, but 3 strains (nos. P25, 487, 171C) yielded growth within 3 days. All group IV strains grew within 3 days. *Mycobacterium tuberculosis* H37Rv did not grow.

Growth in gelatin stabs

Group I strains, group II strains, *Mycobacterium tuberculosis* H37Rv and those group III strains which did not grow on blood and nutrient agar, did not grow in gelatin stabs. The 3 group III strains, which yielded growth on blood and nutrient agar, grew in gelatin stabs within 2 weeks. These strains formed colonies on the top and a filiform growth in the stab; liquefaction was not observed. All group IV strains grew within 2 weeks, forming colonies on the tops and a filiform growth in the stabs, and did not liquefy the medium.

The nicotinic acid test

All strains of atypical mycobacteria yielded a negative nicotinic acid test, while *Mycobacterium tuberculosis* H37Rv yielded a positive result.

Catalase activity

The results of catalase tests are shown in Table 2. All group I, group II and group IV strains showed catalase activity greater than with *Mycobacterium tuberculosis* H37Rv when grown on drug-free medium and on medium containing isoniazid, as did their isoniazid-resistant variants. Group III strains showed a variable pattern of catalase activity, some strains having activities similar to those of the remaining atypical strains and others yielding a pattern more closely resembling that of *Mycobacterium tuberculosis* H37Rv.

Table 2. *Catalase activity of mycobacteria*

		Catalase activity*		
		Original strain		
Group	Strain no.	Growth on drug free medium	Growth on medium containing highest isoniazid concentration allowing growth	Variant resistant to 50 μg. isoniazid/ml.
I	All 16	+ + +	+ + +	+ + +
II	All 4	+ + +	+ + +	+ + +
III	171C, 487, 513, 514, 520, P7, P25	+ + +	+ + +	+ + +
	160, 225, 470, P23	+ + +	+ +	+ +
	248, 277, 509, 585	+ +	+ +	+ +
	170, 477	+ +	+ +	0
	223, M1208	+ +	+	+
IV	All 3	+ + +	+ + +	+ + +
M. tuberculosis	H37Rv	+ +	0	0

* Catalase activity scored by number of + signs.
Catalase activity more marked than that of strain *Mycobacterium tuberculosis* H37Rv, + + +; equal to that of strain H37Rv, + +; present, but less than that of strain H37Rv, +; no activity, O.

Sensitivity tests

The results of the sensitivity tests are set out in Tables 3 and 4. Classifications as sensitive, doubtfully resistant, and resistant are based on experience with strains of *Mycobacterium tuberculosis*. All of the strains of atypical mycobacteria were resistant to isoniazid and all were resistant to *p*-aminosalicylic acid, with three exceptions (group I, strains nos. 0523, 0735 and 260). All group IV strains were resistant to streptomycin, but about one third of the remainder were sensitive. All of the group I strains were sensitive or doubtfully resistant to cycloserine. Of the 22 group II and group III strains tested, one in each group (nos. 0112, 170) was resistant to cycloserine, as were all of the group IV strains. All of the group I and

Table 3. *Sensitivity of atypical mycobacteria to isoniazid,* p-*aminosalicylic acid and streptomycin*

Group	No. of strains tested	Isoniazid minimal inhibitory concentration (μg./ml.) Resistant >50	50	5	Doubtfully resistant 1	Sensitive 0·2	*p*-Aminosalicylic acid resistance ratio* Resistant >16	16	8	Doubtfully resistant 4	Sensitive 2 or less	Streptomycin resistance ratio* Resistant >256	(256–8)	8	Doubtfully resistant 4	Sensitive 2 or less
I	16	3	7	3	3	0	12	1	0	0	3	0	3	4	4	5
II	4	4	0	0	0	0	2	0	1	1	0	0	0	0	3	1
III	19	12	6	0	1	0	18	0	1	0	0	1	8	2	0	8
IV	3	1	1	0	1	0	3	0	0	0	0	1	2	0	0	0

* Resistance ratio: the minimal inhibitory concentration for the test strain: the minimal inhibitory concentration for the standard sensitive strain H37Rv.

Table 4. *Sensitivity of atypical mycobacteria to cycloserine, thioamide and thiacetazone*

Group	Cycloserine resistance ratio* No. of strains tested	Resistant >16	16	8	Doubtfully resistant 4	Sensitive 2 or less	Thioamide resistance ratio* No. of strains tested	Resistant >16	16	8	Doubtfully resistant 4	Sensitive 2 or less	Thiacetazone resistance ratio* No. of strains tested	Resistant >8	8	Sensitive 4 or less
I	12	0	0	0	3	9	12	0	0	0	0	12	11[2]	3	0	8
II	3	0	0	1	0	2	3	0	0	0	0	3	2[3]	2	0	0
III	19	1	0	0	2	16	18[1]	5	0	5	4	4	17[4]	16	0	1
IV	3	3	0	0	0	0	3	0	0	2	1	0	1[5]	1	0	0

* Resistance ratio: the minimal inhibitory concentration for the test strain: the minimal inhibitory concentration for the standard strain of *Mycobacterium tuberculosis.*

[1] Strain no. 170 not tested to thioamide; [2] Strain no. A. 725 not tested to thiacetazone; [3] Strain no. 0112 not tested to thiacetazone; [4] Strains nos. 470, 520 not tested to thiacetazone; [5] Strains nos. 380, 518 not tested to thiacetazone. Strains nos. M1159, M1212, M1246, M1247 (group I) and strain no. M1177 (group II) not tested to any of these three drugs.

group II strains were sensitive to thioamide and the remaining groups showed a variable pattern of sensitivity. Of the 11 group I strains tested, 8 were sensitive to thiacetazone whereas all except one of the remaining strains (no. 514) were resistant. These sensitivity tests were not repeated systematically and, from further experience, a slightly different pattern of resistance, particularly to *p*-aminosalicylic acid and thiacetazone, might have been encountered if several tests had been set up on each strain.

Characteristics of the different groups

Those features of the strains belonging to the four groups which were found to be most consistent and characteristic of the groups, are given below.

Group I. Long, beaded, strongly acid-fast, periodic acid-Schiff positive. Tendency to cord formation. Colonies of the smooth strains (CM type Ia) have central areas of opacity. Colonies of the rough strains (CM type Ib) similar to those of *Mycobacterium tuberculosis*, strain H37Rv. Yellow pigmentation only after exposure to daylight. No growth on blood and nutrient agar at 37° within 3 days, and no growth in gelatin stabs at 22° within 6 weeks. Nicotinic acid test negative. Catalase activity strongly positive even when grown on isoniazid-containing medium. Resistant to isoniazid; sensitive to thioamide; sensitive or doubtfully resistant to cycloserine; often sensitive to thiacetazone.

Group II. Beaded, strongly acid-fast, period acid-Schiff positive organisms of variable length. No tendency to cord formation. Colonies smooth, with entire edges. Pigmented deep yellow when grown in the dark, as well as after exposure to light. No growth on blood and nutrient agar at 37° within 3 days and no growth in gelatin stabs at 22° within 6 weeks. Nicotinic acid negative. Catalase activity strongly positive, even when grown on isoniazid-containing medium. Resistant to isoniazid and thiacetazone; sensitive to thioamide; sensitive or doubtfully resistant to streptomycin.

Group III. Organisms either uniformly shorter than those of *Mycobacterium tuberculosis* H37Rv or pleomorphic. Staining either uniform or beaded. Either all organisms acid-fast or many not acid-fast. No tendency to cord formation (tight cord formed by one strain). Periodic acid-Schiff negative. Colonies of smooth strains (CM type IIIa–IIId) variable in size, with entire edges and uniform structure. (One strain with central area of opacity.) Colonies of rough strains (CM type IIIe) similar to those of *M. tuberculosis* strain H37Rv, but smaller and more variable in size. Colonies of 16 strains pigmented buff, but colonies of the remaining 3 strains pigmented yellow. No growth on blood and nutrient agar at 37° within 3 days, and no growth in gelatin stabs within 6 weeks (3 exceptions). Catalase activity variable, often diminished when grown on isoniazid-containing slopes. Resistant to isoniazid and *p*-aminosalicylic acid; sensitive or doubtfully resistant to cycloserine (1 exception).

Group IV. Bacillary morphology, staining properties (including acid-fastness and periodic acid-Schiff staining), morphology of colonies and pigmentation the same as those of *Mycobacterium tuberculosis*, strain H37Rv. Growth on blood and nutrient agar at 37° within 3 days, and growth in gelatin stabs at 22° within 6 weeks. Nicotinic acid test negative. Catalase activity strongly positive even when grown on isoniazid-containing medium. Resistant to isoniazid, *p*-aminosalicylic acid, streptomycin and cycloserine; resistant or doubtfully resistant to thioamide.

DISCUSSION

Two main problems arise during the course of identifying mycobacteria obtained from material of human origin: (1) the differentiation of *Mycobacterium tuberculosis* from the atypical mycobacteria, and (2) their classification. In these experiments, the most useful method for differentiating *M. tuberculosis* from the atypical mycobacteria was found to be the nicotinic acid test. All of the strains of atypical mycobacteria yielded a negative result, whereas strain *M. tuberculosis* H37Rv gave a positive result. Using the same technique, Gilani & Selkon (1958) found that all of 13 human strains of *M. tuberculosis* yielded a positive nicotinic acid test. Runyon, Selin & Harris (1959), who used a different technique, reported their own findings and those of other workers, and found that the nicotinic acid test was uniformly negative for atypical mycobacteria and uniformly positive for human *M. tuberculosis*. The main disadvantage of the nicotinic acid test is that bovine strains of *M. tuberculosis* yield a negative result, so that strains of mycobacteria which yield such a result must be further characterized, for example by guinea-pig inoculation. Growth at 22° was not reliable as a means of differentiating *M. tuberculosis* from atypical mycobacteria since with the clumped inoculum occasional strains of *M. tuberculosis* yielded growth, and with the dispersed inoculum some of the atypical mycobacteria tested did not grow. Growth at 25° may be more satisfactory (Marks & Trollope, 1960). The following characteristics were also of value in identifying atypical strains: long, beaded organisms; weakly acid-fast; lack of cord formation; bacillary pleomorphism; periodic acid-Schiff positivity; smoothness of colonies; yellow pigmentation of colonies; growth on blood and nutrient agar within 3 days; growth in gelatin stabs cultures; high catalase activity on medium containing isoniazid and a characteristic sensitivity pattern. No one of these characteristics was invariably shown by all of the atypical strains. However, when several of these characters were examined, all of the atypical strains were distinguishable from *M. tuberculosis*.

The following characteristics were found to be of most value in classifying the atypical mycobacteria into the groups proposed by Runyon (1959*a*). *Group I:* formation of yellow pigment after exposure to light for 1 hr. was described as the essential characteristic of strains in this group. However, one of our strains required a longer period of exposure, but it was still included with other strains in this group because of the similarity of their behaviour in other respects. Strains with smooth colonies (CM type Ia) could usually be classified by their colonial morphology, but rough colonies (CM type Ib) could be confused with those of *Mycobacterium tuberculosis* and of group IV strains. Positive periodic acid-Schiff staining was an invariable feature in this group but was also found in group II strains. *Group II:* formation of yellow or orange pigment in the dark (Runyon, 1959*a*) was invariably found with all strains, but also occurred with 3 of the group III strains. Positive periodic acid-Schiff staining was shown by all strains in this group, and also by those in group I. Colonial morphology was also of value. *Group III:* among the 19 strains classified in this group, 3 yielded yellow pigmentation both in the dark and in the light, and on both Löwenstein-Jensen and 7H-10 media. Nevertheless, they resembled the remaining strains in this group in other characteristics and should be retained in it. A further 3 strains grew on nutrient agar and Löwenstein–Jensen medium within

3 days and they also grew in gelatin stab cultures. It would therefore be reasonable to transfer these 3 strains to group IV. With the remaining strains classified in group III, diversity of all of the characteristics studied was noted, and this group cannot be considered as homogeneous. *Group IV:* Runyon did not describe in detail the specific conditions necessary to identify these strains as rapidly-growing ones. In the present work the speed of growth of the strains in all of the groups on Löwenstein–Jensen medium was dependent on the size and type of the inoculum, so that it was not always easy to distinguish group IV strains from the remainder. The ability to grow on blood and nutrient agar plates within 3 days was found only with group IV strains and with the three group III strains mentioned above. The superiority of the test on blood or nutrient agar may be due not only to differences in growth rate, but also the more satisfactory nature of these media for group IV strains. The ability to grow in gelatin stab cultures within 6 weeks was also of value for characterizing these strains. Further experience with 32 strains of saprophytic mycobacteria confirms the value of blood and nutrient agar and of gelatin stab cultures in their identification as group IV strains.

I wish to thank Dr D. A. Mitchison for his many helpful suggestions. Thanks are also due to Miss Janet Lloyd for her excellent technical assistance.

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J. gen. Microbiol. (1961), **24**, 273–289
With 1 *plate*
Printed in Great Britain

Isolation and Classification of a New Series of Azotobacter Bacteriophages

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(*Received* 26 *August* 1960)

SUMMARY

Successful isolation of azotobacter phages from soil samples was accomplished by using a modified Burk's nitrogen-free medium with sucrose as the carbon source. The natural azotobacter flora in the soil samples served as the enrichment strains and the medium was not further enriched by the addition of laboratory cultures of the bacteria. Phage titres as high as $5{\cdot}5 \times 10^9$ plaque-forming units/ml. were obtained when the indicator strain for phage assays was *Azotobacter vinelandii* O. On the basis of plaque morphology, nine phage isolates were obtained and purified by standard techniques. The plaques formed by the phages consisted of a central clear area surrounded by a halo and ranged from 1 to 7 mm. in diameter. Antiphage sera were produced in rabbits against a previously isolated phage and the new isolates; on the basis of cross-neutralization experiments with homologous and heterologous antisera, the 10 phages were placed into four major serological groups. Groups I and II contained four phages each, and groups III and IV contained one phage each. The degree of serological relatedness among the phages within groups I and II was investigated. A survey of 48 azotobacter strains showed that 11 out of 12 *A. vinelandii* strains and 14 out of 25 *A. chroococcum* strains showed plaque formation by one or more of the phages. Strains of *A. agilis*, *A. macrocytogenes*, *A. insigne* and *A. indicus* were not lysed by the phages. The value of the present series of phages in the classification of the genus *Azotobacter* was discussed.

INTRODUCTION

A bacteriophage for certain strains of the genus *Azotobacter* designated *A. vinelandii* was originally isolated and characterized by Monsour (1954), Monsour, Wyss & Kellogg (1955) and Kellogg (1957). Since plaques were not observed with the non-pigmented strains of *A. agilis* or strains of *A. chroococcum* (Monsour *et al.* 1955), the securing of additional phages appeared desirable since they would serve as a supplementary tool in the study of ecology and mutations in these organisms and would be an aid in the species designation of this genus. In addition, most of our present information regarding the bacteriophages has resulted from investigations mainly on the coli phages. Further characterization of additional bacteriophages and phage host systems would aid in confirming and generalizing phenomena already postulated, or might lead to new developments. The azotobacters are especially suited to such an investigation because of their many unique characteristics. They

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are larger than the average bacteria, are capable of abundant growth in a simple medium free from combined nitrogen, possess a high respiratory activity and to a certain extent are genetically stable organisms. The present investigation describes the isolation and classification of a new series of azotobacter phages.

METHODS

Strains. Most of the work was accomplished by using *Azotobacter vinelandii* O, a strain originally obtained from the University of Wisconsin. This strain was subcultured every 4 days on Burk's modified nitrogen-free medium and the colonial morphology of the strain was routinely examined by visual inspection of individual clones, with the use of oblique illumination. On several occasions a smooth clone with the least amount of opaqueness was selected and purified by dilution and plating. Incubation was at 33° for 24–48 hr. Other strains of azotobacter used included the following: *A. vinelandii*, American Type Culture Collection (hereafter referred to as ATCC) strains 478, 7484, 7487, 7489, 7492, 7496, 9046, 9047, 9104, 12837; University of Texas Stock Culture Collection (hereafter referred to as UT) strain K; *A. agilis*, ATCC strains 7494, 9040, 9042, 9570, 12838; UT strain M; *A. chroococcum*, ATCC strains 480, 4412, 7486, 7488, 7490, 7491, 7493, 7497, 7498, 7499, 9043, 9044, 9045, 9048, 9049, 9051, 9335, 9544, 12981; UT strains A, B, C, D, E, F, J, S; *A. indicus*, ATCC strains 9037, 9038, 9039, 9540; *A. macrocytogenes*, ATCC strains 12334, 12335, 12336; *A. insigne* strains 8 and 2, received from Dr Vagn Jensen (University of Lund, Lund, Sweden).

Media. The following media were used:

Burk's modified nitrogen-free medium (Kellogg, 1957). The medium contained (g./l.): Na_2HPO_4, 0·189; KH_2PO_4, 0·011; $MgSO_4.7H_2O$, 0·200; $FeSO_4.7H_2O$, 0·006; MoO_3, 0·0005; $CaSO_4.2H_2O$, 0·02; $SrCl_2.6H_2O$, 0·01; NaCl, 0·01; $NaHCO_3$, 0·05. De-ionized water was used in the preparation of this medium. The medium was at pH 7·8 before autoclaving (18 lb./sq.in. for 20 min.). When sucrose was added as the carbon source, it was autoclaved (18 lb./sq.in. for 20 min.) in 50 % (w/v) solutions and added to the medium to give a final concentration of 0·5 % (w/v). Hereafter, the term 'Nf medium' is used instead of 'nitrogen-free medium'.

ATCC media. The base medium contained (g./l.): K_2HPO_4, 1·0 g.; $MgSO_4.7H_2O_2$, 0·2; NaCl, 0·2; $FeSO_4.7H_2O$, trace; soil extract, 100 ml.; tap water, 900 ml. The soil extract was prepared by adding 77 g. of a commercial African violet potting soil and 0·2 g. Na_2CO_3 to 200 ml. distilled water. The mixture was autoclaved (18 lb./sq.in. for 1 hr.) and filtered through a Buchner funnel. The four media prepared from the above stock were: ATCC-1, adjusted to pH 8·3, 2 % (w/v) mannitol added, autoclaved, final pH 7·6; ATCC-2, adjusted to pH 6·0, 2 % (w/v) mannitol added, autoclaved; ATCC-3, adjusted to pH 6·0, 2 % (w/v) glucose added, autoclaved, ATCC-4, adjusted to pH 7·6, autoclaved, sterile glucose to a final concentration 1·0 % (w/v). All autoclaving was at 18 lb./sq.in. for 20 min.

Phages. Ten phages were used in these studies. One phage, designated 'Original' or 'A-11' for these studies, was reported by Monsour *et al.* 1955; the other nine phages were new isolates made in this laboratory.

Isolation of phage from soil. One-litre flasks containing 50 ml. Nf medium with 0·5 % (w/v) sucrose as carbon source were seeded with 1·0 g. soil and incubated on a shaker at 33° for 4 days. At this time, the contents of the flask were clarified by centrifugation, passed through a membrane filter (Millipore, 047 mm.) and assayed for phage by the double agar layer technique. The indicator strain for the phage assays was *Azotobacter vinelandii* O.

Phage assay. Phage dilutions were made in Nf medium minus carbon source, and plaque counts were made by the overlay method (Adams, 1950) modified by Kellogg (1957) for use with azotobacter phage. Approximately 35 ml. Nf medium containing 1·8 % agar (w/v) were used for the base layer, and 2·0 ml. containing 0·5 % (w/v) agar were used for the semisolid agar layer that contained the mixture of the suspension of bacteria and phage. Unless otherwise stated, both layers of media contained sucrose at a final concentration of 0·5 % (w/v). Incubation was at 33° for 24 hr. or until satisfactory plaque formation was observed (the plates were not inverted).

Preparation of indicator strain. The strain used for the background growth was grown in 15 ml. volumes of Nf medium containing 0·5 % (w/v) sucrose and incubated at 33° on a shaker for 12–18 hr. After growth, the culture was centrifuged at room temperature, the organisms washed, resuspended in de-ionized water and adjusted to a turbidity reading of 35 units on a Klett–Summerson photoelectric colorimeter (blue filter). Unless otherwise stated, *Azotobacter vinelandii* O was used as the indicator strain.

Propagation of phage. For the preparation of stock phage, phages were grown using *Azotobacter vinelandii* O as the propagating strain. Organisms were grown in litre flasks containing 100 ml. Nf medium + 0·5 % (w/v) sucrose. Incubation was on a shaker at 33° until a turbidity reading of 35 units on the Klett–Summerson photoelectric colorimeter (blue filter) was obtained. This turbidity represented 3×10^7 viable organisms/ml. Phages were added to the organisms at a 1:1 ratio of phage:organisms, and the organism + phage mixture allowed to remain static at 33° for 30 min. at which time it was placed on a shaker at the same temperature for 7–13 hr.

A second method for obtaining phage stocks was used for two phages (A-24, A-41). These phages were assayed by the overlay method and the plate from the highest dilution which showed almost complete lysis was selected. The soft agar layer was scraped from the plate and placed in 7·5 ml. Nf media. After overnight extraction at 4°, the agar was removed by filtration through cheesecloth, and the bacterial debris sedimented at low speed in a centrifuge. The supernatant fluid was assayed, and the process repeated until no further increase in phage titre was observed. At this time, a larger quantity of phage stock was obtained by preparing 10–20 plates at the phage dilution which gave complete to partial lysis. Following propagation, phage preparations were stored overnight at 4° to allow debris to settle out. The supernatant fluids were decanted, centrifuged at 700 rev./min. for 30 min. and passed through a membrane filter (Millipore, 047 mm). The phage filtrates were stored at 4°.

Preparation and assay of antiphage sera. For the initial series of immunizations, rabbits were immunized subcutaneously with 5 ml. phage filtrate at 5-day intervals until a series of six injections had been administered. The animals were then bled

at either 6 or 15 days following the last injection. Booster immunizations consisted of two 5·0 ml. subcutaneous injections given 5 days apart and for the majority of the phages were administered 1 month following the last injection of the initial series. All sera were collected in the usual manner and stored at −20° without preservative.

The rates of inactivation of the various azotobacter phages by their homologous and heterologous antisera were determined by the following procedures. All dilutions were made in Nf medium. Stock phages were diluted to contain approximately 10^5 to 10^6 plaque-forming units/ml. (the term 'plaque-forming units' is hereafter referred to as 'PFU'). The sera were diluted to a concentration determined by preliminary tests which permitted approximately 1–10 % survival of the PFU after sera and phage dilutions were mixed and incubated at 37° for 5–10 min. Phage and serum dilutions were preheated to 37° in a water bath. At time zero, 5·0 ml. phage dilution were added to 5·0 ml. serum dilution, and at specified time intervals 0·1 ml. samples were removed and mixed with 9·9 ml. Nf medium. The initial 1/100 dilution was sufficient to stop the neutralizing action of the antisera. Dilutions within the range required were made to detect phage survival by the plaque count method. Plating was done in duplicate by the overlay method in the usual manner. A phage control containing 5·0 ml. Nf medium instead of immune sera was run simultaneously and served as a basis for determining % survival of phage. The results of the assays of surviving phage of each neutralization were plotted on a logarithmic scale against time on a linear scale. A straight line was drawn through the points, and the K value determined (Adams, 1959).

Neutralization technique. Cross-neutralization assays with the various azotobacter phages and antiphage sera were carried out in accordance with the following procedures. All antisera were used at a final dilution of 1/200 except antiserum A-41, which was used at a final dilution of 1/100. Three ten-fold serial dilutions were used for each phage and the dilutions were calculated so that the middle dilution would provide approximately 100 PFU/ml. in the final dilution. Dilutions of phage and antisera were made in Nf medium minus carbon source. Equal quantities of the various phage dilutions and antisera dilutions were mixed and incubated in a water bath at 37° for 30 min. Two controls were run simultaneously: one contained Nf medium instead of antiserum, the second contained normal rabbit serum at the same dilution as the immune sera. Following incubation, the assay tubes were placed in an ice water bath, and samples were plated by the double agar layer method in the usual way. Plaques were counted and a comparison made between the controls and immune sera.

Host specificity assays. A modification of the overlay technique was used in these experiments. Nf medium was used for growth of all strains with the following exceptions: *Azotobacter macrocytogenes* strains 12334, 12335, 12336, medium ATCC-2; *A. indicus* strains 9037, 9038, 9039, 9450, medium ATCC-3; *A. chroococcum* strain 9048, medium ATCC-4, strain 7499, medium ATCC-1; *A. agilis* strains 9042, 9570, medium ATCC-4. Petri plates measuring 14 cm. diam. × 2 cm. were used. Approximately 100–150 ml. agar medium was used for the basal layer, and 10 ml. was used for the soft agar layer which contained 0·5 ml. of a bacterial suspension. Young broth cultures of azotobacter strains were centrifuged, washed, resuspended in water and standardized to a reading of 40 units on the Klett–Summerson

photoelectric colorimeter (blue filter) with the exception of the *A. macrocytogenes* strains which were standardized to 50 units. The plates were subdivided into 20–25 squares, and dilutions of the phages ranging from 1/10 to 1/100,000 were pipetted in 0·02 ml. amounts on to the top layer of agar. Following absorption of the phage suspensions, the plates were incubated at 33° for 18–24 hr.; the time depended upon the growth rate of the various strains.

RESULTS

Isolation of phage from soil

Preliminary experiments indicated that the natural azotobacter flora present in the soil samples under investigation would serve as the enrichment strains and that addition of laboratory cultures of the bacteria to the culture medium was not necessary. Soil samples were obtained from various geographical localities, and phages were isolated according to the procedures described in the previous section. The results of the isolation experiments are presented in Table 1.

Table 1. *Results of azotobacter phage isolations from six different soil samples*

Soil source	Flask no.	Phage assay PFU/ml.	No. different phages isolated	Present phage designation
Maryland	MD-1	$1{\cdot}4 \times 10^9$	1	A-22
	MD-2	$5{\cdot}5 \times 10^9$		
Ohio	OH-1	9×10^8	2	A-12
	OH-2	1×10^7		A-24
Texas	SA	$1{\cdot}2 \times 10^7$	2	A-14
				A-23
Texas	VA	$3{\cdot}7 \times 10^7$	2	A-13
				A-21
Texas	9	1×10^5	1	A-41
Texas	FB	1×10^8	1	A-31

By these procedures, phage titres ranging from 10^5 to 10^9 PFU/ml. were obtained, and on the basis of plaque morphology, nine different phages were isolated and purified by further single plaque selection. From three of the soil samples two distinct phage isolates were made, whereas the remaining samples appeared to have homogeneous plaques.

Plaque morphology

In the selection of plaques during the purification of azotobacter phages by single-plaque selection, an attempt was made to select plaques of extreme size, for example, minute or large; plaques with small clear areas and wide turbid halos; or plaques with large clear areas and small halos. Photographs of the plaques produced by the azotobacter phages are shown in Pl. 1, figs. A–I, and a description of the plaque size is presented in Table 2.

The host strain for these studies was *Azotobacter vinelandii* O, and the plating conditions were the same as those previously described. The plaques consisted of a central clear area surrounded by a halo and ranged in size from 1 to 7 mm. in diameter. This halo was present even in very small plaques in which the central

Table 2. *Plaque size of azotobacter phages with* Azotobacter vinelandii O *as the indicator strain*

Phage	Plaque diameter (mm.)	Halo diameter (mm.)	Diameter of clear area of lysis (mm.)
A-11	1–4	0·5–1·5	0·5
A-12	1	< 1	< 1
A-13	4	1·5	1
A-14	1–2	0·5	1
A-21	6–7	1–1·5	3·5–4
A-22	3–5	1–1·5	2
A-23	3–5	1	2·5–3
A-24	3	1	1
A-31	4	1	2
A-41	3–4	1	0·5

clear area was not very apparent. The plaques observed with these phages were of four types: (1) minute, represented by A-12 and A-14; (2) small central clear area with wide halo, represented by A-13 and A-11; (3) large central clear area with narrow halo, represented by A-21, A-22, A-23 and A-31; (4) large central clear area with irregular or 'frayed' halo, represented by A-41. At early readings this plaque no. 4 gave the appearance of not having a halo; however, when the plates were refrigerated overnight, a more pronounced halo was observed. This observation was also true for the minute plaques of phages A-12 and A-14.

Propagation of phages

Although optimum conditions for maximum phage titre were not determined, high-titre phage stocks were prepared from the original phage (A-11) and eight of the newly isolated phages according to the methods described in the previous section. The results of a propagation experiment are presented in Table 3.

Table 3. *Preparation of phage stocks with* Azotobacter vinelandii O *as the propagating strain*

Phage	PFU/ml.	Phage added (ml.)	Azoto-bacters* added (ml.)	PFU/ml. in flask before incubation	Time on shaker (hr.)	PFU/ml. after incubation
A-11	$1{\cdot}7\times10^{9}$	2	98	$3{\cdot}4\times10^{7}$	13	1×10^{9}
A-12	$1{\cdot}3\times10^{9}$	2	98	3×10^{7}	13	8×10^{8}
A-13	$1{\cdot}3\times10^{9}$	2	98	3×10^{7}	7	$1{\cdot}5\times10^{9}$
A-14	$3{\cdot}2\times10^{9}$	1	99	3×10^{7}	7	1×10^{9}
A-21	$1{\cdot}5\times10^{9}$	3	97	3×10^{7}	7	$1{\cdot}3\times10^{9}$
A-22	$1{\cdot}6\times10^{10}$	0·2	99·8	3×10^{7}	13	5×10^{10}
A-23	1×10^{9}	3	97	3×10^{7}	7	$1{\cdot}3\times10^{9}$
A-31	6×10^{9}	1	99	6×10^{7}	13	4×10^{9}

* 3×10^{7} viable organisms/ml.

The *Azotobacter vinelandii* strain O was used at a concentration of 3×10^{7} viable organisms/ml. and the ratio phage:organisms at the start of the experiment was approximately 1:1. For the majority of the phages, final titre of 10^{9} PFU/ml. was

achieved. Four of the phage preparations showed complete lysis of the bacterial culture after incubation on the shaker for *c.* 7 hr.; for the remaining preparations an arbitrary incubation time of 13 hr. was selected. With the majority of the azotobacter phages a considerable amount of bacterial debris remained after lysis.

Two of the azotobacter phages (A-24, A-41) were not successfully propagated according to the above methods; therefore a modification of the agar layer technique was used. By these procedures, the highest titres obtained for phages A-24 and A-41 were 5×10^8 and 5×10^7 PFU/ml., respectively. All phage preparations appeared stable following Millipore filtration and storage at 4° with the exception of phage A-11; however, non-filtered preparations of this phage appeared to be stable.

Table 4. *Cross-neutralization by representative antisera against various azotobacter phages*

Anti-sera	Phages									
	A-11	A-12	A-13	A-14	A-21	A-22	A-23	A-24	A-31	A-41
A-11	+	+	+	+	−	−	−	−	−	−
A-12	+	+	+	+	−	−	−	−	−	−
A-13	+	+	+	+	−	−	−	−	−	−
A-14	+	+	+	+	−	−	−	−	−	−
A-21	−	−	−	−	+	+	+	+	−	−
A-22	−	−	−	−	+	+	+	+	−	−
A-23	−	−	−	−	+	+	+	+	−	−
A-31	−	−	−	−	−	−	−	−	+	−
A-41	−	−	−	−	−	−	−	−	−	+
Normal serum	−	−	−	−	−	−	−	−	−	−

+ = neutralization; − = no neutralization. All antisera and the normal serum were used at final dilution 1/200, except antiserum A-41 which was used at final dilution 1/100.

Serological classification

The cross-neutralization data (Table 4) indicated that the phages formed four main serological groups; groups one and two contained four phages each, and groups three and four contained one phage each. Although antiserum against phage A-24 was not prepared, this phage was neutralized by the available group two antisera. On the basis of these data, the following serological groups for the azotobacter phages are proposed and will be referred to in subsequent discussions: group I (phages A-11, A-12, A-13, A-14); group II (phages A-21, A-22, A-23, A-24); group III (phage A-31); group IV (phage A-41).

Kinetics of phage neutralization

The neutralization rate constants of the various phages by their respective homologous antisera are presented in Table 5. Since the K value is a characteristic of the particular lot of serum used, the serum data have been included for reference purposes. The phages were neutralized by dilutions of homologous antisera ranging from 1/500 to 1/5000, and the K values ranged from 18 to 1917. As reported for other phage systems, the azotobacter phages differed greatly with respect to their rates of reaction with homologous antisera. As a group, the phages belonging to

Table 5. *Neutralization rate constants (K) of azotobacter phages with homologous antisera*

Phage	Final serum dilution	Serum date	PFU/ml.	K
A-14	1/5000	8/26/58	$7{\cdot}8 \times 10^6$	1917
A-21	1/5000	8/26/58	$3{\cdot}3 \times 10^6$	1642
A-22	1/5000	2/3/58*	$8{\cdot}9 \times 10^6$	1534
A-23	1/2000	8/26/58	$3{\cdot}3 \times 10^6$	742
A-31	1/1000	11/4/57	$8{\cdot}0 \times 10^6$	575
A-13	1/2000	8/26/58	$3{\cdot}7 \times 10^6$	460
A-11	1/1000	10/21/57*	$1{\cdot}7 \times 10^6$	329
A-12	1/200	8/26/58	$5{\cdot}5 \times 10^5$	115
A-41	1/150	9/27/58	$1{\cdot}3 \times 10^5$	18

* Pooled sera.

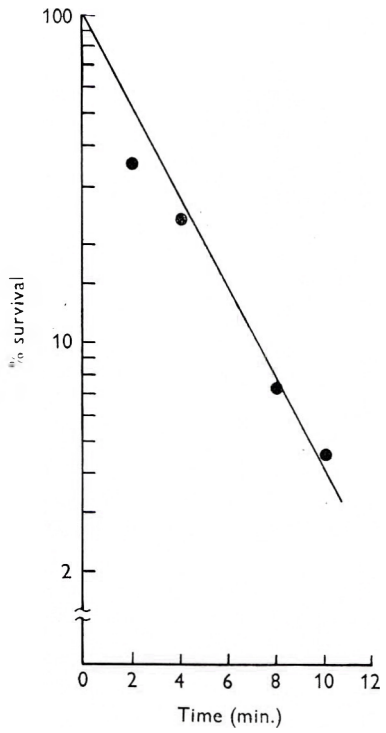

Fig. 1

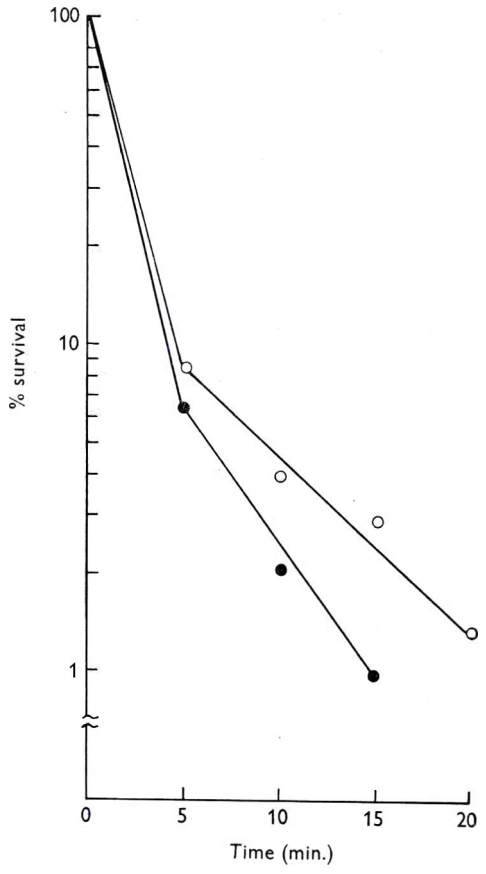

Fig. 2

Fig. 1. Rate of inactivation of phage A-11 by homologous antiserum (diluted 1/1000).

Fig. 2. Rates of inactivation of phage A-12 by homologous antiserum. The antiserum was diluted 1/200. ○, Expt. 1; ●, Expt. 2.

serological group II were among the better antigens, since their antisera showed relatively high K values. The high K value for serum A-14 indicated phage A-14 acted as a much better immunizing antigen than the other phages (A-11, A-12, A-13) of serological group I. The low K value observed with serum A-41 may be attributed to the relatively low concentration of phage used in the immunizing antigen.

The neutralization curves from which the K values were calculated are presented in Figs. 1–7. The values for sera A-21, A-22 and A-23 were obtained from the curves shown for the homologous neutralizations shown in Figs. 5–7.

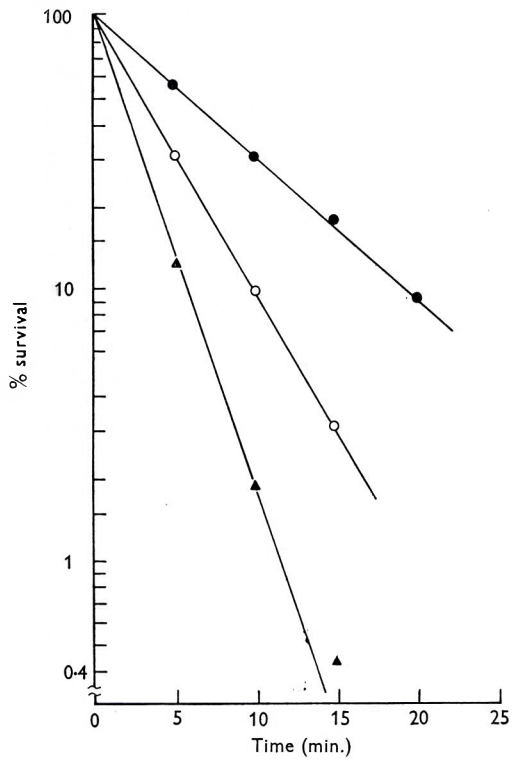

Fig. 3

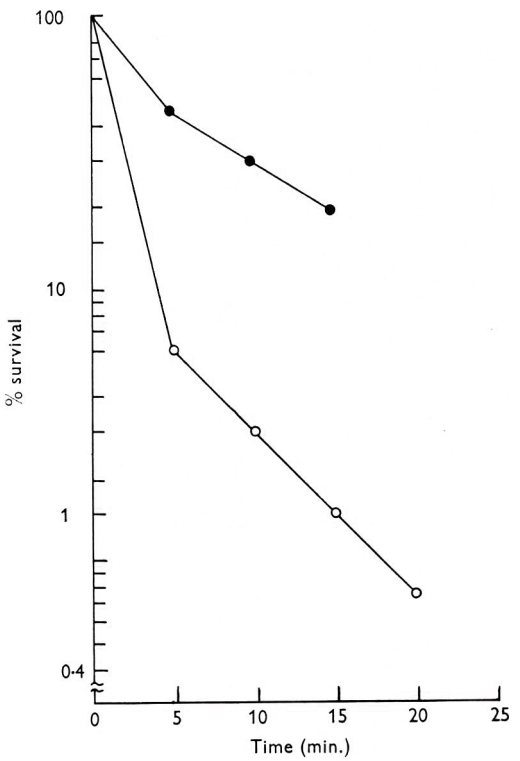

Fig. 4

Fig. 3. Rates of inactivation of phages A-13, A-14 and A-41 by homologous antisera. The antisera were diluted 1/2000 for A-13, 1/5000 for A-14 and 1/150 for A-41. ○, Phage, A-13; ▲, phage A-14; ●, phage A-41.

Fig. 4. Rates of inactivation of phage A-31 by homologous antisera. For Expt. 1, the phage was preparation 3/17/59 and the antiserum was lot number 1/11/58. The antiserum was diluted 1/2000. For Expt. 2, the phage was preparation 6/23/58 and the antiserum was number 4 of 11/5/57. The antiserum was diluted 1/1000. ●, Serum 1/11/58; ○, serum 11/5/57.

With phages A-12 and A-31 neutralization by homologous antisera did not follow first-order reactions. Figure 2 shows two separate neutralization curves obtained for phage A-12 with its homologous antiserum. Neutralization of phage proceeded rapidly to *c.* 96–98 % inactivation of phage and then slowed down rather abruptly. Figure 4 shows that inactivation of phage A-31 behaved similarly. With phage A-31

both the pooled sera (date 1/11/58) and the individual serum from rabbit 4 (date 11/5/57) showed this peculiarity. Also, the use of a different phage preparation in the experiment did not alter the shape of the curve. It should be noted that for these two phage serum systems the initial slopes of the neutralization curves were used to calculate K values to serve as an estimate of the relative neutralizing potencies of the sera.

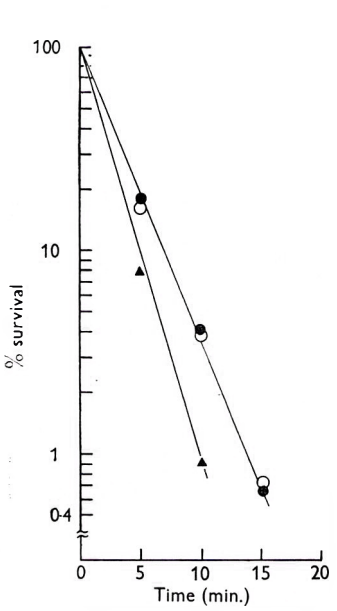

Fig. 5

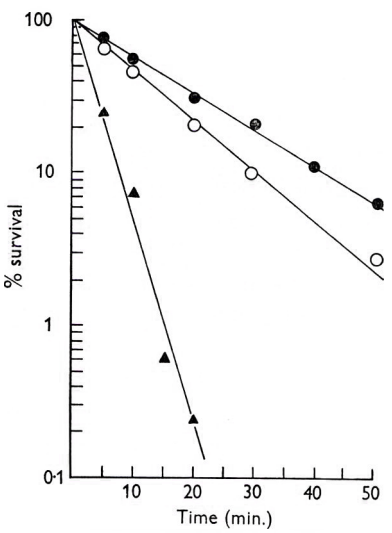

Fig. 6

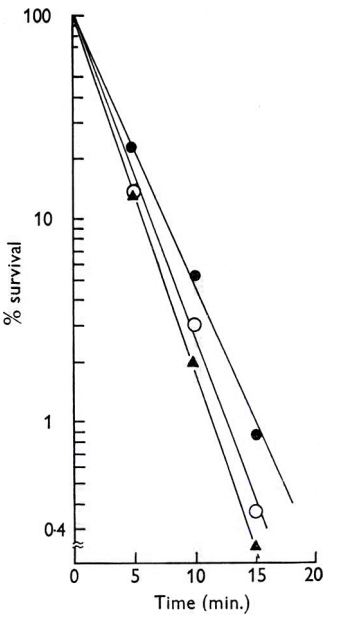

Fig.7

Fig. 5. Rates of inactivation of phages A-21, A-22 and A-23 by A-21 antiserum. The serum was diluted 1/5000 for phages A-21 and A-23 and 1/1000 for phage A-22. ●, Phage A-21; ▲, phage A-22; ○, phage A-23.

Fig. 6. Rates of inactivation of phages A-21, A-22 and A-23 by A-22 antiserum. The antiserum was diluted 1/5000 for the homologous phage and 1/3000 for the heterologous phages. ●, Phage A-21; ▲, phage A-22; ○, phage A-23.

Fig. 7. Rates of inactivation of phages A-21, A-22 and A-23 by A-23 antiserum. The antiserum was diluted 1/2000 for the homologous phage and for phage A-21, and 1/300 for phage A-22.

In order to determine the degree of relatedness among the phages of serological group II, neutralization curves were determined for three of the phages using homologous and heterologous antisera (Figs. 5–7). The K values for the homologous and reciprocal neutralizations are presented in Table 6. The rate of neutralization was greater with homologous phage and antiserum than with the heterologous system, except with antiserum 21 where the neutralization rates for phages A-21 and A-23 were the same. These data indicated that antigenically phages A-21 and A-23 were either very closely related or identical. This conclusion was substantiated with antisera A-22 and A-23. In addition, these data showed that the degree of relatedness of phage A-22 to the other two phages was more distant than the relationship between phages A-21 and A-23.

Table 6. *Neutralization rate constants (K) of representative group II antisera* against group II phages*

Serum	Phages A-21	A-22	A-23
	Neutralization rate constants (K)		
Anti A-21	1642	460	1642
Anti A-22	173	1534	230
Anti A-23	614	26	742

* 8/26/58 sera.

Preliminary investigations have been conducted on the degree of relatedness among the serological group I phages. Kinetic curves of neutralization using heterologous antiphage sera were not determined for these phage + serum systems; instead, an arbitrary end-point was used, namely the % of the initial phage population which survived after contact with serum at a stated concentration and for a specified time. The procedures were essentially the same as those described for the kinetic studies of phage neutralization except that only one time period was used to assay for phage survival. The time of assay varied with the antisera and was as follows: 5 min. for serum A-12, 10 min. for serum A-13, 6 min. for serum A-14. The final dilutions of sera were 1/200 for A-12, 1/2000 for A-13 and 1/5000 for A-14. All phages were diluted to contain approximately 10^5 PFU/ml.

Table 7. *Neutralization of serological group I phages by homologous and heterologous antisera*

Serum	Phages A-12	A-13	A-14
	% survival of phages		
Anti A-12	5	17	3
Anti A-13	75	41	57
Anti A-14	14	39	14

Table 7 shows the % survival of serological group I phages after neutralization by homologous and heterologous antisera. With serum A-12, % survival was essentially the same for phages A-12 and A-14 and greater for phage A-13. The results obtained with serum A-14 were similar to those observed with serum A-12. Serum A-13 inactivated its homologous phage more rapidly than either of the heterologous phages. These data indicated a close antigenic relationship between phages A-12 and A-14. In other experiments, not shown here, serum A-13 neutralized phages A-11 and A-13 at approximately the same rate, indicating a close relationship between these two phages. With phage A-11 it was important to use a newly produced phage stock. Cell-free filtrates of this phage lost their infectivity rapidly upon storage and older preparations sometimes showed anomalous neutralization patterns.

Host specificity assays

Host specificity assays were undertaken for the purpose of defining further the identity of the phages and also to ascertain the range of host infectiveness. Two of the phages, A-21 and A-23, were shown to be either identical or closely related by serological methods and it was of interest to compare their host-range specificity reactions. These data are presented in Table 8 and it may be observed that both phages gave identical results. Since it appeared that these two phages were identical, further studies on host-range specificity included only one of these phages, namely A-21.

Table 8. *Comparison of host-specificity reactions for phages* A-21 *and* A-23

Strains	Phages A-21 lytic activity	Phages A-23 lytic activity	Strains	Phages A-21 lytic activity	Phages A-23 lytic activity
A. vinelandii			*A. chroococcum*		
0	5	5†	A	2*	2*
478	5	5	S	5	4
7487	1*	1*	F	—	—
7489	2*	2*	J	—	—
7492	2*	2*	E	5	5
9046	3*	3*	B	—	—
9047	1*	2*	9335	5	5
12837	3	3	9544	4	4
7496	2*	2*	12981	—	—
9104	5	5			
K	4	4			

† Number 5 means lytic action at $1/10^5$ dilution, etc.
* Indicates plaques were not observed. — indicates no lytic action.

Table 9 shows the lytic action of eight phages for 12 strains designated *Azotobacter vinelandii*. All strains, except 7484, were lysed by one or more of the phages. The strain not lysed by the phages appeared to be a typical member of this species. The phages classified as serological group I (A-11 to A-14), showed identical

Table 9. *Lytic action of phages for* Azotobacter vinelandii *strains*

A. vinelandii strains	Phages A-11	A-12	A-13	A-14	A-21	A-22	A-31	A-41
0	5†	5	5	5	5	5	5	5
478	5	5	5	5	5	5	5	5
7487	2	5	5	4	1*	—	2*	1*
7489	3	4	5	4	2*	—	2*	1*
7492	3	3	5	5	2*	—	2*	1*
9046	2*	2*	5*	2*	3*	2*	4*	5
9047	4	3	5	5	1*	—	2*	1*
12837	2*	2*	2*	5*	3	5	2	—
7496	4	3*	5	3	2*	1*	3*	2*
9104	5	3	4	5	5	5	5	5
K	5	2*	5	4	4	5	5	5
7484	—	—	—	—	—	—	—	—

† Number 5 means lytic action at $1/10^5$ dilution, etc.
* Indicates plaques were not observed. — indicates no lytic action.

host range specificity with the exception of phage A-12 which did not form plaques on strains K and 7496. The phages of serological group II, A-21 and A-22, showed identical host range specificity. The lack of lytic action of phage A-22 on several strains was not considered a major difference, since plaque formation with phage A-21 was not observed on the same strains. In addition, the phages of serological group I appeared to be more specific for the *A. vinelandii* strains than the phages of serological group II. One strain, 12837, was not lysed by any phage of group I but by both members of group II. Phage A-31, the only member of serological group III, showed plaque formation on the same five strains identified with serological group II phages. Phage 41, the only member of serological group IV, differed from the other three groups, and especially from group III, by formation of plaques on strain 9046 and its lack of lytic action on strain 12837. It may be noted that phage A-41 was the only phage to show plaque formation on strain 9046.

Table 10. *Lytic action of phages for* Azotobacter chroococcum *strains*

A. chroococcum strains	Phages							
	A-11	A-12	A-13	A-14	A-21	A-22	A-31	A-41
9335	4†	5	5	5	5	2	5	1
7493	5	5	5	5	5	4	5	2
9043	5	5	5	5	3	3	3*	1*
7491	1*	3	5	—	5	4	1	1
9544	2*	2*	3*	2*	4	5	5	5
7486	3*	4	5	2*	5	5	4	3
7490	1*	2*	4	5	4	4	5	4
9045	2*	4	5	±	5	4	4	4
4412	4	4	5	4	5	5	5	1
7499	±	±	5	1*	—	—	5	—
9049	—	—	—	—	—	—	2*	4
7498	—	—	—	—	—	—	2*	—
7488	—	2*	3*	2*	2*	—	4*	—
A	1*	—	2*	1*	2*	2*	2*	—
S	—	—	—	—	5	1	—	5
F	—	—	—	—	—	—	2*	5
J	—	—	±	—	—	—	3*	—
E	4	4	5	5	5	5	2*	±
B	—	—	±	—	—	2*	—	±
C	—	—	±	—	—	2*	—	±

† Number 4 means lytic action at $1/10^4$ dilution, etc.

* Indicates plaques were not observed. ± means questionable lytic action. — indicates no lytic action. Strains 480, 7497, 9048, 9051 and 12981 showed negative reactions with all phages; strain 9044 was not tested.

Table 10 shows the lytic action of eight phages for 25 strains of *Azotobacter chroococcum*. Five of these strains, listed in the notes beneath Table 10, showed negative reactions with all phages. Six additional strains showed non-specific lysis; that is, plaque formation was not observed. With the *A. chroococcum* strains, the problem of rapid growth and abundant slime production was encountered and plaque formation might have been missed. For example, strain F did not show plaque formation after incubation for 20 hr. at 33°. However, when the plates remained at room temperature during the next 12 hr., plaque formation was observed at *c.* 28 hr., but plaques were covered over by the growth of organism and slime formation at *c.* 32 hr.

These data show that *c.* 50 % of the *Azotobacter chroococcum* strains tested were sensitive to one or more of the phages. The phages A-21 and A-22 (serological group II) had identical host range specificity. Phage A-21 appeared to show greater virulence for strain S than did phage A-22; therefore strains could be used to distinguish between or to identify these phages. Whereas the phages of serological groups II and III showed identical host-range specificity with the *A. vinelandii* strains, a few differences were noted among the *A. chroococcum* strains, namely strains 9043 and 7499. Phage A-41 (group IV) was the only phage to show plaque formation with strains F and 9049. The phages of serological group I showed a few differences. The lack of observed plaque formation with these phages could be due to a combination of poor plaque morphology shown by these phages and the slimy, rapid growth of the strains. Further testing is warranted to determine whether these differences are valid.

A summary of the host-range specificity reactions is presented in Table 11. Only those lytic reactions where plaque formation was observed were included. None of the phages lysed any of the six strains designated *Azotobacter agilis*, the three strains of *A. macrocytogenes* or the two strains of *A. insigne*. Phage sensitivity of the *A. indicus* strains was difficult to assess because of their slow growth and copious formation of gummy material. However, preliminary studies indicated that these strains were not lysed by any of the phages.

Table 11. *Summary of host-specificity reactions of azotobacter phages*

Azotobacter strains	No. of strains tested	Number of strains lysed by phages*								No. of strains not lysed by any phage
		A-11	A-12	A-13	A-14	A-21	A-22	A-31	A-41	
A. vinelandii	12	9	7	9	9	5	5	5	5	1
A. chroococcum	25	5	8	10	6	11	11	9	11	11
A. agilis	6	0	0	0	0	0	0	0	0	6
A. macrocytogenes	3	0	0	0	0	0	0	0	0	3
A. insigne	2	0	0	0	0	0	0	0	0	2
No. of strains tested	48	.	.	.	.	.	.	.	.	.
No. of strains lysed	.	14	15	19	15	16	16	14	16	.

* Only lytic reactions where plaque formation was observed are included.

DISCUSSION

The methods described for the isolation of the azotobacter phages are of special interest since they utilized the natural azotobacter flora for the enrichment strain rather than the usual addition of bacterial cultures. Although the majority of the soil samples were from various parts of Texas, phage isolates were made from Ohio and Maryland soils, indicating that the azotobacter phages are not confined to any particular geographical area of the United States. The fact that the phages from the Ohio and Maryland soils were antigenically related to phages isolated previously from Texas soils may indicate that the number of serological groups is not great. The four serological groups found thus far for the azotobacter phages are comparable to the number of groups reported for other phage systems.

The plaque morphology observed with the azotobacter phages differed markedly from that seen with the coli phages and many other phage systems; however, it

resembled most closely the plaques formed by phage Kp which lyses *Klebsiella pneumoniae* type 2 (Park, 1956). The plaques formed by phage Kp were surrounded by translucent halos which gradually increased in diameter over the course of a week. It was demonstrated that a diffusible enzyme was liberated during lysis of the infected host cell, and that the enzyme was responsible for the large spreading halo that surrounded the phage plaques (Park, 1956; Adams & Park, 1956). It was believed that the enzyme decreased the viscosity of the capsular polysaccharide and stripped it from the surface of the bacterium, thus permitting the phage to penetrate the host cell surface.

The azotobacter phages produce plaques with halos, and since the *Azotobacter vinelandii* O organisms are capsulated, it is possible that a similar enzyme is present in the azotobacter+phage system and might account for the observed plaque morphology.

The most widely applied of the taxonomic criteria recommended for defining the species category in bacteriophages is serological relationship (Adams, 1959). Therefore primary emphasis was placed on serology in classifying the azotobacter phages. Cross-neutralization assays indicated four serological groups for the 10 azotobacter phages in the present collection. These groups were designated I, II, III and IV. The individual phages were given numbers preceded by the letter A which stands for the genus *Azotobacter*. We propose that the phages within a serological group be assigned numbers in the following manner: 11–19 for group I, 21–29 for group II, 31–39 for group III, and 41–49 for group IV. The purpose of using this range of numbers is to permit the incorporation of future phage isolates into any one of the present serological groups. In addition, it allows for inclusion of new serological groups (V, VI, etc.).

The kinetic studies on neutralization of phage with homologous and heterologous antisera indicate that phages A-21 and 23 in serological group II are identical or very closely related. These two phages are similar by plaque morphology and showed identical host-range specificities. If future investigations on additional classification criteria substantiate these findings, it is recommended that one of these two phages be dropped from the present series.

With phages A-12 and A-31 neutralization by homologous antisera did not follow first-order kinetics; however, such anomalous neutralization behaviour has not been uncommon with other phage systems. Andrews & Elford (1933) noted that neutralization of their phages proceeded rapidly until 90–99 % of the phage was inactivated and then slowed rather abruptly. They demonstrated that this phenomenon was not due to exhaustion of antibody since upon removal of phage the remaining serum was still capable of neutralizing phage. They subcultured plaques of phage particles which survived the neutralization experiment and showed that these were susceptible to antiphage serum neutralization and also exhibited similar anomalous neutralization behaviour. Delbrück (1945) observed a similar phenomenon with the T1 coli phage and suggested that antibody might be attaching itself at sites on the phage where it would not interfere with infectivity. Hershey & Bronfenbrenner (1952) believed that these anomalies might be due to an uncontrolled behaviour of complement. Thus, it is not clear whether the neutralization anomalies are a result of inherent heterogeneity of phage preparations or to the properties of specific lots of sera.

It was of interest to consider the value of the present series of phages in defining the bacterial species of the genus *Azotobacter*. Considerable disagreement exists as to whether the group of organisms designated *Azotobacter agilis* (*Bergey's Manual*, 1957) comprise one or two species, namely *A. agilis* and *A. vinelandii*. Some investigations have shown morphological, ecological and physiological differences between these two groups of organisms. The present studies indicate a difference between these strains since 11 out of 12 *A. vinelandii* strains showed lysis by one or more of the phages, whereas none of the six strains of *A. agilis* tested were lysed by any of the phages. Also, the strains designated *A. macrocytogenes* and *A. insigne*, which in many respects are similar to *A. agilis* cultures, were not lysed by any of the present phages. Since the *A. agilis* cultures have a distinct aquatic distribution and have never been isolated with certainty from the soil, it is possible that phages for these strains do not exist, or are rarely present, in the soil. Also, in the described phage isolation procedures the indicator strain used was a culture of *A. vinelandii*, a strain usually isolated from the soil. An exhaustive search for phages which lyse *A. agilis* strains using this culture as an indicator strain has not been made. The azotobacter phages described here are not species-specific since 14 out of 25 strains of *A. chroococcum* tested were lysed by one or more of the phages. Jones (1959) showed that at least one strain of *A. chroococcum* may be lysogenic. Although a considerable amount of discussion regarding taxonomy of the genus *Azotobacter* has centred around the relationship of *A. agilis* and *A. vinelandii*, two classification proposals (Tchan, 1953; Jensen, 1955) were concerned with the relationship of *A. chroococcum* and *A. vinelandii*. On the basis that these two species possessed a dormant stage (microcysts) they were included in the genus *Azotobacter* separate from *A. agilis*, which was placed in the genus *Azotococcus*. The present studies indicate a similar strain relationship, since the phages lysed *A. vinelandii* and *A. chroococcum*, but did not lyse strains of *A. agilis*.

We wish to express our appreciation to Mr Maxwell Nimeck for technical assistance and to Mr Phillip Hodson for taking photographs of the phage plaques. This work forms part of a thesis presented (by J.T.D.) for the Ph.D. degree to the University of Texas.

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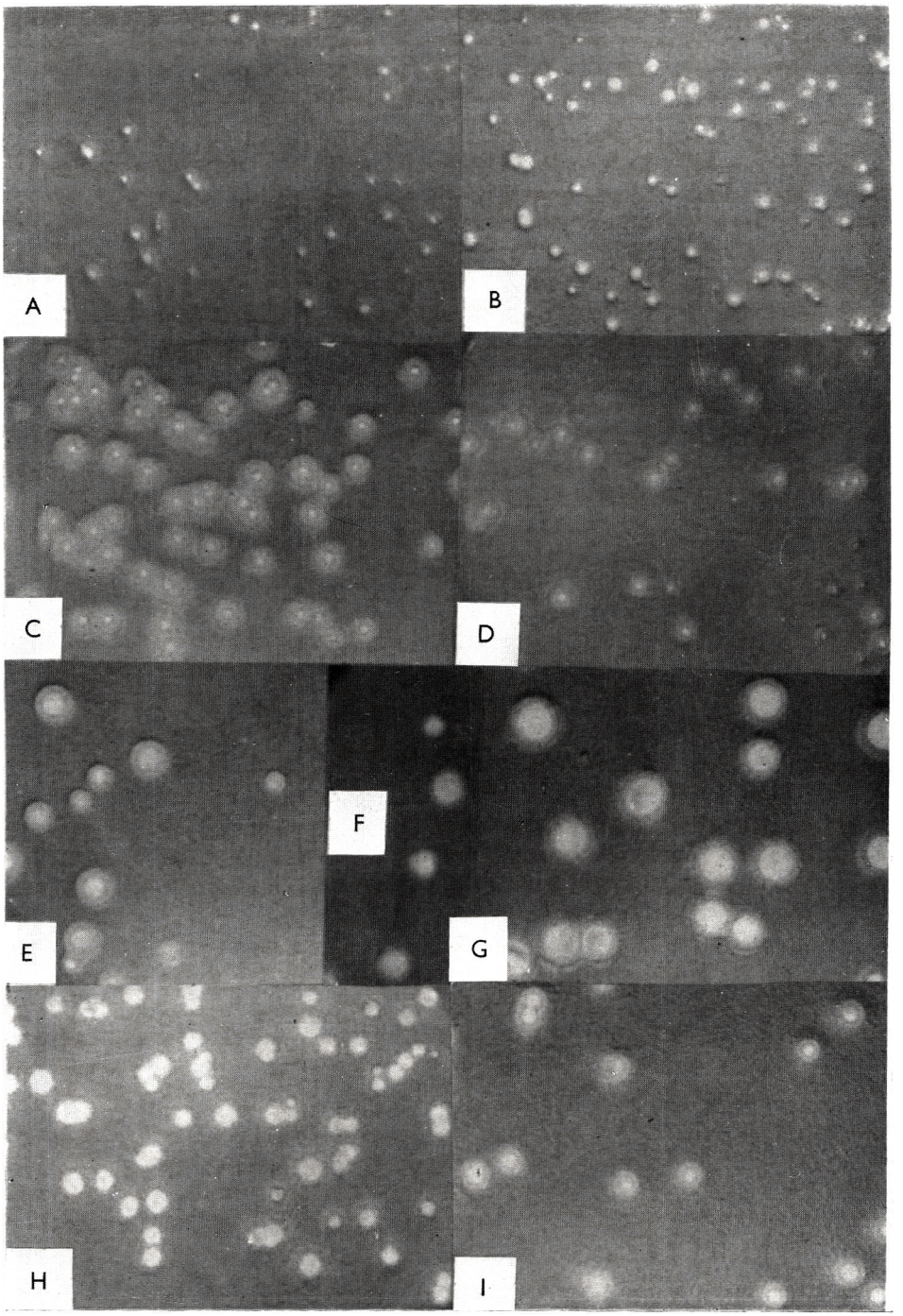

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EXPLANATION OF PLATE

Representative plaque types of azotobacter phages on host strain *Azotobacter vinelandii* O. Fig. A, phage A-12; fig. B, phage A-14; fig. C, phage A-13; fig. D, phage A-11; fig. E, phage A-22; fig. F, phage A-21; fig. G, phage A-23; fig. H, phage A-41; fig. I, phage A-31. Magnification *c.* ×1·5.

J. gen. Microbiol. (1961), **24**, 291–294
Printed in Great Britain

On Sporulation in Sulphate-reducing Bacteria

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(*Received* 29 *August* 1960)

SUMMARY

Enrichment cultures of mesophilic sulphate-reducing bacteria were readily obtained from soils, mud and corrosion products which had been heated to 90°, yet pure cultures of *Desulfovibrio desulfuricans* were killed by heating to 60° even when absorbed on dried sterilized soil. Examination of the populations from heated samples revealed exclusively organisms resembling *Desulfovibrio orientis*; unheated samples contained either *D. orientis* alone or both *D. orientis* and *D. desulfuricans*. The belief that *D. desulfuricans* sporulates is questioned.

INTRODUCTION

Starkey (1938) observed that, though laboratory cultures of mesophilic sulphate-reducing bacteria were killed by heat, such organisms could nevertheless be obtained from soils which had been heated to 90° for ten minutes. Since he was able to demonstrate sporulation among the thermophilic sulphate-reducing bacteria, and since he supported the now obsolete belief that the mesophiles were adapted variants of the thermophiles (see Postgate, 1959*a*, for references), Starkey concluded that the mesophilic species *desulfuricans* formed spores in natural conditions but lost this ability after passage in laboratory media. Pochon & Chalvignac (1952) described a strain which showed such a change on microscopic observation. Starkey proposed the use of the generic name *Sporovibrio* on this account, but the oblique nature of the evidence for sporulation inhibited wide acceptance of this designation, *Desulfovibrio* being generally preferred. Prévot (1948), however, retained the name *Sporovibrio* and, in the third edition of his manual (Prévot, 1957), gave reasons for continuing to retain this generic name and for not accepting the exclusion of the sporulating thermophiles. A mesophilic species showing unequivocal sporulation was isolated by ourselves (Adams & Postgate, 1959) and named *Desulfovibrio orientis*. This species is readily differentiated from *D. desulfuricans* by two additional characters: (i) one or two polar or near-polar flagella (Adams & Postgate, 1959); (ii) absence of a fluorescence reaction typical of *D. desulfuricans* (Postgate, 1959*b*); it is distinguished from the thermophile *Clostridium nigrificans* by its mesophily and by the shape of its spores. It is possible that the heat resistance and sporulation earlier attributed to *D. desulfuricans* was due to the unrecognized presence of *D. orientis*; the present report provides evidence in favour of this view.

METHODS

Organisms and cultivation. *Desulfovibrio desulfuricans*, strain Hildenborough National Collection of Industrial Bacteria (NCIB) 8303, Essex 6 (NCIB 8307) and Beckton (NCIB 8319) were of fresh water origin; strains Canet 41 (NCIB 8393) and El Agheila Z (NCIB 8380) originated in saline environments and their culture media were supplemented with 2·5 % (w/v) NaCl. Stocks were kept in a freeze-dried condition; cultures for routine use were subcultured weekly in medium C of Butlin, Adams & Thomas (1949*b*) at 30° under H_2 99 % (v/v) + CO_2 1 % (v/v). *D. orientis*, strain Singapore (NCIB 8382), was maintained in a similar manner, but sodium thioglycollate (0·01 %, w/v) was added to its culture media.

Enrichment cultures of sulphate-reducing bacteria from natural samples were prepared in medium C supplemented with 0·01 % (w/v) sodium thioglycollate and 0·05 % (w/v) ferrous ammonium sulphate, pH 7·4 ± 0·3, in 1 oz. (*c.* 25 ml.) stoppered bottles filled to overflowing to exclude air. The thioglycollate ensured that *Desulfovibrio orientis* would grow if present (Adams & Postgate, 1959) and it also facilitated growth of *D. desulfuricans*, being below the concentration which showed inhibitory effects (Grossman & Postgate, 1953).

Diagnostic tests. Enrichment cultures were decanted to give bacterial suspensions reasonably free from FeS and the fluorescence test for *Desulfovibrio desulfuricans* (Postgate, 1959*b*) was performed on the decanted material. Flagella were observed microscopically after Casares-Gil staining. Thermophiles were sought by incubation at 55°. Motility was observed in wet preparations under phase contrast. Heat resistance was tested by sealing 1 ml. culture or 1 g. soil in a Durham tube and wholly immersing this in water at 90° for 10 min. Sterility of treated soils was tested by incubating portions aerobically and anaerobically in the glucose + peptone + yeast extract + salts medium of Postgate (1953).

Pure cultures were not in general isolated from the enrichment cultures. After 3–6 days at 30°, when the enrichment cultures were well blackened and rich in H_2S, the populations were assigned to *Desulfovibrio desulfuricans* or *D. orientis* groups according to the following criteria: *D. desulfuricans*, positive fluorescence test, vibrio form, single flagellum, translational motility, failure of subculture to grow at 55°; *D. orientis*, negative fluorescence test, fat vibrio form, more than one flagellum, motility absent or oscillatory, failure of subculture to grow at 55°.

RESULTS

We have repeatedly confirmed Starkey's observation that soils may be heated to 90° and still yield enrichment cultures of sulphate-reducing bacteria. On the other hand, the five pure strains of *Desulfovibrio desulfuricans* mentioned above were all killed when their cultures were heated to 60° or above for 10 min.; *D. orientis* usually survived heating to 90°. To see whether soil exerted a protective effect on *D. desulfuricans*, samples which had yielded sulphate-reducing bacteria after heating were sterilized (by autoclaving or by exposure to ethylene oxide at 4° for 30 min.), dried and then damped with cultures of the Hildenborough strain. The organisms were nevertheless killed when the damped soil was heated to 60° for 10 min. Since commensal spore-formers might have induced heat resistance in the manner that variants

of *Clostridium sporogenes* enhance the heat resistance of *Clostridium welchii* (*Welchia perfringens*; Prévot, Raynaud & Tataki, 1951), three colonial types of sporulating anaerobe were isolated from heated enrichment cultures and mixed with the Hildenborough strain of *D. desulfuricans*. None conferred resistance to 10 min. at 60° upon it. Desiccation conferred a slightly increased heat resistance: a freeze-dried ampoule of the Hildenborough strain as supplied by the NCIB survived heating to 70° for 10 min., though others were killed at 80° and 90°.

It seemed likely that the heat-resistant types of sulphate-reducing bacteria were not of the species *Desulfovibrio desulfuricans*. Nineteen specimens of natural materials likely to contain these bacteria were collected and enrichment cultures made from them before and after heating for 10 min at 90°. All yielded flourishing cultures of sulphate-reducing bacteria. These were then assigned to the *D. desulfuricans* or *D. orientis* groups according to the criteria mentioned above. Although the cultures were not pure, and non-sulphate reducing bacteria were present, all the enrichments contained organisms which fell unequivocally into one or the other groups; intermediate types were not found.

Seven samples yielded cultures of the *Desulfovibrio desulfuricans* group when enriched without heating, but after 10 min at 90° they yielded only the *D. orientis* group. The samples tested comprised: corrosion products from a cooling system and a tubercle within a water pipe (cf. Butlin, Adams & Thomas, 1949*a*); a sewage sample from the Northern Outfalls Sewage Works, Beckton, London; scrapings from a mild steel plate held 1 ft. deep in the Thames at central London; mud samples from the Thames at Teddington and from Byron's pool at Cambridge; a soil sample from Pontardulais, Wales. Twelve specimens yielded the *Desulfovibrio orientis* group both before and after heating. All these were soil samples originating from: the laboratory grounds (2); a local compost heap; the Roman site at Catterick, Yorkshire (3); Singapore; Buenos Aires; Tanganyika; Hawaii; India (2). No soils of marine or brackish origin were tested. None of the enrichments grew on subculture at 55°.

DISCUSSION

These observations suggest that the heat-resistant sulphate-reducing bacteria in soils resemble *Desulfovibrio orientis* rather than *D. desulfuricans*. Consequently, the belief that the latter species sporulates in nature is probably mistaken. The relevance of these considerations to the inclusion of the species *desulfuricans* in a genus named *Sporovibrio* is evident.

We should like to draw attention to the fact that the *Desulfovibrio orientis* group was present in all specimens tested; in a majority of them it was the exclusive type of sulphate-reducing organism. This observation has little statistical value since the number of soils tested was small and their origins were not randomly distributed; nevertheless, it shows an unexpectedly high incidence. We attribute this to the use of thioglycollate in the enrichment media, without which *D. orientis* often fails to grow, whereas growth of *D. desulfuricans* is merely delayed. It seems likely that the *D. orientis* organism is more ubiquitous than was believed at the time of its isolation.

We are indebted to Mr G. D. Giles for technical assistance with part of this work. The paper is published by the permission of the Director of the National Chemical Laboratory.

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J. gen. Microbiol. (1961), **24**, 295–299
Printed in Great Britain

The Occurrence and Location of Teichoic Acids in Lactobacilli

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(*Received* 2 *September* 1960)

SUMMARY

The walls and cell contents of a number of lactobacilli were prepared by mechanical disruption of the organisms followed by differential centrifugation. The nature of the teichoic acid present in the separated fractions was determined by extracting it with dilute trichloroacetic acid, precipitating with ethanol and identifying the precipitated polymer by hydrolysis to ribitol or glycerol phosphates and other recognizable degradation products. All the organisms contained a glycerol teichoic acid within the cell. This and similar observations with other bacteria indicates that glycerol teichoic acids play an important part in cellular metabolism. Both glycerol and ribitol teichoic acids occur in walls. The presence and type of teichoic acid in the walls correlate with the serological behaviour of lactobacilli and provide a useful means of classification. It is suggested that teichoic acids themselves may possess antigenic properties.

INTRODUCTION

The teichoic acids were first obtained from bacteria by extraction from whole defatted organisms with cold dilute trichloroacetic acid, followed by precipitation with ethanol (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). These preparations were mixtures containing the two types of this group of compounds: the glycerol teichoic acids are polymers of glycerophosphate bearing D-alanine residues in ester linkage with hydroxyl groups in the polymer, whereas the ribitol teichoic acids are similar polymers of ribitol phosphate bearing both D-alanine residues and sugars. The number and nature of the sugar residues and the configuration of the glycosidic linkages varies in the ribitol compounds from different organisms. Most of the structural details of the ribitol teichoic acid from *Bacillus subtilis* have now been determined (Armstrong, Baddiley & Buchanan, 1960).

Purified walls of *Bacillus subtilis*, *Lactobacillus arabinosus* 17-5, and *Staphylococcus aureus* H contain large amounts (40–60% of their dry weight) of the appropriate ribitol teichoic acid but no glycerol teichoic acid, and it was concluded that the glycerol derivatives in material from whole organisms came from the cell contents. A more detailed survey of the presence of both types and their location in Lactobacillus was undertaken in an effort to understand their function and possible relationship to biochemical or serological properties of bacteria.

METHODS

Organisms. Most of the organisms studied were obtained originally from the National Collection of Industrial Bacteria (NCIB) and their identity was checked before use by physiological and biochemical tests. According to Sharpe (1957) *Lactobacillus plantarum* NCIB 3254 is *L. casei* in serological group B and *L. bulgaricus* NCIB 76 is *L. casei* var. *rhamnosus* serological group C. *L. delbrueckii* NCIB 8608 has not been classified serologically but it is a strain of *L. casei* var. *rhamnosus* very closely related to the NCIB 76 strain, and almost certainly belongs to serological group C.

Conditions for growth. Organisms were grown in batch culture (9 or 15 l.) in a liquid medium of the following composition: tryptone (Oxoid) 20 g.; yeast extract (Oxoid) 10 g.; sodium acetate 10 g.; glucose 20 g.; KH_2PO_4 4·5 g.; NaOH 1·04 g.; inorganic salts B (Barton-Wright, 1946) 5 ml.; oleic acid 0·01 ml.; Tween 40 1 ml.; L-histidine hydrochloride 100 mg.; distilled water 1000 ml.

Oleic acid was added for *Lactobacillus bulgaricus*, *L. delbrueckii*, *L. helveticus* and *L. lactis*, and L-histidine hydrochloride was added for rapid growth of *L. delbrueckii* (Ikawa & Snell, 1960). *L. bulgaricus*, *L. casei* var. *rhamnosus* (3 strains), *L. delbrueckii*, *L. fermenti*, *L. helveticus* and *L. lactis* were grown at 37° for 16 hr.; *L. plantarum* and *L. casei* at 30° for 16 hr.; *L. brevis* and *L. buchneri* at 30° for 45–48 hr.

Preparation of cell walls. Organisms were harvested in a refrigerated Sharples centrifuge, washed with cold physiological saline and suspended in cold distilled water (about 20 g. wet weight organism/100 ml. water). Disruption was carried out with a Mickle vibrator (Salton & Horne, 1951) and walls were collected by centrifugation. After washing and centrifuging several times with M-phosphate buffer (pH 7) and distilled water to remove traces of cytoplasmic material and whole organisms, the wall suspension was freeze-dried. The purity of the preparations was confirmed by electron microscopy.

Isolation of teichoic acid

From cell walls. Wall preparations were stirred with 10% (w/v) trichloroacetic acid solution (1·5 ml. to 50 mg. cell wall) for 16 hr. at 4°. Walls were removed by centrifugation and extracted again with trichloroacetic acid solution for 24 hr. at 4°. Cold ethanol (2 vol.) was added to the combined extracts which were then kept at 4° for 24 hr. Precipitated teichoic acid was removed by centrifugation and reprecipitated from 10% (w/v) trichloroacetic acid solution (about 5 ml.) by the addition of cold ethanol (1 vol.). After being kept at 4° for 24 hr. the precipitate was collected in a centrifuge, washed with cold acetone, ethanol and ether, then dried in a desiccator (vacuum). More prolonged extraction of walls at 4° yielded further small quantities of teichoic acid.

From cell contents. To the solution obtained after disruption of organisms and removal of walls by centrifuging was added cold 50% (w/v) trichloroacetic acid solution to give a final concentration of 5% (w/v) trichloroacetic acid. After 16 hr. at 4° the precipitate was removed by centrifuging and the clear solution was mixed with cold ethanol (2 vol.). The teichoic acid precipitated slowly at 4°; after 2–4 days it was collected and purified by the reprecipitation procedure described above for cell walls.

Identification. Samples of teichoic acid from walls or cell contents were hydrolysed with 2N-hydrochloric acid for 3 hr. in sealed tubes at 100°. Hydrolysis products were identified by paper chromatography. Whatman no. 4 paper which had been washed in 2N-acetic acid, then water, was used with the solvent systems *n*-propanol + ammonia (sp.gr. 0·88) + water (6 + 3 + 1) and *n*-butanol + ethanol + water + ammonia (sp.gr. 0·88; organic layer) (40 + 10 + 49 + 1). Products were detected by the spray reagents used previously (Armstrong *et al.* 1958). All the appropriate hydrolysis products of glycerol or ribitol teichoic acids were detected and no other products were seen. The presence of alanine ester linkages was confirmed in all samples by treatment with ammonia, followed by paper chromatography of the resulting alanine amide in the propanol solvent system (Armstrong *et al.* 1958).

RESULTS

The distribution of the two types of teichoic acid in the walls and cell contents of a number of *Lactobacillus* spp. is given in Table 1. The results are expressed qualitatively, and in each case refer to teichoic acid which had been isolated by the standard procedure and identified by chromatographic examination of hydrolysis products.

Table 1. *Teichoic acid in walls and cell contents of* Lactobacillus *spp. in relation to serological groups*

R = ribitol teichoic acid; G = glycerol teichoic acid; – = neither R nor G detected.

Organism	Teichoic acid in Cells	Teichoic acid in Walls	Serological group
L. arabinosus 17-5	G	R	D
L. plantarum NCIB 7220	G	R	D
L. brevis NCIB 8169	G	G	E
L. buchneri NCIB 8007	G	G	E
L. lactis NCIB 7278	G	G	E
L. bulgaricus NCIB 2889	G	G	A
L. helveticus NCIB 8025	G	G	A
L. plantarum NCIB 3254*	G	–	B
L. casei ATCC 7469	G	–	C
L. bulgaricus NCIB 76*	G	–	C
L. delbrueckii NCIB 8608*	G	–	C?
L. fermenti NCIB 6991	G	–	F
L. delbrueckii NCIB 8130	G	G	–

* A fuller description of these organisms is given under Methods.

DISCUSSION

All the organisms examined in this survey contained a glycerol teichoic acid in the fraction corresponding to the cell protoplasm and structure beneath the wall. It is concluded that glycerol teichoic acids probably occur in the cells of all *Lactobacillus* spp.; moreover, this type has been found in the cell contents of a variety of Gram-positive bacteria studied in this laboratory (Armstrong *et al.* 1959). In only two or three cases was this material not detected, and it is possible that these failures occurred through the insensitivity of the analytical procedure, which

depends upon actual isolation of precipitated material. Alternatively, the occasional occurrence of fat-soluble teichoic acids containing higher fatty ester residues cannot be discounted; such compounds would escape detection by the methods adopted in this work.

Although the function of teichoic acids is unknown, the widespread occurrence of the glycerol type in Gram-positive bacteria establishes their importance, and the chemical reactivity of their alanine ester residues suggests a metabolic function for these polymers. It has not yet been possible to determine their exact location within the cell, but if they are present in the protoplast membrane or other outer regions of the cell they may be visualized as partners to the wall teichoic acids, the whole system being well suited to such functions as the transport of ions.

Both ribitol and glycerol teichoic acids are found in walls, but in some cases neither type was detected even when large samples of wall were examined. This finding, and the absence of intracellular glycerol teichoic acid in those walls which contained the ribitol compound, indicated that little or no cross-contamination of wall and cell material occurred during the isolation procedure. Faint traces of ribitol derivatives were detected in the cell contents of those organisms which possessed ribitol teichoic acid in their walls. It is not known whether this arose through contamination. Exhaustive chemical studies have not yet been carried out on all samples of teichoic acid obtained from Lactobacillus, but it is possible that structural differences occur within the two general types found in these organisms.

Some correlation was observed between the nature of the teichoic acid in the walls and the metabolic properties of the organisms. It is now generally recognized that, although the classification of *Lactobacillus* spp. solely upon metabolic behaviour is unsatisfactory, they may be classified well by consideration of both metabolic and serological behaviour (Sharpe, 1955; Sharpe & Wheater, 1957). Consequently, the serological groups of organisms studied in this work were compared with the teichoic acid of their walls. The serological groups listed in Table 1 are those described by Sharpe (1955) and Sharpe & Wheater (1957). It is seen that the organisms of group D contained ribitol teichoic acid in their walls, whereas the walls of those in groups E and A contained a glycerol teichoic acid; groups B, C, and F contained no wall teichoic acid. These results suggest a relationship between the serological properties of *Lactobacillus* spp. and the teichoic acid in their walls. It is likely then that these substances themselves would participate in serological reactions. Authentic samples of teichoic acids have not been examined serologically, but a closely related bacterial glycerophosphate polymer was studied by McCarty (1959) who was able to prepare a specific antiserum to it. Clearly, the serological behaviour of *Lactobacillus* spp. would not be determined solely by the antigenic properties of their wall teichoic acids. Thus, groups B, C and F are serologically distinct groups, but their members contain no wall teichoic acids. Nevertheless, a knowledge of wall teichoic acids should be valuable in classifying Lactobacilli, and might be used occasionally to predict the serological grouping of new members.

Ikawa & Snell (1960) detected teichoic acids in the walls of a number of *Lactobacillus* spp. Where identical strains have been used their results agree with those described here. However, they recorded the presence of a ribitol teichoic acid in the walls of *L. delbrueckii* 730 (ACTC 9649) which is understood to be identical with the strain NCIB 8130 (Sharpe, 1959). We found a glycerol teichoic acid in walls of

NCIB 8130 but no trace of a ribitol teichoic acid was detected. This small discrepancy is under investigation. Sharpe was unable to prepare an antiserum to this species but our observations would indicate its similarity to those of groups A or E.

We thank the Nuffield Foundation, the Medical Research Council and the Rockefeller Foundation for financial support, and Mr E. H. Boult of this Department for routine examination of walls under the electron microscope.

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J. gen. Microbiol. (1961), 24, 301–312
Printed in Great Britain

A Mutational Alteration of the Tryptophan Synthetase of *Escherichia coli*

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(*Received* 4 *October* 1960)

SUMMARY

A tryptophan auxotroph of *Escherichia coli* produced an altered tryptophan synthetase which could not convert indole to tryptophan but converted indole-3-glycerol phosphate to indole. As distinct from the normal tryptophan synthetase, which also catalysed this reaction, both pyridoxal phosphate and serine stimulated the activity of the mutant enzyme system. Fractionation and chromatography of the mutant tryptophan synthetase separated it into its two protein components, A and B. Examinations of the separated components showed that the A protein was normal, while the B protein was altered. Studies of the effect of serine on the pH-activity response of mutant preparations in the indole-3-glycerol phosphate → indole reaction demonstrated that different pH-activity responses were obtained, respectively, in the presence and absence of serine. The curves obtained were characteristic of the serine-requiring and serine-non-requiring reactions, respectively, of normal tryptophan synthetase. The saturation curves of the mutant component B by normal component A, with and without serine added, suggest that one role of serine and pyridoxal phosphate in the stimulation of the indole-3-glycerol phosphate → indole reaction is to bind together the A and B proteins in a catalytically effective complex.

INTRODUCTION

Studies performed with washed suspensions of two strains of *Escherichia coli*, a mutant requiring tryptophan for growth (strain 7-4) and a double mutant requiring tryptophan and serine or glycine (strain T2-15), indicated that serine was involved in the synthesis of indole from glucose and ammonium ion (Gibson, Jones & Teltscher, 1956). Although it was also observed that serine was required for anthranilic acid formation, subsequent experiments with bacteria fully adapted to anthranilic acid synthesis showed that serine participated at some point between anthranilic acid and indole, in indole formation by the mutant strains (F. Gibson, unpublished observation). It was not obvious which reaction required serine since in *E. coli* neither the conversion of anthranilic acid to indole-3-glycerol phosphate nor the conversion of indole-3-glycerol phosphate to indole normally involved this amino acid. Recently, DeMoss & Bonner (1959) described a mutationally altered tryptophan synthetase of *Neurospora crassa* (from strain td_{71}) which required serine and pyridoxal phosphate for the conversion of indole-3-glycerol phosphate to indole. Serine and pyridoxal phosphate are not required by the wild

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type enzyme in this reaction. In view of this finding it was thought that the unexplained effect of serine on *Escherichia coli* mutant 7-4 might be due to a similar alteration of its tryptophan synthetase. This possibility was examined and an attempt made to localize the alteration to one of the two protein components of the bacterial tryptophan synthetase.

METHODS

Organisms. Three strains of *Escherichia coli* were used in this study. They were the parent strain (518), a mutant strain which required tryptophan for growth (7-4), and a double mutant derived from strain 7-4 which required tryptophan and serine or glycine for growth. These organisms have been described previously (Gibson *et al.* 1956).

Preparation of cell extracts. Organisms were grown on the mineral salts citrate mixture described by Vogel & Bonner (1956) supplemented with 0·16% (w/v) glucose and 0·005% (w/v) acid-hydrolysed casein (neutralized before use). The glucose was added as a sterile solution after sterilization of the medium. For *Escherichia coli* 7-4, L-tryptophan (5 μg./ml.) was included in the medium. One litre quantities of medium were inoculated with a 6–8 hr. culture in nutrient broth to give an initial population of about 10^6 organisms/ml. The cultures were then shaken vigorously at 37° for 16 hr. After harvesting the bacteria in a Sharples supercentrifuge they were washed once with cold 0·9% (w/v) NaCl solution and resuspended in 0·1 M 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH 7·8, to give a final concentration of about 4×10^{11} bacteria/ml. The suspension was then disrupted in a 10 Kc Raytheon sonic oscillator for 20 min. Cell debris was removed by centrifugation at 144,000 *g* in a Spinco Model L centrifuge for 30 min. The supernatant fluid (crude extract) was stored at −15°.

In addition to crude extracts, preparations obtained by precipitating tryptophan synthetase three times with ammonium sulphate were used (ASP enzyme). Solid ammonium sulphate was added to the crude extract to obtain 50% saturation, the addition being made at 0–5° with stirring, and the stirring continued for a further 10 min. after solution. The precipitate was separated by centrifugation at 4600 *g* for 10 min. and dissolved in an amount of tris buffer (0·1 M; pH 7·8) equal to the original volume of extract. This precipitation was repeated twice and the final precipitate taken up in tris buffer and stored at −15°.

Reagents. Reagents with the exception of those given below were obtained commercially and not further purified. Indole-3-glycerol phosphate was prepared by the method described by Yanofsky (1956) modified to the extent that it was formed by the indole + triosephosphate reaction (Yanofsky, 1959). The solution used contained 1·8 μmole indole-3-glycerol phosphate/ml.; 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate (anthranilic deoxyribulotide) was prepared as described by Smith & Yanofsky (1960).

Normal A and B proteins from *Escherichia coli* T-3 and *E. coli* T-8, respectively, were purified on diethylaminoethyl (DEAE) cellulose columns (Crawford & Yanofsky, 1958). Proteins A and B from *E. coli* mutant strain 7-4 will be referred to as A_{7-4} and B_{7-4}.

Methods for assaying enzymic activity. The details of the methods used for estimating the activity of proteins A and B of tryptophan synthetase in the three

reactions which they catalyse were described previously (Crawford & Yanofsky, 1958; Yanofsky & Stadler, 1958; Yanofsky, 1955, 1956). The conversion of indole-3-glycerol phosphate to indole was carried out in a reaction mixture consisting of indole-3-glycerol phosphate and phosphate buffer (pH 7). DL-serine and pyridoxal phosphate were added where desired. Indole was measured by reaction with Ehrlich's reagent as described by Yanofsky (1955). The conversion indole-3-glycerol phosphate → tryptophan was carried out in a mixture of indole-3-glycerol phosphate, DL-serine, pyridoxal phosphate, NaCl and tris buffer (pH 7·8). The reaction was followed by measuring the disappearance of indole-3-glycerol phosphate. The remaining indole-3-glycerol phosphate was oxidized to indole-3-aldehyde by metaperiodate at pH 5 and the absorption of indole-3-aldehyde formed was measured at 290 mμ. The conversion indole → tryptophan was carried out in the same reaction mixture as that just described except that indole was substituted for indole-3-glycerol phosphate. The reaction was followed by measuring indole disappearance. All tests were carried out in a final volume of 1 ml. at 37°.

One unit of tryptophan synthetase activity is defined as that amount of enzyme which catalyses the disappearance of 0·1 μmole substrate or the formation of 0·1 μmole product in 20 min. at 37°.

Chromatography. Column chromatography was carried out on Reagent Grade 'Selectacel' diethylaminoethyl (DEAE) cellulose (Brown Company, Berlin, New Hampshire, U.S.A.). The preparation and use of such columns for the separation and purification of the components of tryptophan synthetase were described by Crawford & Yanofsky (1958). In the present experiments a column 2×90 cm. was used. The gradient elution system consisted of 1 l. of 0·01 M-phosphate buffer (pH 7·0) in the mixing bottle and 1 l. of 0·4 M-phosphate buffer (pH 8·0) in the inlet bottle. Fractions of 7·5 ml. were collected at 18 min. intervals and the buffer was changed to 0·5 M-phosphate (pH 8·0) at fraction 173. All the buffers contained 2×10^{-5} M-pyridoxal phosphate and 10^{-4} M-reduced glutathione.

The crude extract was prepared for the column as follows. Nucleic acids were precipitated by addition of 1·9 ml. M-$MnCl_2$ to 25 ml. extract. After stirring for 15 min. the mixture was centrifuged and the precipitate removed (15 min., 4600 *g*). Then 9·67 g. $(NH_4)_2SO_4$ was added to 26 ml. of the supernatant solution and the mixture stirred for 10 min. After centrifugation (15 min., 4600 *g*) the precipitate was taken up in dialysis buffer (0·02 M-phosphate) and dialysed for 3 hr. against 1 l. of the same buffer. The final volume was 11·3 ml. of which 11 ml. was chromatographed. All operations were carried out at 0–5°.

Protein estimations. Protein was estimated by using the Folin phenol reagent as described by Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

The site of the serine effect

Indole is now considered to be a breakdown product of indole-3-glycerol phosphate rather than, as previously thought, a normal intermediate in tryptophan synthesis (Yanofsky, 1960). A number of bacterial mutants have been isolated which accumulate indole but, except in the case of *Escherichia coli* strain 7-4, there has been no report of serine being required for indole production. If the effect of serine on the

production of indole by *E. coli* strain 7-4 were due to the fact that, as with *Neurospora crassa* mutant td_{71} (De Moss & Bonner, 1959), it stimulated the conversion indole-3-glycerol phosphate → indole, restriction of the available serine should cause the accumulation of indole-3-glycerol phosphate. Restriction of available serine could be achieved in two ways, either by using the double mutant which also required serine or glycine for growth (*E. coli* strain T2-15) or, alternatively, by using cell-free extracts which could not form serine from the available substrates.

Experiments with whole organisms. From a nutrient agar slope (24 hr., 37°) of the double mutant *Escherichia coli* strain T2-15, three flasks of mineral salts + citrate + glucose medium (10 ml. in 50 ml. flasks), each containing a different concentration of serine, were inoculated to give an initial population of about 2×10^6 bacteria/ml. The flasks were incubated for 18 hr. at 37° with vigorous shaking and then sampled for indole and indole-3-glycerol phosphate determinations. Table 1 shows that as the concentration of serine was decreased 20-fold the ratio of indole-3-glycerol phosphate to indole increased some 100-fold, indicating that serine probably did play some part in the conversion of indole-3-glycerol phosphate to indole by this strain.

Table 1. *The accumulation products of* Escherichia coli *strain T2-15 (double mutant requiring tryptophan and serine or glycine) when grown with limiting amounts of serine*

Organisms grown on basal medium* for 18 hr. with 5 μg. L-tryptophan/ml. and DL-serine as indicated.

Final concentration of DL-serine in basal medium (M)	Product formed (μmole/ml.)		Ratio:
	Indole	InGP†	InGP/indole
2×10^{-2}	9·1	0·45	0·049
10^{-2}	9·6	1·0	0·104
10^{-3}	0·026	0·15	5·8

* Medium as described in methods for preparation of cell extracts with the casein hydrolysate omitted.

† The accumulated material was probably predominantly indole-3-glycerol but was considered equivalent to InGP for the purposes of the experiment.

Experiments with cell extracts. The results given above were confirmed with cell extracts of *Escherichia coli* mutant strain 7-4 which were allowed to metabolize anthranilic deoxyribulotide, the intermediate immediately preceding indole-3-glycerol phosphate (Smith & Yanofsky, 1960). Table 2 shows that indole formation from anthranilic deoxyribulotide was stimulated 25-fold by the addition of serine. Furthermore, the conversion indole-3-glycerol phosphate → indole was increased fivefold when serine was added.

Examination of the effect of serine on indole formation

Indole formation in the presence and absence of serine. Table 2 shows that some indole was formed in the absence of serine when the ASP enzyme was incubated with indole-3-glycerol phosphate. Since the enzyme had been precipitated 3 times with ammonium sulphate it seemed unlikely that this indole formation was due to traces of serine remaining from the crude extract. It appeared more likely that

Table 2. *Effect of serine on the formation of indole-3-glycerol phosphate and indole by a cell-free extract of* Escherichia coli *strain* 7-4

Reaction mixture A consisted of anthranilic deoxyribulotide (0·44 μmole) and phosphate buffer (pH 7·9; 50 μmoles); mixture B contained indole-3-glycerol phosphate (0·18 μmole), phosphate buffer (pH 7·0; 50 μmole) and *Escherichia coli* strain 7-4 ASP extract (0·02 ml.) in a final volume of 1·0 ml. with the additions shown. Incubation was at 37° for 20 min. The contents of the tubes were then assayed for indole and indole-3-glycerol phosphate (InGP; see Methods).

	Product (mμmole)	
	InGP	Indole
Addition to reaction mixture A		
None	84	2
DL-serine (30 μmole)	32	50
Addition to reaction mixture B		
None	—	19
DL-serine (30 μmole)	—	100

some serine-independent indole formation was taking place. The components and additions involved in this serine-independent reaction will be called the basal system. Those mutant preparations and additions required to form indole in the presence of serine will be called the serine system. Hydroxylamine, a potent inhibitor of those reactions of tryptophan synthetase which require pyridoxal phosphate, slightly stimulates as a rule the conversion indole-3-glycerol phosphate → indole (Yanofsky & Rachmeler, 1958). Table 3 shows that the basal system was not affected by hydroxylamine, whereas in the serine system, hydroxylamine acted as an inhibitor, apparently competing with serine for the available pyridoxal phosphate. Inhibition by low concentrations of hydroxylamine provided indirect evidence that pyridoxal phosphate might also be involved with serine in the stimulation of the conversion of indole-3-glycerol phosphate to indole by this altered tryptophan synthetase.

Table 3. *Effect of hydroxylamine on indole formation by the basal and serine systems*

The reaction mixture contained: indole-3-glycerol phosphate, 0·18 μmole; phosphate buffer (pH 7·0), 50 μmole and in Expt. 1, 0·01 ml. crude *Escherichia coli* strain 7-4 extract, or in Expt. 2, 0·02 ml. of ASP enzyme together with various amounts of DL-serine and hydroxylamine as shown. Incubation time, 20 min. Final volume 1 ml.

	Substance added (μmole)		Indole formed
Experiment	DL-Serine	Hydroxylamine	(mμmole)
1	None	None	5
	None	1	4
	60	None	88
	60	1	5
2	None	None	4
	100	None	80
	100	0·01	71
	100	0·1	19
	100	1	5
	10	None	62
	10	0·01	23
	10	0·1	5
	10	1	5

Effect of pH *value on basal and serine reactions.* The pH value of the incubation mixture over the range pH 7—9 (Fig. 1) affected the basal and serine systems differently. The curves obtained may be compared with those in Fig. 2 obtained with normal tryptophan synthetase. The curve for indole formation by the basal system (Fig. 1) resembles that for the conversion indole-3-glycerol phosphate → indole by normal tryptophan synthetase (Fig. 2). The curve for indole formation by the serine system (Fig. 1) more closely resembles that for the serine and pyridoxal

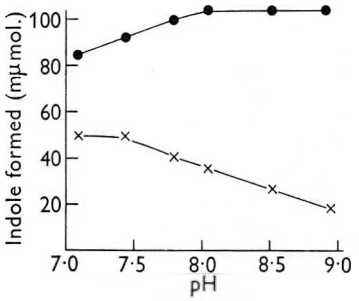

Fig. 1

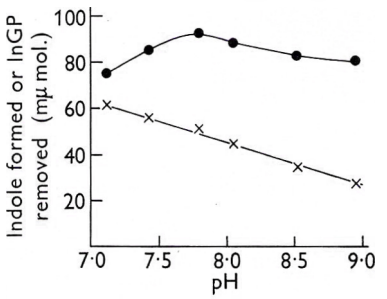

Fig. 2

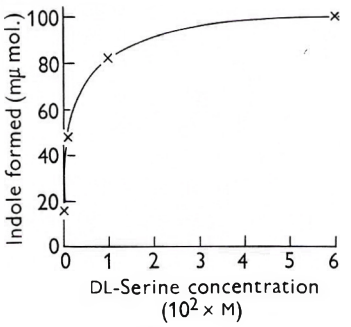

Fig. 3

Fig. 1. The effect of pH on the conversion indole-3-glycerol phosphate → indole by the serine and basal systems with *Escherichia coli* strain 7-4 extract. The reaction mixtures were: for serine system, InGP (0·18 μmole), DL-serine (60 μmole), pyridoxal phosphate (0·03 μmole), normal A (86 units*) crude *E. coli* 7-4 extract (0·01) ml.; for basal system, InGP (0·18 μmole), hydroxylamine (1 μmole), normal A (86 units*) crude *E. coli* strain 7-4 extract (0·04 ml.) together with 0·5 M-tris buffer (50 μmole) in final volume 1 ml. Incubation times: serine system 20 min.; basal system 30 min. Serine system, ●—●; basal system, × — ×.

* Units measured in indole → tryptophan reaction.

Fig. 2. The effect of pH value on the conversion indole-3-glycerol phosphate (InGP) → tryptophan and InGP → indole by normal tryptophan synthetase. The reaction mixtures were: for InGP → tryptophan reaction; InGP (0·18 μmole), DL-serine (60 μmole), pyridoxal phosphate (0·03 μmole), saturated aqueous NaCl solution (0·03 ml.), normal component A (10 units*), normal protein B (2 units*); for InGP → indole reaction; InGP (0·18 μmole), hydroxylamine (1 μmole), normal component A (150 units*), normal component B (40 units*), with tris buffer (50 μmole). Incubation time 20 min. Final volume 1 ml. InGP → tryptophan, ●—●; InGP → indole, × — ×.

* Units measured in indole → tryptophan reaction.

Fig. 3. The effect of serine concentration on the rate of indole formation. The reaction mixture contained indole-3-glycerol phosphate (0·18 μmole), phosphate buffer, pH 7·0 (50 μmole), ASP enzyme from *Escherichia coli* strain 7-4 (0·02 ml.) with serine as indicated. Final volume 1 ml.; incubation time 20 min.

phosphate-dependent formation of tryptophan by normal tryptophan synthetase (Fig. 2).

Specificity of L-*serine.* Some amino acids were tested as possible substitutes for DL-serine in the serine system (Table 4). L-Serine was twice as active as DL-serine on a molar basis, while D-serine had a slight stimulatory effect; this may have been due to contamination of the D-serine with small amounts of L-serine. The other amino acids tested were inactive.

Table 4. *Indole synthesis with various amino acids related to serine*

The reaction mixture was as in Table 3. Enzyme from *Escherichia coli* strain 7-4 (ASP preparation, 0·02 ml.) with the amino acids as shown.

Addition	Final concentration (M)	Indole formed (mμmole)
None	—	10
DL-Serine	0·06	93
L-Serine	0·03	100
D-Serine	0·03	25
DL-Homoserine	0·06	10
DL-Threonine	0·06	8
L-Cysteine	0·03	14
Glycine	0·03	14

Serine saturation curve. Figure 3 shows the effect of increasing concentrations of serine on indole formation from indole-3-glycerol phosphate. The K_A for DL-serine estimated from the curve is about $1{\cdot}3 \times 10^{-3}$ M which is of the same order as the K_M for serine with normal tryptophan synthetase (K_M for L-serine = $1{\cdot}7 \times 10^{-3}$ M; C. Yanofsky, unpublished).

Pyridoxal phosphate and the serine reaction

The inhibition of the serine stimulation by hydroxylamine was strong presumptive evidence that pyridoxal phosphate was concerned in the serine system. However, indole formation by the ASP enzyme was only stimulated to a variable small degree or not at all by added pyridoxal phosphate, despite the fact that ammonium sulphate precipitations usually removed much of the bound pyridoxal phosphate (C. Yanofsky, personal communication). However, after dialysis of 0·5 ml. of ASP preparation for 2 hr. against 1 l. of 0·02 M tris buffer (pH 7·8) it was possible by adding pyridoxal phosphate in the serine system to show stimulation of indole formation (1·5-fold) by mutant preparations.

Separation of the A and B proteins of the tryptophan synthetase of Escherichia coli *mutant strains* 7-4

The two protein components A and B of *Escherichia coli* tryptophan synthetase can be separated by chromatography on cellulose ion exchangers (Crawford & Yanofsky, 1958). To learn more about the peculiarities of the tryptophan synthetase of *E. coli* strain mutant 7-4 the two component proteins were separated and examined individually. After chromatography of a cell-free extract of *Escherichia coli* strain 7-4 on DEAE cellulose (see Methods) the fractions obtained were assayed

for component A activity in the indole → tryptophan reaction in the presence of an excess (10 units) of normal B protein (Fig. 4*a*). After the first peak of component A (A_{7-4}) was located the fractions were examined for component B by measuring the conversion indole-3-glycerol phosphate → indole (in the presence of serine and an excess of A_{7-4}). Figure 4*b* shows that B_{7-4} activity was detected in two peaks. Examination of fraction 154 indicated that the addition of A_{7-4} was not necessary for this fraction to catalyse the conversion indole-3-glycerol phosphate → indole. Therefore, the first B_{7-4} peak probably represented an AB complex (Crawford & Yanofsky, 1958). This was confirmed by assaying fractions 140 to 190 for component A activity using excess normal B protein and measuring the conversion

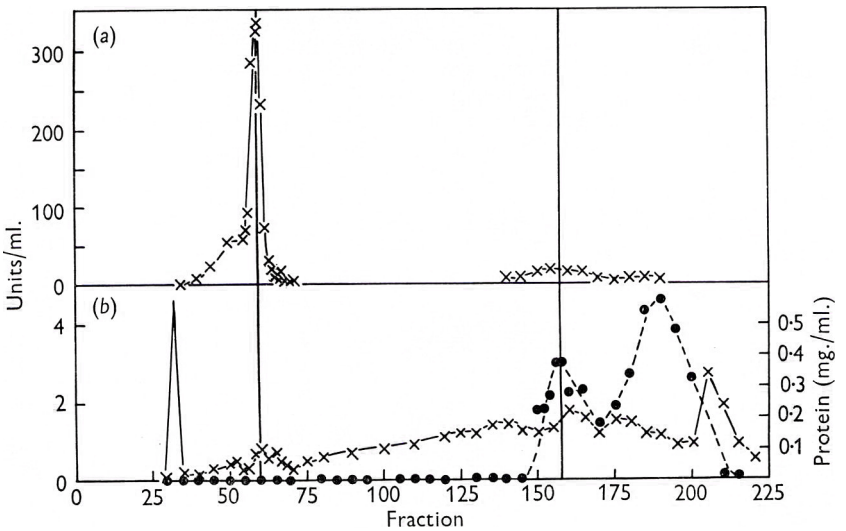

Fig. 4. Chromatography of tryptophan synthetase from *Escherichia coli* strain 7-4 on DEAE cellulose. Details of assay methods in text. (*a*) Component A activity, ×—×; (*b*) component B activity, ●—●; protein concentration, ×—×.

indole → tryptophan (Fig. 4*a*). The second peak of B_{7-4} activity contained only low amounts of A_{7-4}. Both A_{7-4} and B_{7-4} were concentrated by pooling the appropriate fractions, precipitating the proteins with ammonium sulphate at 60% of saturation, and redissolving the precipitates in 0·1 M-tris buffer (pH 7·8). Purified B_{7-4} protein was stored in buffer containing pyridoxal phosphate and reduced glutathione (see Methods).

Experiments with the separated A_{7-4} *and* B_{7-4} *components*

Component A_{7-4} appeared to be a normal A protein; in the presence of normal B protein it catalysed all reactions characteristic of normal A protein. Component B_{7-4} was obviously an altered component; in the presence of A_{7-4} or other normal component A it failed to convert indole to tryptophan. Furthermore, serine stimulated the conversion indole-3-glycerol phosphate → indole by a mixture of B_{7-4} and normal A protein.

The addition of serine to a mixture of normal A and B proteins in the presence of

indole-3-glycerol phosphate and pyridoxal phosphate eliminated indole formation from indole-3-glycerol phosphate because of the much faster conversion of the latter to tryptophan (Table 5).

On the other hand, a mixture of B_{7-4} and normal A protein formed indole from indole-3-glycerol phosphate both in the presence and absence of serine (Table 5) as expected on the basis of the experiments with the ASP extract. Purified B_{7-4} protein had some activity when measured in the serine system without added A protein (Table 5); this can be explained by the small amount of A_{7-4} that was present (see Fig. 4).

Table 5. *The effect of serine on the conversion of indole-3-glycerol phosphate to indole with* B_{7-4} *protein and the components of normal tryptophan synthetase*

The reaction mixture consisted of the reagents listed in the Table together with indole-3-glycerol phosphate (0·18 μmole) and phosphate buffer (pH 7·0; 50 μmole). In Expts. 1 and 3 (*a*), hydroxylamine (1 μmole) was added; in Expts. 2 and 3 (*b*), pyridoxal phosphate (0·03 μmole) was added. Final volume 1 ml. Incubation times: Expt. 1, 80 min.; Expt. 2, 30 min.; Expt. 3, 30 min.

Experiment	Normal A protein (units)*	A_{7-4} protein (units)*	Normal B protein (units)*	B_{7-4} protein (ml.)	Serine added (μmole)	Indole formed (mμmole/ 20 min.)
1 (*a*)	560	—	—	0·02	—	22
(*b*)	—	—	—	0·02	—	< 1
2 (*a*)	56	—	—	0·02	60	87
(*b*)	—	—	—	0·02	60	5
3 (*a*)	—	26	80	—	—	31
(*b*)	—	13	40	—	60	< 1

* Units measured in the indole → tryptophan reaction. In Expts. 1 (*a*) and 2 (*a*) the normal A protein added was sufficient to saturate the B_{7-4} protein used.

It was impossible to obtain reaction rates for the three reactions catalysed by tryptophan synthetase with either of the two components in excess, since the B_{7-4} protein of course failed to catalyse the indole → tryptophan reaction. Further, this B protein could not be used in excess in the basal system, since at the concentration of enzyme required the preparation became inhibitory to the reaction.

The ratio of reaction rates for the indole → tryptophan and indole-3-glycerol phosphate → indole reactions with normal A protein and excess normal B protein in the first case, and normal A and excess B_{7-4} plus serine and pyridoxal phosphate in the second case, was 1:0·05. The ratio of reaction rates for the same two reactions using normal A protein with normal B component was 1:0·03 (Yanofsky, 1960). It is clear then, that the addition of serine to the mutant system restored the ratio to nearly normal but did not accelerate the indole-3-glycerol phosphate → indole reaction to a point where it would be comparable with the serine requiring reactions carried out by normal A and B components.

Saturation of B_{7-4} *by normal A.* With normal tryptophan synthetase components about a three-fold excess of one component was necessary to saturate fully the other component for maximal activity in the conversion indole → tryptophan (Crawford & Yanofsky, 1958). In Fig. 5 it can be seen that the curve for the saturation of normal protein B by normal A in the conversion indole-3-glycerol phosphate →

indole was almost identical with that obtained for the conversion indole → tryptophan (Fig. 5).

It was of interest to examine the saturation curves for B_{7-4} protein in both the serine and basal systems (Fig. 6). A striking difference was detected; in the absence of serine 25 times more protein A was required for half saturation of B_{7-4} than in its presence.

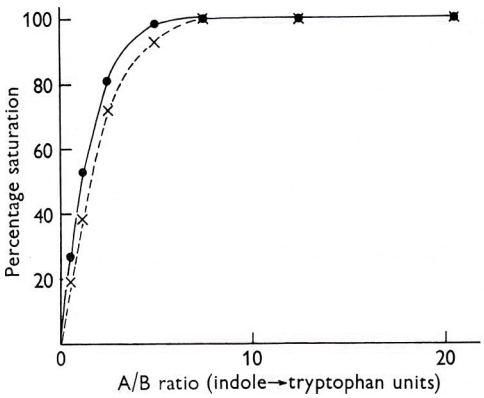

Fig. 5

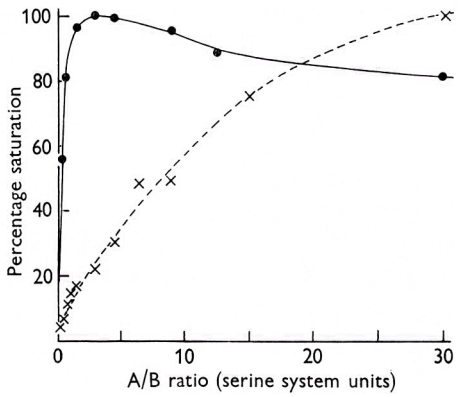

Fig. 6

Fig. 5. The saturation of normal B protein by normal A protein as measured in the conversion indole → tryptophan and the conversion indole-3-glycerol phosphate (InGP) → indole. The reaction mixtures were: (*a*) indole → tryptophan: phosphate buffer pH 7·0 (50 μmole), indole (0·4 μmole), DL-serine (60 μmole), pyridoxal phosphate (0·03 μmole), saturated aqueous NaCl solution (0·03 ml.), normal B (3·2 units), various amounts of normal A protein; (*b*) InGP → indole: InGP (0·18 μmole), phosphate buffer pH 7·0 (50 μmole), hydroxylamine (1 μmole), normal B (26 units), various concentrations of normal A protein. Incubation time 20 min. for both tests. Units are measured in the indole → tryptophan reaction. Indole → tryptophan, ●—●; InGP → indole, ×- - -×.

Fig. 6. The saturation of B_{7-4} protein by normal A protein in the serine and basal systems. The reaction mixtures were: for serine system; indole-3-glycerol phosphate (0·18 μmole), DL-serine (60 μmole), pyridoxal phosphate (0·03 μmole), phosphate buffer pH 7·0 (50 μmole), purified B_{7-4} protein preparation (0·03 ml.), and normal B protein as indicated; for basal system; as above with serine and pyridoxal phosphate omitted and hydroxylamine (1 μmole) added. Final volume 1 ml.; incubation times: serine system 30 min.; basal system 80 min. Serine system, ●—●; basal system, ×- - -×.

DISCUSSION

It seems probable from the findings reported here that the mutation which affects the tryptophan synthetase in *Escherichia coli* 7-4 leads to the production of an altered enzyme system with characteristics similar to those of the tryptophan synthetase of the mutant of *Neurospora crassa* described by DeMoss & Bonner (1959). Experiments with whole organisms and cell extracts of *E. coli* strain 7-4 have shown that serine and probably pyridoxal phosphate have a marked effect on the conversion indole-3-glycerol phosphate → indole although neither of these compounds has any effect on this reaction with normal tryptophan synthetase. The present observations thus extend the similarities of mutational effects on this enzyme system in the two organisms. A major difference between the two systems, however, is the ready dissociation of the tryptophan synthetase from *E. coli* into

two protein components A and B (Crawford & Yanofsky, 1958). This property of the *E. coli* system has permitted the localization of the mutational defect in *E. coli* strain 7-4 to the B protein. The participation of serine and pyridoxal phosphate in a reaction involving an altered B protein lends further support to the suggestion of Crawford & Yanofsky (1958) that protein B provides the surface on which the serine + pyridoxal phosphate complex is combined during those reactions of tryptophan synthetase in which this complex is involved.

Three types of experiments differentiate the conversion indole-3-glycerol phosphate → indole in the presence and in the absence of serine: (i) the serine system is strongly inhibited by a low concentration of hydroxylamine; (ii) the pH-activity curves for the two systems are different; (iii) curves for the saturation of $B_{7\text{-}4}$ protein by normal A protein have marked differences in the serine and basal systems.

The function of serine (presumably as a Schiff base with pyridoxal phosphate) in the serine system is of interest. It does not act as a substrate in the conversion indole-3-glycerol phosphate → indole but it is possible that it is changed to some other compound by the A B protein complex during the course of the reaction. It was thought that if serine were attacked during indole formation, pyruvate would be a likely end product. Experiments designed to detect pyruvate production by the specific method of Friedmann & Haugen (1943) were clearly negative. The affinity for serine is fairly low (K_a about $1{\cdot}3 \times 10^{-3}$ M) and with the concentrations of serine necessary to obtain a significant reaction it was not practicable to look for serine removal,

It has been suggested (DeMoss & Bonner, 1959) that in mutants of *Neurospora crassa* similar to *Escherichia coli* strain 7-4, serine and pyridoxal phosphate might be required to maintain the active site of the altered enzyme in its 'proper' configuration for cleavage of indole-3-glycerol phosphate. This suggestion is supported and extended by the experiment shown in Fig. 4. The amount of normal A protein required for half saturation of $B_{7\text{-}4}$ protein in the basal system is about 25 times that required for the serine system. This suggests that one function of serine and pyridoxal phosphate in the conversion indole-3-glycerol phosphate → indole by the tryptophan synthetase of *E. coli* strain 7-4 is to assist in binding the A and B components into an enzymically effective complex.

One of us (F.G.) is indebted to the Carnegie Corporation of New York which made this work possible while on leave from the University of Melbourne and to the Department of Biological Sciences at Stanford University for its hospitality.

This work was supported by grants from the U.S. Public Health Service and the National Science Foundation (U.S.A).

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