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A Sensitive Assay System for Determination of the Ichthyotoxicity of *Prymnesium parvum*

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SUMMARY

The activity of *Prymnesium parvum* ichthyotoxin and its dependence on various cofactors has been studied. Spermine or other polyamines have a marked synergistic effect on the ichthyotoxic activity. Of the cations studied, Ca^{2+} and Mg^{2+} increased ichthyotoxic activity, while Na^{+} inhibited. Changes in pH value had a profound influence, ichthyotoxicity increasing at least 400-fold as the pH value was increased from 7 to 9. A sensitive bioassay for the estimation of *P. parvum* ichthyotoxin has been developed for use in the laboratory and under field conditions.

INTRODUCTION

Prymnesium parvum, a halophilic Chrysomonad, has been the cause of extensive fish mortalities in many parts of the world (Shilo, 1964). This organism produces a number of soluble toxic principles including an ichthyotoxin, a haemolysin and a cytotoxin (Otterstrom & Steeman-Nielson, 1940; Shilo & Rosenberger, 1960; Yariv & Hestrin, 1961). In spite of its widespread endemic occurrence in Israel in brackish water fish ponds, mortality of fish due to *P. parvum* intoxication is observed only sporadically. It has been postulated that the lack of correlation between numbers of phytoflagellates and the toxicity of the pond water was due to a dynamic state of equilibrium existing between toxin formation and destruction (Shilo & Aschner, 1953), since the ichthyotoxin is readily adsorbed on various colloids and inactivated by external factors such as light (Parnas, Reich & Bergmann, 1962), heat, high pH value or by the action of various bacteria (Shilo & Aschner, 1953). It was further shown that the expression of ichthyotoxic activity was dependent on the presence of cationic cofactors such as Ca^{2+}, Mg^{2+} or streptomycin (Yariv & Hestrin, 1961). The lability of the ichthyotoxin and its cofactor requirements pose special problems in its quantitative estimation. The present paper deals with the effect of different factors on fish toxicity and with the development of a sensitive bioassay for ichthyotoxin estimation.

METHODS

Chemicals. The chemicals used were obtained from the following sources: streptomycin sulphate (Merck and Co., U.S.A.); spermine tetrahydrochloride and propane-1,3-diamine (Fluka AG. Buchs SG Switzerland); neomycin sulphate (Uclaf, Paris, France); 3,3-diaminodipropylamine (Eastman Kodak Co., U.S.A.); diethyl-

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enetriamine, triethylenetetramine, tetraethylenepentamine (L. Light and Co., Ltd., England); putrescine dihydrochloride and cadavarine dihydrochloride (Nutritional Biochemicals Corp., U.S.A.); spermidine trihydrochloride (California Corp. for Biochemical Research, U.S.A.).

Prymnesium parvum culture. The strain of *P. parvum* used in these experiments had been isolated from brackish-water fish ponds in Israel by Reich & Kahn (1954).

Medium and conditions of growth. The growth medium for *Prymnesium parvum* used in this work was the defined medium developed by Droop (1958) in which the amounts of trace elements were modified as follows (per litre medium): $FeCl_3$, 5 mg.; $MnCl_3$, 500 μg.; $ZnSO_4$, 50 μg.; $CaSO_4$, 50 μg.; $CoSO_4$, 5 μg.; $Na_2MoO_4.2H_2O$, 5 μg. The medium was sterilized by autoclaving at 120° for 20 min. Cultures were grown in Erlenmeyer flasks of 250 ml. capacity containing 100 ml. liquid medium in continuous 'fluorescent white' daylight, 220–260 foot-candles, at 26–28°. Cultures were harvested after a period of 30–40 days of cultivation.

Extraction and assay of ichthyotoxin. The ichthyotoxin produced by *Prymnesium parvum* was extracted from the organisms with ethanol as described by Shilo & Rosenberger (1960). The extraction was facilitated by sonic oscillation (Raytheon sonic oscillator Model DF 101, 10 KC) for 7 min at 4°. Such ethanolic extracts were kept at 4° in the dark and were used in concentrations not exceeding 1 % to avoid toxic effects of the ethanol on the test minnows. For titration of ichthyotoxin two-fold dilutions of the extracts of *P. parvum* were prepared in tris buffer (0·05 M) containing cofactor. In the experiments to be described three minnows (1·5–2·0 cm. long) served as test organisms for each dilution. The type and concentration of cofactor were as indicated later and the resulting fish toxicity expressed relative to toxicity under standard conditions of test, as described below in the definition of an ichthyotoxic unit. The pH value of the test solution was readjusted when necessary every hour with N-NaOH.

An 'ichthyotoxic unit' of toxin (ITU) was defined as the minimal amount of *Prymnesium parvum* toxin/ml. which killed within 8 hr all the fish in the test system containing tris buffer (0·05 M) and spermine (0·5 mg./ml.) as cofactor, at pH 8·0 and 25° in the dark.

RESULTS

The effect of different cofactors on the ichthyotoxic activity

Cofactors studied included Ca^{2+}, Mg^{2+} and streptomycin, which have been shown by Yariv & Hestrin (1961) to enhance the activity of *Prymnesium parvum* ichthyotoxin, and spermine, neomycin and several polyamines. Figure 1 summarizes the results of these experiments. A marked effect of spermine in enhancement of ichthyotoxicity occurred; this was 5 times greater than the enhancement with neomycin and 10 times greater than that observed with Ca^{2+} or streptomycin when these substances were tested at 0·0045 M, pH 8·0. The effect of other amines on the ichthytotoxic activity is summarized in Table 1. Additional materials tested for their synergistic activity as cofactors included lysine, arginine, histamine, ornithine, chloramphenicol, and erythromycin (0·5 mg./ml. at pH 8·0 and 9·0). None of these materials showed any cofactor activity except for a slight effect with histamine at pH 9·0. A relationship was found to exist between the concentration of every one of the active cofactors tested and the minimal lethal dose (MLD) of

chthyotoxin of *P. parvum*. This relationship, described by Yariv & Hestrin (1961), can be expressed as cofactor concentration × MLD = a constant; this held up to a concentration of 0·0035–0·0045 M for all the cofactors tested. On further increase of cofactor concentration no additional decrease in the minimal lethal dose was observed.

Table 1. *The effect of various polyamines on ichthyotoxic activity of Prymnesium parvum*

Cofactor used at concentration of 0·0015 M	Formula of the cofactor	Relative activity
Spermine	NH_2—$(CH_2)_3$—NH—$(CH_2)_4$—NH$(CH_2)_3$—NH_2	10
Spermidine	NH_2—$(CH_2)_3$—NH—$(CH_2)_4$—NH_2	5
3,3-Diaminodipropylamine	NH_2—$(CH_2)_3$—NH—$(CH_2)_3$—NH_2	5
Diethylene triamine	NH_2—$(CH_2)_2$—NH—$(CH_2)_2$—NH_2	2
Triethylene tetramine	NH_2—$(CH_2)_2$—NH—$(CH_2)_2$—NH—$(CH_2)_2$—NH_2	5
Tetraethylene pentamine	NH_2—$(CH_2)_2$—NH—$(CH_2)_2$—NH—$(CH_2)_2$—NH—$(CH_2)_2$—NH_2	7·5
Propane 1,3-diamine	NH_2—$(CH_2)_3$—NH_2	2
Putrescine	NH_2—$(CH_2)_4$—NH_2	2
Cadavarine	NH_2—$(CH_2)_5$—NH_2	1
Without cofactor		0·25

Titrations were carried out in the dark at 28°, in 0·05 M tris buffer at pH 8·0. Results were recorded after 4 hr. The toxin employed was 'S' extract having 4000 ITU/ml. Identical results were obtained in all of the five assays made with each cofactor.

Combined effects of various cofactors on ichthyotoxic activity

The possibility that more than one cofactor may be active in the natural environment of the fish led us to investigate the effect of combining different cofactors. Experiments on the effect of increasing concentrations of $CaCl_2$, in presence or absence of spermine, on the ichthyotoxic activity are summarized in Fig. 2. It can be seen that with increasing concentrations of Ca^{2+}, the ichthyotoxicity in the presence of spermine decreased rapidly and approached the value obtained with Ca^{2+} alone. The overall cofactor activity of the Ca^{2+} and spermine mixtures tested on the ichthyotoxicity was found to approach the sum of the activities of the individual cofactors in the mixture each multiplied by its relative concentration (expressed as percentage of total molar cofactor concentration) in the system. No difference in final ichthyotoxicity was found between tests of simultaneous addition of spermine and Ca^{2+} and tests where one of the cofactors was added 30 min. before adding the other.

The study of the effect of increasing concentrations of Ca^{2+} on ichthyotoxicity in presence of a constant concentration of streptomycin (0·001 M) as cofactor showed (Fig. 3) an increase in ichthyotoxicity up to 0·003 M-Ca^{2+}, a concentration at which the maximal effect is obtained when Ca^{2+} served as the single cofactor.

The effect of increasing concentrations of different salts (NaCl; sea water; NaCl + $CaCl_2$ in a ratio of 26:1 as found in sea water; brackish pond water) and of different concentrations of sucrose on the ichthyotoxicity of *Prymnesium parvum* extracts was studied. Results of a typical experiment in presence and absence of spermine are summarized in Fig. 4. It can be seen that with increasing NaCl con-

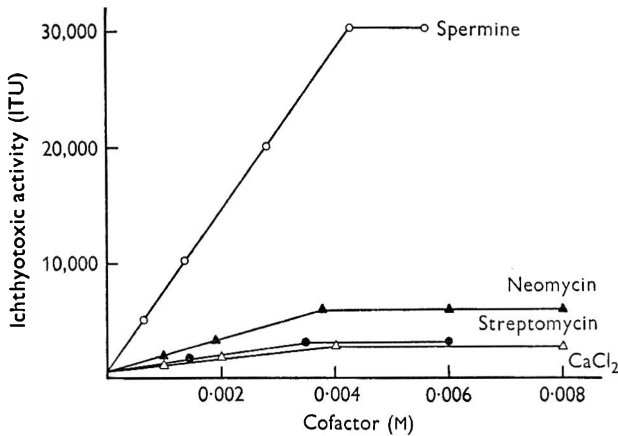

Fig. 1

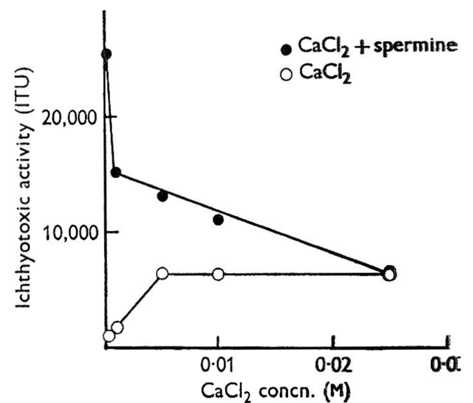

Fig. 2

Fig. 1. The effect of concentration of spermine, neomycin, $CaCl_2$ and streptomycin on activity of *Prymnesium parvum* ichthyotoxin. Titrations were carried out in the dark at 25° with tris buffer (pH 8·0). Results were recorded after 8 hr. The toxin used was extract 'S' having 10,000 ITU/ml.

Fig. 2. The combined effect of spermine and $CaCl_2$ on activity of *Prymnesium parvum* ichthyotoxin. Tests were carried out at pH 9·0 in 0·05 M-tris buffer. Solutions contained various concentrations of $CaCl_2$ and a constant concentration of spermine (0·0014 M). Control solutions contained various $CaCl_2$ concentrations only in 0·05 M-tris buffer (pH 9·0). Tests were carried out at 25° in the dark. Results were recorded after 8 hr. Extract 'S' having activity 6400 ITU/ml. was used.

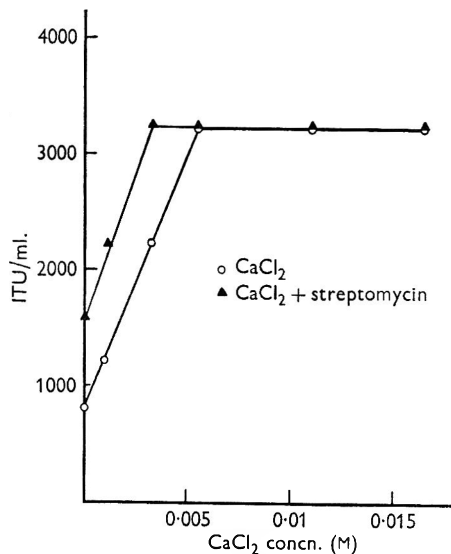

Fig. 3. The combined effect of streptomycin and $CaCl_2$ on activity of *Prymnesium parvum* ichthyotoxin. Tests were made at pH 8·0 in 0·05 M-tris buffer solutions containing various concentrations of $CaCl_2$ and a constant concentration of streptomycin 0·001 M. Control solutions contained various $CaCl_2$ concentrations only, in 0·05 M-tris buffer (pH 8·0). Tests were carried out at 25° in the dark. Results were recorded after 8 hr. Extract 'S' of activity 12,800 ITU/ml. was used.

centrations up to 3·0 mg. Cl^-/ml. or with equivalent salt concentrations in sea or pond water, there was a marked decrease of ichthyotoxic activity in the presence of spermine and a slight decrease in its absence. With further increase in salt concentration a progressive increase in ichthyotoxic activity independent of the presence of spermine was observed.

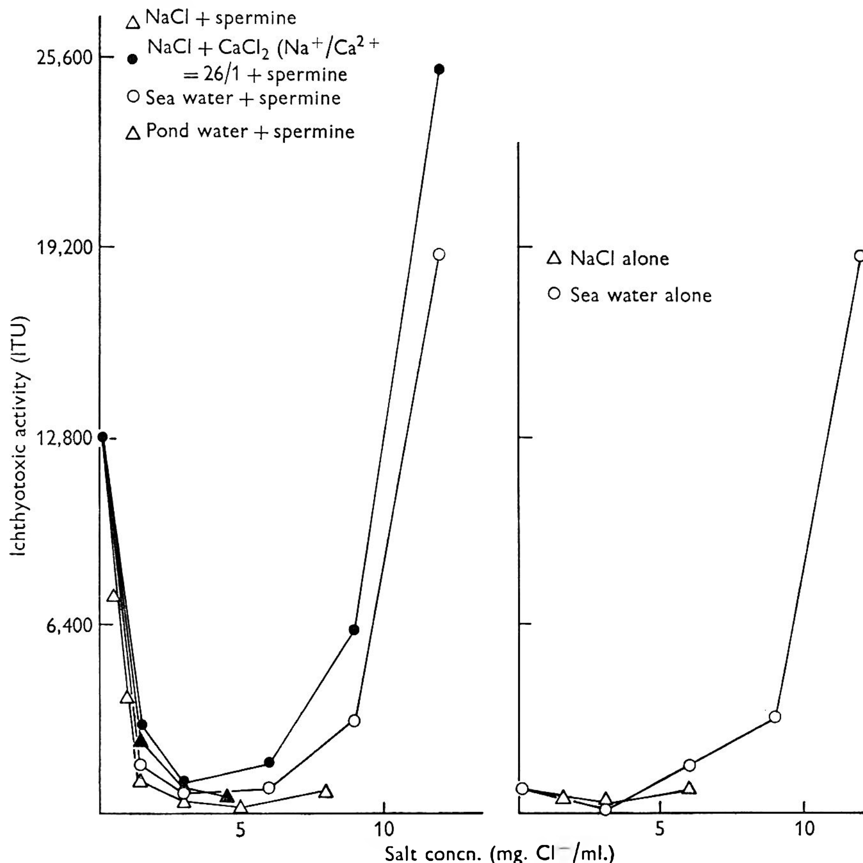

Fig. 4. Effect of various salt concentrations on activity of *Prymnesium parvum* ichthyotoxin. Tests were carried out at pH 8·0 in 0·05 M-tris buffer in the dark; results were recorded after 8 hr at 25°. Concentration of spermine was 0·5 mg./ml. (0·0014 M); NaCl concentration is expressed as mg. Cl^-/ml. The toxin used was extract 'S' of activity 12,800 ITU/ml.

The inhibitory effect of different salt concentrations on the ichthyotoxic activity was reversible. Toxic solutions dissolved in 15 % (v/v) sea water diluted in distilled water, a concentration at which 98 % of the activity observed in the absence of salt was suppressed, was rendered fully toxic by dialysis for 20 hr against distilled water.

In experiments with streptomycin + Ca^{2+} as cofactors, increasing amounts of NaCl up to 3·0 mg. Cl^-/ml. caused a progressive inhibition of the ichthyotoxicity, similar to that observed with spermine. Addition of sucrose up to 0·2 M did not

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affect the activity of ichthyotoxin in presence of spermine, thus excluding the possibility that the suppression of ichthyotoxic activity by different salts was caused by increased osmotic pressure of the suspension medium.

The effect of pH value on the activity of Prymnesium parvum ichthyotoxin

Experiments on the effect of the pH value of the medium on the activity of ichthyotoxin in the presence or absence of added cofactors are summarized in Table 2. An increase in ichthyotoxicity was observed with an increase in pH value of the test medium with all the cofactors studied, and even in the absence of added cofactor. The activity at pH 9·0 was four times greater than that at pH 8·0; no ichthyotoxic activity was observed in the system at pH 7·0.

Table 2. *The effect of pH on ichthyotoxic activity*

Cofactor used at concentration of 0·0015 M	Activity at pH 7·0	Activity at pH 8·0	Activity at pH 9·0
$CaCl_2$	< 100	1000	4000
Streptomycin	< 100	1000	4000
Spermine	< 100	10·000	40·000
3,3-Diaminodipropylamine	< 100	5000	20·000
Neomycin	< 100	2000	4000
Without cofactor	< 100	250	1000

Titrations were carried out at pH 7·0, 8·0 and 9·0 with 0·05 M-tris buffer at 20–25° in the dark. Results were recorded after 8 hr. Identical results were obtained in all of the five assays made with each cofactor.

The effect of temperature on the ichthyotoxic activity of Prymnesium parvum

The effect of temperature in the range of 10–30° at pH 8·0 on the killing of minnows by *Prymnesium parvum* ichthyotoxin in the presence of spermine is shown in Fig. 5. It can be seen from this figure that an increase in temperature caused an increase in the rate of mortality but did not affect the final titre (MLD) of the toxin. Experiments at pH 9·0 showed a similar effect of temperature on the kinetics of killing, but the rate was 30–50 % greater than that observed at pH 8·0. Temperatures higher than 30° were not tolerated by the fish under the test conditions.

ASSAY OF ICHTHYOTOXIN

On the basis of the experiments reported above we have developed the following test for assaying the ichthyotoxin produced by *Prymnesium parvum*. The procedure is essentially the same as described under Methods, with 0·5 mg. spermine/ml. as a cofactor and tris buffer (0·05 M) adjusted to pH 9·0 with HCl. The titration, with three standard size minnows for each test condition, is carried out at 26–28° in the dark, and the results recorded after 3 hr. In cases where the samples tested contain high concentration of salt (e.g. brackish pond water or growth media of *P. parvum*) the medium is dialysed against distilled water for several hours before the test. An examination of the reproducibility of this test under the standard conditions at pH 8 and 9 showed the results to be highly dependable with a standard deviation ranging from 10 to 30 %.

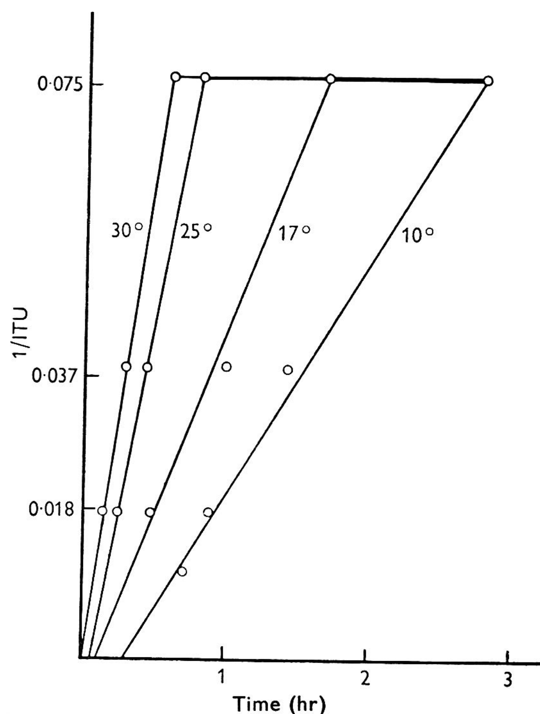

Fig. 5. The effect of temperature on activity at pH 8·0 of *Prymnesium parvum* ichthyotoxin. Tests were carried out at pH 8·0 in 0·05 M-tris buffer+spermine 0·0014 M in the dark. The minnows were incubated in tap water for 12 hr before test at temperatures corresponding to those used in the tests. Average time for loss of equilibrium of all three minnows was recorded. Control fish remained unaffected for 3 hr after the death of all test fishes. Extract 'S' having 12,800 ITU/ml. was used in these tests.

DISCUSSION

The present study confirms and extends the findings of Yariv & Hestrin (1961) on the cofactor requirements of *Prymnesium parvum* ichthyotoxin. In addition to Ca^{2+}, Mg^{2+} and streptomycin described by these workers, other cationic substances, e.g. neomycin, spermine and certain other polyamines, were shown to enhance the ichthyotoxicity of *P. parvum* extract. All the compounds so far found to have cofactor activity in the *P. parvum* ichthyotoxic system have been reported to act as stabilizing agents in a variety of biological systems such as bacterial spheroplasts and protoplasts, mitochondria and microsomes (Tabor, Tabor & Rosenthal, 1961); in all these systems, spermine was the most active.

It has been confirmed that the quantitative relationship between cofactor and ichthyotoxin expressed by Yariv & Hestrin (1961) as $a \times b = Q$ (where a is the ichthyotoxin concentration, b the minimum concentration of cofactor which elicits a lethal effect in presence of a, and Q is a cofactor-specific constant) holds for all the cofactors tested, but that this relationship has an upper limit at cofactor concentration of 0·0045 M.

In the presence of more than one cofactor the resulting toxicity is not necessarily additive but depends on the specific activity of each cofactor and its concentration relative to the other cofactor. Thus, the addition of a cofactor of low activity, e.g.

Ca^{2+}, to a system containing a cofactor of high activity, e.g. spermine, drastically lowers the degree of toxicity to fish. On the other hand, the addition of Ca^{2+} to a system containing a limiting concentration of streptomycin (a cofactor of roughly equivalent activity) increases fish toxicity. This has the result that in high concentrations of Ca^{2+} the effects of spermine and streptomycin are masked. This relationship and the phenomenon of cofactor saturation might be due to competition of cofactors for similar sites.

It has already been emphasized (Shilo & Aschner, 1953) that no correlation was found between the population density of *Prymnesium parvum* in brackish fish ponds and the concentration of ichthyotoxin in the water. It has been suggested that this phenomenon can be explained partly through the continuous destruction of excreted toxin by various inactivating factors. The present findings on the effects of pH value, salt concentration and temperature on the activity of the ichthyotoxin show that various environmental factors may in this manner play a decisive role in determining the overall picture of toxicity. In fact, a survey of the appearance of fish intoxication in brackish-water fish ponds in Israel has shown that a correlation exists between toxicity and elevated pH values and/or low salinity of pond water. Furthermore, the widely differing activity of the various cofactors tested, such as Ca^{2+}, streptomycin and some polyamines, in enhancing ichthyotoxicity suggests that the concentration and nature of cofactor or cofactors in the milieu may have a profound influence on final toxic effect of *P. parvum* in a pond or culture.

The bioassay for the ichthyotoxin developed was suitable for the determination of extracellular, as well as intracellular ichthyotoxin from axenic cultures and from *Prymnesium parvum* blooms in fish ponds. By using this assay method it was possible to detect sublethal amounts of ichthyotoxin in ponds (as low as $\sim 1/30$ of the lethal dose for carp). Since only a small percentage of Prymnesium-infested ponds in nature develop lethal concentrations of ichthyotoxin, this method has economic importance since it limits the need for control of the phytoflagellate to those ponds containing detectable sublethal amounts of toxin.

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A Description of Some Lignanolytic Soil Bacteria and their Ability to Oxidize Simple Phenolic Compounds

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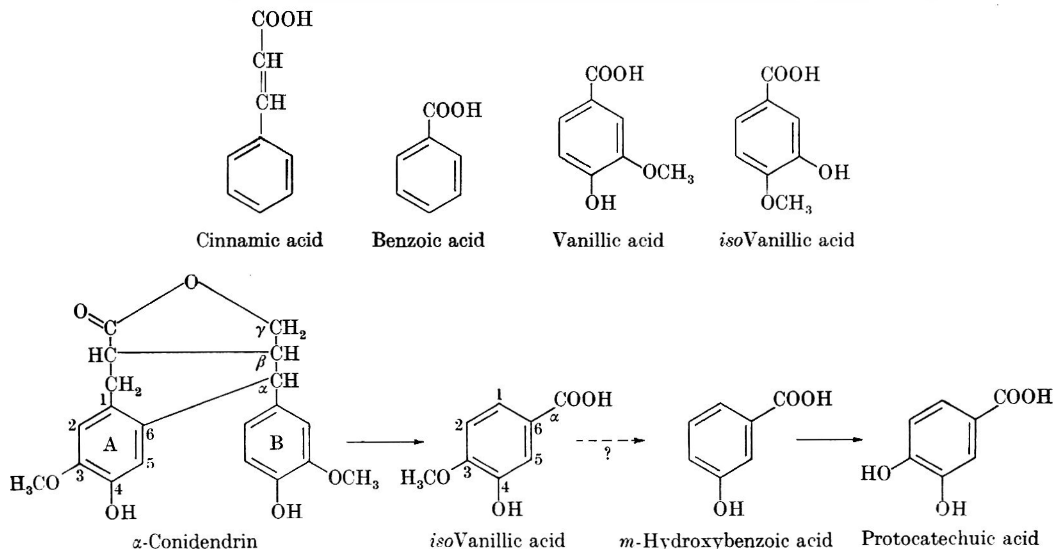
SUMMARY

Bacteria able to decompose α-conidendrin were isolated from three of five soil samples by enrichment on this compound as sole source of carbon and energy. All the isolates obtained were small Gram-negative motile poorly flagellated rods, which were classified as members of the genus *Agrobacterium*. The ability of these bacteria to oxidize phenolic compounds was examined by manometric studies. From calculations of oxygen uptake, it was concluded that the oxidative rupture of the benzene ring of *m*- and *p*-hydroxybenzoic acid by these bacteria could not be explained entirely by the known metabolic paths which lead from these hydroxybenzoic acids to protocatechuic acid or gentisic acid. It was indicated by simultaneous adaptation technique that neither monohydroxybenzoic acids nor monohydroxycinnamic acids were likely to be formed during the oxidative breakdown of α-conidendrin by these agrobacteria.

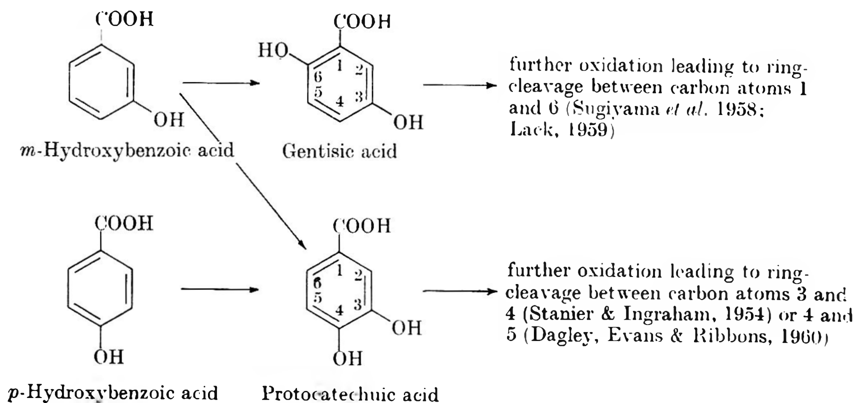
INTRODUCTION

In the first paper of this series (Sundman, 1962) a new type of α-conidendrin degradation by bacteria was described. It was shown that in contrast to earlier reported bacterial attacks on α-conidendrin and other methoxylated aromatic compounds (Konetzka, Woodings & Stove, 1957; Tabak, Chambers & Kabler, 1959), which were interpreted to proceed *via* vanillic acid as an intermediate, the present bacteria produce no vanillic acid but considerable amounts of *iso*vanillic acid during the breakdown of α-conidendrin. The decomposition was assumed to occur according to Fig. 1, with the 6-α-bound guaiacyl group A being conserved in the *iso*vanillic acid, which was believed to be further oxidized, possibly *via* *m*-hydroxybenzoic acid, to protocatechuic acid. In previous work (Konetzka *et al.* 1957; Tabak *et al.* 1959) *p*-hydroxybenzoic acid was reported as intermediate in the bacterial degradation of α-conidendrin *via* vanillic acid, and in the oxidation of trimethoxybenzoic acid by a soil bacterium (Konetzka *et al.* 1957). *p*-Hydroxycinnamaldehyde arises during decomposition of spruce native lignin by various white-rot fungi (Ishikawa, Schubert & Nord, 1963*a*, *b*). It is not known which metabolic sequences could replace a methoxyl group by a hydrogen atom under aerobic conditions thus forming, e.g. *p*-hydroxybenzoic acid from vanillic acid or *m*-hydroxybenzoic acid from isovanillic acid. The position of the mono-substituted benzoic and cinnamic acids in the oxidative breakdown of guaiacyl compounds remains obscure. To acquire more information about the mode of action of the bacteria, this work was enlarged to include the hydroxybenzoic acids and

hydroxycinnamic acids in addition to diphenolic acids and pyrocatechol, which are known as key intermediates in the oxidative breakdown of the aromatic structure (Rogoff, 1961). Further, the oxidation of phenol, benzoic acid and cinnamic acid was investigated. By the simultaneous adaptation technique (Stanier, 1947) organisms



Supposed mode of formation of *iso*vanillic acid from α-conidendrin by the bacteria under investigation (Sundman, 1962).



Metabolic paths of *m*-hydroxybenzoic acid and *p*-hydroxybenzoic acid by bacteria.

Fig. 1. Chemical structures of individual compounds and pathways.

of both α-conidendrin-adapted and non-adapted cultures were examined, and an attempt made to determine whether some of these substances were intermediates in the degradation of α-conidendrin.

The α-conidendrin-decomposing bacteria previously described belong to the genera *Pseudomonas* (Tabak *et al.* 1959) and *Flavobacterium* (Konetzka, Pelczar & Gottlieb, 1952). These genera seem to demand special attention in any attempt to

clarify the ligninolytic microflora of the soil. It has been shown that pseudomonads and flavobacteria originating in soil decompose Braun's native lignin, and proliferate on this material as sole source of energy (Sörensen, 1962) and further, that *Pseudomonas fluorescens* strains are capable of oxidizing a number of lignin preparations (Higuchi, Kavamura & Hayashi, 1956). The conidendrin-decomposing bacteria isolated by the author (Sundman, 1962) showed some resemblance to certain pseudomonads but also features of the genus *Agrobacterium*. The present paper examines the results of attempts to determine the taxonomic position of the lignanolytic bacteria which produce *iso*vanillic acid.

METHODS

Soil samples. Five samples, rich in wood residue, were selected. α-Conidendrin-decomposing bacteria were isolated from soil sample Ks, decaying sawdust (Helsinki), pH 5·5; soil sample Ms, forest soil (Helsinki), under Oxalis and Myrtillus; soil sample O, decaying pile of brush wood (Teisko), pH 5·5. None was obtained from two other samples of forest soil, and a decaying stub, respectively.

Isolation of the bacteria. As previously described (Sundman, 1962), the α-conidendrin-decomposing bacteria were isolated from soil samples by enrichment through several subcultures in a nitrate + mineral salts + vitamin basal medium to which 0·5% (w/v) of α-conidendrin crystals were added as the carbon + energy source. The base solution (K-solution) contained: $NaNO_3$, 2·5 g.; KH_2PO_4, 1·0 g.; $CaCl_2$, 0·1 g.; $MgSO_4.7H_2O$, 0·3 g.; NaCl, 0·1 g.; $FeCl_3$, 0·01 g.; vitamin solution (thiamine 10 mg., Ca-panthothenate 10 mg., biotin 20 μg., B_{12} vitamin 40 μg., pyridoxin 10 mg., nicotinic acid 10 mg., *p*-aminobenzoic acid 10 mg., folic acid 2 mg., dissolved in 200 ml. of water) 5 ml.; water 1000 ml. final pH 6·5. For isolation, the enrichment cultures were plated on KYE agar (K-solution + Bacto yeast extract 0·1% (w/v) + agar 2% (w/v). The isolates were replated on the same medium, and the ability to decompose α-conidendrin was noted as a darkening upon growth in the liquid enrichment medium with α-conidendrin as sole source of carbon + energy. About 10 α-conidendrin-decomposing isolates from each soil sample were kept as stock cultures for further work, with monthly transfers on: (*a*) semi-solid KYE agar (KYE agar with agar content decreased to 0·3% w/v); (*b*) the liquid α-conidendrin-containing medium.

Manometry. The respirometric experiments were made with washed organisms. These were grown at 28° for 2 days in Roux bottles on KYE agar + glucose 0·2% (w/v) + yeast extract 0·1% (w/v). (Organisms Ms 144412 were difficult to handle when grown on glucose-containing medium because of heavy slime production. Glucose was therefore omitted from the medium for growing this bacterium.) For the production of α-conidendrin-adapted bacteria, α-conidendrin 0·05% (w/v) was added to the medium. The bacteria were harvested by the aid of glass beads and water, and the suspension filtered through glass wool to remove large agar particles. The centrifuged organisms were washed three times with 1/15 M-phosphate buffer pH 5·9), and re-suspended in the same buffer, about 6×10^{10} viable organisms/ml. according to plate counts. The Warburg vessels were filled with: 0·5 ml. suspension of organisms; 1·0 ml. of substrate (1–2·8 μmole in water, pH 7·0); 0·2 ml. 20% (w/v) potassium hydroxide in the centre well. The technique recommended by Umbreit,

Burris & Stauffer (1957) was used. In determining the total oxygen consumption, the substrate was added to the main compartment and the bacteria tipped from the side arm to avoid error caused by droplets adhering to the walls of side arm after tipping. The results given are means of duplicate manometer readings.

Growth experiments. To determine whether a compound served as carbon + energy source for the bacteria, washed organisms were inoculated as stab cultures into semi-solid K agar (K-solution + 0·3 %, w/v, Bacto agar), and into similar media with 0·001 and 0·0001 mole/ml., respectively, of compound to be tested. Since these bacteria are not able to utilize agar, the control inoculation into semi-solid K agar served as a check on possible carbon + energy sources transferred with the inoculum or present in the medium. The growth in the tubes provided with compound to be tested is quoted as positive only when the control tube showed no growth.

Phenolic compounds used. α-Conidendrin was kindly supplied by the Crown & Zellerbach Corporation (Camas, Washington state, U.S.A.). The white crystals were dissolved in acetone and re-precipitated from water, m.p. 247–248°. Other compounds were commercially available preparations as follows: *o*-hydroxybenzoic acid (British Drug Houses Ltd, reagent) m.p. 157–158°; *m*-hydroxybenzoic acid (puriss.) m.p. 198–199°; *p*-hydroxybenzoic acid (purum) m.p. 212–214°; *o*-hydroxycinnamic acid (purum) m.p. 207–209°; *m*-hydroxycinnamic acid (purum) m.p. 192–193°; *p*-hydroxycinnamic acid (puriss.) m.p. 214–215°; protocatechuic acid (purum) m.p. 200–201°; gentisic acid (puriss.) m.p. 202–204°; homogentisic acid (purum) m.p. 147–149°, all from Fluka A.G. (Buchs, S.G., Switzerland); pyrocatechol (p.a.) m.p. 104–105°; phenol (p.a. for chromatography) m.p. 39–39·5°, both from Merck Co., Inc. (Darmstadt, Western Germany).

RESULTS

Identification and taxonomy of the α-conidendrin-decomposing bacteria

All the α-conidendrin-decomposing isolates obtained from the three soil samples were motile, non-sporing, Gram-negative single rods, 0·9–1 by 1·3–3·2 μ, with a tendency to uneven staining and Gram variability. No pigment was produced on ordinary media. The isolates from each soil sample showed only minor differences in physiological tests, and were obviously representatives of the same species. One isolate for each soil sample was selected for further study, and replated twice to ensure purity. Of these isolates: Ks 17, O 8, Ms 14, the two first named were used in previous work (Sundman, 1962). The isolates had since been kept as stock cultures for a year. They showed no sign of impurity, but for the identification purposes of the present work their purity was ascertained by repeated platings. Though visually homogeneous, the plates were not considered as proofs of purity until all the colonies from a plate gave visually identical cultures in stabs in semi-solid KYE agar, were positive in the test for α-conidendrin degradation, and showed the same morphology under the phase-contrast microscope. This was achieved after two platings of isolates Ks 17 and O 8, and four platings of isolate Ms 14. The replated cultures are designated Ks 1741, O 811, and Ms 144412.

Each isolate was aerobic, growing as sharply confined surface upon stabbing in semi-solid KYE agar; growth was good at 15° and 28°, the minimum temperature for growth was less than 3°, and the maximum below 37°. Tests for hydrolysis of

gelatin, casein and starch (on KYE agar plates containing 0·4%, w/v, gelatine, 20%, w/v, skimmed milk, and 0·2%, w/v, soluble starch, respectively) were negative. Indole was not produced in the 1% (w/v) tryptone solution, nor hydrogen sulphide in the mannitol tryptone medium of ZoBell & Feltham (1934). In the Hugh & Leifson (1953) test for the type of carbohydrate metabolism, no acid or gas from glucose was detected during the 10 days of incubation; when incubation was extended to 21 days, the open tube turned yellow. Substances tested as sole carbon + energy sources supporting growth were: glucose, galactose, mannose, xylose,

Table 1. *Characteristics which varied in the α-conidendrin-decomposing isolates Ks 1741, Ms 144412 and O 811*

Determinative test	Isolate		
	Ks 1741	Ms 144412	O 811
Flagella staining	1–2 polar (and/or lateral)	1–5 peritrichous	1–3 polar (and/or subpolar)
Oxidase test (dimethyl-*p*-phenylene diamine HCl solution poured on KYE agar surface growth	+	−	+
Nitrite from nitrate (in KYE medium)	+*	−	+*
Litmus milk (21 days, 28°), Hofer (1941)	Slight alkalization, reduction at the bottom. No browning	Slimy serum zone at the surface. Milk yellowish brown	Slight alkalization, reduction at the bottom. No browning
Growth on Caglycerophosphate-mannitol agar (Riker *et al.* 1930)	Brown pigment, surrounded by white precipitate	No colour, abundant white precipitate	Brown pigment, surrounded by white precipitate
Absorption of Congo red	±	+	−
Assimilation of arabinose	−	+	−

* Nitrates completely assimilated, test for nitrite negative upon growth in medium containing glycerol + nitrate (0·025%, w/v)

mannitol, glycerol, citric acid, gluconic acid, protocatechuic acid, hydroxybenzoic acids, hydroxycinnamic acids (see Table 2). Lactose, saccharose, vanillic acid, vanillin, cinnamic acid, ferulic acid, guaiacol, eugenol, isoeugenol, and phenol did not serve as carbon + energy source. All the isolates were negative in the test for alkaline arginine metabolism (Thornley, 1960), did not produce alkalinity when grown in a medium containing potassium nitrate + glycerol (Sagen, Riker & Baldwin, 1934), and were resistant to 100 i.u. penicillin/ml. KYE agar.

Table 1 illustrates the behaviour of isolates Ks 1741, O 811, and Ms 144412 in some additional tests, in which variable results were obtained. The flagellation of these bacteria, as reported in Table 1, represents the conclusions from numerous tests by Leifson's method, with tannic acid + ethanolic fuchsin (Leifson, 1960), and Fontana's method with silver nitrate as described by Rhodes (1958). Some unknown feature of these organisms made clear-cut flagella-stained preparations difficult to obtain by reason of heavy precipitation of stain around the organisms. In the main, isolates O 811 and Ks 1741 had polar flagellation, but lateral and sub-polar flagella

were noted repeatedly. To decide whether these latter were artefacts caused by overlapping of soma and flagellum (Hodgkiss, 1960) *Agrobacterium tumefaciens* (kindly supplied by Professor O. Pohjakallio, Department of Plant Pathology, University of Helsinki) and *Pseudomonas* spp. (isolated in this laboratory from market milk) were included as peritrichously flagellate and polarly flagellate test organisms, respectively, for the flagella staining techniques used. The preparations obtained with *A. tumefaciens* and α-conidendrin-decomposers Ks 1741 and O 811 were almost indistinguishable, showing few flagella, and in situations which were mainly polar and occasionally lateral or sub-polar. The same precipitation of stain around the organisms was noted in all the preparations. The *Pseudomonas* spp. cultures gave preparations with clear-cut polar flagella only, and without the disturbing precipitate around the organisms. All the isolates were thus to be considered as peritrichously flagellate, and a location in the genus *Pseudomonas* excluded. This is supported by the negative test for alkaline arginine metabolism obtained with the isolates. This test was useful for the differentiation of pseudomonads from other Gram-negative organisms, isolated from spoiled chicken meat, (Thornley, 1960), and according to Brisbane & Rovira (1961) of value also in the separation of soil pseudomonads from other Gram-negative soil bacteria.

In a system for the classification of Gram-negative soil bacteria (Holding, 1960), those aerobic organisms which possess peritrichous flagella and are capable of oxidizing glucose and of growth on inorganic nitrogen are allocated to the genus *Agrobacterium*. According to this classification, the α-conidendrin-decomposing bacteria described here would be considered as agrobacteria. Of the seven Agrobacterium species listed in the 7th edition of *Bergey's Manual* (1957), five are plant pathogens, *A. radiobacter* is a common soil organism, and one species, *A. stellulatum*, was isolated from marine mud. On the basis of their growth in litmus milk, and the action on carbohydrates, the present isolates examined here display most resemblance to *A. radiobacter*. Inability to produce hydrogen sulphide, colourless growth on Ca glycerophosphate-mannitol agar (Ms 144412), neutral reaction in nitrate + glycerol medium, growth in litmus milk without production of brown colour (Ks 1741, O 811), and the absorption of congo red (Ms 144412) indicate that none of the isolates listed in Table 1 can be considered as typical *A. radiobacter*. It is believed that they belong to the still rather obscure *A. radiobacter* group of the indigenous soil flora described by Conn (1942, 1948).

Oxidation of aromatic key intermediates

In manometric studies of the oxidative properties of the bacteria, it was found that a common feature of these α-conidendrin-decomposers was a constitutive ability to oxidize protocatechuic acid (PA) and homogentisic acid (HGA) (see Figs. 2, 3). Strains Ms 144412 and O 811 oxidized pyrocatechol (PC) at a comparable rate, and strains O 811 and Ks 1741 also oxidized gentisic acid (GA) at a slower rate (see Fig. 3). No induction was required for the oxidation of these substrates. Organisms grown on yeast extract + glucose started oxygen uptake immediately, with no lag. The *Agrobacterium tumefaciens* strain included lacked the ability to oxidize these phenolic acids. The oxidation of PA, HGA, and PC was viewed in relationship to the ability of the organisms to oxidize various aromatic compounds of lignin-like structure (Sundman, 1964). The simultaneous adaptation technique could

not provide information on whether these aromatic key intermediates were actually intermediates in the α-conidendrin breakdown, since under the experimental conditions the organisms contained the systems for their oxidation independently of whether α-conidendrin was present during growth or not. Protocatechuic acid was indicated by paper chromatography (for technique see Sundman, 1962) in α-conidendrin culture filtrates.

Strain Ms 144412, though constitutively oxidizing homogentisic acid, lacked the ability to oxidize gentisic acid (Table 2). This provided evidence that the homogentisic acid oxidation by this strain did not proceed via gentisic acid formation. This latter compound was assumed to be an intermediate in the oxidation of homogentisic acid by an enzyme preparation of a white-rot fungus (Fukuzumi, 1962).

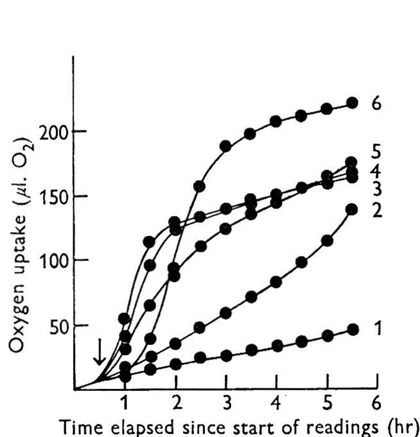


Fig. 2

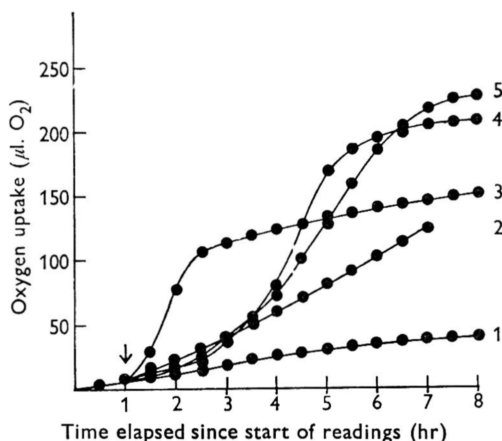


Fig. 3

Fig. 2. Oxygen uptake by α-conidendrin-adapted organisms of strain Ks 1741 with various phenolic acids and α-conidendrin. Number of viable organisms: $3{\cdot}88 \times 10^{10}$. Amount of substrate: phenolic acids, 2 μmole; α-conidendrin, 1 μmole. 1, Endogenous; 2, *m*-hydroxycinnamic acid; 3, protocatechuic acid; 4, homogentisic acid; 5, α-conidendrin; 6, *m*-hydroxybenzoic acid. ↓ addition of substrate.

Fig. 3. Oxygen uptake by organisms of stain O 811 (not adapted to α-conidendrin during growth) with phenolic acids. Number of viable organisms: $3{\cdot}45 \times 10^{10}$. Amount of substrate: 2 μmole. 1, Endogenous; 2, gentisic acid; 3, protocatechuic acid; 4, *p*-hydroxybenzoic acid; 5, *m*-hydroxybenzoic acid. ↓ = addition of substrate.

Oxidation and metabolic paths of monohydroxybenzoic acids and monohydroxycinnamic acids

From the figures for total oxygen uptake given in Table 2, it can be seen to what extent the hydroxybenzoic acids and hydroxycinnamic acids are oxidized by the present bacteria. It was found that when the acids served as substrate, an induction time of 30–90 min. was required before oxygen uptake started (Fig. 3), *m*-hydroxycinnamic acid being an exception. This substrate was oxidized at increasing rate without a well-defined lag (Fig. 2). Adaptation to α-conidendrin during growth could not suppress the induction time required by the agrobacteria to start oxygen uptake with the hydroxybenzoic acids and hydroxycinnamic acids as substrates. Neither was the slow initial oxygen uptake with *m*-hydroxycinnamic acid as

substrate accelerated by presence of α-conidendrin during growth. There was thus no evidence presented for the position of monohydroxybenzoic acids or monohydroxycinnamic acids as intermediates in the decomposition of α-conidendrin by the present agrobacteria. None of these acids were detected by aid of previously described paper chromatographical technique (Sundman, 1962) in filtrates of α-conidendrin cultures.

Table 2. *Total oxygen uptake (mole oxygen/mole substrate) observed in respirometric experiments with α-conidendrin-decomposing Agrobacterium strains, with washed organisms harvested from glucose + yeast extract + minerals*

	Organism					
	Ks 1741		Ms 144412		O 811	
Substrate	Total oxygen uptake	Growth on substrate as sole carbon + energy source	Total oxygen uptake	Growth on substrate as sole carbon + energy source	Total oxygen uptake	Growth on substrate as sole carbon + energy source
phenol	0	.	0	.	0	.
pyrocatechol	0	–	2·16	+	3·14	+
protocatechuic acid	2·73	+	2·22	+	2·40	+
homogentisic acid	3·15	.	2·39	.	3·21	.
gentisic acid	> 2·36	.	0	.	> 1·43	.
benzoic acid	> 0·68*	.	4·90*	.	> 4·50*	.
o-hydroxybenzoic acid	> 1·52*	.	> 1·97*	.	> 1·60*	.
m-hydroxybenzoic acid	4·39*	+	0	–	4·30*	+
p-hydroxybenzoic acid	0	–	3·29*	+	3·85*	+
cinnamic acid	0	–	.	–	0	–
o-hydroxycinnamic acid	0	.	0	.	0·86	.
m-hydroxycinnamic acid	> 2·44	+	0	–	> 4·10	+
p-hydroxycinnamic acid	0	–	> 1·21*	+	0·88	–

*, Oxidation started after a lag period.

>, Total oxygen uptake could not be determined, since the endogenous respiration rate was not reached during the period of experiment, 5 hr. The total uptake thus exceeded the figure given.

0, Oxygen uptake negligible, proceeding at a linear rate slightly exceeding the rate for endogenous respiration, giving a difference between substrate and endogenous of less than 0·40 mole oxygen/mole substrate in 5 hr.

· , not determined.

A comparison of the oxygen uptake with *meta*- and *para*-hydroxybenzoic acids and hydroxycinnamic acids, respectively, as substrates (Table 2), reveals a configurative correlation. The *meta*-hydroxy acids only were oxidized by strain Ks 1741, and utilized as sole carbon + energy sources, whereas strain Ms 144412 was capable of oxidizing and utilizing the *para*-hydroxy acids but not the *meta*-hydroxy acids. Strain O 811 oxidized every one of the four acids, though *p*-hydroxycinnamic acid to a restricted extent, corresponding to 0·88 mole oxygen/mole substrate. This oxygen uptake with *p*-hydroxycinnamic acid was obviously not connected with ring opening and did not provide energy enough to support growth (see Table 2).

The reaction *m*- or *p*-hydroxybenzoic acid → protocatechuic acid (Fig. 1) corresponds to an oxygen uptake of 1 atom oxygen/mole hydroxybenzoic acid. Accordingly the difference in oxygen uptake between hydroxybenzoic acid and protocatechuic acid should be 0·5 mole oxygen/mole substrate, provided that the oxidation of the hydroxybenzoic acids proceeds *via* protocatechuic acid, as has been demonstrated to be the case for soil fungi (Henderson, 1960, 1961) and for soil bacteria (Evans, 1947; Sleeper & Stanier, 1950; Yano & Arima, 1958). The oxygen uptake observed in repeated experiments with washed organisms of the present lignanolytic agrobacteria, as indicated in Table 3, presupposes greater differences. Instead of the theoretical difference, 0·5 mole oxygen/mole substrate, between oxygen uptake with protocatechuic acid and the hydroxybenzoic acids, respectively, 1·4 mole oxygen/mole substrate was found to be the actual difference between oxygen taken up with *m*-hydroxybenzoic acid and with protocatechuic acid as

Table 3. *Total oxygen uptake (mole oxygen/mole substrate) observed in repeated experiments with washed organisms grown on glucose + yeast extract + nitrate + mineral salts, using p-hydroxybenzoic acid, m-hydroxybenzoic acid, and protocatechuic acid as substrate, and differences between obtained means for protocatechuic acid oxygen uptake and hydroxybenzoic acid oxygen uptake*

	Organism		
Substrate	Ks 1741	Ms 144412	O 811
p-hydroxybenzoic acid	—	3·28	3·57
		3·29	3·96
		3·90	3·74
		Mean 3·69	Mean 3·76
m-hydroxybenzoic acid	4·39	—	4·05
	4·02		4·24
	3·93		4·46
	Mean 4·11		Mean 4·25
protocatechuic acid	2·73	2·22	2·30
	2·43	2·32	3·32
	2·92	2·41	2·92
		2·74	
	Mean 2·69	Mean 2·42	Mean 2·85
Difference between *m*-hydroxybenzoic acid and protocatechic acid oxygen uptakes	1·42	—	1·40
Difference between *p*-hydroxybenzoic acid and protocatechuic acid oxygen uptakes	—	1·27	0·91

substrates. The corresponding figure for difference between *p*-hydroxybenzoic acid oxidation and protocatechuic acid oxidation was 0·9 or 1·3 mole oxygen/mole substrate. It is believed that the differences actually found give reason to assume that the metabolism of the hydroxybenzoic acids can be explained only partially by the intermediate formation of protocatechuic acid. Furthermore, the oxygen uptake

with *m*-hydroxybenzoic acid was, on repeated occasions, found to be greater than the amount of oxygen consumed in the presence of *p*-hydroxybenzoic acid (see Tables 2, 3), which might indicate that the substrates were metabolized along different paths.

The transformation *m*-hydroxybenzoic acid → gentisic acid before ring cleavage (Fig. 1) occurs in pseudomonads (Walker & Evans, 1952; Yano & Arima, 1958). As mentioned above, the agrobacterial strains Ks 1741 and O 811, which are capable of oxidizing *m*-hydroxybenzoic acid, oxidized gentisic acid at a comparatively slow rate (see Fig. 3). The rate of gentisic acid oxidation should be limiting for

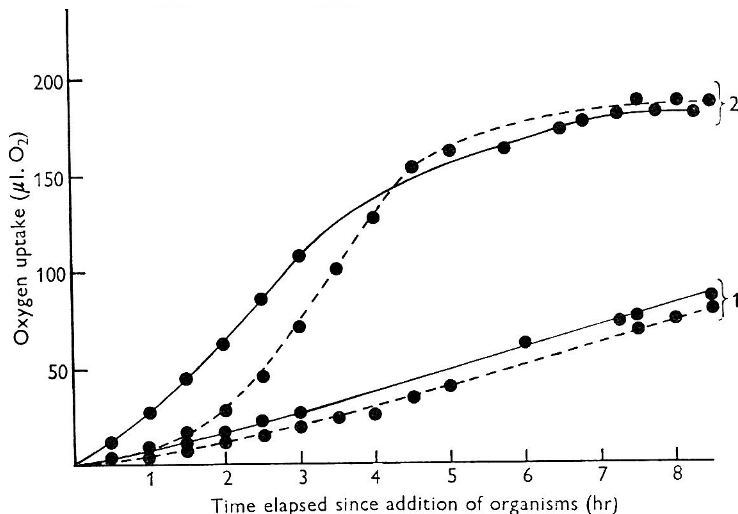


Fig. 4. Oxygen uptake by organisms of strain O 811 (not adapted to α-conidendrin during growth) with gentisic acid and *m*-hydroxybenzoic acid. Effect of adaption to *m*-hydroxybenzoic acid. Number of viable organisms: broken curves, $3 \cdot 5 \times 10^{10}$; whole curves, $1 \cdot 6 \times 10^{10}$. Amount of substrate: 2 μmole. Endogenous value subtracted. 1, Gentisic acid; 2, *m*-hydroxybenzoic acid. Broken curves = untreated organisms, whole curves = organisms adapted to *m*-hydroxybenzoic acids prior to measurement through shaking with 0·001 M *m*-hydroxybenzoic acid pH 5·5 for 90 min. at 28°.

the *m*-hydroxybenzoic acid oxidation, if this latter proceeds *via* gentisic acid. Organisms adapted to *m*-hydroxybenzoic acid through exposure to this substrate prior to measurement of oxygen uptake showed the same slow oxygen uptake with gentisic acid though the lag of *m*-hydroxybenzoic acid oxidation had been suppressed (Fig. 4). The results obtained provide grounds for the assumption that there was some unknown metabolic system which was working more rapidly than the gentisic acid oxidation, which accounted for the oxidative breakdown of *m*-hydroxybenzoic acid.

DISCUSSION

Classification of the Gram-negative bacteria indigenous to soil is rendered difficult by the lack of knowledge of dividing characteristics. There seems to be a need for a more detailed study of the Gram-negative rods of the soil as a group along the taxometric lines indicated by Gyllenberg (1963) and Hill & Silvestri (1962), in order to establish a useful basis for the separation of this important part

of the soil microflora. In the search for separating tests, the ability to utilize phenolic and lignin-related compounds might be of some value. Such reactions could be well suited to divide organisms which are possibly concerned with the breakdown of lignified plant residue in soil and characterized by inertness in ordinary biochemical tests.

The allocation of the present organisms by genus is a matter for discussion. According to the classification scheme of Holding (1960), followed in this work, the only difference between the criteria for placing an isolate in the genus *Agrobacterium* or *Achromobacter* is the ability to proliferate on inorganic nitrogen; when an organism has this ability it is placed in the genus *Agrobacterium*, and when it has not, in the genus *Achromobacter*. About one half of the *Achromobacter* species listed in the 7th edition of *Bergey's Manual* (1957) are described as having the capacity to use ammonium chloride as nitrogen source; the separation on grounds of nitrogen requirement thus puts together agrobacteria and achromobacteria. On tracing the lignanolytic bacteria examined here through the key to the *Achromobacter* species of the *Bergey's Manual* (1957), they will be considered as *A. cycloclastes* or *A. pestifer*. The former organism has been reported as decomposing phenol and naphthalene. Though none of the present bacteria is able to attack phenol (see Table 2), a relationship to this previously isolated, peritrichously-flagellated, non-pigmented, Gram-negative rod lies near at hand. The type of flagellation (few flagella), Gram reaction (granular staining with a tendency to variability), the small size of the organisms, and reactions typical of *Agrobacterium radiobacter*, listed in Table 1, have contributed to consideration of the present organisms as representatives of the genus *Agrobacterium*.

The oxidation of *p*- and *m*-hydroxybenzoic acid by microbes is known to be initiated by the introduction of a second hydroxyl group, resulting in either gentisic acid or protocatechuic acid, which are further oxidized during the opening of the aromatic ring and the formation of energy-yielding metabolites as shown in Fig. 1. These reactions might be affected by the organisms under discussion, but the stoichiometric differences in oxygen uptake observed with washed organisms for *m*- and *p*-hydroxybenzoic acids and protocatechuic acid, indicates that the *p*-hydroxybenzoic acid oxidation by strain O 811 and Ms 144412, consuming roughly 7 oxygen atoms/mole substrate, cannot proceed only along the path *via* protocatechuic acid, which is oxidized to a degree corresponding to the uptake of 5 atoms oxygen/mole substrate. The probability that the oxidation proceeds *via* protocatechuic acid is still less if *m*-hydroxybenzoic acid oxidation by organisms Ks 1741 and O 811 is in question. This substrate is oxidized to a degree which corresponds to 8 or 9 atoms oxygen/mole *m*-hydroxybenzoic acid. Under the prevailing conditions, the oxidation of gentisic acid by the strains which oxidize *m*-hydroxybenzoic acid is too slow to allow of the evaluation of the total consumption of oxygen. Even if the stoichiometric amount of total oxygen consumed were in agreement with the hypothesis that *m*-hydroxybenzoic acid is oxidized *via* the formation of gentisic acid, the rate of gentisic acid oxidation, in connexion with the inability of *m*-hydroxybenzoic acid-adapted organisms to cause increased oxidation of gentisic acid, cannot satisfactorily support such a view.

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The Ability of α-Conidendrin-decomposing Agrobacterium Strains to Utilize Other Lignans and Lignin-related Compounds

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SUMMARY

By the application of respirometric techniques, it was found that the α-conidendrin (I)-decomposing agrobacteria included strains which were adaptively or constitutively lignanolytic. The lignans isotaxiresinol (V), iso-olivil (VI), and olivil (VII) were rapidly oxidized by all the bacteria examined. The adaptive organisms showed a time-lag before oxidation of the lignans if they had been grown on a conidendrin-free medium. The lag required for oxygen uptake with α-conidendrin was shorter than that observed for the other lignans. Matairesinol (IV), which differs structurally from α-conidendrin through the lack of the 'isolignan' bond was not attacked by any of the agrobacteria examined. Olivil and iso-olivil were oxidized identically, giving results which indicate that the organisms bring about the isomerization olivil → iso-olivil. α-Conidendrol (II) was indicated by the simultaneous adaptation to be an intermediate in α-conidendrin decomposition. All the bacteria studied possess a constitutive ability to oxidize aromatic aldehydes (vanillin, isovanillin, veratrum-aldehyde, syringaldehyde). By calculation from the total oxygen uptake, and by paper chromatography, it was found that these oxidations gave the corresponding acids. Simultaneously with oxidation to vanillic acid, a small amount of vanillin was reduced to vanillyl alcohol. Coniferyl alcohol was oxidized to ferulic acid.

INTRODUCTION

α-Conidendrin (Fig. 1, I) like other lignans is synthesized by a variety of plants. It has been assumed to originate in the same simple C_6C_3-precursors as lignin (Erdtman, 1955). Spruce wood contains 0·048 % (w/w) α-conidendrin according to Freudenberg & Knof (1957). During the sulphite cooking process α-conidendrin is enriched in the sulphite waste liquors of various sap woods, and can conveniently be isolated from these sources. Consequently the lignan is readily available, and has found use as a dimeric model compound for the guaiacylpropan unit of lignin, in work on biological lignin degradation. There have been numerous demonstrations of the presence of α-conidendrin-decomposing bacteria in the most diverse surroundings rich in wood residue (Konetzka, Pelczar & Gottlieb, 1952; Tabak, Chambers & Kabler, 1959; Sundman, 1962). The general occurrence of α-conidendrin-decomposing bacteria of different genera in material rich in plant residue might indicate that in nature these organisms also attack lignin-related structures other than α-conidendrin, which as mentioned above represents a diminutive part only of the wooden plant.

Previous workers on the subject have attempted to show, mostly with negative results, that α-conidendrin-degrading bacteria utilize other lignin-related material. Pratt, Konetzka, Pelczar & Martin (1953) studied a flavobacterium, isolated by Konetzka *et al.* (1952) which caused complete degradation of α-conidendrin (supplied as 0·5 %, w/v), in 14 days, but it did not use a variety of lignin preparations and lignin-related monomers as the source of carbon + energy; of a number of lignin-related substances tested, the only compound which permitted luxuriant growth was vanillic acid. Tabak *et al.* (1959), who studied α-conidendrin degradation by mixed and pure cultures of various Pseudomonas, Flavobacterium, and Aehromobacter species, tested commerical lignin residues as sources of energy for the cultures, but found no evidence for utilization of the lignin preparations.

A description has been given of the α-conidendrin-decomposing Agrobacterium strains used in the present work (Sundman, 1962, 1964). These exhibit many metabolic differences from what is known from previous work on α-conidendrin-degrading bacteria. In conformity with its suitability as an energy source for the Flavobacterium of Konetzka, vanillic acid was found to occupy a leading position in the reactions which lead to ring cleavage of the conidendrin molecule brought about by some Flavobacterium and Pseudomonas species (Konetzka, Woodings & Stove, 1957; Tabak *et al.* 1959). In contrast, in the present work, the agrobacteria examined did not grow with vanillic acid as the sole carbon + energy source, and it was found that they did not produce vanillic acid from α-conidendrin but instead, considerable quantities of isovanillic acid (Sundman, 1962). The efficiency to decompose α-conidendrin was rapidly lost by the cultures of Tabak *et al.* (1959) when they were grown, for example, with yeast extract as the carbon + energy source, whereas some of the present organisms have been kept for years on yeast extract without losing the ability to decompose α-conidendrin. The Flavobacterium of Konetzka and the cultures of Tabak *et al.* (1959), synthesized polysaccharides during growth on α-conidendrin but were unable to use simple carbohydrates as a carbon + energy source (Pratt *et al.* 1953). The agrobacteria used in the present work have not been found to produce any polysaccharide concurrently with α-conidendrin degradation, and they can grow on a variety of simple carbohydrates (Sundman, 1964).

Accordingly, there is justification for assuming that there exist different modes of action among the lignanolytic soil bacteria, and different types of bacterial attack on the dimeric guaiacylpropane structure as it occurs in the lignans. It was felt that a more detailed study of the agrobacteria-like α-conidendrin-decomposing bacteria, as regards ability to oxidize other lignans and lignin 'model compounds', might provide data about the part they may play in oxidative metabolism which leads to release of the relatively resistant lignin-carbon back to atmospheric carbon dioxide. The present paper describes the examination of the oxidative activity of washed organisms on a number of lignans and monomeric guaiacyl- and syringyl-derivatives.

METHODS

Organisms. The Agrobacterium strains KS1741, MS144412 and O811 previously described (Sundman, 1964) were used. The technique for the production of suspensions of washed organisms was also outlined in that paper. The rate of oxygen uptake/unit quantity of organism varied from batch to batch, and a decrease in

oxygen-uptake activity with the lignans as substrate was observed during storage in the refrigerator, although the endogenous respiration remained constant during storage up to 10 days. Thus, the results obtained in different experiments are not directly comparable, even when the quantity of organism added to the Warburg flasks was identical. The period of storage in the refrigerator before measurement is stated where the results obtained with organisms of different age are presented in the same figure.

Manometry. Unless otherwise noted, the respirometric experiments were made in M/45—phosphate buffer at pH 5·9. When examining the effect of pH value, the final phosphate-concentration used was M/30. For determinations of the total oxygen uptake suspensions of organism were added from the side arm, the substrate being in the main compartment. Manometer readings were made at 15-min. intervals; the results are presented with intervals of 30 or 60 min., and give means of duplicate manometer readings. For further details see Sundman (1964).

Evaluation of growth on defined carbon source. As described by Sundman (1964), washed organisms were inoculated into semi-solid mineral medium containing 10^{-3} or 10^{-4} mole/ml. of carbon source.

Substances used

Lignin

The lignin preparations used were extracted from samples of brown rotted spruce wood (*Picea excelsa*) with a lignin content of about 80 % according to sulphuric acid lignin determinations. Two per cent NaOH in ethanol at room temperature (20°) was used for extraction in accordance with the method of Phillips (1928) as described by Brauns (1952). The crude lignin fraction, precipitated with hydrochloric acid, represented about 8 % (w/w) of the brown rotted wood on a dry matter basis. After washing until a negative reaction was attained for Cl ion in the wash water, and drying, the lignin precipitate was dissolved in purified dioxan by stirring for 4 hr, to give a solution of about 1 % (w/v). Undissolved particles were removed by centrifugation and the clear supernatant fluid evaporated to small volume in a rotating evaporator under reduced pressure. The lignin was precipitated by dropping the concentrated solution into 10 vol. anhydrous ether, accompanied by vigorous stirring for 30 min. The precipitate was collected in the centrifuge, washed twice with anhydrous ether, once with light petroleum (100–120°) and finally once with light petroleum (40–60°). The final product, after drying to constant weight over

Table 1. *Analytical data on the lignin preparations isolated from brown rotted spruce wood* (*Picea excelsa*)

The figures are percentages on a dry matter basis.

Preparation	ALD_p	ALH_p
Lignin (72 % sulphuric acid method as described by Hägglund (1951))	98·8	96·0
Methoxyl (method of Vieböck & Schwappach as described by Houben & Weyl, 1953)	13·80	13·57
Carbohydrate (determined as glucose with anthron method according to Trewellyan & Harrison, 1952)	0·50	0·49

sulphuric acid and paraffin, was a light brown powder and represented about 2·5 % of the dry wt. of the brown rotted wood specimen. Analytical data of the preparation specimens ALD_p and ALH_p from two samples of brown rotted spruce wood, are given in Table 1.

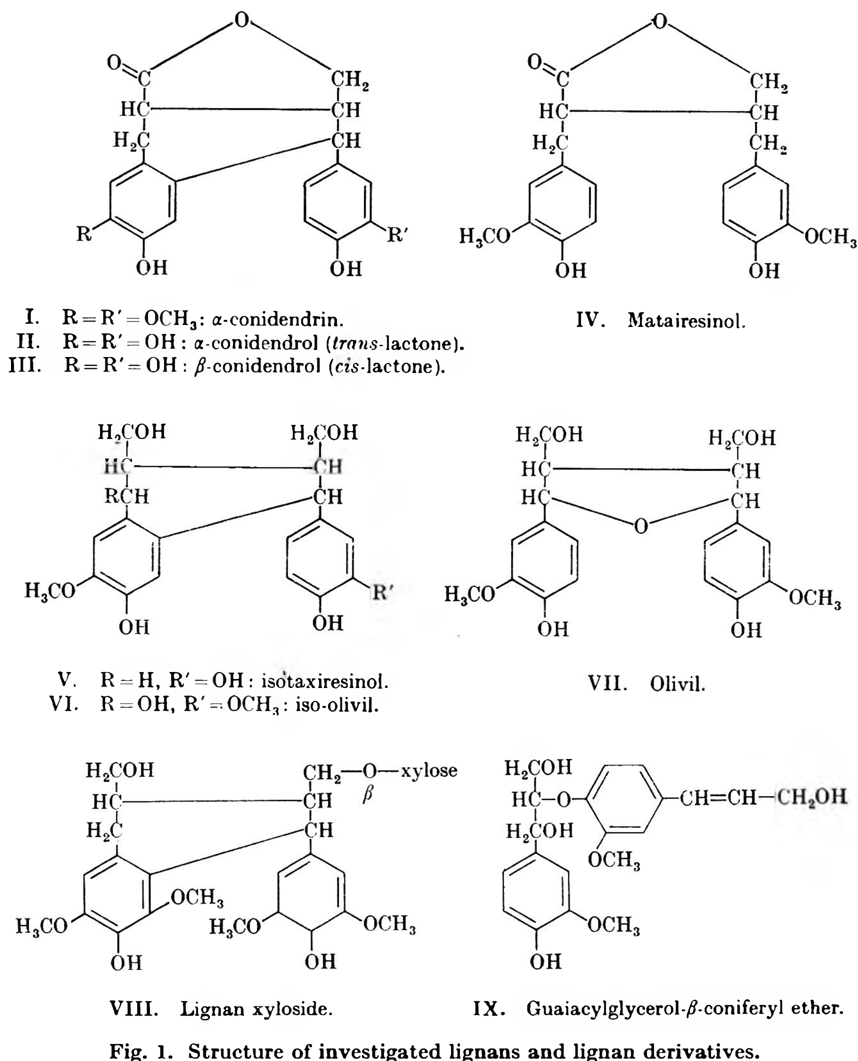


Fig. 1. Structure of investigated lignans and lignan derivatives.

Lignans and lignan derivatives. (The roman numerals refer to the structures presented in Fig. 1.)

α-Conidendrin (I) was supplied by the generosity of the Crown and Zellerbach Corporation, Camas, Wash., U.S.A. Recrystallized from water. m.p. = 247–248°.

α-Conidendrol (II) (*trans*-configuration at the lactone group), purum quality, Fluka AG, Switzerland. m.p. = 157–161°.

β-Conidendrol (III) (*cis*-configuration at the lactone group), purum quality, Fluka AG, Switzerland. m.p. = 244–248°.

Matairesinol (IV), as well as compounds V–VIII, was a generous gift from Professor H. Erdtman, Stockholm. m.p. = 113–114°.

Isotaxiresinol (V), m.p. = 165–168°.

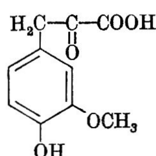
Iso-olivil (VI), m.p. = 154–157°.

Olivil (VII), recrystallized from acetone, m.p. 143–145°.

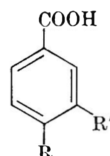
Lignan xyloside (VIII), isolated from the sapwood of *Sorbus aucuparia* (Arya, Erdtman, Krolikowska & Norin, 1962), m.p. = 159–161°.

Guaiacyl-β-coniferyl ether (IX). A small sample synthesized by Dr H. Ishikawa (Department of Organic Chemistry and Enzymology, Fordham University, New York) was kindly supplied by Professor F. F. Nord.

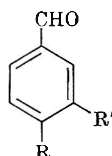
Monomeric compounds. (The roman numerals refer to the structures presented in Fig. 2.)



X. Guaiacylpyruvic acid.

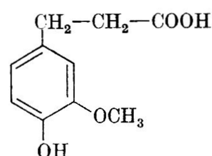


XI. R = OCH$_3$, R′ = OH: isovanillic acid.
XII. R = OH, R′ = OCH$_3$: vanillic acid.
XIII. R = OCH$_3$, R′ = H: anisic acid.
XIV. R = R′ = OCH$_3$: veratric acid.

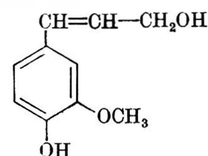


XV. R = OCH$_3$, R′ = OH: isovanillin.
XVI. R = OH, R′ = OCH$_3$: vanillin.
XVII. R = R′ = OCH$_3$: veratryl aldehyde.

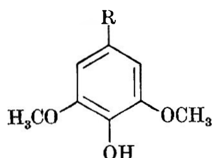
XVIII. R = R′ = OH: caffeic acid.
XIX. R = OH, R′ = OCH$_3$: ferulic acid.
XX. R = OCH$_3$, R′ = OH: isoferulic acid.
XXI. R = R′ = OCH$_3$: 3 4-dimethoxycinnamic acid.



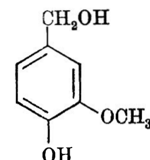
XXII. Hydrocaffeic acid.



XXIII. Coniferyl alcohol.



XXIV. R = COOH: syringic acid.
XXV. R = CHO: syringyl aldehyde.
XXVI. R = CH=CH—COOH: sinapic acid.



XXVII. Vanillyl alcohol.

Fig. 2. Structure of investigated monomeric compounds.

Guaiacylpyruvic acid (X), same source as compound IX.

Isovanillic acid (XI). A preparation was supplied by Professor T. Enkvist, Helsinki. After boiling with active carbon, the isovanillic acid was recrystallized from water. White needles, m.p. = 247–248°.

Substances XII–XXVII listed below were commercial preparations from Fluka AG, Switzerland (except vanillin from British Drug Houses Ltd.) the grades of purity being listed: vanillic acid (XII), purum; anisic acid (XIII), purum; veratric acid (XIV), puriss.; isovanillin (XV), purum; vanillin (XVI), p.a.; veratryl aldehyde (XVII), purum; caffeic acid (XVIII), purum; ferulic acid (XIX), purum; isoferulic acid (XX), puriss.; 3-4-dimethoxy-cinnamic acid (XXI), puriss.; hydrocaffeic acid (XXII), purum; coniferyl alcohol (XXIII), purum; syringic acid (XXIV), purum; syringyl aldehyde (XXV), purum; sinapic acid (XXVI), puriss.; vanillyl alcohol (XXVII), pract., recrystallized, m.p. = 113·5–115°.

RESULTS

Oxidation of α-conidendrin

The enzyme system for oxidation of α-conidendrin was adaptive in organisms of strains KS1741 and MS144412, but present in strain O811 independently of whether α-conidendrin was present during growth or not. This is illustrated in Fig. 3 and Fig. 4, respectively. Figure 3 records the oxygen uptake with α-conidendrin as substrate for organisms of strain MS144412, produced on yeast extract, and yeast extract + α-conidendrin. The figure shows that organisms grown on yeast extract did not oxidize α-conidendrin until after a lag of 3 hr, when oxygen uptake started at a rate comparable to that noted for organisms grown on α-conidendrin. The adaptability to α-conidendrin oxidation was preserved in the cultures of KS1741 and MS144412 during more than 20 monthly transfers into semi-solid KYE-agar (Sundman, 1962) without α-conidendrin.

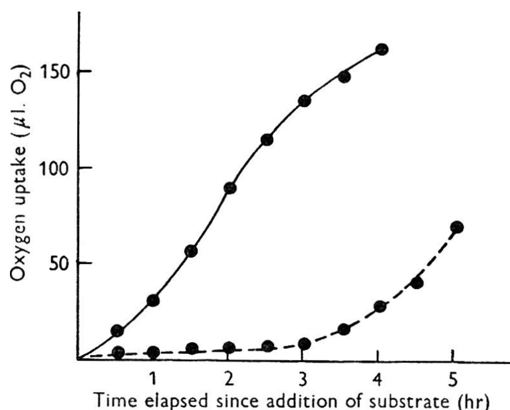


Fig. 3. Oxygen uptake by washed organisms of strain MS 144412 with α-conidendrin as substrate, showing effect of adaptation to the substrate during growth. Endogenous oxygen uptake was subtracted.

Number of viable organisms added to each flask: —, $2{\cdot}5 \times 10^{10}$; ----, $3{\cdot}6 \times 10^{10}$.

Amount of substrate: 1 mg. = 2·8 μmole. —, Organisms produced on a α-conidendrin-containing medium; ---, organisms produced without α-conidendrin.

The type of α-conidendrin oxidation found in strain O811 is illustrated in Fig. 4. It is seen that oxygen uptake with α-conidendrin as substrate starts immediately with both yeast extract-grown organisms and organisms adapted to α-conidendrin during growth. This constitutive ability of lignan oxidation was not found to be a stable characteristic of strain O811; a stock culture kept on semi-solid KYE agar proved after 7 monthly subcultures on this α-conidendrin-free medium to be unable to utilize α-conidendrin, in growth experiments, or to take up oxygen with α-conidendrin as substrate in Warburg experiments. The ability of α-conidendrin oxidation was irreversibly lost by this culture, and could not be restored by growing

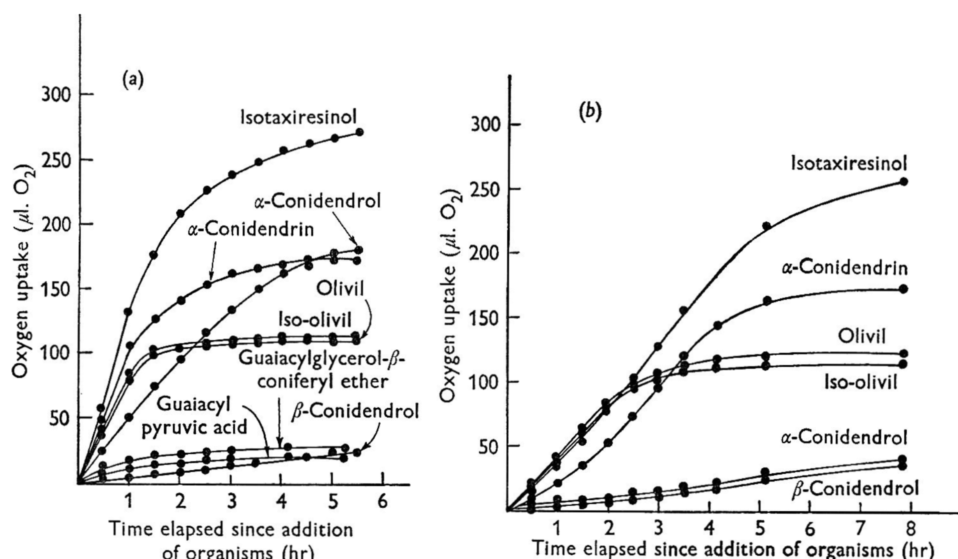


Fig. 4*a*, *b*. Oxygen uptake by washed organisms of strain O 811, with various lignans, guaiacylglycerol-β-coniferyl ether, and guaiacylpyruvic acid, showing the lignanolytic capacity of this strain to be present whether the organisms were produced on α-conidendrin or not. Endogenous oxygen uptake was subtracted. Amount of substrate: 1 μmole. *a*, Organisms produced on α-conidendrin-containing medium. Number of viable organisms added to each flask: $2{\cdot}7 \times 10^{10}$. *b*, Organisms produced without α-conidendrin. Number of viable organisms added to each flask: $3{\cdot}5 \times 10^{10}$.

it for several passages on a conidendrin-containing medium, or by shaking washed organisms of the culture with α-conidendrin crystals for several hours. In another stock culture of the strain O811 kept on α-conidendrin as sole carbon+energy source the ability to oxidize α-conidendrin had been preserved. This culture has since during several passages on conidendrin-free medium preserved the constitutive type of α-conidendrin oxidation illustrated in Fig. 4.

Previously reported results indicate that the adaptive and constitutive attack on α-conidendrin proceed along similar metabolic paths; paper chromatographic analysis of acid ether extracts of α-conidendrin cultures of the constitutively lignanolytic strain O811 and of the adaptive strains KS1741, respectively, has shown identical patterns of phenolic degradation products (Sundman, 1962).

The oxygen uptake with α-conidendrin is unaffected by pH variations in the

range pH 4·6–7·6. In repeated experiments, total oxygen uptakes varying between 13 and 17 atoms of oxygen/molecule α-conidendrin have been noted. This corresponds to 36–39 % of the oxygen required for complete oxidation to CO_2 and H_2O.

Oxidation of other lignans

All the bacteria examined utilized in addition to α-conidendrin the lignans isotaxiresinol (V), iso-olivil (VI) and olivil (VII), whereas matairesinol (IV) and the lignan xyloside (VIII) remained unutilizable. With the adaptive strains KS1741 and MS144412, adaptation to α-conidendrin resulted in simultaneous adaptation to the

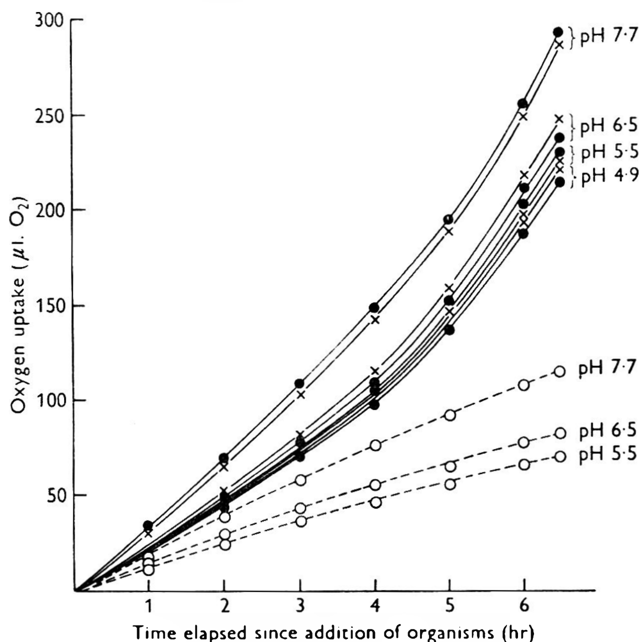


Fig. 5. Oxygen uptake by washed organisms of strain KS 1741 adapted to α-conidendrin during growth, with olivil and iso-olivil at various pH values, showing independence of pH in the range pH 4·9–7·7, and identity of olivil and iso-olivil oxidation. ○----○, Endogenous; ×——×, olivil; ●——●, iso-olivil.

compounds isotaxiresimol, iso-olivil, and olivil. Organisms not adapted to α-conidendrin during growth started oxidation of these lignans after a longer time lag than that required for oxidation of α-conidendrin to start. Strain O811 required no adaptation for oxidation of the lignans (Fig. 4). The above-mentioned culture of strain O811 which had lost the ability of α-conidendrin oxidation had simultaneously lost the ability to oxidize the other lignans.

Matairesinol (IV), which is not oxidized by the present bacteria, differs structurally from α-conidendrin only in the lack of the 6-α carbon-carbon bond (see Fig. 1). Presence of this linkage between the two guaiacylpropane units of the lignan molecule thus seems to be a requirement for the lignanolytic enzyme systems of the bacteria investigated here. It was assumed (Sundman, 1964) that the 6-α bond is preserved in the isovanillic acid formed in the present type of lignan decomposition. Of the lignans other than α-conidendrin, which are oxidized, iso-olivil and iso-

taxiresinol like conidendrin contain the 6-α bond. The variation in side chain structure (see Fig. 1) between α-conidendrin on one side and isotaxiresinol and iso-olivil on the other, does not influence the oxidation of the compounds as far as rate of oxygen uptake is concerned (Fig. 4).

Special interest was paid to the oxidation of olivil in which the 6-α 'isolignan bond' is not present. Repeated measurements of oxygen uptake with olivil and iso-olivil as substrates showed that the compounds are oxidized identically. Examples of this are seen in Fig. 5. The oxygen uptakes with olivil and iso-olivil as substrates at various pH values are presented in the figure without subtraction of the corresponding endogenous values in order to demonstrate the identity of olivil

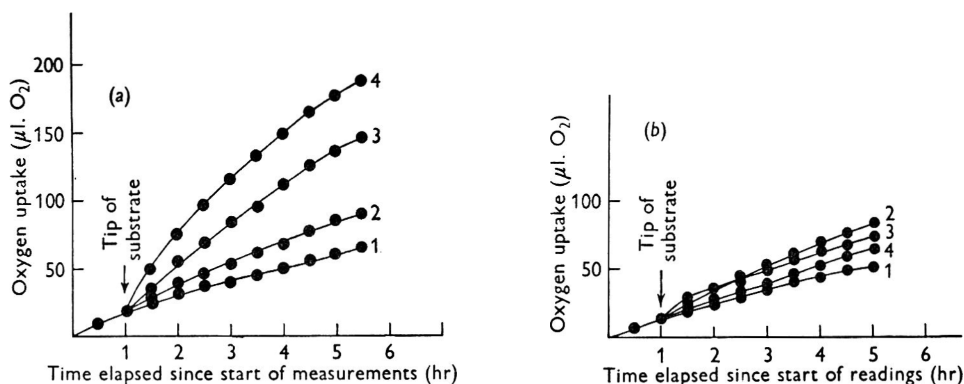


Fig. 6*a*, *b*. Oxygen uptake by washed organisms of strain KS 1741, with α-conidendrin, α- or β-conidendrol as substrate, showing simultaneous adaptation to α-conidendrol, and α-conidendrin.

Amount of substrate: 1 μmole. *a*, Organisms produced on α-conidendrin-containing medium. Number of viable organisms added to each flask: $6{\cdot}15 \times 10^{10}$. Storage of organisms prior to measurement: curves 1–3, 1 day; curve 4, 7 days. *b*, Organisms produced without α-conidendrin. Number of viable organisms added to each flask: $6{\cdot}45 \times 10^{10}$. Organisms stored 3 days prior to measurement. 1, Endogenous respiration; 2, β-conidendrol; 3, α-conidendrol; 4, α-conidendrin.

and iso-olivil oxidation. If the increase of endogenous respiration with increasing pH is taken into account, no influence of the pH variation between 4·9 and 7·7 can be established. This is in full conformity with the results obtained with α-conidendrin.

Olivil and iso-olivil contain different types of carbon skeleton, and it has been thought that they represent two biogenetic categories of lignans. The structural difference is reflected, for instance, in the varying yields of vanillin obtained upon nitrobenzene oxidation: 86 % (w/w) from olivil, and 3 % (w/w) from iso-olivil (Leopold & Malmström, 1951). Olivil is readily subject to isomerization when boiled with dilute acids, and iso-olivil is then obtained (Erdtman, 1955). The oxygen uptake, evidently identical, with olivil and iso-olivil observed for the present lignanolytic bacteria, can be explained by assuming that the bacteria bring about the isomerization olivil $\rightleftarrows$ iso-olivil. Should this explanation prove to be valid, a closer biological relationship exists between the formally different lignan groups than a purely chemical approach might predict. Continued work on the mode of action upon olivil and iso-olivil by the present bacteria is in progress.

The oxidation of the conidendrols is exemplified in Fig. 6. The slight oxygen uptake with β-conidendrol, similar for α-conidendrin-adapted organisms (Fig. 6*a*) and for organisms not adapted to α-conidendrin (Fig. 6*b*) was observed with all the strains studied. The increased oxygen uptake by α-conidendrin-active organisms in the presence of α-conidendrol (Fig. 6*a*) was occasionally observed in experiments with the strains KS1741 and MS144412, but found to be a constant feature of strain O811, if grown on medium containing α-conidendrin. It should be stressed that whereas the ability to oxidize the methoxyl-containing lignans α-conidendrin, olivil, iso-olivil and isotaxiresinol is a constitutive feature of strain O811, the increased oxygen uptake with α-conidendrol requires adaptation to α-conidendrin during growth. The organisms of the α-conidendrin-active O811 culture, produced on yeast extract and glucose, oxidize the lignans without lag, but are incapable of bringing about the increased oxygen uptake with α-conidendrol (Fig. 4*b*). Conversely, organisms of this strain produced on α-conidendrin oxidize α-conidendrol without lag at a rate comparable with that of lignan oxidation (Fig. 4*a*). It was therefore concluded that α-conidendrin decomposition by strain O811 might, as an alternative to the path over isovanillic acid, be initiated by metabolic steps which substitute the methoxyl groups by hydroxyls thus resulting in α-conidendrol formation.

The lignan moiety of lignan xyloside (VIII) has a structure which contains the isolignan 6-α bond and a side chain configuration like isotaxiresinol, but the aromatic rings have a syringyl structure with additional methoxyl groups in the 5 positions. The substance was included in the study with a view to determining whether the lignanolytic activity of the present bacteria is restricted to guaiacylpropane type (soft wood) lignans, or whether syringylpropane (hard wood) lignans are also oxidized. As previously mentioned none of the bacteria was able to oxidize the lignan xyloside. It was found that the bacteria oxidize xylose in respirometric experiments, and proliferate on xylose as the sole energy source. Since the lignan xyloside is not oxidized, it can be concluded that the bacteria do not split the molecule into xylose and lignan. This is in close accord with the inability of the present organisms to proliferate on sucrose, or lactose, although glucose and galactose serve as energy sources (Sundman, 1964), indicating a lack of β-carbohydrases in the studied Agrobacteria.

Guaiacylglycerol-β-coniferyl ether (IX), as Ishikawa, Schubert & Nord (1963*a*, *b*) and Fukuzumi (1960) have demonstrated, is released from lignin, and further metabolized by white-rot fungi. A trial was made with the compound as substrate for organisms of strains O811, actively oxidizing the lignans (Fig. 4*a*). Only a slow oxygen uptake was established.

Oxidation of the lignin preparations

Table 2 gives a summary of the results obtained with lignin preparations ALD_p and ALH_p. As the table illustrates, the preparations were slowly oxidized by strains KS1741 and O811, ALD_p more easily than ALH_p. The oxygen uptake by MS144412 organisms was increased too slightly to allow of positive conclusions. A response to the presence of the lignin preparation during growth was recorded for strain KS1741 as an increased oxygen uptake with both lignin preparations for organisms harvested from the lignin-containing substrate, taken against the oxygen

uptake by organisms produced on yeast extract and glucose. The results for strain O811 were contradictory as far as adaptive response to the presence of lignin in the medium was concerned. The parallel to adaptive *contra* constitutive degradation of lignans in strains KS1741 and O811 is of interest.

Table 2. *Results of respirometric experiments with* 1 *mg. of lignin preparations* ALD_p *and* ALH_p *as substrates for washed, lignin-adapted, and non-adapted organisms*

Strain	Lignin present in growth medium*	Number of organisms $\times 10^{-10}$	Oxygen uptake: Q_{O_2} (10^{10} organisms). Figures in brackets indicate μl. O_2-uptake in 3·5 hr†		Oxygen uptake: Endogenous μl. O_2 in 3·5 hr
			ALD_p	ALH_p	
KS 1741	−	4·8	1·31 (22)	0·71 (12)	39
	+	2·2	4·03 (31)	2·73 (21)	36
O 811	−	3·5	4·07 (50)	0·41 (5)	43
	+	1·9	3·78 (25)	1·51 (10)	32
MS 144412	−	0·9	1·27 (4)	0·32 (1)	32
	+	1·0	2·58 (9)	1·43 (5)	27

* The lignin-free medium KYE-agar contained 0·2 % (w/v) Difco yeast extract, and 0·2 % (w/v) glucose (Sundman, 1964). The lignin-containing medium contained in addition 0·05 % (w/v) of lignin preparations ALD_p and ALH_p.

† Endogenous respiration as in last column and autoxidation of lignin, 3 μl. for ALD_p and 4 μl. for ALH_p, were subtracted.

Oxidation of phenolic monomers

The compounds X–XXVII were included in the investigation partly with a view to finding possible intermediates in the breakdown of α-conidendrin, and partly to acquire knowledge of the oxidative properties of the lignanolytic Agrobacteria. The oxygen uptake with a number of non-methoxylated phenolic compounds has been previously reported (Sundman, 1964).

As regards the possibility that the monomers are intermediates in the metabolic sequences of α-conidendrin decomposition, it should be pointed out that adaptation to conidendrin caused no simultaneous adaptation to any of the monomers, except to isovanillin and to isovanillic acid (Fig. 7*a*, *b*).

Of the aldehydes investigated vanillin, syringylaldehyde, veratrylaldehyde were oxidized in a similar manner by strains KS1741 and O811. The oxygen uptake was completed within 60 min., and corresponded to slightly more than one atom of oxygen per molecule of aldehyde. Isovanillin was oxidized in the same way by organisms of strain KS1741 if not adapted to α-conidendrin during growth. This type of aldehyde oxidation is illustrated in Fig. 7*a* (curve no. 2) and Fig. 7*b* (curves nos. 2 and 5). Similar oxidation of the aldehydes, in spite of differently substituted ring structure, pointed to a common type oxidation restricted to the aldehyde group, which was assumed to be oxidized to carboxyl. When a slight oxygen uptake observed with the corresponding carboxyl compounds was subtracted from the oxygen uptake noted for the aldehydes, on the assumption that the aldehydes are oxidized to corresponding acids, a total oxygen consumption for the reaction aldehyde →acid was obtained, as indicated in Table 3. The figures in the table

indicate that the oxygen uptake observed for strain KS1741 was markedly less than the theoretical amount: 1 atom of oxygen for every molecule of aldehyde.

For study of the reaction products of aldehyde oxidation by strain KS1741, the contents of Warburg flasks were analysed as follows: when manometer readings indicated that the oxygen uptake with aldehyde as substrate had diminished to the same rate as endogenous respiration, the content was pipetted off and centrifuged. The supernatant was evaporated to small volume, and the concentrated supernatant quantitatively applied as two spots on different sheets of paper previously buffered to pH 6·5 with M/15 phosphate buffer. Pure aldehydes, the corresponding acids and vanillyl alcohol were applied as standards. The chromatograms were developed by ascending technique with water-saturated *n*-butanol.

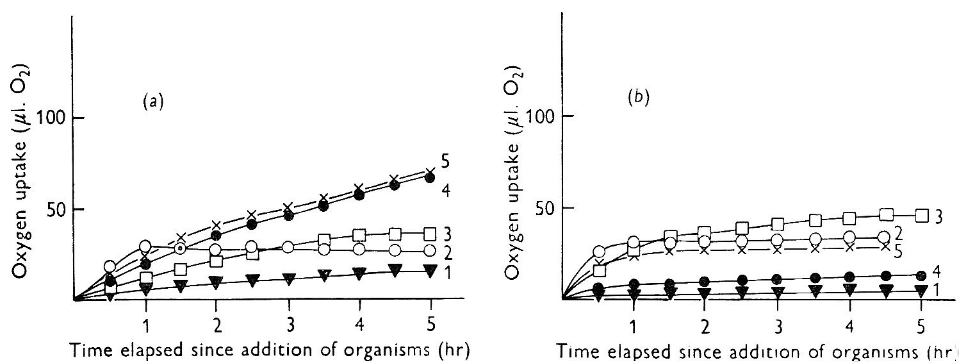


Fig. 7*a*, *b*. Oxygen uptake by washed, organisms of strain KS 1741, with vanillin acid, vanillin, coniferylalcohol, isovanillic acid, or isovanillin as substrate showing adaptation to isovanillin and isovanillic acid upon adaptation to α-conidendrin during growth. Endogenous oxygen uptake was subtracted.
Amount of substrate: 2 μmole. *a*, Organisms produced on α-conidendrin-containing medium. Number of viable organisms added to each flask: $2{\cdot}4 \times 10^{10}$. Storage prior to measurement: curve 1, 4 days; curve 2, 9 days; curve 3, 11 days; curve 4, 4 days; curve 5, 9 days. *b*, Organisms produced without α-conidendrin. Number of viable organisms added to each flask: $4{\cdot}7 \times 10^{10}$. Storage prior to measurement: curves 1, 3, and 4, 1 day; curves 2 and 5, 3 days. 1, ▼, Vanillic acid; 2, ○, vanillin; 3, □, coniferylalcohol; 4, ●, isovanillic acid; 5, ×, isovanillin.

Table 3. *Oxygen uptake evaluated as atoms of oxygen per molecule of aldehyde for reaction RCHO →RCOOH, as calculated from the difference between total oxygen uptakes observed for RCHO and RCOOH*

R	Oxygen uptake, atoms of oxygen per molecule of aldehyde	
	Strain O 811	Strain KS 1741
Vanillyl-	1·04	0·64
Isovanillyl-	—	0·87
Syringyl-	0·81	0·79
Veratryl-	0·89	0·67

One of the runs was sprayed with 2-4-dinitrophenyl-hydrazine (carbonyl reagent) and the other with diazotized sulphanilic acid (phenol reagent). For the veratryl aldehyde run, rhodamin B (reagent for acids) was used for spraying the second run.

It was found that no detectable aldehyde or other carbonyl compound remained

in the samples. Instead, there were strong spots coinciding with the corresponding acids: vanillic, isovanillic, and syringic acid appeared on the chromatograms after spraying with diazotized sulphanilic acid. In addition, a second spot of higher R_f value, detectable with diazotized sulphanilic acid, was present on the runs. In the vanillin run, this spot $R_f = 0{\cdot}84$ was identical with the spot of vanillyl alcohol. From isovanillin, there had been formed a substance with $R_f = 0{\cdot}83$, and from syringyl aldehyde a substance at $R_f = 0{\cdot}75$, through the activity of the bacteria. These two compounds have not yet been identified, owing to the lack of standards available for comparison, but there is reason to believe that they might be the corresponding alcohols.

It appears that an intramolecular dismutation of vanillin to vanillyl alcohol and vanillic acid proceeds simultaneously with oxidation of the aldehyde to acid during oxygen uptake. Reactions of this type, leading to the accumulation of vanillyl alcohol, obviously account for the deviation of oxygen uptake from the theoretical amount: 1 atom of oxygen for every molecule of aldehyde, as indicated in Table 3.

The oxidation of aldehyde to acid as exemplified in Fig. 7*c* for vanillin and in Fig. 7*b* for vanillin and isovanillin does not provide energy to allow the bacteria to proliferate on the aldehydes as the sole energy source. Neither are bacteria which bring about the negligible oxidation of vanillic acid (Fig. 7*a*, *b*) or the comparable oxidation of isovanillic acid (Fig. 7*b*) able to grow on these acids as sole carbon sources. Against this, isovanillic acid can serve as the sole energy + carbon source for organisms adapted to α-conidendrin, hence bringing about the increased-type oxidation of isovanillic acid in Fig. 7*a*.

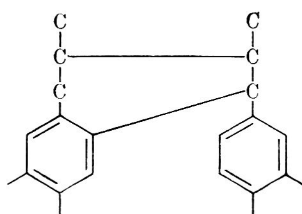
Coniferyl alcohol was oxidized at a comparatively slow rate (Fig. 7*a*, *b*), and a total oxygen uptake corresponding to 1·5–1·98 atoms of oxygen/molecule substrate was recorded. In conformity with the amount of oxygen consumed, ferulic acid was by paper chromatography demonstrated to be the reaction product. No other phenolic compounds except traces of remaining coniferyl alcohol could be demonstrated.

Guaiacylpyruvic acid (X) has been demonstrated as intermediate in the degradation of lignin by white-rot fungi (Fukuzumi, 1959, 1960; Ishikawa *et al.* 1963*a*, *b*) and appears to occupy a central position in the fungal metabolism of lignin. The crude preparation of (X) available was used as substrate with actively lignanolytic O811 organisms (Fig. 4*a*). The slow oxidation does not indicate that guaiacylpyruvic acid plays an important part in the metabolism of lignans by the agrobacteria examined here.

Vanillyl alcohol, anisic acid, caffeic acid, hydrocaffeic acid and syringic acid are oxidized at a negligible rate, comparable with the oxidation of vanillic acid and isovanillic acid in Fig. 7*b*, corresponding to less than one atom of oxygen per molecule of substrate in 5 hr. The cinnamic acid derivatives with the α–β unsaturated side chain: ferulic acid, isoferulic acid, 3-4-dimethoxycinnamic acid and sinapic acid, were oxidized by strain KS1741, isoferulic acid to an extent corresponding to 1·6 atoms of oxygen/mole substrate and the other acids corresponding to 1 atom oxygen/mole substrate. With organisms of strain O811 there could be demonstrated no oxidation of the cinnamic acid derivatives.

DISCUSSION

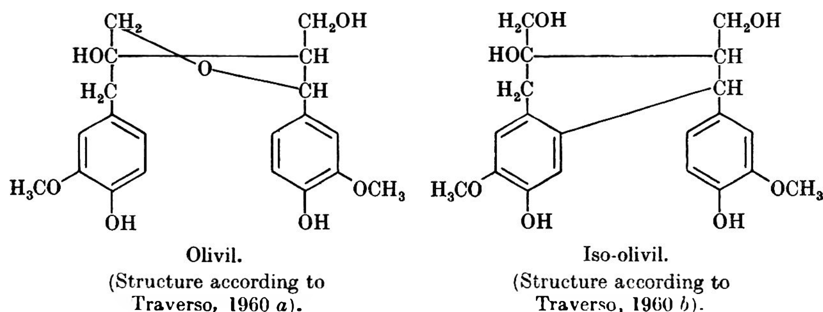
The results presented in this paper, in contrast to previously reported results on the topic, show that conidendrin-decomposing bacteria might also be capable of degrading other lignin-related structures. The experiments with other lignans indicate that the specificity relates merely to the carbon skeleton. The 'isolignan' structure, either present in the substrate or resulting from the isomerizing ability of the bacteria, seems to be a requirement of the organisms under investigation.



Isolignan structure.

The simultaneous adaptation to the other lignans upon growth on conidendrin-containing medium can be explained either by the assumption that the other lignans are intermediates in the conidendrin-decomposition, or, more likely, in accordance with the ideas of Stanier, Sleeper, Tsuchida & MacDonald (1950), as a back adaptation over reversible steps to a possible common substrate for the lignanolytic enzyme system of the bacteria.

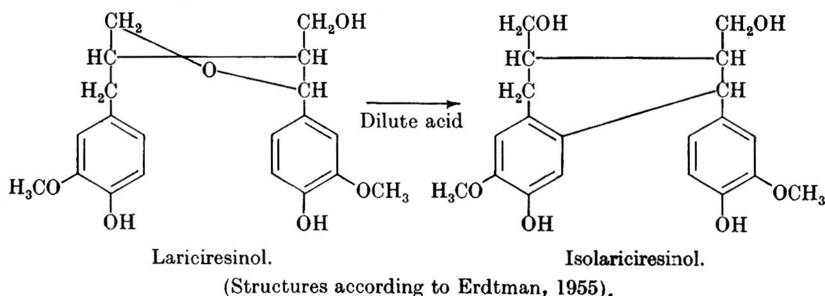
The identical oxidation of olivil (VII) and iso-olivil (VI) confirmed by paper-chromatographic work to be published, indicates that the two isomeric forms are in equilibrium in the presence of the bacteria studied. In work on the constitution of olivil and iso-olivil, Traverso (1960*a*) concluded that olivil has the structure of hydroxylariciresinol, i.e. that the ether linkage is situated between side chain carbons α and γ, and the secondary alcohol group of olivil, accordingly at the side chain carbon β as a tertiary OH-group. By way of agreement with this view, it was demonstrated that the structure of iso-olivil contains a tertiary OH-group at side chain β-carbon (Traverso, 1960*b*):



Olivil.
(Structure according to Traverso, 1960 *a*).

Iso-olivil.
(Structure according to Traverso, 1960 *b*).

According to these structures, the isomerization olivil →iso-olivil is the same transformation as the isomerization of lariciresinol to isolariciresinol, known to occur under the influence of diluted acids:



Lariciresinol. Isolariciresinol.

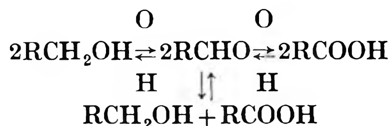
(Structures according to Erdtman, 1955).

There appear to be possibilities for biological confirmation of the structures proposed by Traverso for olivil and iso-olivil, by means of comparative studies of the activity of the lignanolytic agrobacteria on the pairs olivil–iso-olivil and lariciresinol–isolariciresinol. Such work is in progress.

The results reported in Table 2, concerned with oxidation of the lignin used, indicate that the activity of the lignanolytic bacteria on the modified lignins was rather feeble in comparison with the action on the lignans. Since the biological decomposition of lignin-residue in soil is a slow process, it is possible that bacterial attack of the delayed kind observed in this work could contribute to the biological decomposition of lignin, at least in the modified form in which it occurs in brown-rotted wood.

The reduction of aromatic aldehydes under aerobic conditions to alcohols by the present agrobacteria which has been demonstrated to occur when vanillin serves as substrate, and probably working also with isovanillin, syringic aldehyde and veratric aldehyde, has previously been demonstrated with soil fungi which utilize aromatic compounds (Henderson, 1961), with white-rot fungi (Ishikawa *et al.* 1963*b*) and with *Aspergillus niger* (Raman & Shanmugasundaram, 1963). The reduction of aldehyde to alcohol, occurring under aerobic conditions simultaneously with the rapid oxidation to acid reported in this paper, forms only a minor part of the aldehyde metabolism of the agrobacteria, under the prevailing conditions. The main part of the aldehyde is transformed to acid.

It appears that the oxidative transformations of aromatic aldehydes, brought about by lignanolytic fungi and bacteria, are the result of reversible processes in which alcohols, aldehydes, and acids are in equilibrium according to the following scheme:



The equilibrium in the reversible processes will favour the more oxidized compound.

The manometric results reported in this paper concerning the oxidation of vanillyl alcohol and vanillin by organisms which are incapable of oxidizing vanillic

acid indicate that, in comparison with the transformation of aldehyde to acid, the oxidation of alcohol to aldehyde is a slow reaction. The appearance of alcohol at the time when the rapid oxidation of aldehyde to acid has ceased, can be explained by assuming, either that a Cannizzaro-type dismutation of the aldehyde provides the alcohol, or that a small fraction of the aldehyde is reduced to alcohol, this being accumulated in the medium by reason of relatively slow oxidation.

The author is indebted to Professor F. F. Nord and Dr H. Ishikawa for the gift of samples of guaiacylglycerol-β-coniferyl ether and 4-hydroxy-3-methoxyphenyl-pyruvic acid. She further wishes to thank Professor H. Erdtman for the samples of lignans. The work was supported by grant No. FG-Fi-108-60 from the U.S. Department of Agriculture. This is the third paper in a series on microbial decomposition of lignins. For previous papers see Sundman (1962, 1964).

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With 1 *plate*
Printed in Great Britain

Production of Amidase and β-Lactamase by Bacteria

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SUMMARY

Fresh clinical isolates of Gram-positive and Gram-negative bacteria were tested for the production of β-lactamase and amidase. Techniques for identifying and studying the latter enzyme are described, in relation to its action upon penicillins and other substrates. Various commensal and pathogenic Gram-negative bacilli produce a 'pen-amidase' of relatively narrow specificity, though no species invariably produces it. A specific pen-amidase was not formed by the Gram-positive bacteria examined, though non-specific amidases were present. *Escherichia coli* and paracolon bacilli produced pen-amidase more often than β-lactamase but the Klebsiella-aerogenes group did the reverse. Among other organisms, enzyme production was very variable but absence of either enzyme did not necessarily connote sensitivity of an organism to any penicillin. Administration of penicillin to a patient promotes colonization of the gut and oropharynx by β-lactamase-forming coliforms, but not of amidase-forming organisms. There is a strong association between resistance to the penicillins and formation of β-lactamase by an organism; with pen-amidase, the association is less strong. Neither enzyme accounts completely for bacterial resistance to the penicillins, even within any one species of organism, but the lower resistance of coliforms to ampicillin may be related to the lesser susceptibility of this derivative to amidase.

INTRODUCTION

Viewed collectively the various penicillins now available act upon a wide range of Gram-positive and Gram-negative bacteria but, unlike other drugs, their action is influenced by inactivating enzymes produced by some of these species. It is generally agreed that penicillinase acting in this way accounts for the resistance of *Staphylococcus aureus* to penicillin, but the extent to which inactivating enzymes explain the resistance of other organisms is much less certain, especially since it is now known that at least two penicillin-splitting enzymes can be formed. One is a β-lactamase (Pollock, 1961) acting upon the lactam ring of the molecule. The other is an acylase (English, McBride & Huang, 1960) or amidase (Batchelor, Chain, Richards & Rolinson, 1961) acting at the peptide linkage of the prosthetic group. Apart from the resistance of individual bacteria, formation of penicillinase by commensal or collateral organisms may interfere with the action of penicillin upon the target organism. In the present work, we studied the production of inactivating enzymes by pathogenic and commensal organisms isolated from human sources.

METHODS

The coliform and other organisms used in these studies were isolated from routine specimens submitted to the bacteriological laboratory. Each organism was titrated in broth in inocula of about 10^7 organisms/ml. against increasing concentrations of various penicillins over an unusually wide range of concentrations (1–500 μg./ml.) so that the limited sensitivity of some strains could be detected.

Penicillinase (β-lactamase) was detected in four ways: (i) by bore-hole assay of bacteria-free filtrates of broth containing subinhibitory concentrations of drug, after growth of the organism; (ii) by adding whole-cell sediments from these cultures to solutions of the penicillins, which were then assayed against controls after 1 hr at 37° C; (iii) by the membrane-plate technique, in which various penicillins were incorporated as substrates in 6 % agar buffered to pH 7·6, overlaid by a nutrient cellulose-acetate membrane upon which coliform organisms under test were inoculated in marked positions; after overnight incubation, the membranes were removed and the plates flood-inoculated with a suspension of indicator organisms (*Staphylococcus aureus*, NCTC 6571 (Oxford) or *Sarcina lutea* NCTC 8340) which grew, after further incubation, only in sites where the penicillin had been destroyed; these methods have been described elsewhere (Stewart, Coles, Nixon & Holt, 1961; Holt & Stewart, 1963); (iv) by applying the hydroxylamine method (see below) to detect disruption of the lactam ring.

Techniques for the detection of amidase

Use of differential penicillin substrates. The membrane-plate technique mentioned above (iii) was adapted by using 6-aminopenicillanic acid (6-APA) and penicillin G as substrates in the agar layer. Since it was unaffected by amidase 6-APA retained its activity and inhibited the indicator organism on further incubation after removal of the membrane; whereas penicillin G was de-acylated to 6-APA but in an ineffective concentration at which the indicator organism grew freely (Pl. 1, figs. 1, 2). When a β-lactamase was present, both substrates were inactivated; hence, by this test alone, amidase could not be detected in the presence of β-lactamase. It was necessary in this adaptation of the membrane-plate technique to use different indicator organisms in order to allow for the difference in intrinsic activity of each substrate. The Oxford staphylococcus (NCTC 6571) was inhibited in 6 % agar at pH 7·6 by low concentrations ($<0{\cdot}1$ μg./ml.) of several therapeutically used penicillins (penicillin G, penicillin V, ampicillin) but withstood 6-APA at 50–100 μg./ml. whereas *Sarcina lutea*, though inhibited by the penicillins within the same range, was much less resistant to 6-APA, being inhibited by 2 μg./ml. Hence the Oxford staphylococcus was suitable for detecting amidase-destruction of the penicillins, since these compounds could thereby be used over a range of substrate concentrations as high as 50 μg./ml., which excluded any possibility of inactivation by extraneous factors and yet left a reasonably high, though not inhibitory, residue of 30 μg./ml. 6-APA. For detecting destruction of 6-APA, however, *Sarcina lutea* was a more suitable indicator in view of its higher sensitivity to this compound. In some experiments, as described in the text, the residue left after test organisms had acted upon penicillin G was treated with phenylacetyl chloride to demonstrate resynthesis of benzylpenicillin and thereby prove that

the residue was 6-APA. This was not done with all strains since additional methods of proof were also being used (Holt & Stewart, 1964).

Use of other substrates. Theoretically, amidases should attack many substrates. To test the specificity of amidases of bacterial origin in this respect, leucylnaphthylamide and L-leucinamide were also used as substrates, the products of reaction being identified by colour changes and chromatography, as described in the experimental results. Crude aminopeptidase from a non-bacterial source (hog stomach, Sigma Chemical Co., 3500 Dekalb Street, St Louis 18, Mo., U.S.A.) was also used for purposes of comparison on these substrates and on penicillin.

Hydroxylamine method. The method of Ford (1947) was applied as follows. Washed bacteria were added to benzylpenicillin or 6-APA (100–500 μg./ml.) at pH 7·0 or 5·6, respectively, and kept at 37° for 1 hr. After centrifugation, 0·5 ml. water, 6 ml. hydroxylamine reagent, 0·25 ml. 5 N-H_2SO_4 and 2 ml. ferric sulphate solution were added to 2 ml. samples of the supernatant fluids; 0·25 ml. NaOH and 0·25 ml. 5 N-H_2SO_4 were added to 2 ml. blanks. Benzylpenicillin and 6-APA were used as standards, 6-APA being estimated (after amidase hydrolysis) by extracting with butyl acetate at pH 2·0 to remove residual benzylpenicillin. Readings were made in an S.P. 500 spectrophotometer (Unicam, Cambridge) at 495 mμ.

RESULTS

Production of penicillin-inactivating enzymes by Escherichia coli

Of 310 strains of *Escherichia coli* tested (Table 1), 125 (40 %) inactivated penicillin G but not 6-APA by the membrane-plate technique, and were therefore classified as specific amidase-producers. Thirty-four strains (12 %) inactivated both substrates and therefore produced β-lactamase; it is possible that some of these 34 strains also formed amidase for, in presence of β-lactamase, proof of an independent amidase cannot readily be obtained. About half the strains tested produced neither enzyme.

Table 1. *Production of amidase and β-lactamase by Escherichia coli*

No. of strains tested	310
No. forming amidase	125 (40 %)
No. forming β-lactamase	34 (12 %)
No. forming neither enzyme	151 (48 %)

These 310 strains had all been isolated from patients who were receiving no form of penicillin therapy; at the same time, a smaller number of strains isolated from the faeces or oropharynx of patients receiving penicillin was similarly tested (Table 2). The proportion of organisms forming β-lactamase was significantly higher in the latter group. There was no difference in amidase-production.

A group of 23 serotypes of *Escherichia coli*, isolated from children with gastro-enteritis, was also examined (Table 3); here again, the proportions of amidase-formers and β-lactamase-formers did not differ significantly from the 310 miscellaneous strains. When these 310 strains, in turn, were grouped according to their sites of isolation, or whether they came from a lesion (e.g. infected urine, peritoneal pus) or from normal faeces, no differences in enzyme production were apparent. Tested by the hydroxylamine method (Table 4), amidase-forming coliform organisms did

not attack the 6-APA nucleus, whereas β-lactamase-forming coliform organisms opened the lactam ring.

Independently of their source, coliform organisms which produced β-lactamase were all highly resistant to the various penicillins, requiring 500 μg./ml. or more for inhibition of growth, even with ampicillin, which is more active against Gram-negative bacilli than the other therapeutically used penicillins. Amidase-forming

Table 2. *Production of amidase and β-lactamase by Escherichia coli from patients receiving and not receiving penicillin therapy*

E. coli from patients	No. of strains tested	No. of strains (observed/expected) forming		
		Amidase	β-lactamase	Neither enzyme
Receiving a penicillin	55	23/22	17/8*	15/25
Not receiving a penicillin	310	125/123	34/37	151/150
Total	365	148	51	166

*$P < 0{\cdot}01$.

Table 3. *Production of amidase and β-lactamase by 23 Escherichia coli serotypes isolated from children with gastro-enteritis*

No. of strains (observed/expected) forming		Minimal inhibitory concentration (μg./ml) of	
		Penicillin G	Ampicillin
Amidase	9/10*	500	50–100
β-lactamase	6/4*	> 500	> 500
Neither enzyme	8/9*	50–200	10–100
No. tested	23		

* Difference not significant.

Table 4. *Action of β-lactamase and amidase from Escherichia coli upon 6-aminopenicillanic acid (6-APA) (estimated by hydroxylamine method)*

Strain no.	% 6-APA destroyed	Enzyme formed
C. 11	79	β-lactamase
C. 48	50	
C. 14*	Nil	Amidase
C. 33*	Nil	
C. 256	Nil	None
C. 257	Nil	

* These strains and two others have been accepted by the Department of Scientific Research (National Collection of Industrial Bacteria), Torry, Aberdeenshire, with reference numbers as follows: *E. coli* type 1: C. 14 = NCIB 9464; C. 15 = NCIB 9465; C. 33 = NICB 9466; C. 48 = NCIB 9472.

strains were less resistant to ampicillin (Tables 3 and 5), though highly resistant to penicillin G. Strains forming neither enzyme were much more readily inhibited, especially by ampicillin. Fifty-six strains were tested simultaneously against 5 different penicillins. This showed (Table 6) that there were some further differences between these representative penicillins, both as inhibitors and as substrates: in most instances, ampicillin was less vulnerable to amidase than penicillins G and V,

but was susceptible to the amidase of a minority of strains against which it was much less inhibitory; methicillin withstood hydrolysis by two β-lactamase strains, but was susceptible to that enzyme from another strain; organisms which formed neither enzyme were more readily inhibited by all the penicillins, but only ampicillin, penicillin G and penicillin V, in that order, were sufficiently inhibitory for therapeutic purposes and, even then, were sometimes borderline.

Table 5. *Relationship between resistance to penicillins and formation of enzymes by Escherichia coli* (134 *strains*)

Minimal inhibitory concentration (μg./ml.)			Production of	
Penicillin G	Ampicillin	No. of strains	Amidase	β-lactamase
50–200	5–100	83	0	0
> 500	50–200	36	36	0
> 500	> 500	15	See text	15

Table 6. *Inactivation of various penicillins by 56 strains of Escherichia coli in relation to minimal inhibitory concentrations*

		Inactivation (+ or −) and minimum inhibitory concentration (μg./ml.) of				
Enzyme activity	No. tested	Penicillin G	Penicillin V	Methicillin	Cloxacillin	Ampicillin
Amidase	16	+ (> 500)	+ (> 500)	− (> 500)	− (> 500)	− (20–200)
Amidase	4	+ (> 500)	+ (> 500)	− (> 500)	− (> 500)	+ (> 500)
β-lactamase	2	+ (> 500)	+ (> 500)	− (> 500)	± (> 500)	+ (> 500)
β-lactamase	1	+ (> 500)	+ (> 500)	+ (> 500)	+ (> 500)	+ (> 500)
Neither	33	− (20–100)	− (20–100)	− (200)	− (200)	− (5–100)

The susceptibility of methicillin to the β-lactamase from one coliform organism clearly required further investigation. This was done by performing the same tests on 250 unselected strains of *Escherichia coli*, of which 22 formed a β-lactamase active against penicillin G and 6-APA. With 4 strains, this β-lactamase also inactivated 200 μg./ml. methicillin and 50 μg./ml. cloxacillin. This meant either that the β-lactamase from these strains was different from the usual coliform β-lactamase, or that an amidase was acting concurrently. To test the latter possibility, 50 μg./ml. methicillin and 10 or 50 μg./ml. cloxacillin were exposed to amidase from coliform organisms grown on membranes, and then to β-lactamase from various sources (coliform organisms, *Staphylococcus aureus* and *Bacillus cereus* 5B). In each case, both compounds were destroyed. When tested by the same procedure in different order, i.e. β-lactamase followed by amidase, neither compound was destroyed.

Action of coliform amidase on other substrates

All the coliform organisms listed above were tested also for activity against *l*-leucyl-β-naphthylamide on the assumption that amidase would liberate a naphthalene residue by attacking the peptide linkage in this substrate. Membranes were impregnated with 1 % substrate solution and laid on nutrient agar; they were then heavily inoculated with coliform organisms from solid cultures and kept at 37° for

2 hr, after which the membranes were removed. A drop of saturated aqueous diazonium dye (Fast blue B) was added to the reverse sides. With all 310 strains tested, addition of the dye produced a blue colour, intensified by treatment with 0·01 M-copper sulphate solution. This substrate was therefore susceptible to amidases from all coliform organisms, independently of the ability of some of these organisms

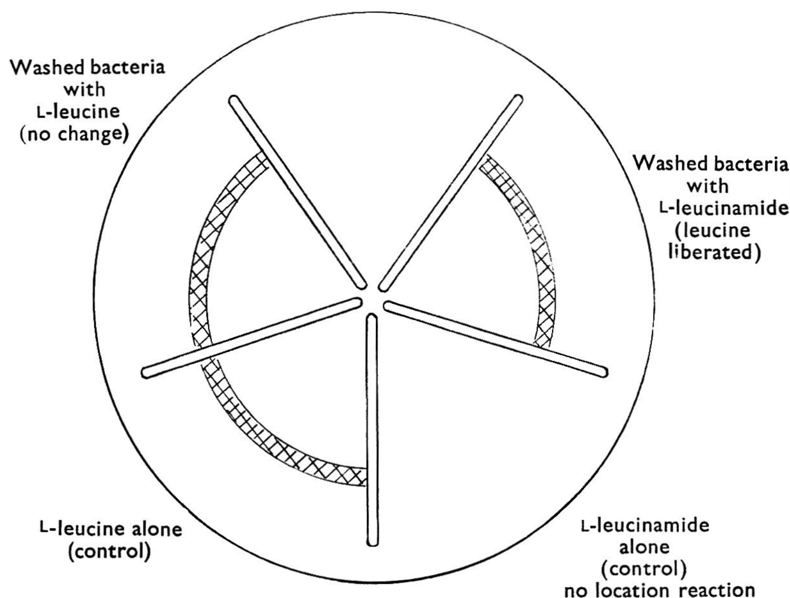

Fig. 1. Paper chromatography (Kawerau jar) showing effect of coliform organism c. 14 upon leucinamide as substrate. Washed bacteria and/or buffered substrate were applied at the apices of the divided paper segments. Leucine was liberated from the substrate by amidase formed by the bacteria.

to form a specific amidase, active against the penicillins as substrates. *Staphylococcus aureus* and many other organisms also attacked leucyl-naphthylamide, independently of their ability to produce any form of penicillinase. Conversely, this compound, in common with penicillin G but not 6-APA, was susceptible to the enzyme complex present in crude aminopeptidase from intestinal mucosa, proving that this enzyme-complex could behave as an amidase.

The locus of action of the amidase was then investigated by adding washed coliform organisms to L-*leucinamide* (0·05 %, 0·1 %, w/v). After 2 hr at 37° filtrates were applied to Whatman no. 1 paper for chromatography, as follows (by vol.): (*a*) two-dimensional run in ethanol 40 + butanol 40 + water 20 + propionic acid 8, followed by acetone 40 + water 20 + dicyclohexylamine 8 as solvents; (*b*) one-dimensional run on segmented paper in a Kawerau jar, using butanol 40 + acetone 40 + water 20 + dicyclohexylamine 8 as solvent. In each case the papers were developed in 0·2 % (w/v) ninhydrin in acetone. All coliform organisms tested by this method liberated free leucine from the substrate (identified by R_F value and colour: (Fig. 1)), though many other organisms did not do so.

These results showed that amidase was present in all the coliform organisms as an

enzyme complex of low specificity presumably capable of attacking C:N or CO:NH linkages in different loci. When penicillins were used as substrates, it was therefore necessary to prove directly that the CO:NH linkage was attacked, leaving 6-APA as the principal residue. This was done by saturating 8 cm. disks of Whatman no. 1 paper with penicillin G 200 μg./ml., 200 μg./ml. penicillin V and 100 μg./ml. 6-APA. These were laid on 6 % agar and covered by nutrient cellulose-acetate membranes, each of which was heavily inoculated at 4 marked points with a penicillin-amidase-forming coliform organism; a further set similarly treated was inoculated with a coliform organism which did not form penicillin-amidase. After overnight incubation the membranes were removed. Each of the underlying drug-loaded papers, into which any enzyme formed had meanwhile diffused, was then divided into halves. One half was sprayed with aqueous sodium bicarbonate (5 %, w/v), then with phenylacetylchloride, then with sodium bicarbonate again. The two halves were then placed in sterile Petri dishes and covered with 10 ml. melted agar at 60° incorporating a light inoculum (10^5 cocci/ml.) of *Staphylococcus aureus* NCTC 6571 as indicator organism. The dishes were then incubated overnight (Pl. 1, figs. 3, 4). On the penicillin G (and V) plate, the lower sprayed half showed inhibition of indicator organisms over the marked point (4), whereas the unsprayed half showed growth at points (1) and (2), corresponding to the positions of de-acylation by the penicillin-amidase-forming coliform organism. This meant that an active benzylpenicillin had been resynthesized in the sprayed paper from the residue left after the action of the coliform enzyme at point (4) and that the said residue was therefore 6-APA. When 25,000 units β-lactamase/ml. from *Bacillus cereus* 5B were added to the sprayed paper before covering it with melted agar and indicator organism, growth was not inhibited at the marked point (3), showing that the substance at point (4) resynthesized at alkaline pH from the 6-APA residue was a benzylpenicillin. With the 6-APA plate (same figure) growth occurred over the whole of the unsprayed half of paper, since the concentration therein was subinhibitory, but was inhibited over the sprayed half by synthesis of benzylpenicillin except where (Pl. 1, fig. 3, point 3) β-lactamase had been applied.

De-acylation and resynthesis of penicillins G and V was similarly demonstrated with two other coliform organisms which likewise did not attack 6-APA, but not with coliform organisms which were inactive against these penicillins in the membrane plate technique; this therefore appeared to be a valid screening test for the presence of amidase as well as β-lactamase.

Production of enzymes by other Gram-negative bacteria

A total of 126 strains of various species was tested (Table 7). Amidase active against penicillin was produced by 29, including some strains of paracolon bacilli, Klebsiella-aerogenes, Proteus, Shigella and *Pseudomonas pyocyanea*. β-Lactamase was formed by 22 organisms, including the majority of strains from the Klebsiella-aerogenes group, and occasional strains of paracolon bacilli, Proteus and *P. pyocyanea*. Of the 126 strains tested, 75 (including all strains of Salmonella and Bacteroides) produced neither enzyme. When tested against L-leucyl-β-naphthylamide as substrate, all 126 strains liberated a naphthalene residue which reacted with the diazonium dye. Leucinamide was decomposed by some strains of Proteus, but free leucine was not a product of this reaction.

Table 7. *Production of amidase and β-lactamase by various Gram-negative bacteria*

Organism	No. tested	No. producing		
		Amidase	β-lactamase	Neither enzyme
B. paracolon	17	9	2	6
'Klebsiella-aerogenes'	18	2	12	4
Proteus mirabilis	25	5	4	16
P. vulgaris	4	0	1	3
P. morganii	19	4	1	14
P. rettgeri	1	1	0	0
Shigellas	7	5	0	2
Salmonellas	18	0	0	18
Pseudomonas pyocyanea	9	3	2	4
Bacteroides*	8	0	0	8
Escherichia coli (for comparison)	310	125	34	151

* Tested anaerobically.

Table 8. *Production of amidase and β-lactamase by Gram-positive bacteria*

Organism	No. tested	No. producing		
		Amidase	β-lactamase	Neither enzyme
Staphylococcus aureus	43	0	28*	15
S. albus	14	0	6	8
Streptococcus faecalis	21	0	0	21
S. hominis	3	0	0	3
S. viridans	6	0	0	6
Clostridia†	11	0	0	11
Lactobacilli†	4	0	0	4

* Including 11 methicillin-resistant strains. † Tested anaerobically.

Production of enzymes by Gram-positive bacteria

None of 102 strains tested produced a penicillin-amidase (Table 8). Twenty-eight strains of *Staphylococcus aureus* and 6 of *S. albus* formed β-lactamase, including 11 which were resistant to all forms of penicillin. None of the other organisms formed β-lactamase, but all of them split the leucyl-naphthylamide substrate.

DISCUSSION

Among the various factors responsible or thought to be responsible for resistance of bacteria to penicillin, production of penicillinase has long been known (Abraham & Chain, 1940: Bondi & Dietz, 1944). One form of this enzyme, now known to be a β-lactamase (Pollock, 1961), is undoubtedly responsible for the resistance of *Staphylococcus aureus* to penicillin G (Barber, 1947); but it does not explain the high resistance commonly encountered among coliform organisms and other Gram-negative bacilli (Stewart, 1947; Chain, Florey & Jennings, 1949), nor does it explain the resistance of some staphylococci to methicillin (Stewart, 1961; Barber & Waterworth, 1962; Stewart & Holt, 1963). The work of Sakaguchi & Murao (1950) suggested that some organisms formed an enzyme capable of attacking benzylpenicillin at another locus; this was later shown (Batchelor *et al.* 1959) to be an amidase capable of liberating 6-aminopenicillanic acid, with lesser antibacterial activity, from penicillin G or

penicillin V. Since then, other workers have shown that acylase or amidase is produced by coliform organisms (English, McBride & Huang, 1960).

The results reported here show that a variety of human commensal and pathogenic Gram-negative bacilli produce a specific amidase, acting at the linkage of the prosthetic side-chain to the NH group of 6-aminopenicillanic acid, though no single species invariably produces it. *Escherichia coli* and paracolon bacilli produce amidase more often than β-lactamase, but many strains produce neither enzyme, even under the conditions of inducement by presence of substrate which the membrane-plate technique provides. Organisms of the Klebsiella-aerogenes group, on the other hand, produce β-lactamase more often than amidase. *Pseudomonas pyocyanea* can produce either enzyme. Strains of *Proteus* vary considerably, but the most resistant type, *P. morgani*, does not appear to form inactivating enzymes more frequently than the less resistant *P. vulgaris* and *P. mirabilis*. Salmonellas appear to be unable to form either enzyme. Penicillin-amidase production was not detected in various species of Gram-positive bacteria.

It is evident that the administration of penicillin favours selective survival or colonization in the gut or oropharynx of coliform organisms which can form β-lactamase. There is however no evidence that amidase-forming coliform organisms behave similarly, even in patients in whom heavy colonization of the oropharynx occurred during penicillin therapy in the present survey. Pathogenic coliform organisms do not differ from those present commensally in the faeces in the production of either enzyme.

There is a close relationship between enzyme-production by *Escherichia coli* and resistance to some of the penicillins. Strains which form β-lactamase are invariably resistant to more than 500 μg./ml. of any penicillin, though methicillin can withstand inactivation by the otherwise typical enzyme formed by the majority of these strains; this does not however mean that such organisms are in the slightest degree sensitive to methicillin. Amidase-forming strains are resistant to penicillins G and V, methicillin and cloxacillin, but the majority of strains in the present series are unable to inactivate ampicillin and are more sensitive to this compound; here again, a paradox is evident, for this amidase does not readily inactivate methicillin or cloxacillin, though the organism is still highly resistant. When these compounds are subjected to high concentrations of both enzymes, destruction occurs, but this could only be demonstrated by a two-stage technique which may or may not bear any relation to natural happenings.

The present results therefore confirm and extend those of English, McBride & Huang (1960) and Sabath & Finland (1963). The former workers showed that many coliform organisms form acylase or amidase capable of inactivating various penicillins; the latter group showed that 26 out of 55 Gram-negative bacteria inactivated one or more of 5 penicillins, but they did not try to identify amidase. The action of this enzyme against ampicillin was studied by Ayliffe (1963) who reported that it was not formed by any of 109 strains of coliform organisms, including 49 which were resistant to ampicillin. He concluded that, if ampicillin was inactivated, this was attributable to β-lactamase. Our results do not entirely support these conclusions; we would agree that amidase does not always inactivate ampicillin and that high coliform resistance to this compound is associated with production of β-lactamase, but moderate degrees of resistance (50–200 μg./ml.) were found among amidase-

forming strains which were frequently encountered in our series, unlike his. The difference in this respect may be one of technique. Ayliffe identified amidase by reconverting the degraded substrate to benzylpenicillin, whereas we relied upon the use of different substrates in the screening procedure, and then tested the products of degradation where applicable by resynthesis and hydroxylamine reactivity. Ideally, the products of inactivation should be examined as well as the inactivating process in all such experiments.

Penicillinases from Gram-negative as well as Gram-positive bacteria were examined by Smith & Hamilton-Miller (1963). They found, and we would agree, that the β-lactamase from different Gram-negative bacteria varied in their pattern of specificity and did not show the inducibility characteristic, for example, of the enzymes from staphylococci. Amidase as well as β-lactamase may fall into this category. The susceptibility of substrates like leucylnaphthylamide and leucinamide to enzymes from many bacterial species shows that amidases are widespread, perhaps as part of complex aminopeptidases which practically all free-living micro-organisms must possess. Only a few of these amidases attack penicillins, despite the presence in all therapeutic penicillins of a theoretically-vulnerable CO:NH linkage. Amidase is obviously a less powerful enzyme which operates effectively within a limited pH range and, though active against penicillin G, may not inactivate closely-related derivatives of 6-aminopenicillanic acid such as ampicillin. In view of this narrow specificity of penicillin-splitting amidase, the enzyme should be referred to more precisely in terms of the substrate, as is done with esterases: for ready reference, the term 'penamidase' might be appropriate, to distinguish the enzyme from those others possessed by many bacteria which attack C:N or CO:NH linkages.

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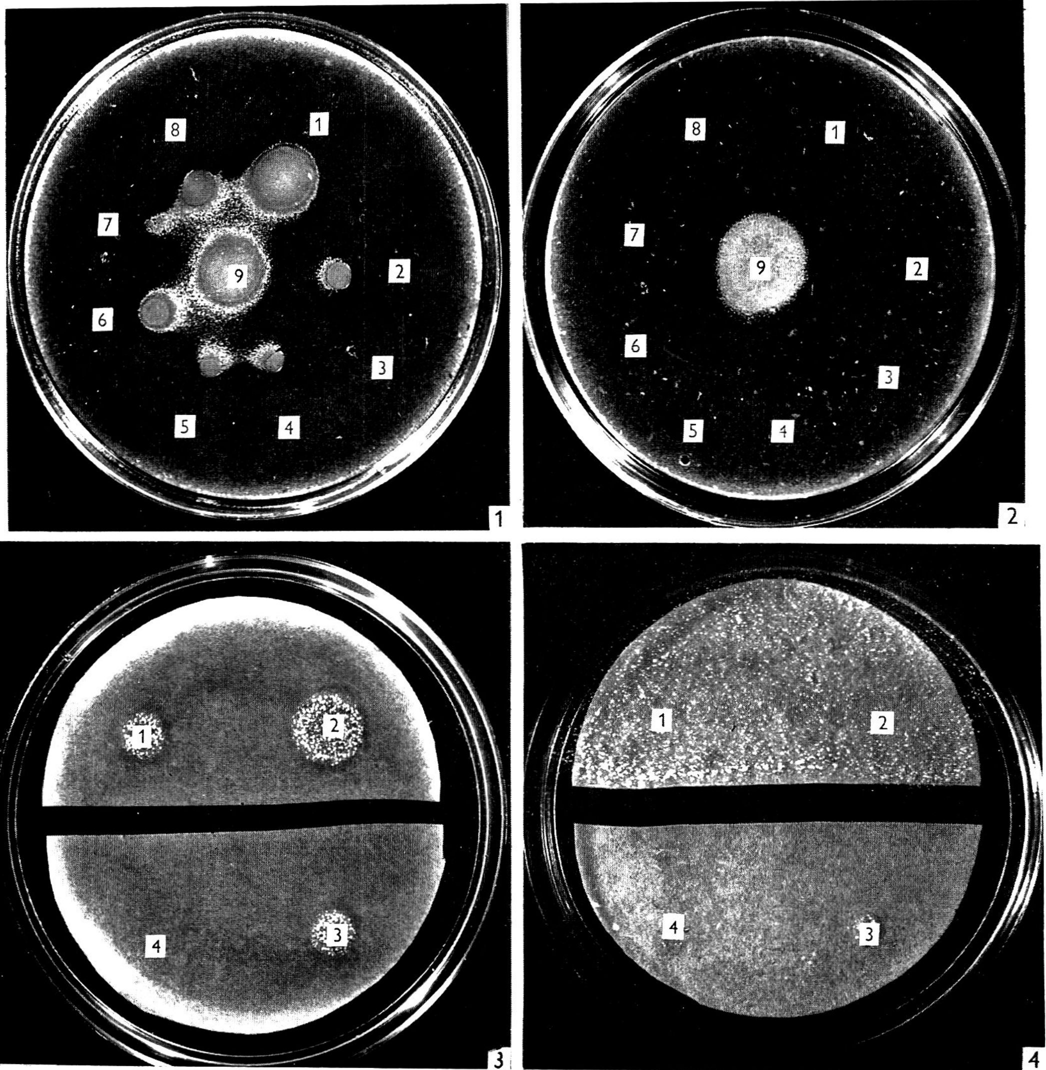

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EXPLANATION OF PLATE

Figs. 1, 2. Membrane plate technique for detection of amidase and β-lactamase by growing cultures or suspensions of bacteria (coliform organisms). Appearance of plate after removal of membrane, showing growth of indicator organisms at sites of destruction of substrate (see text).

Fig. 1. Substrate penicillin G (50 μg./ml.)

Fig. 2. Substrate 6-APA (100 μg./ml.). 1, 2, 4, 5, 6, 7, 8, Destruction of penicillin G but not 6-amino-penicillanic acid (6-APA) by amidase. 9, Destruction of penicillin G and 6-APA by β-lactamase. 3, Destruction of neither.

Figs. 3, 4. Resynthesis of benzylpenicillin following action of amidase-forming bacteria. Sprayed paper technique (see text). The lower half of the paper in each plate was sprayed with phenyl-acetyl chloride.

Fig. 3. Substrate benzylpenicillin (penicillin G). Unsprayed: indicator organism killed except at points (1) and (2), where amidase-forming coliform organisms were inoculated on membrane. Sprayed: (4) No growth of indicator organism (resynthesis of benzylpenicillin). (3) Growth (resynthesized benzylpenicillin hydrolysed by addition of β-lactamase)

Fig. 4. Substrate 6-aminopenieillanic acid (6-APA). No inhibition of indicator organism. (4) No growth of indicator organism (synthesis of benzylpenicillin). (3) Growth (synthesized benzyl-penicillin hydrolysed by addition of β-lactamase).

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Differentiation of Amidase and β-Lactamase by Infrared Absorption

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SUMMARY

The carbonyl-β-lactam absorption band of penicillins and cephalosporins at 1760 cm.$^{-1}$ is detectable when the antibiotics are mixed with enzymes or bacterial protein. This band can therefore be used for differentiating the two inactivating enzymes β-lactamase and amidase. Examined thus, methicillin, cloxacillin, amino-cephalosporanic acid (7-ACA) and thienylacetamido-7-ACA were stable to staphylococcal β-lactamase but less stable to coliform β-lactamase. Only quinacillin among a range of therapeutic derivatives tested resisted both types of β-lactamase. The other inactivating enzyme, amidase, can exist independently of β-lactamase in coliform organisms. When methicillin and cloxacillin were treated with amidase. they lost their stability to β-lactamase.

INTRODUCTION

In earlier papers (Holt & Stewart, 1964*a*, *b*) we described microbiological and chemical methods for the identification of amidases of bacterial origin which act specifically on penicillin substrates by de-acylating them to 6-aminopenicillanic acid (6-APA). This enzyme can be identified with certainty, since the resultant 6-APA can be reconverted to benzylpenicillin by treatment with phenylacetylchloride. When the inactivating enzyme is a β-lactamase, however, identification is to some extent presumptive. We therefore sought a more direct method to differentiate β-lactamase from amidase, and to examine the effect of both enzymes upon a wider range of β-lactam antibiotics. It is known that the lactam ring of the various penicillins absorbs infrared emissions strongly and characteristically at a wavelength of 5·6 μ and wave frequency of 1760 cm.$^{-1}$ (Thompson, Brattain, Randall & Rasmussen, 1949). This absorption band probably depends upon the carbonyl linkage in the intact β-lactam ring. This band is in an unusual position and, in some preliminary experiments, we found that it was clearly discernible by infrared spectrophotometry of 6-APA and benzylpenicillin, even in the presence of bacteria and enzyme protein. We therefore used this finding to study the effect of inactivating enzymes upon these and other β-lactam antibiotics.

METHODS

Infrared spectrophotometry. Pure 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA) were made up in strong stock solutions in distilled water at 100,000 μg./ml., with the addition of sufficient potassium hydroxide to

raise to pH 7·0. For experiments, these stock solutions were diluted to 100–500 μg./ml. in M/15 phosphate buffer at (pH 6·0), at which both amidase and β-lactamase were active. Various derivatives of 6-APA and 7-ACA were similarly prepared without alkalinization. Solid potassium bromide (AR quality) was added to the solutions in the proportion of 200 times the weight of drug present. The solutions were then rapidly freeze-dried *in vacuo*, yielding an intimate fine-grained mixture of salt and drug. This was then moulded under high pressure in a hand-press with die to obtain a transparent disk, suitable for direct infrared spectrophotometry. With the use of slit micro-frame holders in a Perkin-Elmer Model 137 spectrophotometer, Perkin-Elmer Ltd, Beaconsfield, Bucks., England, as little as 100 μg. benzylpenicillin was adequate for a clear-cut absorption spectrum.

To examine the effect of enzymes, these solutions were mixed, as described in the experimental results below, with washed coliform organisms. In some experiments a cell-free semi-purified preparation of the enzymes (Holt & Stewart, 1964*a*) was used, instead of washed bacteria, in the proportion of 50 μg. preparation to 500 μg. substrate. After incubation for 2–6 hr at 40°, one drop of 0·1 N-potassium hydroxide was added to form the potassium salt and thus ensure that no free carboxylic acid was present to obscure the β-lactam carbonyl peak. At stated intervals, the mixtures were centrifuged and the cell-free supernatant fluids, together with the appropriate blanks and standards, compressed into disks after the addition of potassium bromide.

Attempts were made to obtain better separation of the bands given by the OH and NH stretching bonds in the 3000–4000 cm.$^{-1}$ frequency region. It was hoped by this means to show alteration in the stretching vibrations of the NH linkage of the side-chains of 6-APA derivatives; striking alterations of this nature might be anticipated as a result of amidase action on this link. Despite the use of various liquid vehicles as well as solid state disks, and examination with another spectrophotometer of much greater resolution at this wave-frequency, no alterations were clearly discerned. This failure was almost certainly due to the presence of traces of water vapour which could not be eliminated.

Tests for inactivation. The same solutions of penicillins and cephalosporins, with and without the addition of bacteria, were assayed by bore-hole bio-assay plate techniques for loss of antibacterial potency. Each organism examined for enzyme production was also re-tested by the membrane-plate technique against the same range of β-lactam substrates (Holt & Stewart, 1963).

RESULTS

Effect of coliform organisms upon penicillin substrates

Benzylpenicillin and 6-aminopenicillanic acid. Fresh isolates of *Escherichia coli* were obtained from the routine laboratory. Eighteen-hr broth cultures of these organisms were centrifuged at 3700 *g* for 15 min., and the deposit repeatedly washed with sterile distilled water to remove as much medium as possible. Samples (0·1 ml.) of a very dense suspension (now about 8×10^{10} organisms/ml.) of these washed organisms were added to 1 ml. portions of the various penicillin solutions, at 500 μg./ml. in M/15 phosphate buffer at pH 6·0. After 2 hr at 40° with frequent agitation, a trace of potassium hydroxide was added, the bacteria removed by centrifugation and excess potassium bromide added to the supernatant fluid. The mixtures were

freeze-dried, compressed into solid disks, and examined by infrared spectrophotometry. As controls, identical preparations were made without incubation, and also standards of penicillins at the same strength to which no bacteria had been added. A background control of potassium bromide + buffer only was also examined, to ensure that no unsuspected absorption bands were present.

Twenty successive isolates of coliform organisms were treated in this way; each organism was incubated with both benzylpenicillin and aminopenicillanic acid (6-APA). The organisms were also re-tested against these two compounds on membrane plates. This experiment (Table 1; Fig. 1) showed that 12 strains did not change the infrared absorption spectra or the activity of either substance; 5 strains abolished the activity of benzylpenicillin without affecting the carbonyl peak of the lactam ring, proving thereby that inactivation was not attributable to β-lactamase; 3 strains destroyed 6-APA and the lactam ring absorption spectrum, and were therefore producers of β-lactamase.

Table 1. *Examination of coliform organisms for amidase and β-lactamase production by membrane-plate technique and infrared spectrophotometry*

No. of strains tested	Membrane-plates Destruction of		Infra-red absorption (carbonyl peak at 1760 cm.$^{-1}$)
	Benzylpenicillin	6-APA*	
12	Nil	Nil	Present unchanged
5	+ +	Nil	Present unchanged
3	·	+ +	Absent

* 6-Aminopenicillanic acid.

Table 2. *Reconstitution of benzylpenicillin after de-acylation by enzyme in Escherichia coli*

Tube*	Content as benzyl penicillin (μg./ml.)	Percentage† recovery	Infra-red absorption (carbonyl peak at 1760 cm.$^{-1}$)
A	410	90	Present
B	Nil	Nil	Present
C	275	60	See text

* Tube A contained washed organisms of *E. coli*, no incubation, tube B contained washed organisms of *E. coli*, incubated 2 hr, tube C contained washed organisms of *E. coli*, incubated 2 hr, then treated with phenylacetylchloride.

† Original concentration after adding 1/10 vol. organism = 455 μg./ml.

Reconstitution of benzylpenicillin after de-acylation. To tubes A, B, C containing 1 ml. lots of benzylpenicillin at 500 μg./ml. in pH 6·0 buffer were added 0·1 ml. of washed coliform organisms shown by the above technique to form an amidase but no β-lactamase. Tube A was now centrifuged (4000 rev./min. for 15 min.). A trace of N-KOH was added to the cell-free supernatant fluid, 0·1 ml. fluid removed, diluted 1500, and heated at 75° for 10 min. Potassium bromide (0·125 g.) was immediately added to the remaining 1·0 ml. The two other tubes, B and C, were incubated for 2 hr at 40°; tube B was then treated in exactly the same way as tube A. After tube C had been centrifuged, one drop of 5 % $NaHCO_3$ was added to the supernatant fluid, followed by one drop phenylacetylchloride, and the tube shaken for

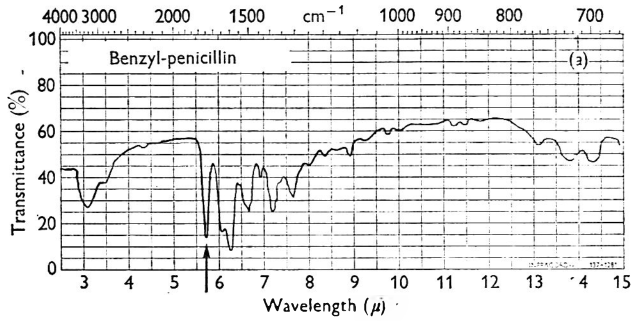


Fig. 1 *a*

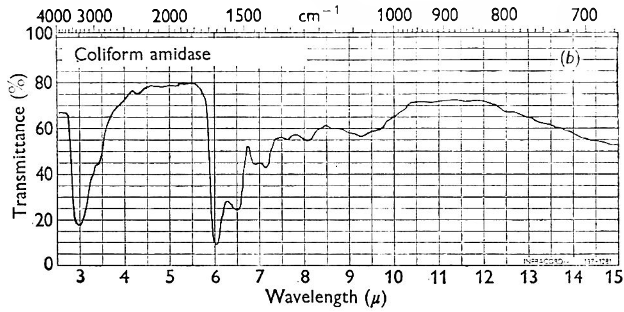


Fig. 1 *b*

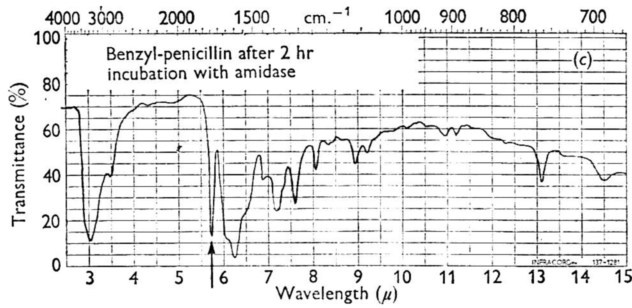


Fig. 1 *c*

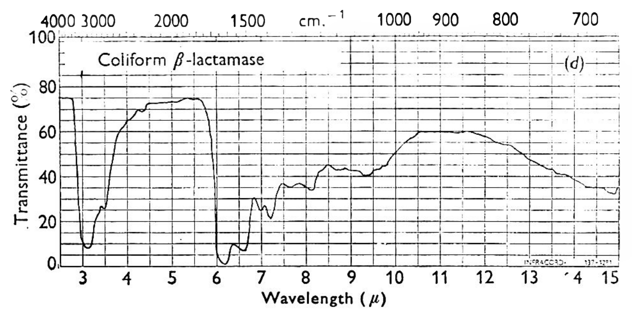


Fig. 1 *d*

For legend and Fig. 1*e* see opposite page.

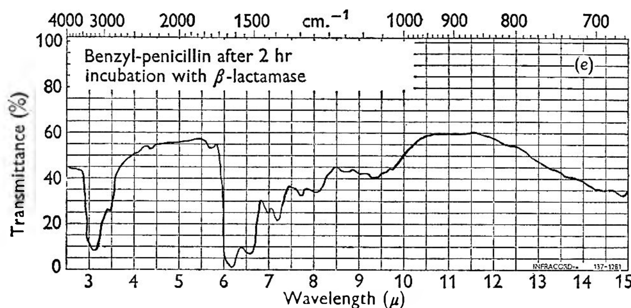


Fig. 1*e*

Fig. 1. Effect of enzymes from *E. coli* on benzylpenicillin

10 min. Finally, one more drop of 5 % $NaHCO_3$ was added and the fluid now treated as tube A. Plate bio-assay of the 1/500 dilutions was carried out against a standard range of benzylpenicillin dilutions similarly heated. Infrared spectra of the bromide preparations (Table 2) showed preservation of the lactam ring in tube B with total loss of benzylpenicillin activity. No infrared trace was obtainable from tube C, the whole spectrum being obscured by the presence of phenylacetylchloride which had re-acetylated benzylpenicillin to 60 % of the original concentration, as shown in the bio-assay. The above experiment was repeated with a different coliform strain

Table 3. *Effect of inactivating enzymes upon β-lactam substrates*

Substrate	Infra-red absorption at 1760 cm.$^{-1}$ Coliform amidase	Coliform β-lactamase	Staph. β-lactamase
6-Aminopenicillanic acid	Present	Absent	Absent
Benzylpenicillin	Present	Absent	Absent
Methicillin	Present	Weakened	Present*
Cloxacillin	Present	Weakened	Present*
Ampicillin	Present	Absent	Absent
Quinacillin	Present	Present	Present
7-Aminocephalosporanic acid (ACA)	Present	Weakened	Present
7-substituted 7-ACA deriv.	Present	Weakened	Present
3,7 subst. 7-ACA deriv.	Present	Absent	Absent

* The absorption band was abolished when methicillin and cloxacillin were previously treated with amidase.

possessing similar properties. Here again the carbonyl absorption band was still present after the microbiological activity of benzylpenicillin had been decreased to a very low value; this activity was largely restored by subsequent treatment with alkaline phenylacetylchloride. These experiments served to differentiate coliform organisms which produced β-lactamase from those which produced amidase. In the experiments which follow, washed organisms from two representative strains were used as enzyme preparations for testing against other substrates.

Semi-synthetic derivatives of 6-aminopenicillanic acid. The results are summarized in Table 3.

Neither enzyme preparation alone had any effect upon the carbonyl band of

methicillin after 2 hr or 4 hr. After 6 hr, β-lactamase caused less intense absorption at 1760 cm.$^{-1}$, although the band was still clearly present. In a mixture of both enzyme extracts, the band was much weaker after 2 hr; after 6 hr, the band was almost absent.

The carbonyl band of cloxacillin was unaffected by the action of either enzyme alone after 2 hr, but the joint action showed considerable weakening of the band after incubation for 2 hr. In 6 hr the band was weakened by β-lactamase acting alone.

Amidase alone had no effect upon the carbonyl band shown by ampicillin, even after incubation for 6 hr; within 2 hr β-lactamase had markedly weakened the band, and in 6 hr there was no absorption at 1760 cm.$^{-1}$.

The very strong carbonyl band of quinacillin was quite unaffected by either enzyme, even after incubation for 6 hr.

Effect of cell-free amidase from Escherichia coli

The derivatives of 6-aminopenicillanic acid listed above were tested also against semi-purified enzymes prepared from *Escherichia coli* (strains c. 11, c. 15, c. 33, c. 48, c. 236) as described elsewhere (Holt & Stewart, 1964*c*). The results were identical to those obtained with washed concentrates of organisms from these strains, as described above, in respect of the effect of the enzyme upon the carbonyl band at 1760 cm.$^{-1}$. There was, however, some masking of fine detail in other regions of the infrared spectra of the mixtures, due to the presence of a larger proportion of enzyme protein.

Effect of coliform organisms upon 7-aminocephalosporanic acid and its derivatives

The results are summarized in Table 3.

Apart from the parent compound 7-aminocephalosporanic acid itself, two derivatives were available for examination. One was a 3,7 substituted compound 3-pyridine 7 thienylacetamido-cephalosporanic acid: No. 87/4) and the other substituted in the 7 position only (thienylacetamido-cephalosporanic acid).

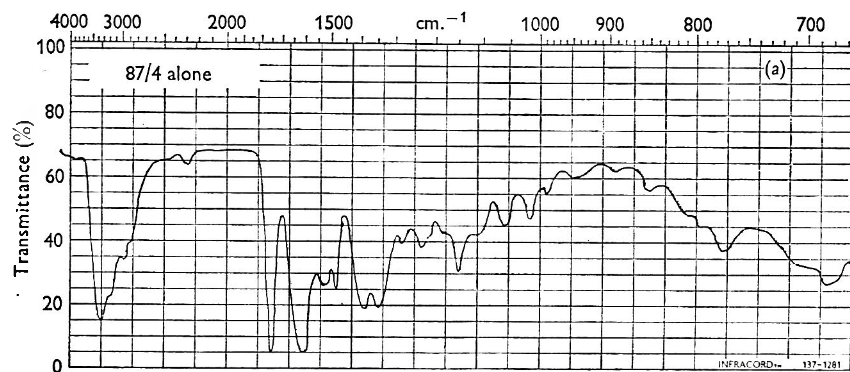
Amidase had no effect upon the strong carbonyl band of 7-aminocephalosporanic acid; β-lactamase showed a slight weakening of the band after 2 hr.

Amidase had no effect upon the carbonyl band of 3,7-pyridine-thienylacetamido-cephalosphoranic acid, but incubation for 2 hr with β-lactamase showed complete removal of this absorption band from the infrared spectrum (Fig. 2).

The carbonyl band of 7-thienylacetamido-cephalosporanic acid was unaffected by amidase alone, but β-lactamase materially weakened the band after incubation for 2 hr.

Effect of staphylococcal β-lactamase upon derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid

Similar experiments were carried out with heavy suspensions of washed cocci of two strains of *Staphylococcus aureus*. One of these was a methicillin-resistant β-lactamase-producing strain (Stewart & Holt, 1963), the other a methicillin-sensitive β-lactamase-producing strain. Each strain was incubated at 40° for 4 hr with solutions of 6-APA and 7-ACA and their various derivatives, at 500 μg./ml. concentration. Both strains of *S. aureus* were able to remove completely the β-lactam



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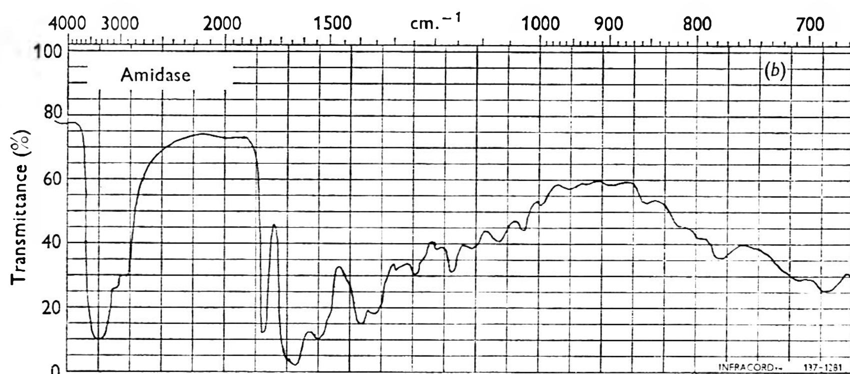

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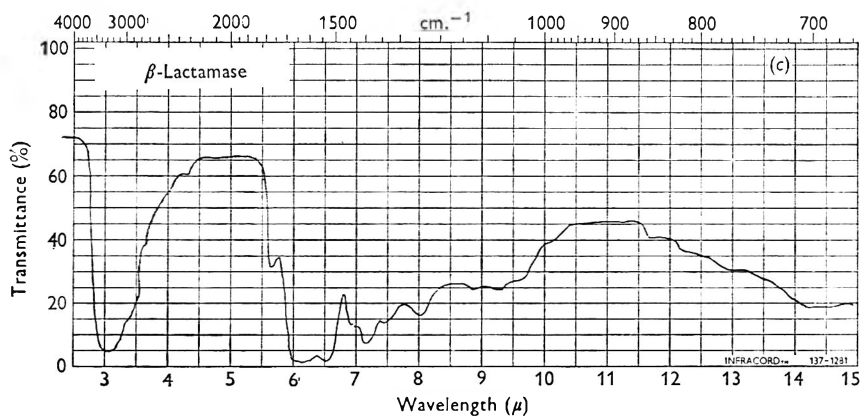

c

Fig. 2. Effect of enzymes from *E. coli* on 3,7 substituted derivative of 7-ACA (compound 87/4).

carbonyl band from infrared spectra of 6-APA, benzylpenicillin and ampicillin; neither caused any alteration in this band in methicillin, cloxacillin or quinacillin within the stated time. Both strains caused complete loss of absorption in the carbonyl band from the 3,7 substituted 7-ACA derivative, but appeared to have no effect on the band with the parent compound or the 7-substituted derivative.

DISCUSSION

The experiments described here provide a direct means of differentiating the enzymic products of coliform organisms and staphylococci, with regard to their site of action on penicillins and cephalosporins. Coliform organisms may produce either of two inactivating enzymes: (i) a β-lactamase which abolishes all the antibacterial activity of 6-aminopenicillanic acid (6-APA), 7-aminocephalosporanic acid (7-ACA) and certain derivatives thereof, by hydrolysing the lactam ring; (ii) an amidase which does not alter the lactam-ring structure of 6-APA or 7-ACA but causes a lesser degree of inactivation by de-acylating the side-chains of some derivatives at the peptide linkage.

The β-lactamase-forming organisms can be identified directly by incubating whole-cell concentrates with substrate for a few hours; loss of the characteristic carbonyl absorption band at an infrared frequency of 1760 $cm.^{-1}$ is conclusive evidence of this. Some organisms, however, inactivate derivatives of 6-APA and 7-ACA without altering this absorption band; the substrate residue can then be reactivated by phenylacetylation, proving that enzymes in this category have no action upon the lactam-ring and that de-acylation is clearly the consequence of a specific amidase present in coliform cells acting at the CO:NH linkage of the side-chain.

The β-lactam antibiotics vary in their susceptibility to enzymes which attack the β-lactam ring. 6-aminopenicillanic acid, benzylpenicillin and ampicillin are completely hydrolysed, under given experimental conditions, by β-lactamases from coliform organisms as well as from staphylococci; whereas methicillin and cloxacillin, though resistant to the staphylococcal enzyme, are partially hydrolysed fairly rapidly by β-lactamase from coliform organisms. Of the penicillins tested, quinacillin is most resistant to each form of β-lactamase. 7-aminocephalosporanic acid and its 7-substituted thienyl-derivative is stable to staphylococcal β-lactamase but partially hydrolysed by the coliform enzyme; but a more active 3,7-substituted derivative is hydrolysed by both forms of β-lactamase. The stability of methicillin and cloxacillin to β-lactamase is clearly of a high order but it is not complete. When exposed before incubation with an amidase, both compounds are rendered susceptible to the hydrolytic action of β-lactamase.

The results reported here relate to the interaction of bacterial enzymes and β-lactam antibiotics *in vitro*. There are undoubtedly differences, imperfectly understood at present, between any such findings and the behaviour of these compounds *in vivo*. It seems likely, however, that the readiness with which coliform organisms of human origin inactivate these compounds finds some counterpart in the interaction between the compounds and organisms in the body, especially when mixed infections are being treated.

We are indebted to Beecham Research Laboratories Ltd., Glaxo Laboratories Ltd. and Boots Pure Drug Co. Ltd. for supplies of penicillins and cephalosporins; to Dr J. H. C. Nayler and Miss E. M. Tanner for valuable technical advice.

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With 1 *plate*
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Biochemical Effects of Ethidium Bromide in Micro-organisms

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SUMMARY

Growth of *Escherichia coli* was partially inhibited by $1{\cdot}2\times10^{-4}$ M-ethidium bromide, a phenanthridinium trypanocide. In the presence of manganese the drug's effect was decreased. During growth in the presence of ethidium, RNA and protein contents were relatively unaffected when comparison was made between experimental and control cultures at similar turbidities; DNA content, on the other hand, was considerably decreased. A differential effect of ethidium on the formation of polynucleotide pyrimidines from labelled uracil and orotic acid was observed. Oxygen uptake continued almost unchanged during growth whether in the presence or absence of drug.

Bacillus cereus was extremely sensitive to the growth-inhibitory action of ethidium (10^{-5} M) and morphological changes were observed. Manganese protected the organisms from the drug's actions. RNA and DNA biosynthesis were both suppressed during inhibition of growth to a greater extent than was total protein formation, whereas diaminopimelic acid incorporation into cell wall and oxygen uptake continued almost unaffected. Some evidence was obtained that the pattern of protein synthesis was disturbed.

It was concluded that the drug's actions were species dependent, and that the effect on *Escherichia coli* resembled that described for a flagellate, while that on *Bacillus cereus* did not. Evidence for compartmentation of nucleic acid synthesis, as obtained with the drug in tumour cells, was not shown for either micro-organism.

INTRODUCTION

Recent investigations in this laboratory revealed that the trypanocidal phenanthridinium, ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide), produced a remarkable inhibition of the incorporation of labelled purines into polynucleotides of Ehrlich ascites cells *in vitro*, while nucleic acid synthesis continued at an only slightly slowed rate (Kandaswamy & Henderson, 1962). Since acid-soluble purine nucleotides were formed from the exogenous purines, it was postulated that the drug prevented the incorporation of these purine nucleotides into nucleic acids, while the corresponding compounds from endogenous precursors were condensed into these macromolecules. Additional evidence for this concept of compartmentation of purine nucleotides was the observation that under hypotonic conditions intracellular integrity was not maintained, and the differential effect on nucleic acid synthesis was no longer demonstrable (Kandaswamy & Henderson, 1963). It was of interest to determine whether the drug could supply

information on similar compartmentation in micro-organisms. The compound had been shown to inhibit DNA synthesis preferentially in a trypanosomal flagellate (Newton, 1957), and to depress RNA and DNA formation to a greater extent than that of protein in yeast cells (Kerridge, 1958). The drug's actions were therefore evaluated in a Gram-positive organism, *Bacillus cereus*, and a Gram-negative organism, *Escherichia coli*, in an effort to uncover dissociation of biosynthetic processes during inhibition of growth. Preliminary reports have been presented (Tomchick & Mandel, 1962, 1963).

METHODS

Organisms, media, drug, and labelled compounds. Bacteria studies were made with *Bacillus cereus* strain 569 H and *Escherichia coli* strain B (ATCC 11303). The organisms were maintained on sterile nutrient agar slopes at 4°. *Bacillus cereus* was grown in Casamino acids medium (Smith & Matthews, 1957) and *E. coli* was usually grown in C medium (Roberts *et al.* 1955).

Ethidium bromide (subsequently called ethidium, see Fig. 1) was furnished by Dr M. R. Gurd of Boots Pure Drug Co., Ltd., Nottingham.

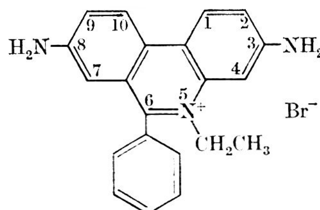

Fig. 1. Ethidium bromide.

The following labelled compounds were used in uptake studies: 8-^{14}C-adenine, 2-^{14}C-uracil, 2-^{14}C-orotic acid, 1-^{14}C-D,L-valine, 2-^{14}C-D,L-methionine, and 1-^{14}C-D,L-lysine (from Isotopes Specialties Co., Inc., Burbank, Calif.) 2-^{14}C-glycine, from Tracerlab Inc., Boston, Mass. The 4-^{14}C-guanine was previously synthesized in this laboratory (Mandel & Carló, 1953) and ^{3}H-diaminopimelic acid (^{3}H-DAP) was obtained from Dr J. L. Strominger (Washington University, St Louis, Mo.).

Growth and uptake studies. Sterile medium (50 ml.) was inoculated from an agar slope and the culture allowed to incubate overnight at 37°. After centrifugation the pellet was resuspended and shaken in a gyratory shaker at 37° until a turbidimetric reading (OD_{620}) of 0·400 at 620 mμ (Beckman Spectrophotometer, Model DU) was reached. At this wavelength interference from light absorption by ethidium was minimal. The bacterial suspension was then diluted with fresh medium to OD_{620} 0·150 and grown again. At OD_{620}, 0·200 ethidium bromide and radioactive precursors were added and incubation continued.

For rapid determination of radioactive precursors in various chemical fractions of bacteria, organisms were selectively extracted by reagents, followed by filtration through a collodion membrane. Distribution into protein, DNA, and RNA was calculated by difference (Roodyn & Mandel, 1960*a*). Generally, 2 ml. samples of exponentially growing bacterial cultures were sampled at OD_{620} readings of approximately 0·200, 0·250, 0·300, 0·350, 0·400. These samples were placed in tubes

containing 2 ml. 10 % (w/v) trichloroacetic acid (TCA) solution and allowed to stand 15–30 min. in the cold. The material was then filtered through a coarse filter membrane (Type A, Schleicher and Schuell Co.) and washed twice with 1 % TCA. The residue on the membrane represented the acid-insoluble fraction (proteins, nucleic acids, etc.) and the filtrate the acid-soluble fraction.

To determine the incorporation of precursors into the protein portion, samples of culture were mixed with TCA as before and were heated at 100° for 30 min. This treatment solubilized polynucleotides but not proteins. The protein fraction was recovered quantitatively on the filter.

To estimate DNA of *Bacillus cereus*, a 5 ml. sample of the original bacterial suspension was treated with 1 ml. of 3N-KOH and allowed to stand overnight at room temperature. DNA remained insoluble and was recovered quantitatively after filtration of the KOH hydrolysate, while RNA was hydrolysed and solubilized during the process, and was removed during the filtration (Roodyn & Mandel, 1960*a*). Filters were attached to aluminium planchets with rubber cement and, when dry, counted in a gasflow proportional counter. The total radioactivity was estimated directly for ^{14}C-labelled compounds which were plated essentially at infinite thinness. In the case of soft radiation from ^{3}H-DAP the relative total activity was computed by multiplying the specific activity values (obtained from counting the filters directly) by the turbidity reading (OD_{620}) of the bacterial suspension, a function of cell mass (Alpen & Mandel, 1960). To show reproducibility of results, duplicate or triplicate samples were taken of control and drug-treated cultures at the various turbidimetric readings.

Separation of RNA pyrimidine nucleotides. Twenty-five ml. samples of bacterial suspensions were centrifuged at 17,300*g* for 10 min. and the resulting pellet extracted with 80 % (v/v) ethanol in water at 80–85° for about 1 min. Upon cooling, the supernatant ethanolic extract was decanted. A drop of N-KOH was added to the residue to hydrolyse the RNA to mononucleotides (overnight, 25°). The samples were then adjusted to pH 4 with 10 % perchloric acid (PCA) at 0°, centrifuged in the cold, and the supernatant fluid subjected to paper electrophoresis at pH 3·5 to separate the mononucleotides (Markham & Smith, 1952). Molar activities were calculated from radioactivity and ultraviolet extinction coefficients of uridylic acids.

Chemical determination of proteins, RNA and DNA. One ml. samples of growing cultures were mixed with an equal volume of 10 % TCA and centrifuged. The pellet was then analysed for protein, using the method of Oyama & Eagle (1956). Bovine serum albumin (Armour Pharmaceutical Co.) was used as the standard.

RNA was determined by the colorimetric method of Schneider (1945). One ml. samples of bacterial suspension were centrifuged, washed with saline, and mixed with 5 % PCA. After heating for 15 min. at 100° to hydrolyse polynucleotides, 1 ml. of the filtrate was reacted with the ferric chloride orcinol reagent. Purified yeast nucleic acids (Nutritional Biochemical Co.) served as standard.

Ten ml. samples of bacterial suspension were taken for DNA analysis (Burton, 1955). Salmon sperm DNA (Nutritional Biochemical Corp.) was used as standard.

Manometric experiments. One ml. of bacterial suspension was placed in manometric flasks and oxygen uptake measured by the usual manometric procedure of Umbreit, Burris & Stauffer (1957).

RESULTS

Effect of ethidium on growth

Ethidium bromide produced inhibition of growth of *Bacillus cereus* at much lower concentrations than with *Escherichia coli*. Although the two organisms were usually grown in different media, ethidium bromide was equally inhibitory to *E. coli* when grown in either medium, indicating that the difference in susceptibility to the drug

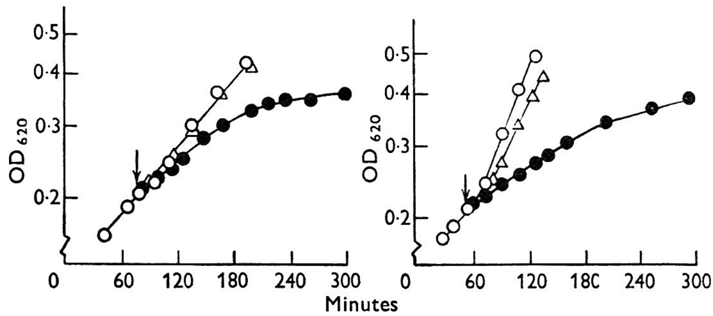

Fig. 2. Growth measured turbidimetrically at 620 mμ, in the presence of ethidium bromide added at arrow. Left, *Escherichia coli*: Control, ○——○; ethidium bromide 0·025 mM, △——△; and 0·12 mM, ●——●. Right, *Bacillus cereus*: Control, ○——○; ethidium bromide 5 μM, △——△; and 10 μM, ●——●.

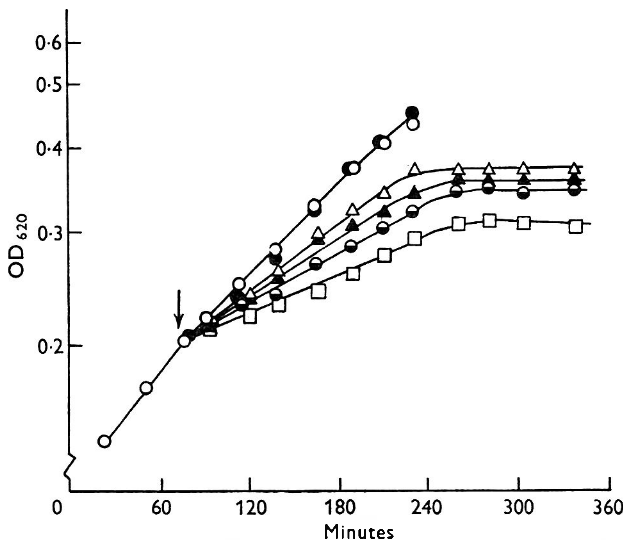

Fig. 3. Antagonism of ethidium bromide-induced growth inhibition (0·12 mM) of *Escherichia coli* by manganese; ethidium bromide and manganese added at arrow. Control, ○——○; 0·33 mM-$MnSO_4$, ●——●; ethidium bromide plus 0·33 mM-$MnSO_4$, △——△; ethidium bromide + 0·07 mM-$MnSO_4$, ▲——▲; ethidium bromide + 7 μM-$MnSO_4$, ◒——◒; ethidium bromide only, □——□.

was related to the organisms. Marked slowing of growth of *B. cereus* was observed at 10^{-5} M-ethidium, whereas a corresponding concentration of $1 \cdot 2 \times 10^{-4}$ M was necessary for *E. coli*. The characteristic pattern of the drug's effect on growth is recorded in

Fig. 2. With *E. coli* the onset of inhibition occurred more gradually than with *B. cereus*, but only with *E. coli* did growth cease altogether, as measured turbidimetrically. Organisms were still viable upon subculturing after exposure for 3 hr to the drug.

Associated with the inhibition of growth were some morphological changes of the organisms. *Escherichia coli* appeared to be slightly larger and thicker after drug treatment, while *Bacillus cereus* showed major modifications in structural morphology. Cell contents were disorganized and less aggregated, and the cells, which appeared more granular, were elongated in shape, upon Gram staining (Pl. 1, fig. 1–4). A characteristic colour shift was noted a few minutes after the addition of the orange-red drug, and the bacterial suspension became pink.

Growth inhibition by ethidium depended on the concentration of manganese ion in the medium, and could be partially overcome in both microbial systems at a molar concentration twice that of the drug. Figure 3 shows the effect of different concentrations of the metal ion on the drug-induced growth inhibition of *Escherichia coli*. Antagonism of growth inhibition by manganese became less pronounced when the interval between the addition of drug and Mn^{2+} was increased. Occasionally guanine produced antagonism of the inhibition of growth in *Bacillus cereus*.

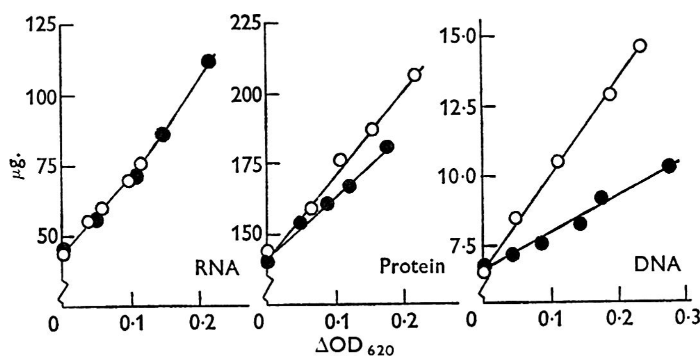

Fig. 4. Effect of ethidium bromide on content of RNA, protein, and DNA of *Escherichia coli* in organisms/ml. suspension. Colorimetric assays described in Methods. Control, ○——○ ; 0·12 mM-ethidium bromide, ●——●.

Effects on biosynthesis

Escherichia coli. Figure 4 shows that for the same change in turbidity reading of the culture, RNA synthesis was unaffected by drug treatment, whereas only a very slight depression of protein biosynthesis was observed. On the other hand, DNA synthesis was significantly decreased during inhibition of growth. Studies with labelled precursors were usually in agreement with the measurements of content of the major constituents. Thus, 8-^{14}C-adenine uptake into nucleic acids (mainly RNA) was essentially unaffected, as was that of 2-^{14}C-methionine, 2-^{14}C-lysine, or 1-^{14}C-valine into TCA-insoluble protein.

An unexpected finding was the differential effect of ethidium on the incorporation of 2-^{14}C-uracil and 2-^{14}C-orotic acid into pyrimidines of RNA. Figure 5 indicates that more uracil was used for nucleic acid synthesis in the presence of ethidium than

in its absence, results which were confirmed by measurement of molar radioactivities of isolated uridylic and cytidylic acids from RNA (Table 1). (During these studies, the separation of mononucleotides following KOH digestion of cell residues revealed the presence of a strongly fluorescent compound which was identical in its electrophoretic mobility and R_F to ethidium.) The uptake of orotic acid into polynucleotide pyrimidines, however, was sharply curtailed.

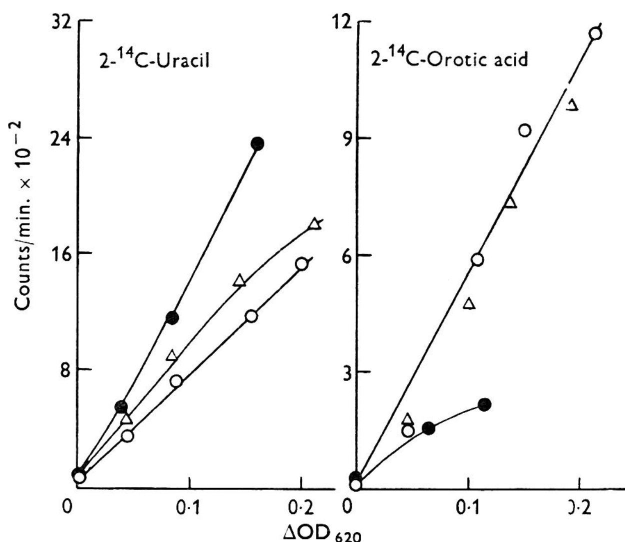

Fig. 5. Uptake of radioactivity from 2-^{14}C-uracil and 2-^{14}C-orotic acid into the cold-TCA insoluble fraction of a 2 ml. culture sample of *Escherichia coli*. Control culture, ○-----○ ; ethidium bromide 0·025 mM, △——△ ; 0·12 mM, ●——●.

Table 1. *Radioactivity of RNA mononucleotides from Escherichia coli grown with ethidium bromide in the presence of* 2-^{14}*C-uracil*

The control and 0·025 mM concentrations have been corrected to the final culture turbidity of the 0·12 mM concentration.

	Expt. 1. Ethidium bromide (mM)			Expt. 2. Ethidium bromide (mM)		
	0	0·025	0·12	0	0·025	0·12
	Counts/min./mmole			Counts/min./mmole		
Cytidylic acid	1298	2431	3614	4545	5376	6815
Uridylic acid	1460	2397	3414	4893	5638	8300

Bacillus cereus. In contrast to the results with *Escherichia coli*, RNA content of *B. cereus* was decisively decreased by ethidium treatment, whereas the effect on total protein biosynthesis again was quite small. DNA content also was decreased (Fig. 6).

The incorporation of various labelled precursors is shown in Fig. 7. The utilization of adenine for RNA, and also DNA synthesis, was sharply decreased immediately after treatment with ethidium, although eventual recovery from this effect did occur. Similar results were obtained in studies with labelled hypoxanthine, uracil

and, to a lesser extent, orotic acid. The very slight depression of the incorporation of 4-^{14}C-guanine may be related to the antagonism of growth inhibition observed when this purine was added.

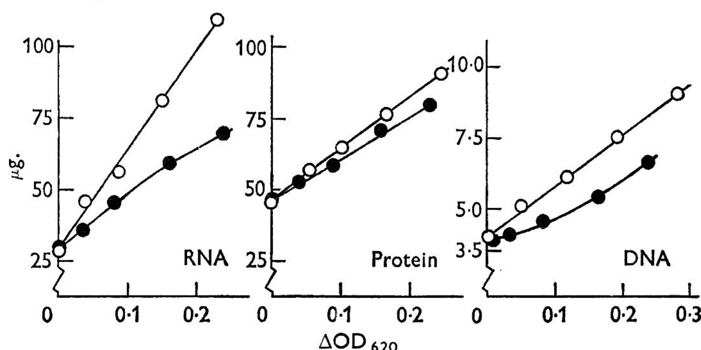

Fig. 6. Effect of ethidium bromide on content of RNA, protein, and DNA of *Bacillus cereus* in organisms/ml. of suspension. Colorimetric assays described in Methods. Control, ○——○; 10 µM-ethidium bromide, ●——●.

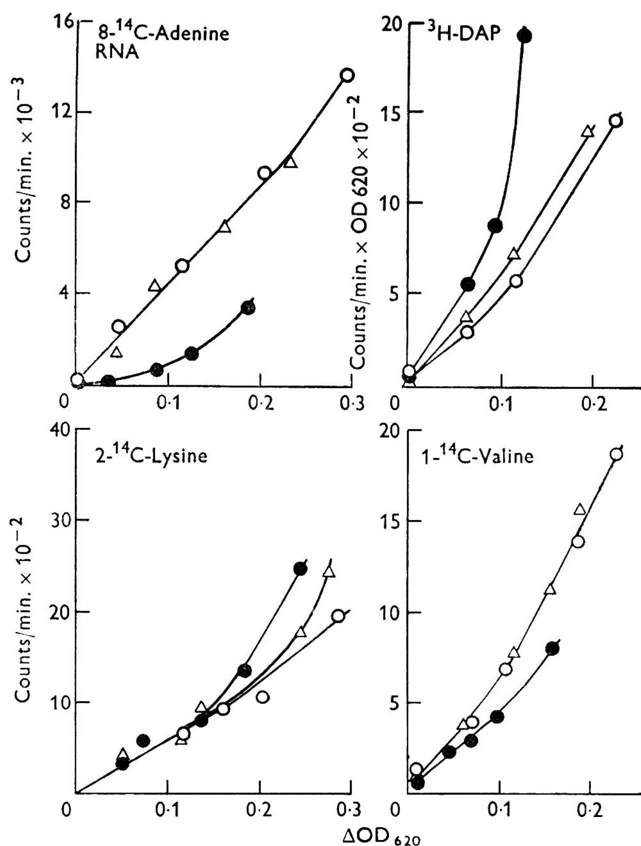

Fig. 7. Uptake of radioactivity from various precursors into the cold-TCA insoluble fraction of *Bacillus cereus*. Control, ○——○; ethidium bromide 5 µM, △——△; 10 µM, ●——●. Top left: 8-^{14}C-adenine into RNA; top right: ^{3}H-DAP into cell wall; bottom left: 2-^{14}C-lysine into protein; bottom right: 1-^{14}C-valine into protein.

Diaminopimelic acid, a characteristic component of the cell wall of various micro-organisms (Work, 1951), has been used as an index to measure the formation of that constituent (Roodyn & Mandel, 1960*b*). The increased incorporation of that precursor for the same change in culture turbidity in the presence of ethidium implies that more cell wall was being formed in relation to the total mass of organism synthesized. When plotted on a rate basis, the uptake of diaminopimelic acid continued at essentially the same rate as before the addition of ethidium.

Considerable variation was observed in the uptake of individual amino acids into bacterial protein (Fig. 7). Thus, the incorporation of lysine was increased, that of valine decreased, and that of methionine unchanged by treatment with ethidium, comparisons being made at similar turbidity of the cultures. It would appear that ethidium disrupted the normal pattern of protein biosynthesis.

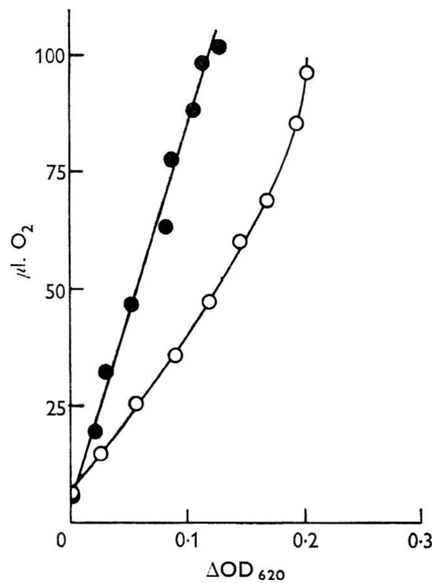

Fig. 8. Uptake of oxygen by cultures of *Escherichia coli* growing in the presence and absence of ethidium bromide. Control, ○——○ : 0·12 mM-ethidium bromide, ●——●.

Effects on oxygen consumption

Ethidium bromide had little effect on oxygen consumption in comparison to its depressant actions on growth and biosynthesis. With *Escherichia coli*, the rate of oxygen consumption was essentially unaffected by drug treatment; since growth was slowed, the utilization of oxygen for similar turbidimetric changes was increased in the presence of the drug (Fig. 8). With *Bacillus cereus* also, but to a somewhat lesser extent, oxygen consumption was greater for similar increments of culture turbidity during inhibition by ethidium. These results suggest the possibility that ethidium may also be capable of uncoupling oxidative phosphorylation.

DISCUSSION

Certain major differences in the effect of ethidium bromide on the two strains of micro-organisms used were apparent. Growth of *Bacillus cereus* was inhibited at about one-twelfth the concentration of ethidium needed with *Escherichia coli*. This observation is in agreement with a previous report (Brownlee *et al.* 1950) that Gram-positive micro-organisms are more readily affected by phenanthridinium compounds than Gram-negative organisms. In addition, the extensive morphological changes probably are related to changes in the ribosome-containing chromophilic granules in cytoplasm of trypanosomes treated with a related drug, dimidium (the 5-methyl analogue of ethidium), as first reported by Ormerod (1951).

Various distinctions in the biochemical actions of ethidium on the two organisms were also noted. During inhibition of growth of *Escherichia coli*, the most noticeable change was a sharp decrease in DNA content while the overall effects on RNA and protein biosynthesis were minor. The system resembles that of the trypanosomal flagellate *Strigomonas oncopelti* described by Newton (1957), where the most pronounced drug effect was also directed at DNA synthesis. The differential effect of ethidium on the formation of tumour cell polynucleotides *in vitro* from exogenous and endogenous precursors (Kandaswamy & Henderson, 1962) was not demonstrable in the *E. coli* system since neither ^{14}C-adenine uptake nor RNA content were affected during growth to the same cultural turbidity in the presence or absence of the drug.

It is difficult to explain the observed effects of ethidium on pyrimidine biosynthesis in *Escherichia coli*. The increased utilization of exogenous uracil, in contrast to that of orotic acid (Fig. 5), is probably unrelated to endogenous nucleic acid pyrimidine biosynthesis, which was not affected (Fig. 4). Neither of the two precursors is required for growth, although both compounds can serve to form nucleic acid pyrimidines. Although several possible alternatives exist, compartmentation of pyrimidine pools may account for the differential actions by ethidium on the incorporation of the precursors.

Inhibition of the *Bacillus cereus* system by ethidium resulted in greater depression of nucleic acid biosynthesis than that of protein. Cell wall biosynthesis, as observed during growth inhibition with 8-azaguanine (Roodyn & Mandel, 1960*b*) and chloramphenicol (Mandel & Altman, 1961) in the same bacterial system, continued at almost the same rate as before the addition of ethidium. The effects on *B. cereus* resemble those on the yeast, *Saccharomyces carlsbergensis*, as described by Kerridge (1958), who observed a preferential inhibition of nucleic acid biosynthesis by ethidium in comparison with its effect on total protein formation. With *B. cereus*, ethidium depressed ^{14}C-adenine uptake into nucleic acids and endogenous RNA synthesis to approximately the same extent, thus differing from the results of Kandaswamy & Henderson (1962) with tumour cells, who reported the almost complete suppression of adenine incorporation by ethidium in contrast to a relatively minor inhibition of nucleic acid formation from endogenous precursors such as ^{14}C-glycine.

The differential effects of ethidium on the utilization of various amino acids by *Bacillus cereus* is of interest. The amino acids chosen were precursors of protoplasmic protein rather than of cell wall (Roodyn & Mandel, 1960*b*). The results obtained suggest a major derangement of protein biosynthesis. It is possible that particular proteins are made in abnormal quantities, or that the ethidium changed the amino

acid composition of certain proteins. In this connexion, the inhibition by ethidium of lytic enzyme synthesis in a strain of *B. subtilis* may be of interest, although the specificity of this effect on protein formation and inhibition of all processes of cellular growth is not clear (Richmond, 1959).

The interaction of phenanthridiniums closely related to ethidium bromide with nucleic acids, particularly DNA, was reported (Brownlee *et al.* 1950; Seaman & Woodbine, 1955). In the present experiments ethidium was bound extensively to bacteria, and was usually recoverable following KOH digestion of the organisms and separation of mononucleotides. The similarity in structure of ethidium to acridine derivatives, such as acriflavin, acridine orange, and proflavin, and the reported binding of the latter group of drugs to polynucleotides, certainly suggest similarities in action. Ethidium (Elliott, 1963), like the acridines (Leith, 1963), is believed to form a complex with DNA, thereby interfering with DNA polymerase activity. Although little information has been reported on the effect of the acridines on biosynthetic processes, it is believed that the intercalation of these drugs in the DNA molecule (Lerman, 1963) is responsible for their growth inhibitory and mutagenic properties. A similar possibility should be considered with ethidium bromide. Another mutagenic drug, 5-bromouracil (Michaelson & Mandel, 1962), which was studied in an *Escherichia coli* system, was shown to inhibit DNA formation, to be incorporated into bacterial DNA, and to produce effects indicative of disruption of the pattern of protein biosynthesis.

The mechanism of reversal of the drug's effects by manganese is unclear. Since it is doubtful that ethidium chelates the metal, perhaps the divalent cation interferes with the drug's binding to polynucleotides or ribosomes. In the two bacterial systems requiring different concentration of drug for inhibition of growth, the effective concentration of Mn^{2+} was found to be related to that of ethidium. The reversing effect of guanine may be more closely related to the observed action of nucleic acids in preventing the drug's actions, perhaps by binding the drug before it has a chance to act in the cell.

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EXPLANATION OF PLATE

Micrographs of *Escherichia coli* and *Bacillus cereus* all at the same magnification.

Fig. 1. *Escherichia coli* control
Fig. 2. *E. coli* grown in 0·12 mM ethidium bromide.
Fig. 3. *Bacillus cereus* control.
Fig. 4. *B. cereus* grown in 10·0 μM ethidium bromide.

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In vitro and *In vivo* Effects of Deoxyribonucleic Acid Degradation Products on Virulent and Avirulent Group A Streptococci

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SUMMARY

A deoxyribonuclease-treated deoxyribonucleic acid (DNase and DNA), further supplemented with all eight of the naturally occurring deoxynucleosides and deoxynucleotides enhanced the rate and extent of multiplication of several virulent strains of group A β-haemolytic streptococci (*Streptococcus pyogenes*) without affecting the multiplication of related avirulent strains. Ribonucleic acid (RNA) degradation products or yeast extract did not exert such selective effects. An enhancement of DNA synthesis occurred in suspensions of virulent strains augmented with the DNA degradation products, whereas avirulent strains did not respond. In addition, DNA synthesis was enhanced selectively in comparison to RNA and protein syntheses. The rate at which mice succumbed to infection by streptococci was enhanced considerably by the DNA degradation products, but the LD 50 remained unchanged.

INTRODUCTION

Several reports have cited the stimulatory effects of certain DNA degradation products on multiplication and virulence of pneumococci (*Streptococcus pneumoniae*) and staphylococci (*Staphylococcus aureus*) (Firshein & Braun, 1958, 1960; McKee & Braun, 1962). An interesting aspect of this work was the lack of any significant effects of the DNA degradation products on multiplication of avirulent variants of these organisms. With pneumococci, it was possible to correlate the growth-promoting effects of the DNA degradation products with a selective stimulation of DNA synthesis (Firshein, 1961*a*). The DNA degradation products which caused these effects consisted of deoxyribonuclease-treated DNA further supplemented with all eight of the naturally occurring deoxynucleosides and deoxynucleotides (hereafter called supplement-1). Ribonucleic acid derivatives were found to be inactive in stimulating multiplication of virulent pneumococci. Preliminary experiments with group A β-haemolytic streptococci showed that supplement-1 stimulated multiplication of virulent strains in a manner similar to that which occurred with pneumococci. The present report is concerned with the further elucidation of these observations, with a study of the effects of supplement-1 on multiplication of avirulent strains, and with the effects of the supplement *in vivo*.

METHODS

Organisms. All the strains of *Streptococcus pyogenes* used, except one avirulent strain, were obtained either from Dr S. S. Barkulis, or from the Hebrew University Hadassah Medical School, Jerusalem, Israel. One avirulent strain was freshly

derived from its parental strain by picking smooth variant colonies which emerged after prolonged incubation of the parental strain on Difco brain heart infusion blood agar plates. Avirulence or virulence were ascertained by mouse infection in all cases. All strains showed β-haemolysis on blood agar, but only the virulent strains contained M protein, an important component of virulence in this group (Foley, Smith & Wood, 1959; Wiley & Wilson, 1961). The organisms were maintained on Difco brain heart infusion agar plates supplemented with 0·3 % (w/v) Difco neopeptone and 5 % (v/v) defibrinated sheep blood. Their designations follow:

Strain M-6 (virulent), strain 6AM$^-$ (avirulent, derived previously);

Strain M-18 (virulent), strain 18AM$^-$ (avirulent, freshly isolated);

Strain Q-43x (virulent, derived by 43 mouse passages of avirulent strain), strain Q-496 (avirulent).

Media for growth studies. In addition to the maintenance medium described above, brain heart infusion neopeptone broth supplemented with 0·5 % (v/v) defibrinated sheep blood was used routinely for growth studies. The medium was prepared double strength and diluted with the appropriate supplements dissolved in distilled water. In some experiments, Difco Todd-Hewitt broth supplemented with 0·5 % (v/v) defibrinated sheep blood was used. Lastly, a medium containing casitone, tryptone, yeast extract (all Difco), and albumin (Fraction V from bovine serum albumin, Armour and Co.; Marmur & Hotchkiss, 1955) was prepared for growth studies and for obtaining large numbers of viable organisms for suspension studies.

Growth studies. Viable counts were made by the serial dilution method and checked by direct microscopic counts. Since virulent and avirulent streptococci form chains and clump in growth media, errors in determining the actual number of viable cocci may occur. These errors can be minimized by vigorous mixing of the dilution tubes before plating. However, the relative viable counts of virulent and avirulent streptococci in response to the supplement were considered to be the important point. Therefore, it was examined whether the supplement affected the number of chains and the number of cocci per chain. It was found that the added supplements had no effects on these variables.

Preparation of DNA, DNase-treated DNA and other substances. Highly polymerized calf-thymus Na-DNA was prepared by the method of Kay, Simmons & Dounce (1952). Deoxyribonuclease-treated DNA, deoxynucleosides and deoxynucleotides were prepared as described previously (Firshein, 1961 *a*). Concentrations used for all strains were as follows: (μg./ml. medium), DNA, 130; deoxyribonuclease, 25; deoxynucleosides, 800 (200 each of deoxyadenosine, deoxyguanosine, deoxycytidine, thymidine); deoxynucleotides, 200 each of the phosphorylated deoxynucleosides. Ribonuclease-treated RNA, nucleosides and nucleotides were prepared in the same manner and used in the same concentrations as the DNA degradation products, except that Mg^{2+} was omitted in the preparation of ribonuclease-treated RNA.

Suspension studies, extraction and measurement of nucleic acids and protein. The methods used were as described previously (Firshein, 1961 *a*). The analyses were accurate to within 3 % for protein (Lowry, Rosebrough, Farr & Randall, 1951), and 5 % for DNA and RNA (Brody, 1953; Drury, 1948, respectively). Percentage

increase of DNA over protein was determined in the following way. For a given measurement, an amount of DNA (in μg./ml.) was calculated whose % increase over zero time was identical with that observed for the % increase in protein over zero time. This calculated amount was compared to the actual amount of DNA obtained experimentally. When the latter figure was higher, the % increase of the actual value was determined over the calculated value.

Manometric experiments. The Warburg apparatus was used with 20% (w/v) KOH in the centre well. Supplement-1 and glucose were added to the sidearms and organisms (in 0·02M Na-K phosphate buffer, pH 7·5) were added to the centre compartment of the Warburg flask. To prevent toxic effects of H_2O_2 accumulation, catalase (Nutritional Biochemicals) was added to 0·005% (w/v). Manometers were tipped 10 min. after equilibration of the flasks at 37°.

Animal experiments. Albino Swiss mice weighing about 18 g. were injected intraperitoneally with dilutions (in 0·9% saline) of organisms of the different strains, at the same time as intraperitoneal injection of supplement-1. Ten mice were used per dilution of infecting organism. The total concentration of supplement-1 injected into each animal was identical with that used in culture media 1·73 mg./ml. Injection of twice the usual amount of supplement-1 without streptococcal infection did not affect the mice in any observed manner.

RESULTS

Effects of supplement-1 on multiplication

Initial experiments were concerned with the effects of supplement-1 on multiplication of the various virulent and avirulent strains in brain heart infusion neopeptone blood broth. The concentrations chosen for the various experiments corresponded with those found to exhibit optimum responses in pneumococcal cultures (Firshein & Braun, 1960). It was observed (Figs. 1–3) that the virulent and avirulent strains showed strikingly dissimilar responses to the supplement. The supplement stimulated the rate and extent of multiplication of virulent strains, but had no significant effects on proliferation of avirulent strains. After 12 hr, the enhancement for virulent strains with respect to final yield of viable organisms was 11-fold for strain Q-43x, 15-fold for strain M-18, and 9-fold for strain M-6. The DNase-treated DNA (DNA digest) accounted for a substantial portion of the stimulation with all three virulent strains, despite its low concentration in the supplement (8%, w/w, see 'Methods'). Deoxynucleosides and deoxynucleotides augmented this response; by themselves, they produced only a small enhancement.

The rate of multiplication of virulent strains was also increased from control rates in the presence of the supplement, as evidenced by decreases in the generation time. The generation time was decreased from 55 to 37 min. for strain Q-43x, from 70 to 38 min. for strain M-18, and from 100 to 61 min. for strain M-6.

Table 1 demonstrates that the selective effects of supplement-1 on the multiplication of the virulent strains were not restricted to brain heart infusion neopeptone blood broth, but occurred in other media that supported growth of the streptococci. Only the results obtained with two pairs of virulent and avirulent strains are shown, the third pair acted in the same manner.

With a pair of virulent and avirulent strains, the maximum stimulation of multiplication of virulent organisms by supplement-1 occurred at a concentration of

1200μg./ml. (Fig. 4). Avirulent organisms did not respond to any concentration of supplement-1.

Table 1. *Effects of DNA degradation products on multiplication of Group A streptococci in different media*

Procedures are described in the Methods. Inocula ($\times 10^{-5}$): strain Q-43x, 9; strain Q-496, 12·5; strain M-18, 8; strain 18AM⁻, 9·5.

Medium	Strain of organism	Additions: Supplement-1	Additions: Control	Viable count after 12 hr ($\times 10^5$)
Difco Todd-Hewitt broth +0·5 % (v/v) defibrinated sheep blood	Q-43x (virulent)	+	−	11,000
		−	+	405
	Q-496 (avirulent)	+	−	600
		−	+	580
CAT medium (Marmur & Hotchkiss, 1955)	Q-43x	+	−	8,500
		−	+	400
	Q-496	+	−	1,000
		−	+	800
Difco Todd-Hewitt broth +0·5 % (v/v) defibrinated sheep blood	M-18 (virulent)	+	−	3,200
		−	+	310
	18AM⁻ (avirulent)	+	−	900
		−	+	1,050
CAT medium	M-18	+	−	905
		−	+	150
	18AM⁻	+	−	670
		−	+	900

Effects of supplement-1 in vivo

McKee & Braun (1962) and Firshein & Braun (1958) observed that the DNA degradation-products enhanced the infectivity of virulent staphylococci and pneumococci for mice. Therefore, it appeared of interest to ascertain whether such products would also influence the course of a streptococcal infection. Two of the strains containing M protein (strains M-18, Q-43x) were used to infect mice and the mice were treated immediately after infection with supplement-1 as described under Methods. The results (Table 2) show that supplement-1 enhanced the rate at which mice succumbed, but the LD 50 dose was not decreased significantly. The ET 50 dose was calculated for one strain (M-18) using Litchfield's nomographs (Litchfield, 1949). Figure 5 shows a clear difference between controls and treated animals, with the ET 50 39 hr for the controls and only 25·2 hr for the treated animals.

Effects of supplement-1 on nucleic acid and protein syntheses in suspensions of cocci

Firshein (1961*a*) found that the selective enhancement of multiplication of virulent strains of pneumococci in medium containing supplement-1 involved an effect on DNA metabolism. It was of interest, therefore, to test the effects of the supplement on DNA synthesis in streptococci. As in the experiments with pneumococci, measurements of DNA were made in suspensions of streptococci (Firshein, 1961*a*) in the presence of supplement-1 or its component parts (the DNA digest

alone, and mixtures of deoxynucleosides and deoxynucleotides by themselves). RNA and protein syntheses were also estimated. The results of these experiments, replicated several times (Table 3), showed that DNA synthesis was enhanced selectively in comparison to RNA and protein syntheses in suspensions of virulent streptococci containing supplement-1. In contrast, suspensions of avirulent streptococci showed no response to the supplement. Tests with parts of the supplement

Table 2. *Effects of supplement-1 on the survival of mice infected with group A streptococci*

Procedures are described in the Methods. See Fig. 5 for a further analysis of the effects of supplement-1 *in vivo*.

Treatment	Strain of organism	No. of infecting organisms	Time (hr) of observation: Cumulative % mortality 18	22	26	30	34	38	42	46	50	54
Supplement-1 dissolved in 0·9 % (w/v) saline	M-18	10^2	0	0	0	0	10	→				
		10^3	0	0	20	30	30	40	→			
		10^4	10	50	60	100	→					
0·9 % (w/v) saline	M-18	10^2	0	0	0	0	0	0	0	10	→	
		10^3	0	0	0	0	0	10	10	20	30	→
		10^4	0	20	20	30	30	30	60	90	100	→
Supplement-1 dissolved in 0·9 % (w/v) saline	Q-43x	$10^{1·5}$	0	0	0	10	20	40	→			
		$10^{2·5}$	0	20	40	40	70	80	→			
		$10^{3·5}$	20	70	100	→						
0·9 % (w/v) saline	Q-43x	$10^{1·5}$	0	0	0	0	10	20	→			
		$10^{2·5}$	0	0	0	0	30	40	40	40	60	90→
		$10^{3·5}$	0	0	20	30	60	70	100	→		

Table 3. *Effects of DNA degradation products on nucleic acid and protein syntheses by group A streptococci*

Procedures are described in the Methods

Strain of organism	Additions: DNA + DNase	Additions: Deoxynucleosides + deoxynucleotides	Increase (%) over zero time after 70 min.: DNA	RNA	Protein	Increase (%) of DNA over protein
Q-43x (virulent)	−	−	65	60	70	.
	+	+	150	78	80	50
	+	−	115	74	65	33
	−	+	87	70	70	13
Q-496 (avirulent)	−	−	78	76	80	.
	+	+	85	90	81	2
	+	−	85	75	75	3
	−	+	65	73	70	.
M-6 (virulent)	−	−	20	20	19	.
	+	+	70	25	25	36
	+	−	52	26	24	22
	−	+	38	23	22	12
6 AM⁻ (avirulent)	−	−	31	28	30	.
	+	+	28	31	33	.
	+	−	30	33	28	1
	−	+	25	25	27	.

revealed that the DNA digest stimulated DNA synthesis selectively to a greater extent than did mixtures of deoxynucleosides and deoxynucleotides, thus paralleling their effects in growing cultures with respect to enhancement of multiplication of virulent strains (see Figs. 1–3).

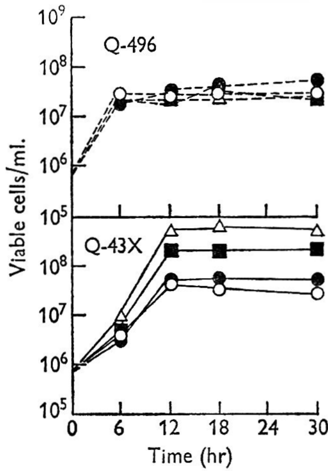

Fig. 1

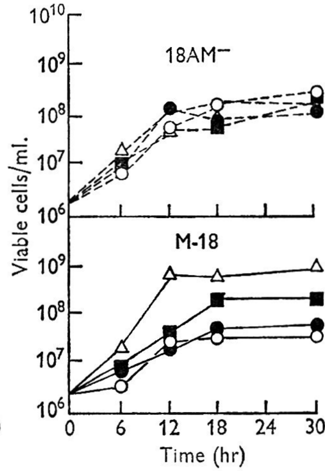

Fig. 2

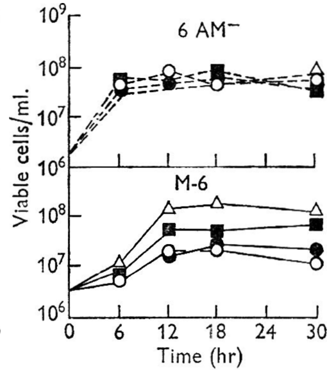

Fig. 3

Figs. 1–3. Viable counts of virulent and avirulent strains of Group A streptococci in the presence or absence of supplement-1. For concentrations of supplement-1 and its moieties, see Methods. Medium was brain heart infusion neopeptone blood broth. Virulent: solid line; avirulent, broken line. Supplement-1 (△); DNA + deoxyribonuclease (■); deoxynucleosides + deoxynucleotides (●); control (○).

Table 4. *Effects of RNA degradation products and yeast extract on multiplication and DNA synthesis of group A streptococci*

Procedures are described in the Methods. Concentration of yeast extract (Difco) in suspending or in culture medium was 0·3% (w/v). Medium for viable count determinations was brain heart infusion neopeptone blood broth. Inocula for culture medium were as follows (× 10⁵): strain M-18, 9; strain 18AM⁻, 10; strain M-6, 13; strain 6AM⁻, 16.

Additions	Strain of organism	Viable count after 12 hr in complete medium (× 10⁵)	Increase (%) of DNA over protein in suspensions
RNA derivatives	M-18 (virulent)	525	1
Yeast extract		1200	1
Supplement-1		2000	40
None		400	.
RNA derivatives	18AM⁻ (avirulent)	3500	.
Yeast extract		8000	2
Supplement-1		3100	.
None		2200	.
RNA derivatives	M-6 (virulent)	300	.
Yeast extract		950	2
Supplement-1		1100	32
None		190	1
RNA derivatives	6AM⁻ (avirulent)	1000	4
Yeast extract		1800	3
Supplement-1		900	.
None		1300	.

Effects of other nutrients on DNA synthesis and multiplication

It may be argued that the supplement contained components which affected the overall metabolic rate in virulent streptococci, and that no specific effects were being observed. Table 4 summarizes the effects of yeast extract and RNA degradation products on DNA synthesis and multiplication of virulent and avirulent strains. In no instance were effects similar to those elicited by the DNA degradation products

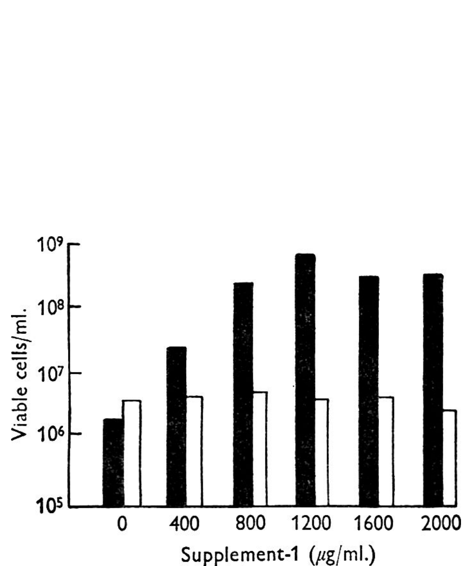

Fig. 4

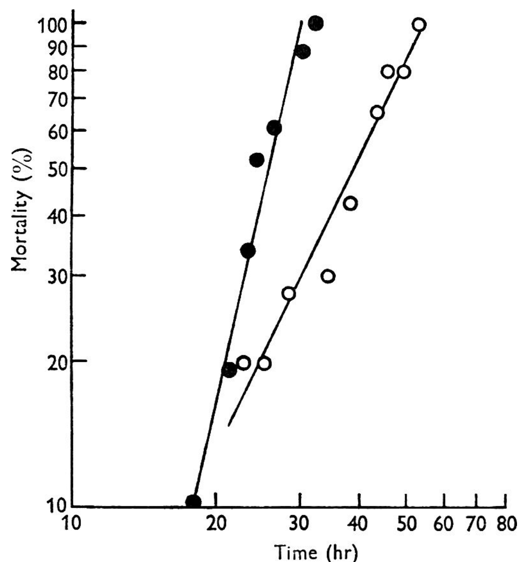

Fig. 5

Fig. 4. Effects of concentration of supplement-1 on the number of viable organisms of a virulent and avirulent strain of Group A streptococci after incubation for 12 hr. Medium was brain heart infusion neopeptone blood broth. Initial inocula: ($\times 10^4$), virulent (strain Q-43x), 6; avirulent (strain Q-496), 10. Shaded bars, virulent; unshaded bars, avirulent.

Fig. 5. Cumulative % mortality of mice as a function of time, plotted on log probability paper for strain M-18. Forty mice/group, 10^4 organisms injected into each animal. Conditions of treatment, inoculation of organisms, concentration of supplement-1 were as described in Methods. The confidence limits for the ET50 (median effective time) with a 19/20 probability found by using Litchfield's nomographs (Litchfield, 1949) were as follows: ET50 for treated was 25·2 hr (26·7–23·7). The slope of the line was 1·21 (1·26–1·15). ET50 for control was 39 hr (44·4–34·2). The slope of the line was 1·54 (1·69–1·53). (●), mice treated with supplement-1 at time of infection with strain M-18; (○), infected with strain M-18 only.

obtained. Yeast extract stimulated multiplication of avirulent and virulent streptococci in complete medium to approximately the same extent, and had no effects on DNA synthesis in coccal suspensions. The RNA degradation products were almost without effect on multiplication or DNA synthesis of virulent and avirulent strains.

Effects of supplement-1 on respiration

The ability of supplement-1 to enhance respiration of pneumococci (Firshein, 1961*b*, 1962), and the importance of such effects in the enhancement of DNA synthesis and multiplication in this organism, suggested a study of the effects of

supplement-1 on respiration of streptococci. It was found (Fig. 6) that the supplement did not enhance oxygen uptake in glucose-containing suspensions of virulent streptococci. However, the supplement was oxidized extensively in the absence of glucose in suspensions of virulent organisms. With avirulent organisms, oxygen uptake was depressed in suspensions containing glucose and supplement-1, as compared with suspensions containing glucose only. Avirulent streptococci were also capable of oxidizing supplement-1 in the absence of glucose, but the values obtained were not as high as those with virulent organisms.

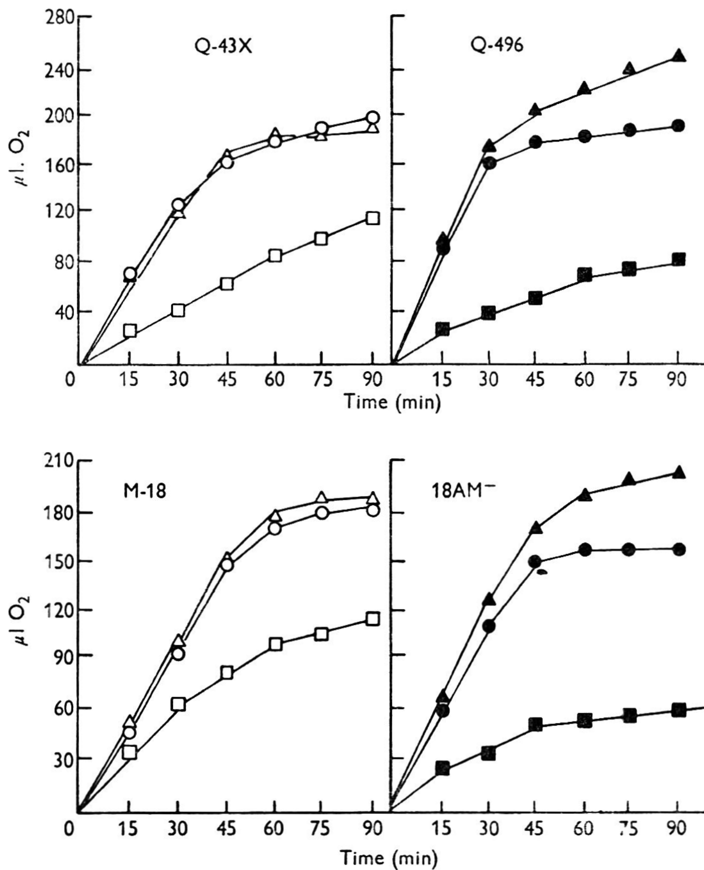

Fig. 6. Effects of supplement-1 on oxygen uptake of virulent and avirulent strains of group A streptococci in suspensions containing glucose. For concentration of supplement-1 see Methods. Concentration of glucose was the same as that in washed suspensions of cocci (50 μM). Endogenous rates are not shown, they were negligible for all strains tested. (○), (●), supplement-1 + glucose; (□) (■), supplement-1 alone; (△) (▲), glucose. Inocula for all strains were adjusted to the same value, $8 \cdot 5 \times 10^8$ viable cocci/ml.

DISCUSSION

To some extent, the interest of the results described in the paper (Firshein & Zimmerman, 1964) concerning Mn^{2+} lies in the contrast between them and those described in the present paper. It is convenient to discuss the findings of both papers here.

The observations made with DNA degradation products extend the generality of the selective effects of such products on multiplication and DNA synthesis of virulent strains of Gram-positive pathogenic cocci. The similarities can be summarized conveniently in Table 5.

Table 5

	Addition of supplement-1				
Organism	Stimulation of multiplication of virulent strains	Effects on avirulent strains	Stimulation of infectivity	Stimulation of DNA synthesis by virulent strains	References
Pneumococci	+	−	+	+	Firshein & Braun (1958), Firshein (1961*a*)
Staphylococci	+	−	+	?	McKee & Braun (1962)
Streptococci	+	−	+	+	—

'+', positive effect; '−', no effect.

The results described in the previous paper (Firshein & Zimmerman, 1964) concerning Mn^{2+} suggest that the effects of Mn^{2+} and DNA degradation products on multiplication of pneumococci (Firshein, 1962) and streptococci may be different. Some question had arisen previously with pneumococci because both augmentations stimulated the multiplication of virulent strains. However, DNA degradation products have now been shown to act in the same manner in pneumococci and streptococci, whereas Mn^{2+} acts differently in these two species. Actually, the results obtained with virulent pneumococci and Mn^{2+} may be unique, since effects of Mn^{2+} on multiplication of avirulent pneumococci (Firshein, 1962) and staphylococci (Ramsey & Wilson, 1957) have also been found to be inhibitory. An interesting point in the present studies with streptococci is that both Mn^{2+} and the DNA degradation products exert their effects selectively on virulent and avirulent strains. However, the effects of the former are inhibitory with respect to multiplication, while the effects of the latter are stimulatory.

As with pneumococci (Firshein, 1961*a*), it appears that the stimulation of multiplication of virulent streptococci by DNA degradation products is associated with increased rates of DNA synthesis. This is supported by the observation that the effects of components of supplement-1 on DNA synthesis closely parallel their effects on multiplication. The greater the enhancement of DNA synthesis by moieties of the supplement in suspensions of cocci, the greater the stimulation of multiplication.

Although DNA synthesis may represent the primary process affected by the degradation products in streptococci and pneumococci, some important secondary effects of the products on respiration may differ in both species. For example, with pneumococci (Firshein, 1961*b*) components of supplement-1 stimulate the oxidation of glucose by virulent strains. This stimulation provides excess energy needed for incorporation of deoxynucleosides and deoxynucleotides, and enhanced DNA synthesis. The supplement itself is not oxidized significantly by virulent pneumococci. In contrast, with streptococci the supplement is oxidized extensively by

virulent strains, and it is incapable of stimulating the oxidation of glucose. In fact, a slight depression of oxygen uptake occurs when the supplement and glucose are present together in suspensions of streptococci, in comparison to glucose alone. Thus, no excess energy is available to the virulent organisms. However, the fact that enhanced DNA synthesis does occur in suspensions under these conditions shows that: (1) the available energy is sufficient for metabolizing the DNA degradation products; and (2) enough of the supplement remains unoxidized for synthetic purposes, in spite of the oxidation of the degradation products.

Oxygen uptake is inhibited to a greater extent in suspensions of avirulent streptococci containing glucose and DNA degradation products than in suspensions of virulent streptococci containing such supplements. This sensitivity in both types of organisms may involve the lack of sufficient enzymic raw material to oxidize both supplement-1 and glucose when these are present together. The depression of oxygen uptake would result from the fact that a proportion of the available oxidative-enzyme supply normally used in glucose oxidation would be used to act on substrates (supplement-1) which were not as efficiently oxidized. If this hypothesis be valid, then the pool of oxidative enzymes in avirulent streptococci may be less than that found in virulent strains. Experiments with cell-free extracts are now under way to examine this possibility.

Mn^{2+} appears to exert its inhibitory effects differently in virulent and avirulent streptococci. In suspensions of virulent streptococci a general depression of respiration, nucleic acid synthesis and protein synthesis occurs, whereas in suspensions of avirulent streptococci, a selective inhibition of nucleic acid synthesis is observed. Protein synthesis is largely unaffected and respiration is only slightly depressed in comparison to that noted in suspensions of virulent organisms. It is conceivable that much of the inhibition of multiplication of virulent strains by Mn^{2+} is due to a depression of respiration, since it is hard to conceive that the cation would be involved directly in all of the metabolic systems concerned with nucleic acid and protein synthesis. Rather, it is possible that an inhibition of respiration could result in a suppression of other metabolic systems including nucleic acid and protein syntheses. The inhibition of multiplication of avirulent strains by Mn^{2+} may be due to a primary effect on nucleic acid synthesis and to a secondary effect on respiration. This is supported by two observations: (*a*) Mn^{2+} inhibits nucleic acid synthesis to a much greater extent in avirulent than in virulent suspensions, while respiration in avirulent strains is not affected nearly as much as nucleic acid synthesis; (*b*) protein synthesis is largely unaffected in avirulent suspensions.

The specific sites of inhibition of respiration and nucleic acid synthesis by Mn^{2+} are unknown. Mn^{2+} is known to be involved in respiratory processes (Ochoa & Weisz-Tabori, 1948), but the effects are stimulatory, rather than inhibitory as with streptococci.

In certain respects the effects of supplement-1 on the virulence of streptococci in mice differ from the results obtained with pneumococci (Firshein & Braun, 1958) and staphylococci (McKee & Braun, 1962). Both the LD50 and ET50 values are decreased in the latter two species by supplement-1; but with streptococci, only the ET50 is decreased, the LD50 remaining about the same in controls and supplements. The reason for this is unknown. A point may be made about the possible contribution of DNA degradation products in the outcome of an infection by Gram-

positive pathogenic cocci. It is conceivable that the occurrence, *in vivo*, of compounds such as the degradation products might enhance the multiplication of the infecting organisms in susceptible hosts and thus contribute to the pathological effect. Whether these compounds may occur naturally *in vivo*, or may occur as a result of cellular necrosis during an infection, is still largely unknown. Tillett, Sherry & Christensen (1948) found that deoxyribonucleases produced by virulent streptococci were effective in hydrolysing deoxyribonucleoprotein from necrotic cells *in vivo*. However, no correlation was made between the occurrence of the DNA degradation products and multiplication of streptococci *in vivo*. Although effects of Mn^{2+} on virulence were not studied, the cation has been found to contribute significantly to virulence of pneumococci in mice by stimulating the multiplication of virulent strains *in vivo* (Hitchings & Falco, 1946; Firshein, 1962). Thus, by analogy, it may be that Mn^{2+} will exert a deleterious effect on streptococcal virulence. Studies are under way to examine this possibility.

Ca^{2+} can antagonize the effects of Mn^{2+} whether such effects are inhibitory in the case of streptococci, or stimulatory in the case of pneumococci (Firshein, 1962); such interactions have been observed by other investigators. Fry (1955) reported that glutamine synthesis in cultures of staphylococci was activated by Mn^{2+} and was annulled by Ca^{2+}. Gerloffe & Folke (1957) noted that toxic concentrations of Mn^{2+} for certain algae in lakes could be rendered less deleterious by addition of Ca^{2+}. The wide variety of environmental conditions under which this interaction can exist suggests that there is a competitive relationship between the two cations, but the nature of this competition, at least in streptococci, is unknown.

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Differential Toxicity of Manganese for Virulent and Avirulent Group A Streptococci

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SUMMARY

Mn^{2+} depressed the rate and extent of multiplication of several virulent strains of group A beta-haemolytic streptococci (*Streptococcus pyogenes*), and caused a 6–12 hr increase in the lag phases of related avirulent strains. Several other cations did not exert such differential inhibitory effects. Ca^{2+} overcame the inhibitory effects of Mn^{2+} in both kinds of organism. In suspensions of virulent strains, Mn^{2+} depressed both nucleic acid and protein syntheses below the control values, but only nucleic acid synthesis was depressed in avirulent-organism suspensions; protein synthesis was largely unaffected. The oxidation of glucose was depressed below control values by Mn^{2+} to a much greater extent in virulent strains than in avirulent strains.

INTRODUCTION

In previous experiments with pneumococci (*Streptococcus pneumoniae*, Firshein, 1960, 1962), Mn^{2+} was shown to stimulate the multiplication of virulent strains and to produce either inhibitory or no effects in the case of avirulent strains. In this respect, Mn^{2+} acted in a manner similar to that of certain deoxyribonucleic acid (DNA) degradation products, which also enhanced multiplication of virulent strains of pneumococci. However, Mn^{2+} inhibited the multiplication of several avirulent strains of pneumococci, whereas DNA degradation products did not depress the multiplication of any avirulent strains tested. It was ascertained (Firshein, 1962) that the stimulatory effects of Mn^{2+} on multiplication of virulent pneumococci were related to an enhancement of respiration, whereas the toxic effects of Mn^{2+} on multiplication of avirulent pneumococci involved, in part, a depressing effect on respiration. Recent results (Firshein & Zimmerman, 1964) have shown that DNA degradation products also stimulate multiplication of virulent strains of group A streptococci. However, in contrast to pneumococci, these virulent group A streptococci showed a depressed rate and extent of multiplication in the presence of Mn^{2+}. The present report is concerned with an analysis of this Mn^{2+}-effect on virulent streptococci as well as with a study of the effects of Mn^{2+} on multiplication of avirulent streptococci. The effects of Mn^{2+} on respiration, nucleic acid synthesis, and protein synthesis of both cell types will also be described. In part, the interest of the results with Mn^{2+} lies in their contrast to the results described in the accompanying paper (Firshein & Zimmerman, 1964) with DNA degradation products. The results of both papers are discussed together in the next paper (Firshein & Zimmerman, 1964).

METHODS

General. Three pairs of virulent and avirulent strains of group A streptococci were used. They were identical to those used by Firshein & Zimmerman (1964). Maintenance of these strains, medium, viable counts, techniques of preparing and performing experiments with suspensions of organisms, were as reported by Firshein & Braun (1960), Firshein (1961) and Firshein & Zimmerman (1964). Mn^{2+} (as $MnSO_4$) and Ca^{2+} (as $CaCl_2.2H_2O$) were dissolved in distilled water before use. Other cations

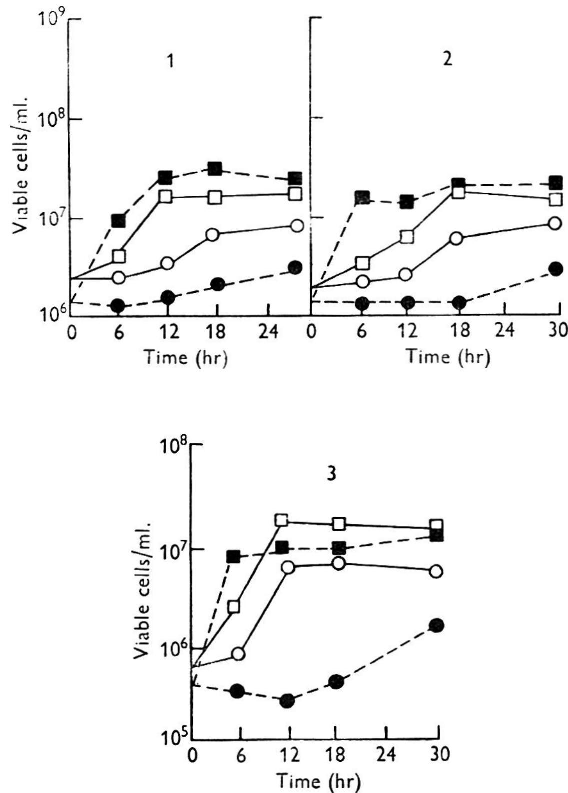

Fig. 1. Viable counts of virulent and avirulent group A streptococci in the presence and absence of Mn^{2+}. Concentration of Mn^{2+} was the same for all strains tested (4·0 μM). Medium was brain heart infusion neopeptone blood broth (Firshein & Zimmerman, 1964). Virulent, solid lines; avirulent, broken lines. (○) (●), with Mn^{2+}; (□) (■), control. 1. Strain M-18 (virulent) and 18AM⁻ (avirulent). 2. Strain M-6 (virulent) and 6AM⁻ (avirulent). 3. Strain Q-43X (virulent) and Q-496 (avirulent).

that were tested included Mg^{2+} (as $MgSO_4.7H_2O$), Zn^{2+} (as $ZnSO_4$), and Fe^{2+} (as $FeSO_4$). Manometric experiments were made as described in Firshein & Zimmerman (1964). As in this latter report, it was necessary to determine the effects of Mn^{2+} on the length and percentage of chains of cocci in liquid cultures An effect of Mn^{2+} on either variable could produce an error in counting the actual number of viable cocci (when this number is determined by plating serial dilutions of the cultures). It was found that Mn^{2+} had no effects on these variables.

RESULTS

Effects of Mn^{2+} on multiplication

Figure 1 shows the effects of Mn^{2+} on the multiplication of virulent and avirulent strains of group A streptococci in broth cultures. In all cases, the same concentration of Mn^{2+} (4·0 μM) depressed the rate and extent of multiplication of virulent strains and caused a 6–12 hr increase in the lag phases of avirulent strains. Table 1

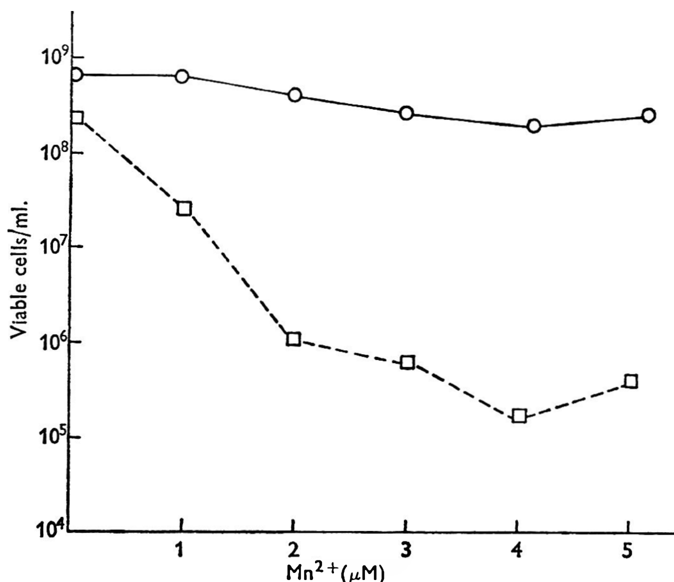

Fig. 2. Effects of concentration of Mn^{2+} on the number of viable cocci of a virulent and avirulent strain of group A streptococci after incubation for 18 hr. Medium was brain heart infusion neopeptone blood broth (Firshein & Zimmerman, 1964). Inocula ($\times 10^5$); strain Q-43x (virulent), 50; strain Q-496 (avirulent), 35. Virulent, solid line; avirulent, broken line.

Table 1. *Analysis of total yields of organisms and generation times for virulent and avirulent Group A streptococci in the presence and absence of Mn^{2+}*

Data are based on results of Fig. 1. Generation times are determined in the log phase of growth, which varied from strain to strain.

Organism	Yield of cocci after 18 hr (Count $\times 10^5$/ml.)		Relative decrease from control value*	Generation time (min.)		Relative increase from control value*
	Mn^{2+}	Control		Mn^{2+}	Control	
Q-43x (virulent)	80	300	3·75	52	48	1·1
Q-496 (avirulent)	6	200	33·3	144	23	6·4
M-18 (virulent)	50	170	3·1	190	60	3·1
18 AM⁻ (avirulent)	20	500	25	276	45	6·1
M-6 (virulent)	65	130	2·0	180	70	2·6
6 AM⁻ (avirulent)	15	150	10·3	90	18	5·0

* These values connote the number of times greater or less, the Mn^{2+}-supplemented values differ from the control values.

compares the generation times and total yields of avirulent and virulent organisms in the presence and absence of Mn^{2+}. After incubation for 18 hr, viable counts in the presence of Mn^{2+} were much smaller for avirulent cultures than for virulent cultures. Similarly, the rates of multiplication of avirulent strains were lower than those of virulent strains, as evidenced by changes in the generation times. The actual duration of the lag and log phases remained essentially unaltered in Mn^{2+}-supplemented and unsupplemented cultures of virulent strains. However, with avirulent strains, the length of these two phases was drastically altered in the presence of Mn^{2+}. Thus, by the time avirulent cocci from unsupplemented cultures had been in the stationary phase for 6–12 hr, avirulent cocci from cultures containing Mn^{2+} were just beginning to enter the log phase.

Table 2. *Effects of Mn^{2+} on multiplication of group A streptococci in different media*

Concentration of Mn^{2+} was 4·0 μM. Inocula ($\times 10^5$): strain Q-43x, 10; strain Q-496, 14; strain M-18, 6·8; strain 18 AM⁻, 12.

Medium	Organism	Additions		Viable cell count after 12 hr ($\times 10^{-5}$)
		Mn^{2+}	Control	
Difco Todd-Hewitt broth +0·5 % (v/v) defibrinated sheep blood	Q-43x (virulent)	+	−	50
		−	+	310
	Q-496 (avirulent)	+	−	15
		−	+	240
CAT medium (Marmur & Hotchkiss, 1955)	Q-43x	+	−	24
		−	+	105
	Q-496	+	−	4
		−	+	125
Difco Todd-Hewitt broth +0·5 % (v/v) defibrinated sheep blood	M-18 (virulent)	+	−	43
		−	+	305
	18 AM⁻ (avirulent)	+	−	7
		−	+	850
CAT medium	M-18	+	−	56
		−	+	110
	18 AM⁻	+	−	4
		−	+	710

By using a related pair of virulent and avirulent strains (Q-43x, Q-496), it was determined that maximum inhibition of multiplication for both strains occurred at a concentration of 4·0 μM Mn^{2+}, although, as suspected, the inhibition was much greater in avirulent cultures (Fig. 2).

Table 2 shows that the inhibitory effects of Mn^{2+} on multiplication of virulent and avirulent streptococci were not restricted to one medium, but occurred in other media that supported the multiplication of streptococci.

Effects of other cations on multiplication

It was found (Firshein, 1962) that the stimulatory effects of Mn^{2+} on multiplication of pneumococci were annulled by the addition of Ca^{2+}. Similarly, in studies with streptococci strains Q-43x and Q-496 (Table 3), a substantial portion of the inhibition of multiplication of both organisms by Mn^{2+} could be prevented by the addition of Ca^{2+} to a level of 4·0 μM.

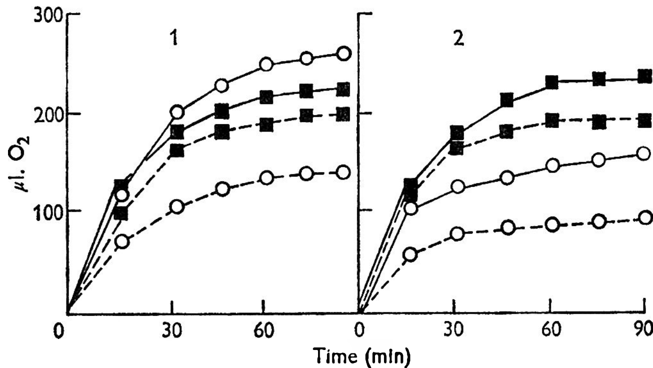

Fig. 3. Effects of Mn^{2+} on oxygen uptake of virulent and avirulent strains of group A streptococci in suspensions containing glucose. For concentration of Mn^{2+}, see Table 1. Concentration of glucose was the same as that in washed suspensions (50 μM). Endogenous rates are not shown on the graph because they were negligible for all strains tested. 1. Strain Q-43x (virulent) and Q-496 (avirulent). 2. M-18 (virulent) and 18 AM⁻ (avirulent). Solid lines, glucose alone; dashed lines, glucose + Mn^{2+}. (○), virulent; (■), avirulent. Inocula for all strains were adjusted to the same number, $8·5 \times 10^8$ viable cocci/ml.

Table 3. *Reversal of the inhibitory effects of Mn^{2+} on multiplication of streptococci by Ca^{2+}*

Medium was brain heart infusion neopeptone blood broth (Firshein & Zimmerman, 1964). Inocula: ($\times 10^5$), strain Q-43x, 24; Q-496, 15.

Organism	Additions (to μM) Ca^{2+}	Mn^{2+}	Viable count after 12 hr ($\times 10^{-5}$)
Q-43x (virulent)	·	·	340
	·	4·0	45
	0·4	4·0	80
	4·0	4·0	290
	8·0	4·0	200
	0·4	·	300
	4·0	·	245
	8·0	·	190
Q-496 (avirulent)	·	·	200
	·	4·0	10
	0·4	4·0	30
	4·0	4·0	250
	8·0	4·0	150
	0·4	·	200
	4·0	·	410
	8·0	·	300

Several other divalent cations did not exert inhibitory effects on multiplication of group A streptococci (Table 4). One cation, Mg^{2+}, enhanced the multiplication of virulent and avirulent strains slightly.

Effects of Mn^{2+} on respiration, nucleic acid synthesis, and protein synthesis

The ability of Mn^{2+} to affect respiration of pneumococci (Firshein, 1962) suggested a study of the effects of Mn^{2+} on respiration of group A streptococci. Using oxidation of glucose as a parameter of respiration, it was found (Fig. 3) with two related pairs of virulent and avirulent strains that Mn^{2+} depressed oxygen uptake in both virulent

Table 4. *Effects of Mg^{2+}, Zn^{2+}, and Fe^{2+} on multiplication of group A streptococci*

Since no inhibitory effects on multiplication were exerted by the cations tested, only the results with one concentration (indicated in parentheses) are shown. Medium was brain heart infusion neopeptone blood broth (Firshein & Zimmerman, 1964). Inocula ($\times 10^5$); strain Q-43x, 8; strain Q-496, 5; strain M-18, 15; strain 18AM⁻, 15; strain M-6, 20; strain 6AM⁻, 12.

Organism	Cation tested	Viable count after 12 hr ($\times 10^5$)
Q-43x (virulent)	Mg^{2+} (2·0 μM)	520
	Zn^{2+} (4·0 μM)	210
	Fe^{2+} (4·0 μM)	220
	None	300
Q-496 (avirulent)	Mg^{2+}	250
	Zn^{2+}	100
	Fe^{2+}	150
	None	105
M-18 (virulent)	Mg^{2+}	950
	Zn^{2+}	550
	Fe^{2+}	440
	None	550
18AM$^-$ (avirulent)	Mg^{2+}	1000
	Zn^{2+}	730
	Fe^{2+}	680
	None	700
M-6 (virulent)	Mg^{2+}	210
	Zn^{2+}	95
	Fe^{2+}	90
	None	100
6AM$^-$ (avirulent)	Mg^{2+}	450
	Zn^{2+}	220
	Fe^{2+}	160
	None	200

and avirulent-cell suspensions containing glucose. However, the inhibitory effects were more pronounced in virulent than in avirulent-cell suspensions, particularly for one pair (Q-43x and Q-496). These results were surprising in view of the fact that multiplication was depressed to a greater extent in avirulent strains than virulent strains. To test whether other metabolic systems in avirulent strains may have been affected by Mn^{2+}, measurements of nucleic acid and protein synthesis

were made in washed-cell suspensions in the presence and absence of the cation. In addition, effects of Mn^{2+} on nucleic acid and protein syntheses of virulent strains were determined. The results of these experiments, replicated several times (Table 5), demonstrated that: (1) nucleic acid synthesis and protein synthesis were depressed equally from control levels in suspensions of virulent strains; (2) only nucleic acid synthesis was depressed in suspensions of avirulent strains, protein synthesis was largely unaffected; and (3) nucleic acid synthesis was depressed to a much greater extent from control levels in suspensions of avirulent than virulent strains.

Table 5. *Effects of Mn^{2+} on nucleic acid and protein synthesis of Group A streptococci*

Concentration of Mn^{2+} was 4·0 μM. Preparation of washed cocci, suspending medium, extraction and measurement of nucleic acids and protein were as described previously (Firshein, 1961).

Organism	Additions		% increase over zero time after 70 min.		
	Mn^{2+}	Control	DNA	RNA	Protein
Q-43x (virulent)	+	−	50	45	42
	−	+	69	54	64
Q-496 (avirulent)	+	−	25	23	65
	−	+	68	71	74
M-18 (virulent)	+	−	14	10	15
	−	+	21	18	23
18AM⁻ (avirulent)	+	−	1	—	14
	−	+	13	14	16
M-6 (virulent)	+	−	21	20	18
	−	+	34	40	31
6AM⁻ (avirulent)	+	−	—	1	21
	−	+	24	25	19

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The Effect of Moisture on Gram Differentiation, and its Relation to Proposed Gram-Positive Substrates in Yeast and Other Organisms

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SUMMARY

Wet and dry Gram staining procedures have different characteristics in respect to their rates of decolorization. It has been proposed that this difference is due to a Gram-positive substrate which dissociates (becomes Gram-negative) in the presence of water. However, the differences between wet and dry Gram procedures can also be explained on the hypothesis that water influences the rate of solvent permeation through cell envelopes. Since no direct proof exists for the reality of the proposed Gram-positive substrate, and since evidence is presented here which cannot be explained by such a hypothesis, it is felt that the solvent permeation concept should be considered seriously as an explanation for the decolorization differences observed between wet and dry Gram procedures.

INTRODUCTION

The mechanism of the differentiation of bacterial cells by the Gram staining procedure is not clear. Some believe that Gram differentiation may be a permeability or permeation phenomenon (Benians, 1920; Burke & Barnes, 1929; Bartholomew, Cromwell & Finkelstein, 1959; Salton, 1963); some believe it to be due to differences in basic dye uptake (Stearn & Stearn, 1924; Barbaro & Kennedy, 1954; Lamanna & Mallette, 1954); and some believe it to be the result of some specific Gram-positive chemical component in the cell such as lipids (Schumacher, 1928), proteins (Deussen, 1921), nucleic acids (Henry & Stacey, 1943), carbohydrate (Webb, 1948), sulfhydryl groups (Fischer & Larose, 1952) and glycerophosphate (teichoic acids; Mitchell & Moyle, 1950, 1954). This variety of proposed Gram-positive components leads to the suspicion that no single component can adequately explain the phenomenon of Gram positivity.

Chelton & Jones (1959) supported the concept of a Gram-positive substrate. They observed that ruptured cells of Gram-positive bacteria and yeast would stain Gram-negative when water wet slides were subjected to a Gram procedure, but Gram-positive if a dry slide was processed. Intact cell preparations gave a Gram-positive result with either procedure. They proposed, therefore, that Gram-positive organisms contain a substrate which could form a complex with dye and iodine. This complex remained undissociated (Gram-positive) in the presence of 90 % (v/v) ethanol in water, but became dissociated (Gram-negative) in the presence of more water such as with 70 % (v/v) ethanol in water. Water wet slides would supply

sufficient water to dissociate this complex, even though a 90 % (v/v) ethanol decolorizer was used. Intact cells would not be subjected to this difference between wet and dry slides since their intact cell envelopes enabled them to control the ethanol concentration inside of the cell so that it never fell below 90 %. The mechanism of Gram-positivity was interpreted, then, as a combination of the presence of a Gram-positive component, and selective permeability to ethanol of the intact cell envelopes.

Bartholomew (1962) showed that intact cells, both Gram-positive and Gram-negative, were influenced by wet and dry Gram procedures, as well as by the water content of the decolorizer, in respect to the rate of decolorization. This presents the possibility that the differences observed by Chelton & Jones for ruptured cells can be explained without resorting to the concept of a Gram-positive component. The present paper presents the results of experiments to test this possibility.

METHODS

In the duplication of the work of Chelton & Jones (1959) all cell preparations and staining procedures were as described by them. However, since it was desired to have a more quantitative comparison of decolorization characteristics than that used by Chelton & Jones, a Gram procedure described by Bartholomew (1962) also was used. Briefly, the latter involved the substitution of Hucker's crystal violet for 0·5 % methyl violet 6 B, the use of 95 % (v/v) ethanol in water as a decolorizer rather than 90 % (v/v) ethanol in water and the use of a quantitative decolorization procedure. The quantitative decolorization procedure used 3 Coplin dishes (25 × 75 mm.) each filled with 70 ml. of ethanol + water and each used for one third of the total decolorization time, which was varied in each experiment from 5 sec. to 60 min. The water content of the decolorizer was prevented from increasing by use of a dish rotation system, in which after four slides the ethanol water mixture in the first dish was discarded, the dish refilled, and placed last in the sequence.

All organisms were from 24 hr cultures grown on an enriched nutrient agar, except for *Clostridium perfringens* which was grown on a semi-solid thioglycollate medium, and *Saccharomyces cerevisiae* which was grown on Sabouraud's agar, or obtained from yeast cakes. Organisms were disintegrated in a Mickle apparatus, with cooling in ice water after every minute of treatment. The time of treatment varied from 8 to 20 min. which was sufficient time, as observed with the microscope, to produce disruption of 90–99 % of the organisms. Centrifugation was done with a Servall Centrifuge at 3000 ***g*** for 15 min., followed by resuspension in ice water. After two such centrifugations and resuspensions in ice water the cell fragments were recentrifuged and only the upper portion of the pellet was resuspended in ice water. This suspension was centrifuged at 3800 ***g*** for 15 min. and the supernatant fluid decanted. After resuspension of the pellet in ice water, the suspension was used for preparation of the slides. In the reproduction of the Chelton & Jones experiments, the suspensions were very concentrated and resulted in areas in which cell fragments and other cell debris made the slide almost opaque. In our Gram procedures, the cell fragments were diluted so that they did not overlap.

In the dry Gram procedure the smears were treated with crystal violet solution, followed by iodine, washed in water, and then blotted and air dried. Just before

passage of these slides through the decolorizer, they were lightly flamed and cooled. In the wet Gram procedure, the smears were treated with crystal violet solution, iodine, then washed in water and processed wet through the decolorizer. In some experiments it was desired to have wet smears, but with no excess of water on the surface of the slide. For this, a modified wet Gram procedure was used. This consisted of staining the slides with crystal violet, treating with iodine, washing and then thoroughly drying. Following this, the slide was exposed to a strip of No. 1 Whatman filter-paper saturated with water. This wet filter-paper was pressed to the surface of the smear to assure moisture contact, and removed just before decolorization.

A Beckman model DB automatic recording spectrophotometer was used to obtain the light extinction curves.

RESULTS

The wet and dry Gram procedures gave different results for ruptured yeast cells, as reported by Chelton & Jones (1959). The last line of results in Table 1 shows that when using their procedure (procedure II, Table 1) ruptured yeast cells were Gram-positive when a dry procedure was used, but were Gram-negative when a wet procedure was used. Almost identical results were obtained, for a 2 min. decolorization time, when a quantitative Gram procedure (procedure I, Table 1) was used. The quantitative procedure, however, showed several additional facts. When the decolorization time was reduced to 15 sec. for the wet procedure, the yeast cell fragments could then be considered to be Gram-positive (as compared with the control organisms) despite the Gram-negative results obtained with a 2 min. decolorization time. Also, despite the Gram-positive nature of the ruptured yeast cells following a dry Gram procedure and a 2 min. decolorization time, they were Gram-negative following a 30 min. decolorization time. It was thus clear that the Gram-positive or Gram-negative characteristic of ruptured yeast cells, following a wet or dry Gram procedure, was a quantitative rather than a qualitatively determined characteristic.

Chelton & Jones reported that ruptured yeast cells stained Gram-negative with a dry Gram procedure when 70 % (v/v) ethanol in water was used as a decolorizer. They suggested that the 70 % (v/v) ethanol in water supplied sufficient water to allow the dissociation of the Gram-positive complex. It can be seen in Table 2 that when a dry Gram procedure and a 2 min. decolorization time were used ruptured yeast cells were Gram-negative when 70 % (v/v) ethanol in water was used as decolorizer. However, by decreasing the decolorization time to 30 sec., the ruptured cells were Gram-positive. According to the Chelton & Jones concept, a wet Gram procedure also using 70 % (v/v) ethanol in water as decolorizer would have two exposures to water sufficient to produce a Gram-negative result for ruptured yeast cells. Yet Table 2 shows that such a procedure produced a Gram-positive result when the decolorization time was decreased to 15 sec. It would appear that Gram differentiation was not solely a function of the effect of water on the presence or absence of a Gram-positive complex, but would be at least in part a function of the proper choice of a decolorization time to compensate for factors which influence rate of decolorization.

Chelton & Jones interpreted the Gram-negative results for ruptured yeast cells stained by a wet Gram procedure as due to the dilution of the decolorizer by the

Table 1. *A comparison of the Gram decolorization characteristic of intact Gram-positive and Gram-negative organisms with ruptured yeast cells, using wet and dry Gram procedures*

The Gram stain procedures used were those of Bartholomew (1962).

		Percentage of cells (or cell fragments) retaining the primary stain when stained by the indicated Gram procedure.											
		Dry Gram procedure						Wet Gram procedure					
		Intact Gram-positive organisms				Intact Gram-negative organisms		Intact Gram-positive organisms				Intact Gram-negative organisms	
Procedure	Total time of decolorization	*Sl*	*Bs*	Y	Ruptured yeast	*Ec*	*Sm*	*Sl*	*Bs*	Y	Ruptured yeast	*Ec*	*Sm*
	5 sec.	100	100	100	100	100	10	100	100	100	100	10	5
	15 sec.	100	100	100	95	20	10	100	100	100	95	0	0
	30 sec.	100	100	100	90	10	0	100	100	100	20	0	0
	60 sec.	100	100	100	90	0	0	100	100	100	0	0	0
	2 min.	100	100	100	90	0	0	95	90	100	0	0	0
	3 min.	100	100	100	70	0	0	95	85	100	0	0	0
	6 min.	100	100	100	50	0	0	90	85	90	0	0	0
	15 min.	95	95	100	40	0	0	20	70	80	0	0	0
	30 min.	70	90	100	0	0	0	10	20	70	0	0	0
	60 min.	20	40	100	0	0	0	0	0	60	0	0	0
II	2 min.	.	.	100	80	0	.	.	.	100	105	0	.

I = Bartholomew (1962) Gram procedure using Hucker's crystal violet, Burke's iodine, 95 % ethyl alcohol as the decolorizer, in 3 Coplin dishes, and 0·25 % safranin as counter stain.

II = As per Chelton & Jones (1959). Jensen's Gram procedure using 0·5 % methyl violet 6B, Lugol's iodine, 90 % ethyl alcohol on the slide for 2 min. to decolorize, and 1/20 Ziehl-Neelson carbol fuchsin as the counter stain.

Sl = *Sarcina lutea*; *Bs* = *Bacillus subtilis*; Y = yeast, *Saccharomyces cerevisiae*; *Ec* = *Escherichia coli*; *Sm* = *Serratia marcescens*. Wet Gram procedure = wet slide processed through the decolorizer. Dry Gram procedure = slide air dried and flame dried before processing through the decolorizer.

Table 2. *The effect of water in the decolorizer on the Gram decolorization characteristic of intact and ruptured organisms as shown by wet and dry Gram procedures and using 70 %, 95 % and 100 % ethyl alcohol as the decolorizer*

The numbers shown represent the percentage of cells or cell fragments which retained the primary stain (Gram-positive).

Total time of decolorization	Dry Gram 70% ethanol			Wet Gram 70% ethanol			Dry Gram 95% ethanol			Wet Gram 95% ethanol			Modified wet Gram 95% ethanol			Modified wet Gram 100% ethanol		
	Intact yeast	Ruptured yeast	Intact *E. coli*	Intact yeast	Ruptured yeast	Intact *E. coli*	Intact yeast	Ruptured yeast	Intact *E. coli*	Intact yeast	Ruptured yeast	Intact *E. coli*	Intact yeast	Ruptured yeast	Intact *E. coli*	Intact yeast	Ruptured yeast	Intact *E. coli*
5 sec.	100	100	95	100	95	0	100	100	100	100	100	10	100	100	20	100	100	10
15 sec.	100	95	10	100	90	0	100	95	20	100	95	0	100	95	0	100	90	0
30 sec.	100	95	5	100	20	0	100	90	10	100	40	0	100	10	0	100	10	0
60 sec.	100	50	0	100	0	0	100	90	0	100	0	0	100	5	0	100	5	0
2 min.	100	20	0	70	0	0	100	90	0	100	0	0	100	0	0	100	0	0
3 min.	100	0	0	50	0	0	100	70	0	100	0	0	100	0	0	100	0	0
6 min.	100	0	0	30	0	0	100	50	0	90	0	0	95	0	0	100	0	0
15 min.	100	0	0	10	0	0	100	40	0	80	0	0	85	0	0	95	0	0
30 min.	100	0	0	10	0	0	100	10	0	80	0	0	80	0	0	85	0	0
60 min.	100	0	0	5	0	0	100	0	0	70	0	0	70	0	0	70	0	0

The wet and dry Gram procedures were the same as procedure I, Table 1. Modified wet Gram procedure was the same as for the Dry Gram procedure. However, just before decolorization the smears were exposed to water by contact with a small piece of filter paper saturated with water. After pressing to the slide the paper was removed and the slide immediately processed through the decolorizer.

Table 3. *A comparison of wet and dry Gram procedures in the demonstration of the Gram characteristic of intact cells, and cell fragments*

95% (v/v) ethanol in water used as the decolorizer.

Total time of decolorization	Dry Gram procedure: Intact cells						Dry Gram procedure: Ruptured cells						Wet Gram procedure: Intact cells						Wet Gram procedure: Ruptured cells					
	Sa	*Sl*	*Bs*	*Clp*	Y	*Ec*	*Sa*	*Sl*	*Bs*	*Clp*	Y	*Ec*	*Sa*	*Sl*	*Bs*	*Clp*	Y	*Ec*	*Sa*	*Sl*	*Bs*	*Clp*	Y	*Ec*
5 sec.	100	100	100	100	100	100	+	+	+	+	+	+	100	100	100	100	100	50	+	+	+	+	+	+
15 sec.	100	100	100	100	100	20	+	+	+	+	+	−	100	100	100	100	100	0	+	+	+	+	+	−
30 sec.	100	100	100	95	100	10	+	+	+	±	+	−	100	100	100	100	100	0	−	+	+	−	−	−
60 sec.	100	100	100	90	100	0	+	+	+	±	+	−	100	100	100	80	100	0	−	−	−	−	−	−
2 min	100	100	100	85	100	0	+	+	±	±	+	−	60	95	90	70	100	0	−	−	−	−	−	−
3 min.	95	100	100	50	100	0	+	+	±	−	+	−	20	95	85	40	100	0	−	−	−	−	−	−
6 min.	50	100	100	50	100	0	±	+	−	−	+	−	20	90	80	30	90	0	−	−	−	−	−	−
15 min.	5	50	80	10	100	0	−	−	−	−	±	−	0	20	50	5	80	0	−	−	−	−	−	−
30 min.	0	20	50	5	100	0	−	−	−	−	±	−	0	20	20	0	80	0	−	−	−	−	−	−
60 min.	0	0	40	0	95	0	−	−	−	−	±	−	0	10	0	0	70	0	−	−	−	−	−	−

Wet and dry Gram procedures were the same as procedure I, Table 1. Figures given represent the percentage of intact cells staining Gram-positively. + = Significant amount of Gram-positive areas as described by Chelton & Jones (1959). − = All material Gram-negative. *Sa* = *Staphylococcus aureus*, *Sl* = *Sarcina lutea*, *Bs* = *Bacillus subtilis*, *Clp* = *Clostridium perfringens*, Y = Yeast, *Saccharomyces cerevisiae*, *Ec* = *Escherichia coli*.

water on the slide, such dilution supposedly resulting in dissociation of the Gram-positive complex. The wet Gram procedure was therefore modified to assure that the decolorizer was in no significant way diluted by water from the slide. The slide itself was dry except for the smear area which had been exposed to wet filter-paper. The slide was then processed through three Coplin dishes, each containing 70 ml. of 95 % (v/v) ethanol in water in some experiments, and absolute ethanol in other experiments. It would have been impossible for the water in the cells to have diluted the 95 % or 100 % ethanol to any significant degree. Yet under these conditions, as shown in Table 2, the results were typical for the usual wet procedure. Certainly, the decolorization differences observed between the modified wet procedures and dry procedure cannot be explained by water dilution of the decolorizer.

Table 3 presents the results obtained with intact and ruptured organisms of many species of Gram-positive bacteria, as compared with *Escherichia coli*. With the 2 min. decolorization time of Chelton & Jones, and 95 % (v/v) ethanol in water as decolorizer, the ruptured organisms were Gram-positive with a dry Gram procedure and Gram-negative with a wet procedure, as Chelton & Jones would have predicted. However, whether ruptured organisms were Gram-positive or Gram-negative depended on the decolorization time. With the dry Gram procedure, ruptured organisms were Gram-positive with a 2 min. decolorization time, but Gram-negative with a decolorization time of 15 min. or longer. With the wet Gram procedure, ruptured organisms were Gram-negative for a 2 min. decolorization time, but Gram-positive for a 15 sec. decolorization time. It would thus appear that, as for intact and ruptured yeast cells, the length of the decolorization time was more relevant to Gram differentiation than was the presence or absence of water in the smear. Our results which show the proper Gram differentiation of ruptured Gram-positive organisms are of particular interest since it has often been reported that such ruptured organisms are Gram-negative (Benians, 1912, 1920; Burke & Barnes, 1929). It can be seen (Table 3) that such differentiation would not have been detected if a wet Gram procedure were used with a single 2 min. decolorization time. However, it was detected with either a wet or dry Gram procedure, when a quantitative decolorization procedure was used. The ability to properly differentiate ruptured organisms demonstrates the fact that the cell envelopes by themselves could be an important contributing factor to Gram differentiation.

Chelton & Jones (1959) further supported their concept of a Gram-positive substrate as part of the mechanism of Gram positivity by presenting evidence which they believed disproved the concepts involving permeability. They (also Burke, 1922) reported that ethanolic solutions of the dye iodine precipitate would stain intact Gram-positive organisms. This was interpreted as showing that the large dye iodine molecule could permeate through the envelopes of Gram-positive organisms, therefore no permeability barrier to it existed. Such a barrier to the loss of the dye iodine precipitate inside of a Gram-positive organism, therefore, could not be used to explain the mechanism of Gram positivity. While such a barrier has been proposed (Benians, 1920; Burke & Barnes, 1929), this concept has long been discounted by Stearn & Stearn (1930) who reported that ethanolic solutions of the precipitate gave identical light extinction peaks as for ethanolic solutions of the iodine and crystal violet alone. This, along with boiling point data, led them to conclude that no new molecule was produced on precipitate formation, which on redissolution in ethanol

did not revert to the original dye and iodine molecules as independent entities. Stearn & Stearn did not publish light extinction curves and therefore such curves are shown in Fig. 1. It can be seen that the extinction curves for Burke's iodine, or crystal violet alone, were identical with those obtained when the dye iodine precipitate was dissolved in ethanol. Thus, the ability of ethanolic solutions of the precipitate

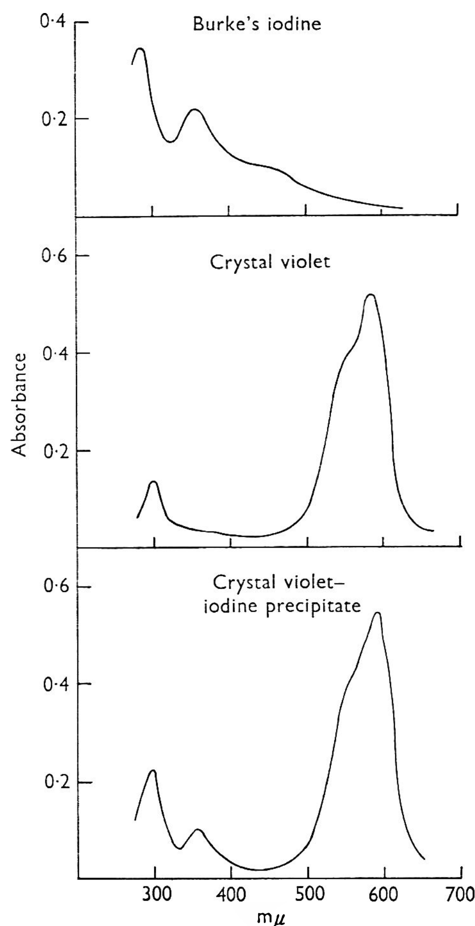

Fig. 1. Extinction spectra of Burke's iodine, crystal violet, and crystal violet + Burke's iodine precipitate dissolved in 95 % (v/v) ethanol in water.

to stain intact Gram-positive organisms would not be evidence against a permeability concept since the hypothetical large dye iodine molecule does not exist in ethanolic solutions. However, the non-existence of such a large dye iodine molecule does not destroy all permeability concepts, since permeability differences could exist for the dye or iodine molecules alone, or for the solvent itself.

DISCUSSION

It is not necessary to propose a Gram-positive component with the dissociation characteristics suggested by Chelton & Jones (1959) to explain the differences in decolorization speeds of wet and dry Gram procedures. Another explanation would be that water in the cell envelopes, or in the decolorizer, could have a marked speeding effect on decolorization. That water has this effect can be well documented (Benians, 1920; Burke, 1922; Burke & Barnes, 1929; Wensinck & Boevé, 1957; Tucker & Bartholomew, 1962; Bartholomew, 1962). Thus, if a single decolorization time were used for both wet and dry Gram procedures (as Chelton & Jones) the results obtained might be misleading concerning the Gram nature of an unknown, even though comparisons were made with known organisms. Table 1 shows that when a single 2 min. decolorization time was used, ruptured yeast cells appeared to be Gram-positive with a dry procedure, and Gram-negative with a wet, as compared to known controls. However, had a 15 sec. decolorization time been used, ruptured yeast cells would have appeared to be Gram-positive, and if a 30 min. time had been used Gram-negative, with both wet and dry procedures. In both examples, although the opposite Gram results were obtained the results appeared to be valid as compared to the control organisms included in the experiments. The Gram-positive (dry) and Gram-negative (wet) results reported by Chelton & Jones for ruptured yeast cells, then, were the result of the choice of a single decolorization time for two very different Gram procedures, rather than to any real change from a Gram-positive to a Gram-negative state. The true state of affairs as to the Gram nature of ruptured yeast cells is shown in Tables 1–3. Here it can be seen that with either wet or dry procedures ruptured cells of Gram-positive organisms decolorized faster than corresponding intact cells, and slower than either the intact or ruptured cells of Gram-negative organisms. Thus, the ruptured cells were more Gram-positive than intact Gram-negative cells, but more Gram-negative than intact Gram-positive cells. This fact could not be determined from the results obtained with only one decolorization time, but could be determined only by comparing the results obtained from a series of decolorization times. The question which arises then, is what criteria can be used to evaluate the validity of results obtained with any single Gram procedure. The answer is simple if one is only concerned with intact cells and if one closely follows the ordinarily recommended Gram procedures (Bartholomew, 1962). However, if one studies ruptured organisms, or used heat in staining, or changes the decolorizer or decolorization procedure, and if at the same time one sticks to a single decolorization time, then all that emerges blue is not necessarily Gram-positive. Some of the criteria for the recognition of true Gram-positivity (in addition to rate comparisons) have been presented by Bartholomew, Mittwer & Finkelstein (1959).

A Gram-positive substrate, with the dissociation characteristics proposed by Chelton & Jones, was not confirmed by our results. Wet Gram procedures which prevented any significant water dilution of a 100 % ethanol decolorizer gave results (Table 2, modified wet Gram) as expected for wet procedures, not dry. Thus, the rapid decolorization effect of 'wet' procedures could not be due merely to the dissociation of a Gram-positive complex when exposed to ethanol (70 %, v/v) diluted by water. Furthermore if, as proposed, the Gram-positive substrate is dissociated in the presence of 70 % (v/v) ethanol in water, then when such a decolo-

rizer is used it should not be possible to obtain a Gram-positive result for ruptured yeast cells. Yet Table 2 shows that this result could be obtained for both wet (15 sec. decolorization) and dry (30 sec. decolorization) Gram procedures. Our results do not support the suggestion that intact Gram-positive organisms would not be influenced by water in the decolorizer because of their ability to control the ethanol concentration inside of the cell. If this were true then intact Gram-positive organisms should not be influenced in their decolorization rate by wet or dry Gram procedures, or by water in the ethanol decolorizer. Our results, however, showed that all organisms, intact or ruptured, Gram-positive or Gram-negative, were decolorized more rapidly by a wet than by a dry Gram procedure. A very conclusive argument against the formation of a Gram-positive substrate dye iodine complex is the fact that it has been demonstrated that cells stained with iodine alone (Bartholomew, Cromwell & Finkelstein, 1959) or dye alone (Benians, 1920; Bartholomew & Mittwer, 1950) could be properly Gram differentiated if care were taken with the decolorization procedure. Thus, the Gram-positive characteristic could not be due entirely to any such proposed complex.

If a dissociating Gram-positive complex is not responsible for the differences between wet and dry Gram procedures, how can the differences be explained? They can be explained by a concept involving the permeation rate of solvent through cell envelopes. If the dye iodine precipitate were dispersed through the whole cell or cell fragments, and if ethanol permeated the cell envelopes of Gram-positive organisms, then ruptured as well as intact Gram-positive organisms should reflect this difference in their speed of decolorization. That is, intact organisms would decolorize slower than ruptured organisms due to the lesser cell envelope surface exposed for ethanol permeation, and to the greater amounts of dye iodine precipitate present in the intact cell as compared to the fragmented envelopes. Intact or fragmented Gram-positive organisms would be slower to decolorize than intact or fragmented Gram-negative organisms since the envelopes of Gram-negative organisms possess very little resistance to ethanol permeation.

Finally, Gram differentiation is basically a rate difference in the speed of decolorization between Gram-positive and Gram-negative organisms. Therefore, under conditions where fine differences must be detected, this rate difference must be quantitatively determined. Such quantitative determinations are not possible using the usual Gram procedures with a single decolorization time. If all of the known variables of the Gram stain procedure were kept constant (Bartholomew, 1962), then a single decolorization time known to give valid differentiations could be used with a fair degree of confidence. If, however, the known variables are changed, and if determinations of the Gram nature of an unknown substrate under different conditions are to be made, then the only valid experimental procedure is to use some form of a quantitative Gram procedure.

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Nuclear Division in the Dinoflagellate *Gonyaulax tamarensis*

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SUMMARY

The interphase nucleus of *Gonyaulax tamarensis* is U-shaped with a lens-shaped central body lying between the arms of the U. At the beginning of division the chromosomes become arranged around the central body and can be seen to be split into pairs of chromatids which are held together by relational coiling. The pairs lie across the plane of the equator. The beginning of anaphase is obscure but as the chromatids separate into the daughter nuclei they exhibit a variety of arrangements which seem to exclude the possibility of localized centromere and normal spindle. The central body divides into two at this stage. During telophase the ball of chromosomes breaks open and the chromosomes lose some condensation as they twist together and the U-shaped interphase nucleus is re-established. In some of these features Gonyaulax differs from other dinoflagellates which have been studied as well as exhibiting a vastly different nuclear division to that found in higher organisms. The central body behaves like the endosome of the Euglenophyta but also has similarities with the central spindle reported from certain flagellates and diatoms.

INTRODUCTION

Recent studies of the nuclear structure and division in the Dinophyceae (= algal division Pyrrophyta) have shown (Skoczylas, 1958; Dodge, 1963*a*) that the nucleus differs in many respects from the normal state found in most animals and plants. The differences are seen in the construction of the nucleus, and its component parts, and also in its behaviour during mitotic division. Earlier workers in this group reported that the nucleus had many strange attributes and some of these have now been confirmed, but many others have of necessity to be discarded. The present need is for detailed studies of a wide range of species to make it possible to see whether the nuclear behaviour of say Ceratium or Prorocentrum is the typical pattern throughout the group or alternatively whether some variety exists.

The present paper sets out to describe in some detail the nuclear division of *Gonyaulax tamarensis* Lebour, the nucleus of which has not been studied before. A few comparisons are made concerning the closely related species *G. polyedra* Stein. The nucleus in this genus, whilst showing many Dinoflagellate characteristics, also poses several new problems for investigation and interpretation. The more important of these are the considerable change in the shape of the nucleus, the anaphase segregation of chromatids and the function of what is called here the 'central body'. The genus *Gonyaulax* is indeed interesting and problematical.

METHODS

Unialgal cultures of *Gonyaulax tamarensis* (Plymouth number 173) were supplied by Dr M. W. Parke of the Plymouth Laboratory of the Marine Biological Association of the U.K. They originated as single cell isolates made from sea water collected in the Tamar estuary where Lebour (1925) obtained material for the first description of the species. Cultures were maintained (with some difficulty) in enriched sea water under artificial illumination by the methods already described (Dodge, 1963*a*). Fixed material of *G. polyedra* was supplied by Dr B. M. Sweeney of Yale University.

Gonyaulax tamarensis was fixed in methanol, mordanted with ferric acetate and stained with aceto-carmine. Examination and photography were carried out on temporary preparations as previously described. The chromosomes stained readily and no special methods were needed.

RESULTS

The interphase nucleus

The non-dividing nucleus of Gonyaulax (both species) must be one of the most unusual yet recorded for any organism, perhaps with the exception of the macronuclei of certain ciliates. The typical form can be seen in Fig. 1A and Pl. 1, fig. 1, which show the horseshoe or U-shape. This form is lost during division. Only one other member of the Dinophyceae, a freshwater Peridinium species, is known to have a similar nucleus. The chromatin consists of long intertwined threads which are clearly chromosomes, although often they are not so strongly condensed as are the interphase chromosomes of Prorocentrum (Dodge, 1963*a*). They appear to be arranged more or less spirally in the nucleus, but the large number makes it impossible to follow the orientation of any individual chromosomes. The smooth outline of the nucleus with no projecting chromosomes (unlike Prorocentrum) suggests the presence of a membrane around the nucleus at this stage.

The interphase chromosomes stain readily with the Feulgen stain, which Hastings & Sweeney (1964) have also observed for *Gonyaulax polyedra*, and with methyl green. As with other Dinoflagellates (Dodge, 1964) the chromosomes appear to contain a considerable amount of deoxyribose nucleic acid (DNA).

Situated between the arms of the nucleus is a large, almost structureless, lens-shaped body (Fig. 1; Pl. 1, fig. 1). This body is probably homologous with the nucleolus of other organisms. However, because of its strange behaviour during mitosis (see later) and its position adjacent to the interphase nucleus but not within it, the non-committal term 'central body' will be used to describe it. The central body contains no DNA but does appear to contain ribose nucleic acid as evidenced by a certain amount of staining with pyronin.

The nucleus in division

The first indication that nuclear division is about to commence is seen when the nucleus begins to change its shape. The U-shape is obviously an inconvenient one for the processes involved in mitosis. Thus the distal ends of the nucleus come together and the whole nucleus forms a ball around the central body (Fig. 1B, C). The chromosomes, whilst becoming more condensed, are also reorientated and take up

the position of lines of longitude around a globe, the central body being visible at the top and bottom as 'polar caps'. Towards the end of this process it becomes clear that the chromosomes have split longitudinally into pairs of relationally coiled chromatids. A simplification of such a stage is shown in Fig. 1 D and two focal levels of an

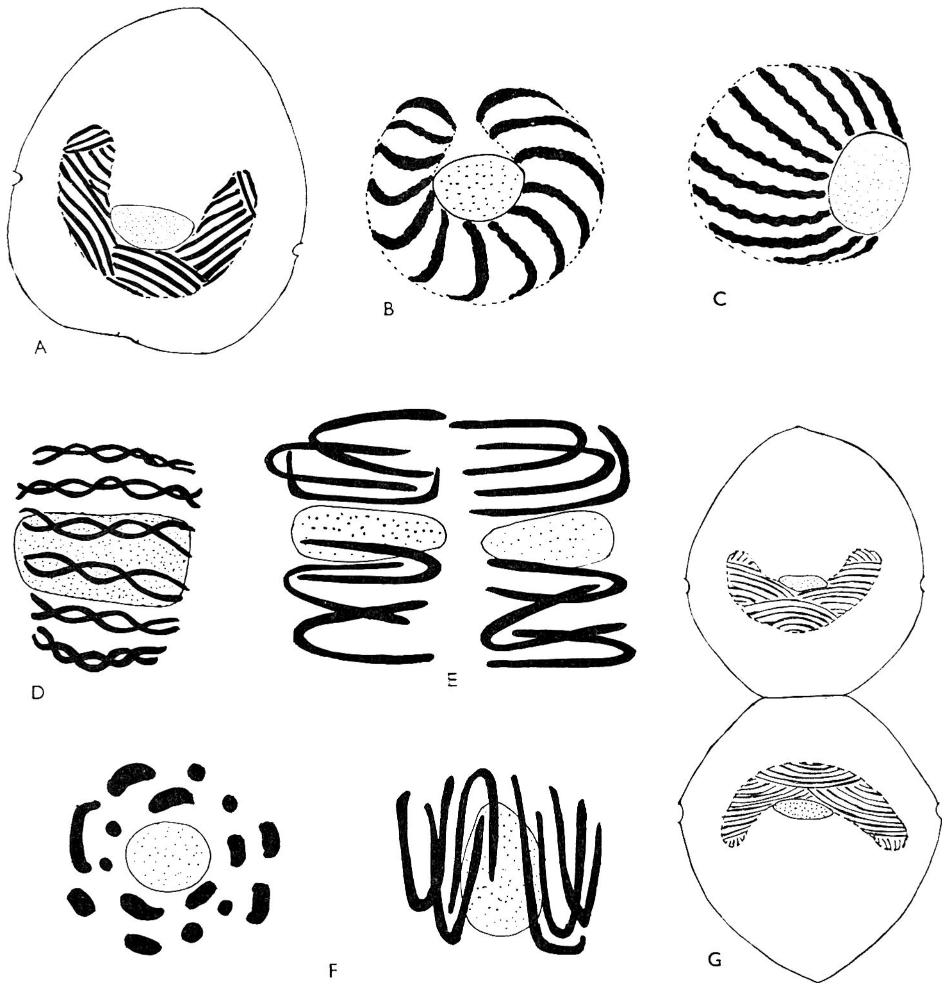

Fig. 1. Diagrams to illustrate some of the processes taking place in the mitosis of Gonyaulax. For clarity only a small number of chromosomes are shown (not all to same scale). A. Cell with interphase nucleus and central body. B. Early prophase: the arms of the nucleus begin to wrap around the central body; the chromosomes become more distinct. C. Mid-prophase 'globe' formation now complete. D. Late prophase or pre-metaphase, chromosomes split into pairs relationally coiled together and lying parallel to the central body. E. Mid-anaphase, showing some of the shapes of the separating chromatids. F. Late anaphase: change of orientation of daughter nuclei has begun; one nucleus in polar view, other in side view (cf. Pl. 4, fig. 13). G. Telophase. The interphase state of nucleus almost re-established but cell cleavage is not quite complete.

actual nucleus are shown in Pl. 1, figs. 2, 3. In Pl. 1, fig. 3, the focal level passes through the central body, which does not stain with aceto-carmine and so is observed only as a region lacking chromosomes. This mitotic figure shows the considerable tangle which

exists due to the large number of very long chromosomes. However, it is clear that the orientation of the pairs of chromatids is mainly at right-angles to the 'equator' and parallel to the central body. This stage might best be described as a pro-metaphase. If a nuclear membrane is present around the interphase nucleus it has clearly disappeared by now.

The chromatids probably contract somewhat in length, and by rotation lose the relational coils which have been holding sister chromatids together. The anaphase separation possibly begins whilst this process is taking place. Plate 1, fig. 4, shows an early anaphase where separation has just begun, but the tangling of the chromatids and the depth of the nucleus effectively conceals the method being employed.

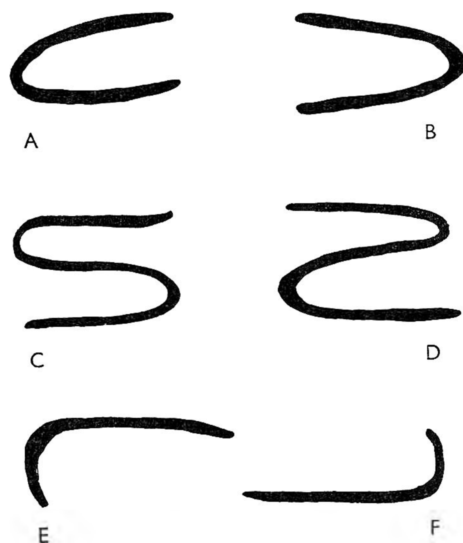

Fig. 2. Diagrams illustrating some of the shapes shown by separating chromatids at mid-anaphase, *in situ* the 'equator' would be situated to the right of the figure. A, B are very common types and C–F are rather rare. (Details in text.)

When the daughter groups of chromatids have completely separated from one another at mid-anaphase the orientation of individuals can be more clearly discerned (Fig. 1E; Pl. 2, figs. 5–8; Pl. 3, figs. 9–12). A careful analysis of such stages has shown the chromatids to be arranged in at least six different ways (Fig. 2A–F). The most frequent form is the U-shape (Fig. 2A, B), which can have the open end of the U pointing either towards the 'equator' or towards the 'poles'. The S-shapes (Fig. 2C, D) are less frequent and could conceivably be misinterpretations of two oppositely orientated U-shaped chromosomes lying partly over each other. Alternatively, it may be that the S is the normal form and the U is a misunderstanding of the parts of the S. Another arrangement is the right-angle type (Fig. 2E, F) with the short arm either at the polar or the equatorial end of the daughter nucleus.

At first it was thought that the normal arrangement here was the very unusual one (type A or B) of the chromatids in one nucleus with their open end (of the U) facing the equator and in the other the open end facing the pole. This was found to be an over-simplification for when a careful analysis was made of all the U-shaped

chromatids which could be clearly distinguished in photographs of eight anaphases, results were obtained which made this seem unlikely (see Table 1).

Assuming that any chromatid shape found in one daughter nucleus would be either the same or the opposite of what its sister chromatid formed in the other daughter nucleus we can suggest four possible combinations of chromatids all of which should give rise to a one to one ratio between the two nuclei. From the table it can be seen that the two unusual types A and B gave ratios of 3:1 and 1:2 respectively. These therefore appear unlikely to be the normal arrangements of the separating chromatids. The third type (X), which is also the usual type found in higher

Table 1. *The shape and arrangement of certain chromatid types in the mid-anaphase of Gonyaulax*

Arrangement of chromatids in the daughter nuclei	No. of chromatids		Ratio, left nucleus:right	
	Left nucleus	Right nucleus	Expected	Found
A ⊂ ⊂	45	15	1:1	3:1
B ⊃ ⊃	22	43	1:1	1:2
X ⊂ ⊃	45	43	1:1	1:1
Y ⊃ ⊂	22	15	1:1	4:3

plants (although there the form is more V-shaped), will be seen to give a clear 1:1 ratio, which is also very nearly given by the opposite of the usual arrangement in the fourth possibility (Y). As two-thirds of the chromatids which were analysed could be said to be in type X and as this gave the expected ratio it would seem likely that this is the normal manner of mid-anaphase separation. However, from the large number of chromatids giving other shapes, such as the 37 in the table and the S and right-angle forms mentioned earlier, it is clear that the process is not uniform and that the division mechanism used here allows much scope for variety of chromatid form.

The central body is in existence throughout the anaphase and can clearly be discerned (Pl. 3, figs. 9, 10) as a chromosome-free region running through the centre of the nucleus. At this mid-anaphase stage it had become divided into two and one half remained in each daughter nucleus.

The late anaphase exhibits two processes, the further separation of the two daughter nuclei combined with a change in orientation of each through ninety degrees. Such a stage is shown in Pl. 4, fig. 13, where one daughter nucleus can be seen in a 'polar' view and the other from the side. In the former the central body is surrounded by concentric rings of chromosomes, perhaps as many as 150 in number (Dodge, 1963*b*). In the latter a focal section gives a picture of the chromosomes lying alongside the central body.

The final stage of the division, telophase, is occupied as is usual by the reconstitution of the interphase nucleus. This takes place by a reversal of the early prophase changes; the chromosomes of each daughter nucleus separate opposite the anterior end of the new cell and, perhaps whilst becoming less condensed and being arranged in a somewhat spiral fashion, are partially detached from the central body to give the two arms of the nucleus. In Pl. 4, fig. 14, is shown a pair of cells in which this process has just occurred and the two new interphase nuclei can be seen to be mirror images of each other. Finally the two daughter cells, which have been acquiring

their own cell walls since late anaphase, separate and the nuclear and cell division is complete.

Gonyaulax polyedra has been briefly examined (and by Hastings & Sweeney, 1964). The stages which were observed appeared to be identical with those described above for *G. tamarensis*.

DISCUSSION

The nuclear division of Gonyaulax is unusual if not unique. Not only does it possess the characteristic Dinoflagellate features of permanent condensation of the chromosomes and the pre-anaphase arrangement of the pairs of chromatids across the plane of the equator, but it also has its own distinctive changes in the shape of the nucleus and the strange behaviour of the central body. At present very few representatives of the Dinophyceae have been studied and although these represent a fair range of genera it is quite possible that a similar behaviour will be found in other organisms belonging to this group.

One of the major problems in Gonyaulax is the process of anaphase separation of the sister chromatids. The vital early stage is made so complicated by the large number of long chromatids that little detail has so far been observed. It is obviously insufficient to say that 'the nucleus pulls apart like a skein of yarn' (Hastings & Sweeney, 1964), but do the chromatids separate by a gliding movement as seems to happen in Prorocentrum (Dodge 1963*a*), or are they pulled apart first from their mid-points leaving the distal arms to unwind as reported for Ceratium (Skoczylas, 1958), or is a completely different process in operation here? At a slightly later stage of anaphase it is possible to make out the configuration of many of the separating chromatids. This has revealed that, as in certain members of the Euglenophyta (Leedale, 1958), a considerable variety of chromatid shapes are present. Although the majority of the chromatids counted in the present study showed what may be called the typical U-shape, facing the equator, it would be unwise to make any deductions about the mechanism of anaphase movement for, as has been found in Prorocentrum, this shape can quite easily be due to secondary factors after the main separation has taken place. One other important fact is that neither in the Euglenophyta (Leedale, 1958) or in Gonyaulax is there any evidence for diffuse centromeres or a normal spindle. This fact is disputed by Skoczylas (1958) and von Stosch (1958), who recognize both localized centromere and spindle in Ceratium, but the variety of chromatid arrangement in Gonyaulax definitely argues against this for spindle and centromeres should surely result in a more or less uniform, ordered, anaphase segregation.

The central body provides another interesting problem. In appearance and behaviour it is not unlike the central spindle which has been described for many members of the Hypermastigina (as Barbulanympha—Cleveland, 1958), but in these organisms the spindle arises from prominent centrioles which do not appear to be present in Gonyaulax or any other Dinoflagellate. Central spindles have also been described for a number of diatoms (Lauterborn, 1896; Geitler, 1927) and for the strange flagellate (perhaps Dinoflagellate) Noctiluca (Ishikawa, 1894; Calkins, 1899). The function of the central spindle in Barbulanympha is said to be purely passive, providing a track for the chromosomes to be pulled along by traction fibres rather like those found in a normal spindle.

In several respects the central body is similar to the endosome (= persistent nucleolus) which is universally present in the Euglenophyta (Leedale, 1958; and earlier workers) and which is also present in at least one member of the Dinophyceae, *Oxyrrhis marina* (Hall, 1925; Dodge, 1963*a*). Although the endosome usually becomes stained in cytological preparations, which the central body does not, during division its behaviour is almost identical with that of the central body as it normally runs through the centre of the dividing nucleus and breaks into two at anaphase. In Euglena and perhaps Oxyrrhis a persistent nuclear membrane surrounds chromosomes and endosome. In Gonyaulax, however, there is clearly no membrane around the dividing nucleus and there is little evidence for the presence of a membrane during the interphase.

It is easy to visualize some form of connexion between certain of the chromosomes and the central body perhaps like the nucleolar-organizing chromosomes known from many other organisms. If this is in fact the case in Gonyaulax and if further investigation suggests that the central body plays an active part in the division processes of anaphase separation and the changes in shape of the nucleus, then it may be necessary to revert to the traditional terminology of 'central spindle' for this body.

I am grateful to Dr M. W. Parke for supplying the cultures of *Gonyaulax tamarensis* and to Dr B. M. Sweeney for the fixed material of *G. polyedra*.

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EXPLANATION OF PLATES

All photographs are of aceto-carmine-stained cells in temporary preparations of *Gonyaulax tamarensis* × 2000.

Plate 1

Fig. 1. Interphase nucleus showing the appearance of the tightly wound chromosomes and the large central body between the two arms of the nucleus.

Figs. 2, 3. Two focal levels of a late prophase nucleus. Note the central body in fig. 3 and the pairs of chromatids lying more or less parallel to this.

Fig. 4. A very early anaphase showing the beginning of chromatid separation (top focal level).

Plates 2 and 3

Figs. 5–12. Here four pairs of photographs illustrate four focal levels through a single mid-anaphase division stage. In the second print of each pair (right-hand side) some of the chromosomes have been overdrawn with indian ink to make more clear the variety of shapes exhibited at this stage (for further explanation see text). In figs. 9, 10 note the clear central region in both daughter groups of chromosomes which is due to the presence of the divided central body.

Plate 4

Fig. 13. A late anaphase with the daughter nucleus at the left in 'polar' view and at the right in side view and optical section.

Fig. 14. The end of division with the interphase nuclei now re-established and cell cleavage almost complete.

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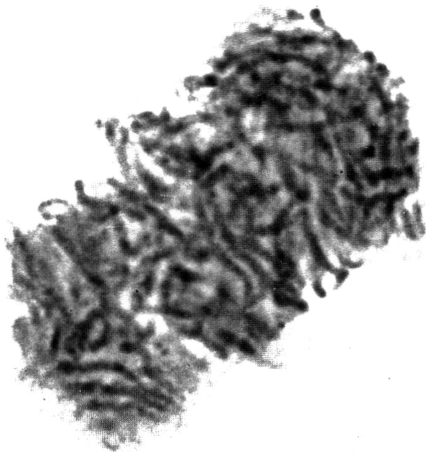

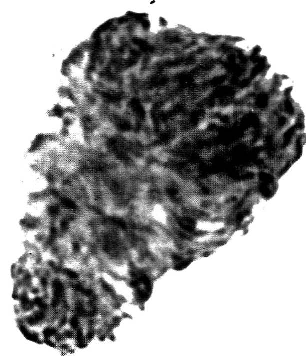

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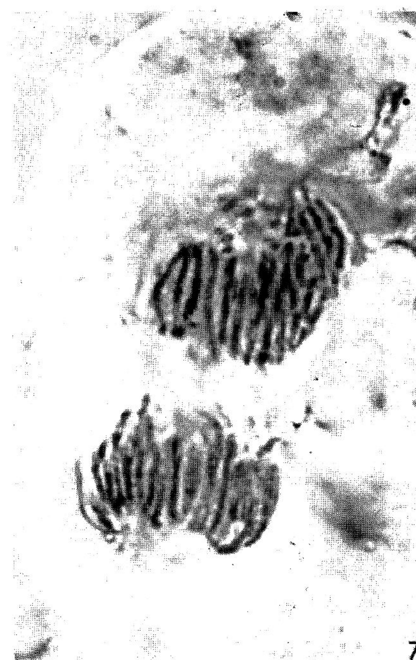

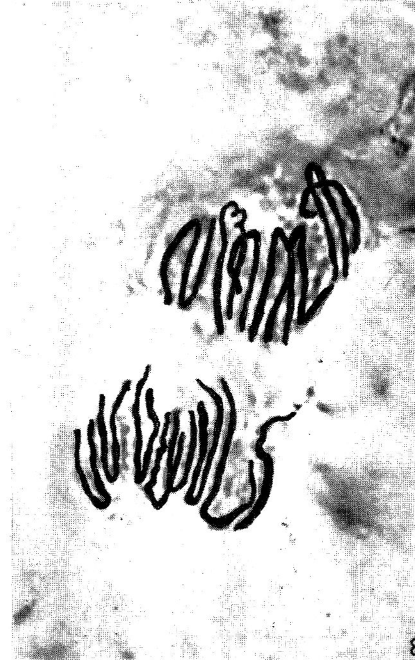

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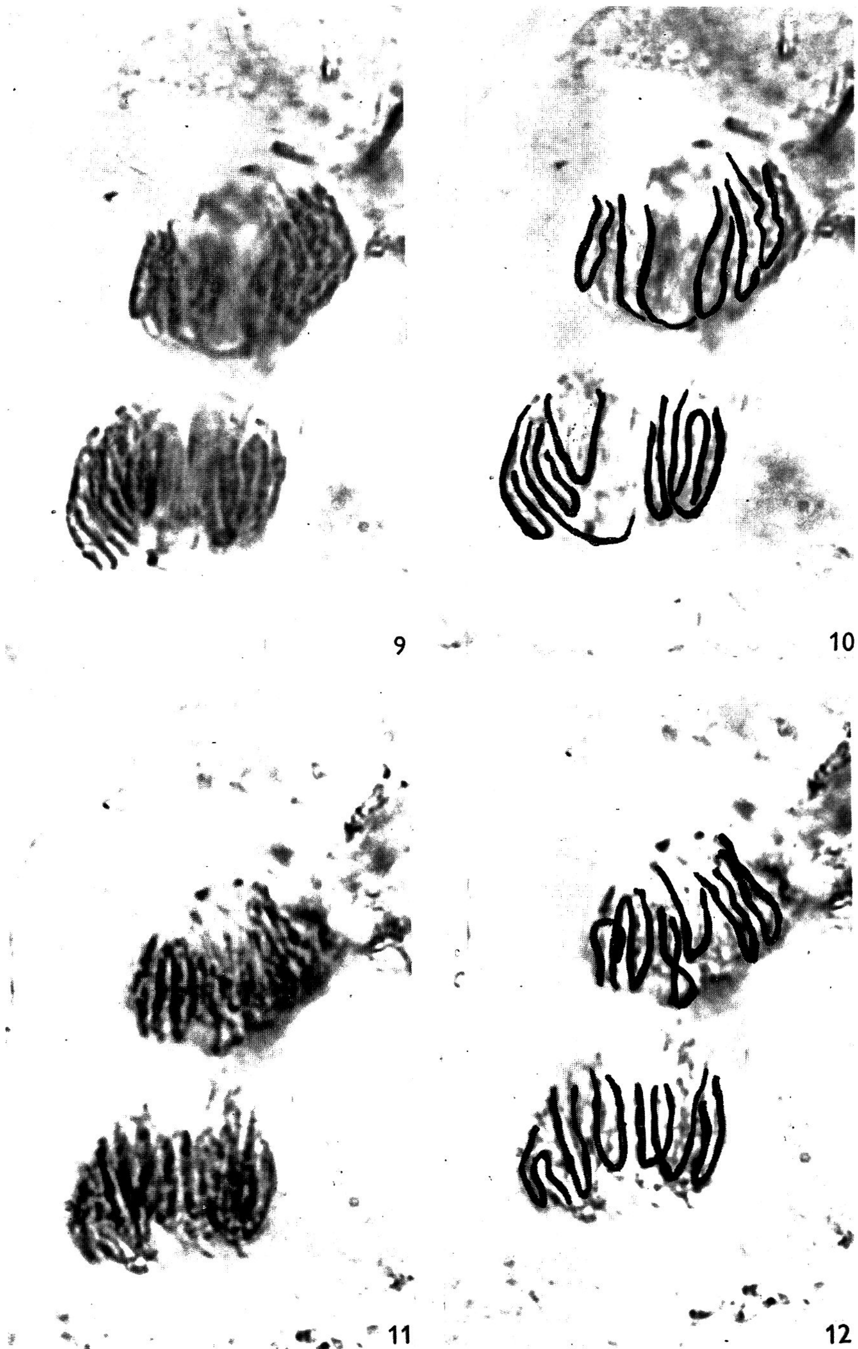

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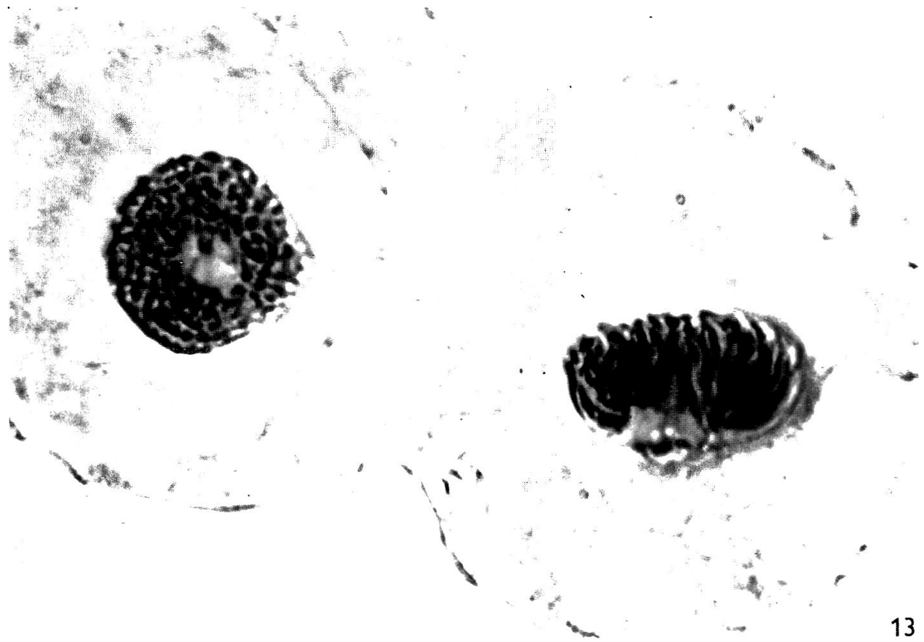

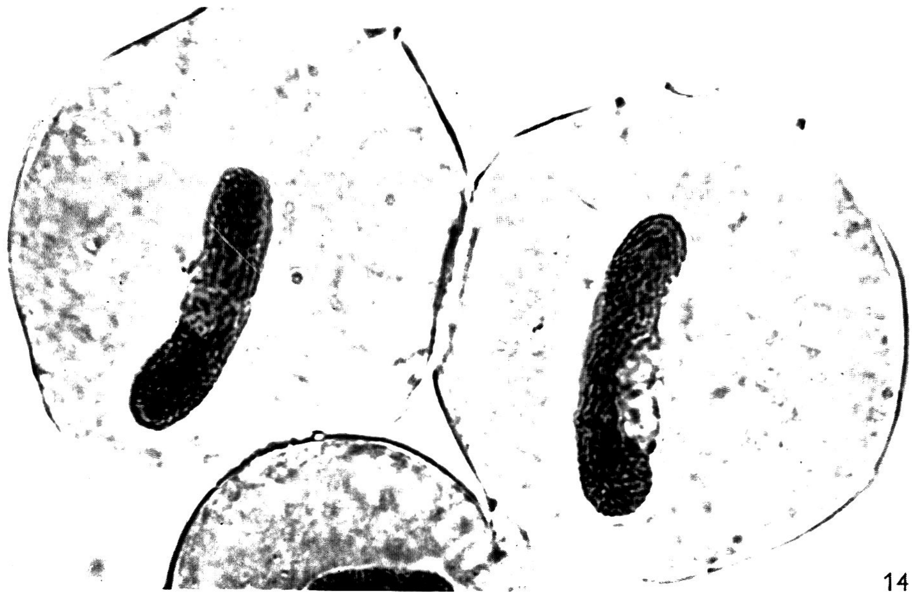

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Pigments, Growth and Photosynthesis in Cultures of Two Chrysomonads, *Coccolithus huxleyi* and a *Hymenomonas* sp.

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SUMMARY

The two chrysomonads, *Coccolithus huxleyi* and a *Hymenomonas* sp., contained chlorophylls *a* and *c*, carotene and fucoxanthin, and a number of minor xanthophylls. *Coccolithus huxleyi* was rich in chlorophyll *c* and had a chlorophyll *a*:*c* ratio of 1·5:1·0; in Hymenomonas the ratio was 5:1. Incubation of broken-cell preparations at high light intensities resulted in the decomposition of chlorophyll *a*, whereas chlorophyll *c* was stable under these conditions. This photochemical bleaching of chlorophyll *a* shifted the spectrum from 678 to 674 mμ. with a difference spectrum showing a maximum at 680 mμ. Maximum photosynthetic rates of 150–200 μmoles CO_2/mg. chlorophyll $a+c$/hr were reached in 10-day cultures. Maximum populations of 10^6 organisms/ml. for Hymenomonas and 10^7 for *C. huxleyi* were reached in about 14 days. Hymenomonas grew best at light intensities of 800 ft.c. or higher, whereas growth of *C. huxleyi* was independent of light intensity above 60 ft.c. Maximum photosynthetic rates were obtained at a light intensity of 3500 ft.c.

INTRODUCTION

The chrysomonads, which include the coccolithophores and the golden-brown flagellates, are widely distributed in temperate and tropical oceans; in some areas they may comprise the dominant members of the phytoplankton (Bernard & Lecal, 1950; Knight-Jones, 1951; McAllister, Parsons & Strickland, 1960). Some chrysomonads, such as the freshwater Ochromonas, contain only chlorophyll *a*, but most organisms in this class which have been examined also contain chlorophyll *c* and fucoxanthin as the major accessory pigments (Allen, Goodwin & Phagpolngarm, 1960; Parsons, 1961; Jeffrey, 1961). Although a great deal is now known about the photosynthetic mechanisms of higher plants and green algae which contain chlorophylls *a* and *b* (Arnon, 1961; Bassham & Calvin, 1960) and the photosynthetic bacteria (Stanier, 1961), little is known of photosynthesis as it occurs in the marine algae which contain chlorophyll *c* and either fucoxanthin (chrysomonads, diatoms, brown algae), peridinin (dinoflagellates) or phycobilins (cryptomonads) as additional accessory pigments. The mechanisms of light energy conversion and the products of carbon fixation are unknown in these organisms. Action spectra studies of brown algae and diatoms have suggested a photosynthetic function for chlorophyll *c* and fucoxanthin (Haxo & Blinks, 1950; Tanada, 1951), but more detailed studies are needed before the precise role of these pigments is known.

In the present work the physiology of cultures of two chrysomonads was studied as a preliminary to more detailed investigations of photosynthetic mechanisms and accessory pigment function. The organisms chosen were *Coccolithus huxleyi* and a *Hymenomonas* sp. whose nutrition in pure culture was investigated by Pintner & Provasoli (1962). These authors found that *C. huxleyi*, which is a widely distributed oceanic organism, is a photolithotrophic organism practically devoid of heterotrophic abilities, whereas the Hymenomonas, which inhabits euryhaline inshore environments, can utilize a wide range of organic carbon and nitrogen sources, although they are not essential for growth. The present paper describes the pigment composition of both organisms, the stability of the chlorophylls in whole organisms and in broken-cell preparations under different conditions, and the relationships between growth and photosynthetic rates under a range of light intensities. A later communication will describe the photochemical activities of chloroplast preparations from these two organisms.

METHODS

Cultures. Axenic cultures of *Coccolithus huxleyi* and *Hymenomonas* sp. were obtained from Dr L. Provasoli (Haskins Laboratories, New York). *Coccolithus huxleyi* in our cultures was non-motile, about 5 μ in diameter, and possessed its coccolith plates. The Hymenomonas was an actively motile flagellate, about 15–20 μ in diameter. The organisms were grown at 14–16° under 500 ft.c. of 'cool white' fluorescent lights (40 watts). Light intensities were measured with a General Electric Light Meter, Type 213, which was colour and cosine corrected, and was calibrated in foot-candles (ft.c.). The Hymenomonas was grown in 1 l. Erlenmeyer flasks containing 500 ml. of medium; *C. huxleyi* was grown in Roux bottles containing 250 ml. medium. The medium, here designated DDC, was adapted from the DC medium of Provasoli, McLaughlin & Droop (1957), by omitting all organic supplements except vitamins. The cultures were static and were not supplied with additional carbon dioxide during growth, since the organisms clumped badly on agitation.

Experiments in which the organisms were grown at different light intensities were carried out in 125 ml. Erlenmeyer flasks, containing 50 ml. medium. Neutral filters were made by exposing Eastman medium contrast lantern slides to light for different times to obtain an appropriate range of filters of different intensities. Percentage transmission of each filter over the visible range was checked in a Cary Recording Spectrophotometer, Model 14 R (Applied Physics Co., Monrovia, California). The filters were fixed to the bottoms of the flasks to obtain the required light intensity, and the flasks were illuminated from below by 40 watt Ultralux reflector fluorescent lights covered with a single surface ground-glass diffusing screen to provide a uniform light field. The sides of the flasks were protected from stray light by covering the sides of the flasks with black electrical tape.

Extraction and chromatography of pigments. Pigments were extracted from the organisms and separated by two-dimensional paper chromatography for qualitative and quantitative analyses (Jeffrey, 1961). After extraction with 90% (v/v) acetone in water, the pigments were transferred to ether in a measuring cylinder, by adding ether to the extract and washing with 10% NaCl solution. The ether layer containing

the pigments was then used directly for chromatography without further drying. For quantitative measurements, the volume of the ether extract was measured directly in the cylinder before samples were taken for chromatography. Chromatograms were run in the dark at 5–8°; the chromatography jars contained a beaker of silica gel to keep solvents and atmosphere dry.

Pigment spots were quantitatively eluted by means of an L-shaped glass elution tube kindly given by Dr T. R. Parsons (Fisheries Research Board, Nanaimo, British Columbia). The horizontal arm was a capillary tube, 10 cm. long, at the end of which was attached a needle point, and the vertical arm, also 10 cm. long, which acted as the solvent reservoir, was 1 cm. in diameter. The paper circle containing the pigment spot was attached to the needle point so that the paper touched the end of the capillary. Solvent was run into the vertical arm and flowed down the horizontal capillary tube to the paper, rapidly eluting the pigment. Chlorophyll *a* and carotenoids were eluted with acetone; chlorophyll *c* with acetone + methanol mixtures. Eluates were collected, and the concentrations of pigment determined spectrophotometrically. For identification, absorption curves of the pigments in different solvents were recorded with a Cary Recording Spectrophotometer (Model 14 R). Derivative spectra were carried out according to the method as developed by French *et al.* (1954) with a tachometer attachment to the Cary Spectrophotometer to measure the speed of pen travel. Detailed estimation of individual pigment fractions was carried out by chromatography and elution (see Table 3), and a recovery of 80% for the chlorophylls and 85–90 % for the carotenoids was achieved. The extinction values were those given previously (Jeffrey, 1961) except for chlorophyll *c* (Jeffrey, 1963) and fucoxanthin (Parsons & Strickland, 1963). Where the extinctions were unknown (the minor xanthophylls) the extinction for carotene was used.

Routine spectrophotometric determination of chlorophylls a and c. For simple routine determinations of chlorophylls *a* and *c* samples of the cultures were centrifuged, and the sedimented organisms extracted with a small volume (3–4 ml.) of 90% (v/v) acetone in water. After centrifugation, absorbances of the clear acetone extract were measured at 663 and 630 mμ, and the concentrations of chlorophylls *a* and *c* were calculated in μg./ml. according to the equations of Humphrey & Jeffrey (manuscript in preparation):

$$\text{chlorophyll } a = 13\cdot31\ E_{663\,\text{m}\mu} - 0\cdot27 E_{663\,\text{m}\mu};$$
$$\text{chlorophyll } c = -8\cdot37 E_{630\,\text{m}\mu} + 51\cdot72 E_{630\,\text{m}\mu}.$$

(E = extinction in l/g. cm.)

Measurement of photosynthesis. Centrifugation or shaking the cultures caused the organisms to clump to a compact mass that could not be resuspended without breaking the organisms. Rates of photosynthesis were therefore measured by $^{14}CO_2$ uptake. For ^{14}C studies, 3·0 ml. suspension of organisms were taken directly from growing cultures and were pipetted into Warburg flasks containing 0·1 ml. 0·1 M-$Na_2{}^{14}CO_3$ (10^6 counts/min.) in one side arm and 0·2 ml. 20% trichloroacetic acid (TCA) in the other. Dark controls were run with each experiment in Warburg flasks covered with black tape, and graded light intensities were obtained when needed by attaching neutral filters of different densities to the bottom of the flasks and covering the sides with black tape. The flasks were equilibrated at 14° ± 0·5

for 5 min. without shaking, and the reaction started by tipping in the $Na_2{}^{14}CO_3$ and by turning on the lights. A bank of ten 150-watt reflector flood lamps under the bath provided a maximum intensity of 3500 ft.c. Incubation was for 15 min. without shaking. The reaction was terminated by tipping in the TCA, which also liberated the excess $^{14}CO_2$ which had not been utilized. The $^{14}CO_2$ was subsequently released into a trap, and samples (0·1 ml.) of the suspension were then plated on to aluminium planchets, dried under infra-red lamps and the radioactivity counted with an automatic Nuclear Chicago thin-window counter (Nuclear Chicago Corp., Des Plaines, Illinois). Self-absorption was corrected for by counting standards with a known amount of radioactivity to which 0·1 ml. DDC medium was added. The total CO_2 added to each flask was determined manometrically in a separate experiment, and from the total counts added and the counts recovered in each flask the values of μmoles CO_2/mg. chlorophyll $a+c$/hr were calculated. The rates of ^{14}C fixation were linear over a 2-hr period, and therefore the 15 min. reaction time gave an accurate indication of steady-state photosynthetic rates.

Broken-cell preparations. In certain experiments broken-cell preparations were used because of the tendency of the organisms to clump in dense suspensions. Cultures (about 250 ml.) were centrifuged at 2000 rev./min. for 5 min. and the supernatant fluid discarded. The sedimented organisms were then transferred to a glass Potter–Elvehjem homogenizer in a small volume (8–10 ml.) of 0·35 M-NaCl solution containing 0·15 M-tris buffer (pH 7·8). The organisms were kept at 5° throughout the procedure. With Hymenomonas, the organisms were homogenized gently for 2 min., and the small proportion of unbroken organisms was removed by centrifugation at 1000 rev./min. for 2 min. The Coccolithus, being smaller and more difficult to disrupt, was placed in distilled water for 1–2 min. after the initial centrifugation to break the cell walls, and immediately transferred to tris + NaCl buffer solution. Unbroken organisms were then removed by centrifuging at 1500 rev./min. for 2 min. These broken-cell preparations retained good Hill reaction activity, but did not fix CO_2 photosynthetically.

RESULTS

Pigment composition

Chromatograms of the pigments from *Coccolithus huxleyi* and the *Hymenomonas* sp. are shown in Fig. 1. Both organisms contained chlorophylls *a* and *c*, carotene, fucoxanthin, and a number of minor xanthophylls. Table 1 gives the absorption maxima of the carotenoids in different solvents and their probable identification. Table 2 gives R_F values in the two solvent systems. The xanthophyll fraction of *C. huxleyi*, more complex than that of the Hymenomonas, contained six xanthophylls, two of which remain unidentified. Table 3 gives the concentration of each pigment fraction in both organisms. The Hymenomonas was rich in chlorophyll *a*, and contained five times more chlorophyll *a* than *c*. On the other hand, *C. huxleyi* was rich in chlorophyll *c*, with a concentration almost equal to that of Chlorophyll *a*.

Stability of pigments

Because of the lability of chloroplast pigments, the effect of incubating whole organisms and broken-cell preparations under high light intensities for various periods of time was studied. The pigments in the intact organisms were stable for at

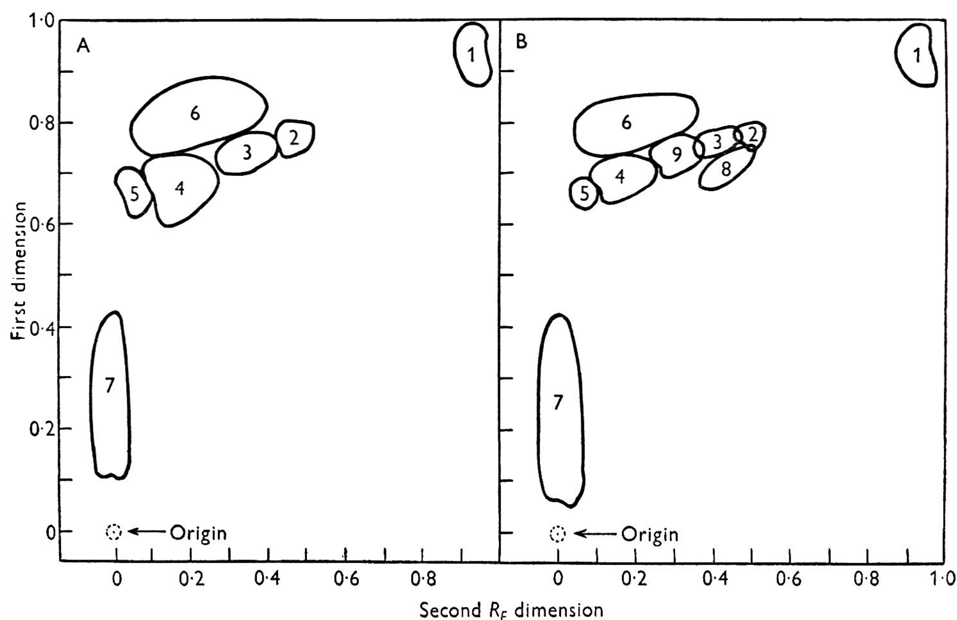

Fig. 1. Two-dimensional chromatograms of pigments from (A) *Hymenomonas* sp. and (B) *Coccolithus huxleyi*. Chromatographic solvent system: first dimension, 4 % propan-1-ol in light petroleum (60–110°); second dimension, 30 % chloroform in light petroleum. 1, Carotene (orange); 2, diatoxanthin (pale orange); 3, diadinoxanthin (yellow); 4, fucoxanthin (deep orange); 5, neofucoxanthin A and B (orange); 6, chlorophyll *a* (blue-green); 7, chlorophyll *c* (light green); 8, unknown xanthophyll$_1$ (pink); 9, unknown xanthophyll$_2$ (yellow-orange).

Table 1. *Absorption maxima of carotenoids from Hymenomonas sp. and Coccolithus huxleyi*

Organism	Fraction no. (see Fig. 1)	Carotenoid	Colour	Absorption maxima (mμ)	Solvent
Hymenomonas sp.	1	Carotene	Orange	‾430,451,478	Light petroleum (60–110°)
	2	Diatoxanthin	Pale orange	451,480	Ethanol (absolute)
	3	Diadinoxanthin	Yellow	446,478	Ethanol
	4	Fucoxanthin	Deep orange	452	Ethanol
	5	Neofucoxanthin A and B	Orange	447	Ethanol
Coccolithus huxleyi	1	Carotene	Orange	‾430,450,477	Light petroleum (60–110°)
	2	Diatoxanthin	Pale orange	453,480	Ethanol (absolute)
	3	Diadinoxanthin	Yellow	447,476	Ethanol
	4	Fucoxanthin	Deep orange	451	Ethanol
	5	Neofucoxanthin A and B	Orange	445	Ethanol
	8	Unknown xanthophyll$_1$	Pink	455	Ethanol
	9	Unknown xanthophyll$_2$	Yellow-orange	448,472	Ethanol

least 6 hr, whereas with broken-cell preparations in buffered 0·35 M-NaCl solution (containing 0·15 M-tris buffer, pH 7·8), 30–40 % of the chlorophyll *a* was lost during incubation for 3 hr in air under a light intensity of 3500 ft.c. Chlorophyll *c* did not break down under these conditions. At lower light intensities (400–850 ft.c.) the breakdown of chlorophyll *a* was not quite as extensive. Chromatograms of pigment extracts from broken-cell preparations incubated at high light intensities showed no trace of decomposition products (pheophytins, chlorophyllides, pheophorbides) which suggested that chlorophyll *a* was photo-oxidized to colourless products.

Table 2. *R_F values of pigments from Coccolithus huxleyi and Hymenomonas sp.*

Fraction no. (see Fig. 1)	Pigments	R_F: 1st dimension*: *C. huxleyi*	R_F: 1st dimension*: *Hymenomonas* sp.	R_F: 2nd dimension*: *C. huxleyi*	R_F: 2nd dimension*: *Hymenomonas* sp.
1	Carotene	0·95	0·96	0·95	0·96
2	Diatoxanthin	0·62	0·62	0·33	0·33
3	Diadinoxanthin	0·60	0·61	0·20	0·23
4	Fucoxanthin	0·59	0·60	0·11	0·10
5	Neofucoxanthin A and B	0·59	0·60	0·05	0·05
6	Chlorophyll *a*	0·85	0·86	0·24	0·23
7	Chlorophyll *c*	0·29	0·27	0	0
8	Unknown xanthophyll$_1$ (pink)	0·53	—	0·24	—
9	Unknown xanthophyll$_2$ (yellow-orange)	0·60	—	0·17	—

* Solvent systems: first dimension: 4% (v/v) propan-1-ol in light petroleum (60–110°); second dimension: 30% (v/v) chloroform in light petroleum (60–110°).
Chromatograms were run in the dark at 5°.

Table 3. *Pigment composition of Coccolithus huxleyi and Hymenomonas sp.*
Values given are mg. pigment/g. dry wt. organisms.

	Coccolithus huxleyi	*Hymenomonas* sp.
Chlorophyll *a*	7·25	21·4
Chlorophyll *c*	4·68	3·7
Carotenes	0·09	1·35
Diatoxanthin	0·09	3·20
Diadinoxanthin	0·40	1·13
Unknown xanthophyll$_1$	0·17	—
Unknown xanthophyll$_2$	0·57	—
Fucoxanthin	2·97	8·94
Neofucoxanthin A and B	0·70	2·10
Total chlorophylls	11·93	25·1
Total carotenoids	4·99	16·72
Ratio chlorophyll *a*:chlorophyll *c*	1·55	5·78

Chlorophyll *a*, E = 84·0 l/g. cm. (Smith & Benitez, 1955); chlorophyll *c*, E = 15·8 l/g. cm. (Jeffrey, 1963); carotene, $E^{1\%}_{1cm.}$ = 2500 (Goodwin, 1955); fucoxanthin, $E^{1\%}_{1cm.}$ = 880 (Parsons & Strickland, 1963); minor xanthophylls, E unknown, therefore E for carotene was used.

Chlorophyll absorption spectra in vivo

Absorption spectra of whole organisms were impossible to obtain since these delicate organisms aggregated irreversibly when concentrated. Absorption spectra of broken-cell preparations are shown in Fig. 2. The chlorophyll *a* maximum was at 678 mμ in both *Coccolithus huxleyi* and the *Hymenomonas* sp. Since derivative curves were simple and symmetrical (Fig. 3), one could infer that the red band of chlorophyll *a* in two organisms may consist of a single component. However, the chrysomonad *Ochromonas danica* shows a very complex chlorophyll band in whole

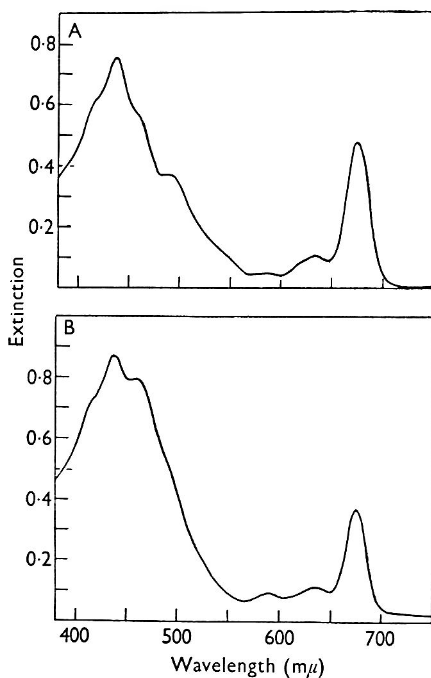

Fig. 2

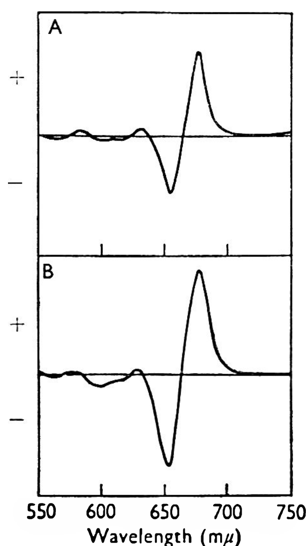

Fig. 3

Fig. 2. Absorption spectra of broken-cell preparations of (A) *Hymenomonas* sp.; (B) *Coccolithus huxleyi* in 0·35 M-NaCl containing 0·15 M-tris buffer (pH 7·8).

Fig. 3. Derivative spectra of broken-cell preparations of (A) *Coccolithus huxleyi*; (B) *Hymenomonas* sp.

organisms but in broken cells shows only a single component (Allen, French & Brown, 1960). That the chlorophyll *a* band in *C. huxleyi* and the Hymenomonas may actually be more complex was indicated by the spectral changes which occurred on bleaching (Fig. 4). At high light intensities the absorption maximum of chlorophyll *a* in broken-cell preparations shifted from 678 to 674 mμ, and the difference spectrum showed a maximum at 680 mμ. It is not clear whether this shift represents selective bleaching of a chlorophyll complex that for some reason was not detectable in the original derivative spectrum, or a breakdown of a single normal chlorophyll complex by the high light intensity.

Growth and photosynthesis

Growth, photosynthesis and chlorophyll content of the Hymenomonas and *C. huxleyi* cultures are shown in Fig. 5. Maximum population density was achieved in about 14 days at 14°. The Hymenomonas reached a density of about 10^6 organisms/ml., and *C. huxleyi* 10^7 organisms/ml. Since, as in all stationary cultures, growth may have been limited by the diffusion of carbon dioxide from the air, the population densities achieved may not be the maximum attainable at the temperature and light intensity used.

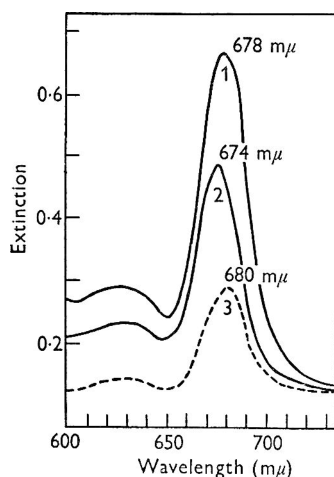

Fig. 4

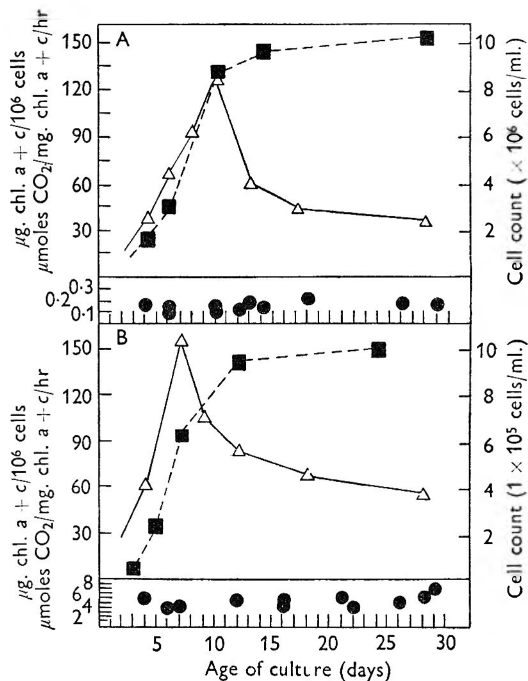

Fig. 5

Fig. 4. Absorption spectra of broken-cell preparations of *Hymenomonas* sp. after incubation at 3500 ft.c. at 14° for 3 hr. 1, Dark incubation (identical with spectrum before incubation); 2, light incubation; 3, difference spectrum.

Fig. 5. Growth, carbon fixation and chlorophyll content of cultures of (A) *Coccolithus huxleyi*; (B) *Hymenomonas* sp. ■---■, Count of organisms; △—△, CO_2 fixation; ●, chlorophyll content of several different cultures.

The chlorophyll content per organism did not vary significantly throughout growth of the cultures, nor was there any consistent change in the ratio of chlorophylls *a* to *c* as the cultures aged. The rate of photosynthesis, by contrast, did vary with the age of the culture, and reached a maximum of 150–200 μmole CO_2/mg. chlorophyll $a+c$/hr in 10-day cultures, near the end of the period of most rapid growth. After this, although the cultures continued to grow slowly, the carbon dioxide uptake declined to low values.

In several experiments the organisms were separated from the medium by centrifugation immediately after the 15 min. period of incubation, but before the addition

of trichloroacetic acid, to determine whether all the carbon fixed was retained by the organisms or whether soluble products were present in the medium. After resuspending the organisms in fresh medium, trichloroacetic acid was added to both organisms and supernatant fluid, and samples of each fraction counted. All the radioactivity was recovered in the organism fraction, showing that these chrysomonads did not 'leak' soluble photosynthetic products during short periods of incubation.

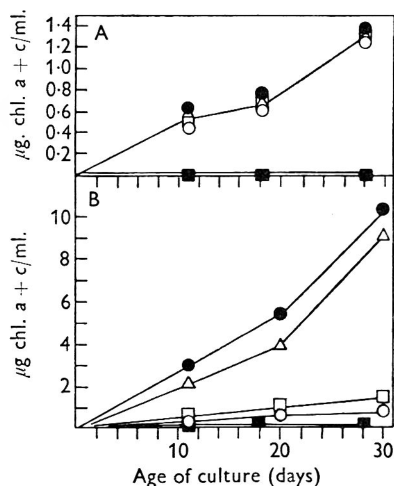

Fig. 6

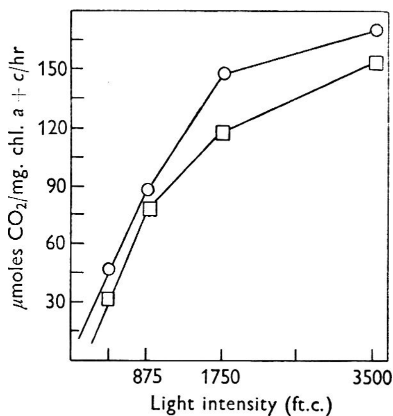

Fig. 7

Fig. 6. Growth of cultures of (A) *Coccolithus huxleyi*; (B) *Hymenomonas* sp. under different light intensities (ft.c.): ■—■, 60 ft.c.; ○—○, 200; □—□, 400; △—△, 600; ●—●, 800.

Fig. 7. Carbon fixation of cultures of *Coccolithus huxleyi* and *Hymenomonas* sp. under different light intensities. Identical light saturation curves were obtained with both young and old cultures. ○—○, *Coccolithus huxleyi*; □—□, *Hymenomonas* sp.

Light saturation curves for growth and photosynthesis of the two organisms are shown in Figs. 6 and 7. Optimal growth of the Hymenomonas was obtained at light intensities of 800 ft.c. or higher, whereas growth of *Coccolithus huxleyi* was independent of light intensity over a wide range, and became saturated at low light intensities (60 ft.c.). Photosynthetic carbon dioxide fixation by both organisms was light saturated at 3500 ft.c.; these light saturation curves for carbon fixation were similar at all stages of growth of the cultures.

DISCUSSION

Carbon fixation rates and chlorophyll content of sea-water samples have been used to assess the productivity of the marine algae in the oceans (Strickland, 1960). Similar studies applied to organisms in pure culture provide a guide to the interpretation of results with naturally occurring organisms, particularly in areas where one class of marine alga may predominate. With regard to the chrysomonads, the pigments of eight species have now been examined in some detail. A summary of the proportions of the major pigments in these organisms is given in Table 4. The

total chlorophyll concentration in these eight chrysomonads was in the same range as that found for other types of marine algae (diatoms, dinoflagellates, etc., Jeffrey, 1961). The ratios of chlorophyll a:chlorophyll c ranged from 1·5 to 5·7. A distinguishing feature of the chrysomonads was the variety of minor xanthophylls which was encountered; these included diadinoxanthin, diatoxanthin, dinoxanthin, and several others not yet identified.

Table 4. *Major pigments of some chrysomonads*

Values given are mg. pigment/g. dry wt. organisms.

	Isochrysis galbana†	*Sphaleromantis* sp.†	*Coccolithus huxleyi*	*Hymenomonas* sp.	*Monochrysis lutheri*‡	*Syracosphera carteri*‡	*Ochromonas danica*§	*Prymnesium parvum*§
Chlorophyll a	2·96	2·43	7·25	21·4	5·5	6·5	Not given	Not given
Chlorophyll c	1·61	0·95	4·68	3·7	0·3*	1·5*	Not given	Not given
Total chlorophylls	4·57	3·38	11·93	25·1	5·8	8·0	Not given	Not given
Carotene	0·38	0·10	0·09	1·35	0·42	0·48	0·73	0·70
Fucoxanthin and isomers	2·41	2·32	3·67	11·04	0·69	1·45	3·16	7·91
Other xanthophylls	0·80	0·23	1·23	4·33	0·66	1·07	0·40	1·96
Total carotenoids	3·59	2·65	4·99	16·72	1·77	3·00	4·29	10·57
Ratio chlorophyll a:chlorophyll c	1·84	2·50	1·55	5·78	18·0	4·3	—	—
Ratio total chlorophylls: total carotenoids	1·28	1·27	2·39	1·50	3·28	2·67	—	—

† Jeffrey (1961).
‡ Parsons (1961).
§ Allen, Goodwin & Phagpolngarm (1960).
* Calculated from the equations of Richards & Thompson (1952).

Paper chromatographic analyses of pigments from marine algae in sea-water samples taken over a 12-month period in Australian coastal waters have shown the predominance of chlorophylls a and c, fucoxanthin, peridinin and carotene in such waters (Jeffrey, unpublished); the pigments found correlated well with the classes of organisms which were present. In similar geographical regions chlorophylls were estimated spectrophotometrically over a 3-year period, and some exceptionally high chlorophyll c:a ratios were observed (Humphrey, 1960, 1963). However, no marine alga is yet known which contains more chlorophyll c than a. An alternative explanation for hith c:a ratios may be found in the ready photo-oxidation of chlorophyll a in broken organisms (of which there is always a significant proportion in any natural population), and the stability of chlorophyll c under these conditions. Furthermore, the inaccuracies of the spectrophotometric method of Richards & Thompson (1952) which was used for the estimation of the chlorophylls may also have contributed to high values for chlorophyll c in ocean waters. This subject will be discussed in more detail elsewhere.

The photochemical bleaching of chlorophyll a was first described for higher plants (Krasnovsky & Kosobutskaya, 1955) and later for Chlorella (Brown & French, 1959). In both types of plant a shift in the absorption peak from longer to shorter wavelength forms was noted. In the present work, a similar effect was observed in

broken-cell preparations incubated at light intensities from 500–3500 ft.c. Both Krasnovsky, and Brown & French deduced that the 683 mμ component of chlorophyll *a* bleached more rapidly than the 673 mμ component. A similar explanation might be used to explain the spectral shifts observed during photochemical bleaching of chlorophyll *a* in the Hymenomonas and in *Coccolithus huxleyi*. The difference spectrum, with a maximum at 680 mμ, suggested the loss of some component absorbing in that region.

The carbon fixation rates, observed in whole organisms of the two chrysomonads, reached maxima of 150–200 μmole CO_2/mg. chlorophyll $a+c$/hr. The change in photosynthetic rates during growth of the cultures was not a consequence of changes in pigment concentration, since this remained constant throughout the growth period. The reasons for the changes in photosynthetic rates during growth of the cultures remain to be studied. From the range of values for carbon fixation rates which have been published (Rabinowitch, 1951; Wassink, 1946), it appears that higher plants fix carbon at rates higher than have so far been observed for marine organisms.

Preliminary experiments on the photochemical activity of chloroplasts prepared from the two chrysomonads indicated that the chloroplasts retained good Hill reaction activity and some carbon fixation ability. Detailed descriptions of the preparation and properties of chloroplasts from these organisms will be discussed in a later communication.

The chrysomonads resemble the brown algae and the diatoms in the pigment pattern of the main components, but the variety of their minor xanthophylls distinguishes the chrysomonads from the other groups of marine algae. Whether these minor xanthophylls have a special photosynthetic function as has been postulated for chlorophyll *c* and fucoxanthin (Tanada, 1951), or simply represent intermediates in the formation or degradation of fucoxanthin, is as yet unknown.

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Preparation and Properties of a Hyphal Wall Fraction from *Pithomyces chartarum*

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SUMMARY

A low-sporing isolate of *Pithomyces chartarum* was grown in submerged liquid culture in defined medium, and a hyphal wall fraction isolated by mechanical disruption. It contains about 20 % protein, 40 % bound hexoses, 10 % bound glucosamine, 10 % lipid and 5 % ash.

INTRODUCTION

The chemical nature of the spicules which invest the conidia of *Pithomyces chartarum* was recently elucidated (Bertaud, Morice, Russell & Taylor, 1963). Continuing such studies in chemical morphology, we wished to investigate the spore wall that underlies the layer of lipid and depsipeptide. Because comparative data about the composition of hyphal and conidial walls do not exist for any fungus, we began by investigating the hyphal walls of *P. chartarum*. We aimed to find conditions for isolating, in quantity, a hyphal wall fraction free from conidial contamination, to analyse it in terms of its total content of carbohydrate, lipid and protein, and to determine the qualitative composition of these groups of substances in terms of their monosaccharide, fatty acid and amino acid contents, respectively.

METHODS

Organism. An isolate of *Pithomyces chartarum* (Ellis, 1960; Dingley, 1962) found growing on debris of *Holcus lanatus* at Virginia Water, Surrey, in September 1961 (Lacey & Gregory, 1962), and kindly provided by Dr P. H. Gregory, F.R.S. (Rothamsted Experimental Station, Harpenden, England) was designated isolate E. It formed moderate numbers of spores in surface culture in potato carrot broth at 25°, and sectored frequently on potato glucose agar. Single hyphal tips from the edges of sectors of 3-week cultures were plated to fresh agar. After five such subcultures, two out of the many isolates obtained formed very few spores. One, designated E-HT/1, was chosen for use.

Colonies of E-HT/1 grown from single hyphal tips on potato glucose agar at 25° for 21 days were dark green to black on the under surface. The superficial mycelium was grey-green except for a central portion which was buff and floccose. The edges of the colonies were olive-green and adpressed. Sectoring occurred rarely and only towards the edges of the colonies. The aerial mycelium was thin-walled and almost hyaline, with frequent septa; hyphae, 2·0–4·0 μ diam. Two other types of mycelium were observed; in one type the hyphae, 2·0–3·6 μ diam., were brown, evenly septate, and appeared to bear conidia. The other type was composed of massive hyphae,

4·0–6·6 μ diam., thick-walled, dark, and with more frequent septa. Conidia were few, brown, 9·5–15 μ × 19–25 μ, with two or three transverse septa, and one longitudinal septum or none.

Agar discs (3 mm. diameter) cut from potato glucose agar colonies of isolate E-HT/1 were plated on to each of the five solid media listed below, and incubated for 3 weeks at 15, 20, 25, 30 or 37°. No growth occurred at 37°. No sectoring was seen on the minimal medium at any temperature, but on all other media sectors were observed, most abundantly on the 'complete' medium at 25°. No colony sectored at 15°. Most rapid radial extension of colonies on potato glucose agar took place at 25°.

Media. Potato carrot broth (Done, Mortimer, Taylor & Russell, 1961) contained total solids 25 mg./ml., reducing sugars 20 mg./ml. (adjusted with glucose) and nitrogen 1 mg./ml. (adjusted with glycine). Ross's medium was a solution of salts, glucose and asparagine (Ross & Thornton, 1962; Butler, Russell & Clarke, 1962).

Five solid media were used: (*a*) potato glucose agar (Oxo Limited, London, England); (*b*) potato carrot agar (Done *et al.* 1961); (*c*) the minimal medium of Pontecorvo (1953); (*d*) minimal medium + peptone (2 g./l.), casein hydrolysate (3 g./l.), yeast nucleic acid (0·5 g./l.) (these products were supplied by British Drug Houses Ltd., Poole, Dorset) and Yeastral (1 g./l.) (Brewer's Food Supply Co. Ltd., 189–191 Fountainbridge, Edinburgh 3); (*e*) Ross's medium containing agar 2% (w/v).

Media were sterilized by autoclaving at 120° for 20 min.

Fermentation vessel. The fermentor was a 5 l. glass aspirator, with a mouth 4·0 cm. diam., closed by a rubber bung. A glass tube fitted with a cottonwool plug passed through the bung, terminating 5 cm. below it and serving as an outlet for gases and as a means of withdrawing samples for analysis. Two further glass tubes passing through the bung each bore at the lower end an upward-facing sintered glass disc, porosity 1, placed at about one-third of the distance from the bottom of the vessel to the surface of the medium (3·7 l.). Air from a compressor was filtered through cottonwool to remove oil mist, then sterilized by passage through a ceramic filter (Aerox Ltd., Glasgow, Scotland) and passed at about 8 l./min., first through sterile water and then through the sintered glass discs. Additional stirring was by a Teflon-covered magnet, 6 × 1 cm., turned at about 500 rev./min. by a magnetic rotor beneath the fermentor. Two vortices of fine air bubbles were formed in the medium, one travelling upwards and the other downwards. The fermentor was immersed in a bath at 25 ± 0·2°.

Growth of organism. To sterile water (100 ml.) contained in a sterile stainless steel 'Atomix' blender (50–200 ml.; Measuring and Scientific Equipment Ltd., Spenser St., London, S.W. 1) were added six discs (6 mm. diam.) cut aseptically from a 10-day culture of isolate E-HT/1 on potato glucose agar grown at 25°. Blending was carried out for 1 min. at half speed. A portion (10 ml.) of the resulting suspension was added to each of three Ehrlenmeyer flasks (2 l.) containing Ross's medium (200 ml.). The plugged flasks were incubated for 65 hr on a 'Gyrotory' Incubator Shaker (New Brunswick Scientific Inc., P.O. Box 606, New Brunswick, N.J., U.S.A.) at 26° and 250 strokes/min. The mycelial dry weight was determined in two flasks whilst the contents of the third were transferred aseptically to the

fermentor containing sterile Ross's medium (3·5 l.). An average inoculum contained about 7 mg. dry-wt. mycelium/ml. The organism was incubated at 25° for 4 days.

The growth in the fermentor, examined microscopically at the end of incubation, consisted of straight, hyaline hyphae, 1·6–2·0 μ diam. and regularly septate. Some darker mycelium which formed a ring adherent to the wall of the vessel at the level of the liquid surface was discarded. In more than three dozen fermentations conidia were never observed.

Small portions of the mycelium obtained at the end of the incubation period were used as inocula for potato glucose agar plates which were incubated for 10 days at 25° and then examined for the presence of contaminants. Meanwhile, when no obvious contamination was detected by microscopic examination, the contents of the fermentor were used for the preparation of the wall fraction.

Harvesting and preparation of the hyphal wall fraction

The culture (3·5 l.) was dispersed in a Waring Blendor (Waring Products Corporation, New York, N.Y.) at 19,000 rev./min. for 45–60 sec. at 4°, and then centrifuged in a pre-cooled Continuous Flow Centrifuge (Sharples Centrifuges Ltd., Camberley, Surrey, England). The mycelium was redispersed in water (3·5 l.) at 4° and collected once more.

Washed mycelium (120 g. wet-wt.) was dispersed in the Waring Blendor in ice-cold 0·1 M-NaCl solution (150 ml.; Crook & Johnston, 1962), and portions (30–40 ml.) were passed at 16,000 lb./in^2. through a fine needlevalve of a type similar to that described by Milner, Lawrence & French (1950). The wall fraction was recovered from the suspension of broken hyphae by centrifugation for 10–15 min. at 1400*g*, suspended in M-sucrose and collected by centrifugation. Resuspension and centrifugation were repeated twice more with M-sucrose, three times with 0·1 M-NaCl and three times with water, all below 4°. Preparations contained no unbroken hyphae (absence of intense staining with methylene blue 0·01 %). The yield of wall obtained by freeze-drying the final plug was usually 4–6 g.

Analytical methods

Analyses of medium constituents and mycelial dry weights were made as previously described (Done *et al.* 1961).

Total nitrogen was estimated by the micro-Kjeldahl procedure of Chibnall, Rees & Williams (1943).

Total carbohydrate (bound hexose) was determined by a modification of the orcinol H_2SO_4 method of Winzler (François, Marshall & Neuberger, 1962).

Total hexosamine was quantitatively determined, after hydrolysis of the samples in 4 N-HCl for 4 hr at 110°, by the Elson-Morgan reaction (Blix, 1948), with glucosamine as standard.

Lipids were extracted into chloroform + methanol (2 + 1, by vol.), washed with 0·2 vol. water and evaporated to dryness. Analysis of constituent fatty acids after methanolysis (Metcalfe & Schmitz, 1961) was done on a Pye-Argon Gas Chromatograph (W. G. Pye and Co., Cambridge, England), with Apiezon L or polyethylene glycol adipate as the stationary phase.

Ash was estimated in samples heated at 600° to constant weight.

Phosphorus was estimated by the method of Berenblum & Chain (1938).

Tryptophan was determined on unhydrolysed samples by the method of Opieńska-Blauth, Charęziński & Berbeć (1963).

Paper chromatography was done on Whatman No. 1 paper. The following solvent systems were used: (*A*) ethyl acetate–pyridine+water (10+4+3, by vol.); (*B*) butan-1-ol+pyridine+water (6+4+3, by vol.). Detection methods: for reducing sugars, alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) or aniline hydrogen phthalate (Partridge, 1948); and for amino acids, 0·2% (w/v) ninhydrin in acetone.

Hydrolysis of hyphal wall

(1) For monosaccharide constituents, the wall fraction (5 mg.) was suspended in 0·1 ml. of 72% (v/v) H_2SO_4; after 18 hr at room temperature the solution was diluted with water (0·7 ml.), heated at 100° for 4 hr in a sealed tube, then neutralized (pH 6) with barium carbonate, filtered and the filtrate evaporated to dryness at 25° under vacuum.

(2) For amino acids, the wall fraction (30 mg.) was hydrolysed by 6 N-hydrochloric acid (50 ml.) at 110° for 18 hr. The hydrolysate was evaporated to dryness under reduced pressure and dried over solid sodium hydroxide. Quantitative analysis of the amino acids was carried out by the method of Spackman, Stein & Moore (1958) in an Automatic Analyser (Evans Electroselenium Ltd., Halstead, Essex).

(3) For nucleic acids, the wall fraction (10 mg.) was hydrolysed by N-HCl for 1 hr and the hydrolysate chromatographed in butan-1-ol+acetic acid+water (4+1+5, by vol.). The chromatogram was examined in ultraviolet light for the presence of purines and pyrimidines.

RESULTS

Although no isolate of *Pithomyces chartarum* tested by Dingley, Done, Taylor & Russell (1962) formed more than a few conidia in submerged culture it was desirable, for work on hyphal walls, to use an asporogenous isolate. It was also desirable to obviate batch-to-batch variation due to adaptive behaviour of a heterokaryon, by using a homokaryotic strain. Neither ideal was achieved; all the isolates obtained by hyphal-tip subculture sectored under favourable conditions, and none was completely asporogenous. However, the isolate E-HT/1 did not sector on the minimal medium used, and no spores were detected in submerged cultures. This isolate also had the advantage that its walls were only lightly pigmented. Preliminary work with the conidia of *P. chartarum* has shown that characterization of other wall constituents is complicated by the presence of pigment.

Because of the morphological heterogeneity resulting from surface culture, submerged liquid culture was used to produce a homogeneous mycelium free from conidia. Although the organism grew well in shake flasks, a more even and finely divided growth was obtained in a simple fermentor. In Fig. 1 the growth and utilization of medium constituents are shown for a typical culture; the incubation was terminated after 4 days, when the mycelium had become so dense that stirring and aeration were impaired.

Preparation and analysis of the hyphal wall fraction

After early experiments on disruption of hyphae with glass beads (Korn & Northcote, 1960; Crook & Johnson, 1962) we changed to a method in which the mycelial suspension was passed under pressure through a fine needle-valve. This, followed by differential centrifugation to remove cell contents, gave a cleaner product in higher yield than was obtained by similar treatment of bead-disrupted hyphae. The wall fraction was obtained in larger fragments, up to 20 μ long. No

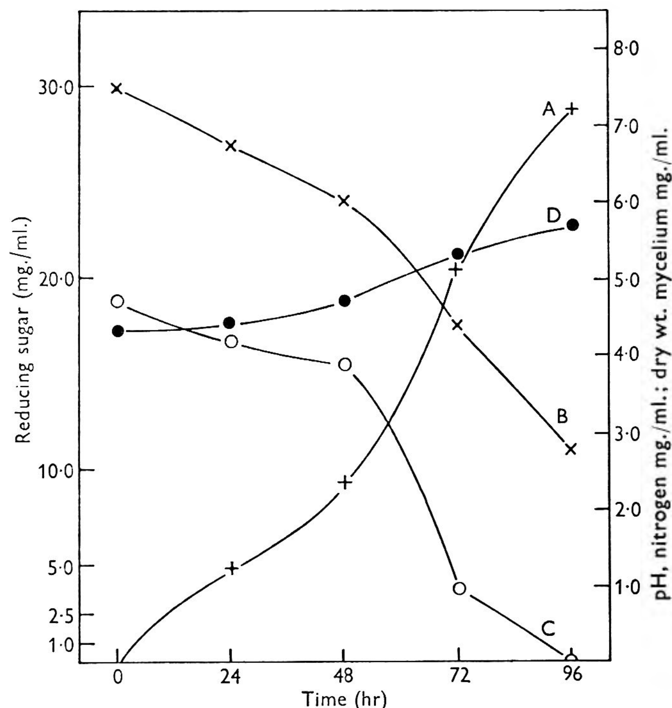

Fig. 1. Growth of *Pithomyces chartarum* isolate E-HT/1, on defined medium in deep culture at 25°, as a function of culture age. A, Dry-wt. mycelium; B, reducing sugar in culture filtrate; C, nitrogen in culture filtrate; D, pH value of medium.

Table 1. *Analysis of freeze-dried hyphal wall fraction of Pithomyces chartarum isolate* E-HT/1

	%
Bound hexose	37–41
Total nitrogen	3·8–4·6
Bound hexosamine	9–10
Protein nitrogen (by difference)*	3·1–3·9
Protein nitrogen (by amino acid analysis)†	2·4–2·9
Lipid	8·5–8·7
Phosphorus	0·5–0·6
Ash	3–5
Moisture	5–6

* Calculated by subtracting bound hexosamine nitrogen from total nitrogen.

† Calculated by adding together the nitrogen of the amino acids determined by ion-exchange chromatography.

cytoplasm was detected either in the optical microscope after mounting the material in methylene blue solution, or in electron micrographs (kindly prepared by Dr Hilary Griffiths) of unsectioned fragments fixed with phosphotungstic acid (Plate 1). In Table 1 are listed the results of analyses of many batches of wall fraction prepared in this way. Nucleic acids were not detected.

Samples were hydrolysed by acid and the hydrolysates examined by paper chromatography for the presence of sugars; only hexoses and hexosamine were found. Other hydrolysates were submitted to amino acid analysis by ion-exchange chromatography, kindly carried out by Mr L. Lester. Tryptophan, which is acid-labile, was determined separately. In view of the known mutual destruction of amino acids and sugars during acid hydrolysis, the results, listed in Table 2, are presented only semiquantitatively.

Table 2. *Monosaccharide and amino acid composition of acid hydrolysates of hyphal wall fraction of Pithomyces chartarum isolate* E-HT/1

Constituent	Amount*	Constituent	Amount
Glucose	+ + + + +	Valine	+ + + + +
Galactose	+	Methionine	+
Mannose	+	Isoleucine	+ +
Glucosamine	+ + + +	Leucine	+ + + + +
		Tyrosine	+
Aspartic acid	+ + + +	Phenylalanine	+ + +
Threonine	+ + + +	Lysine	+ + +
Serine	+ − + + +	Histidine	+
Glutamic acid	+ + + +	Arginine	+
Proline	+ + + +	Tryptophan	+
Glycine	+ + + + +	Cystine + cysteine	−
Alanine	+ − + + +	Ammonia	+ + + + +

* For sugars, the size of spots on chromatograms, judged by eye, varied from + + + + + (maximum size) to + (small spot). For amino acids, the range in μmoles/mg. nitrogen, was + − + + + (> 2·0), + + + + (1·5–2), + + + (1–1·5), + + (0·5–1), + (< 0·5), − (not detected).

A small peak on the ion-exchange chromatogram, estimated to be about 0·5 % of the total hexosamine, may have been due to galactosamine. Estimation of galactosamine in the presence of glucosamine becomes inaccurate when the minor component represents less than 2 % of the total hexosamine (Gardell, 1953; Cessi & Serafini-Cessi, 1962; Johnston, 1963). An artificial mixture of 2·4 mg. glucosamine + 100 μg. galactosamine was cleanly separated on a column of Zeo-Karb 225 (60 × 1 cm.) by the method of Crumpton (1959), but no such evidence was found for the presence of galactosamine in wall hydrolysates containing similar concentrations of glucosamine. We conclude that more than 99 % of the total wall amino sugar was glucosamine. The identity of glucosamine was confirmed by the Morgan & Elson (1934) reaction, by its position relative to the amino acids on ion-exchange chromatograms, and by its degradative deamination to arabinose (Stoffyn & Jeanloz, 1954). No evidence was found for the presence of any other hexosamine.

The lipid was not analysed completely. Vapour-phase chromatography of the methyl esters of the constituent fatty acids, kindly carried out by Miss Julia Cullen, showed the presence, in descending order of abundance, of C_{16}-saturated, C_{18}-di- and mono-unsaturated, C_{18}-saturated and C_{16}-mono-unsaturated fatty acids. Small unidentified peaks appeared in the C_{12}–C_{14} region.

DISCUSSION

This paper records the preparation and properties of a fraction obtained by mechanical disruption of *Pithomyces chartarum* hyphae. In avoiding chemical or enzymic treatment we sought to isolate the 'whole wall'; we are aware that, in so doing, the ideal of 'nothing but the wall' may have eluded us. Nevertheless, the fraction so prepared is reproducible, is obtained in good yield, and corresponds in morphology to what is generally termed cell wall. Having noted the pertinent comments of de Duve (1964), we have called our material the 'hyphal wall fraction'.

Cell walls may vary in composition even when the organism is grown under controlled conditions. For this reason alone, precise quantitative estimates of amino acids and sugars would be of little significance. In fact, the precision obtainable is low, because chemical changes take place during hydrolysis. This limitation is not serious, for careful fractionation gives homogeneous materials, more amenable to chemical study. The results of such studies will be reported elsewhere.

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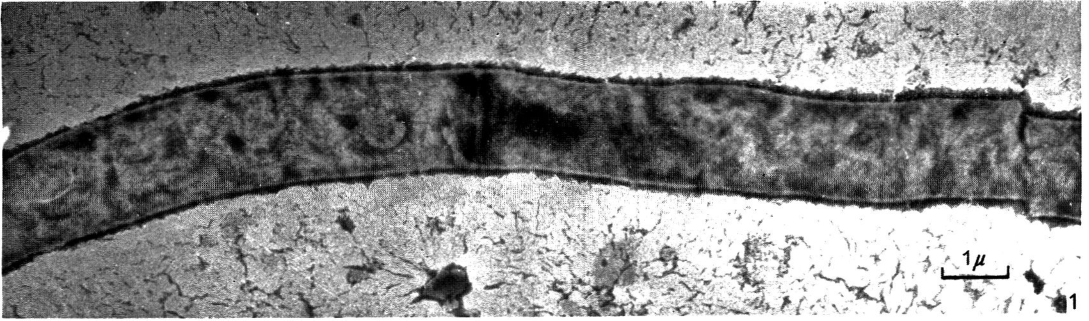
EXPLANATION OF PLATE

Electron micrographs of *Pithomyces chartarum* hyphae during preparation of the cell wall fraction.

Fig. 1. Hypha after passage through the needle valve but before washing and centrifugation. Note fracture at extreme right.

Fig. 2. Hypha after washing and centrifugation in M-sucrose and 0·1 M-sodium chloride.

Fig. 3. Hypha after washing and centrifugation in M-sucrose, 0·1 M-sodium chloride and water.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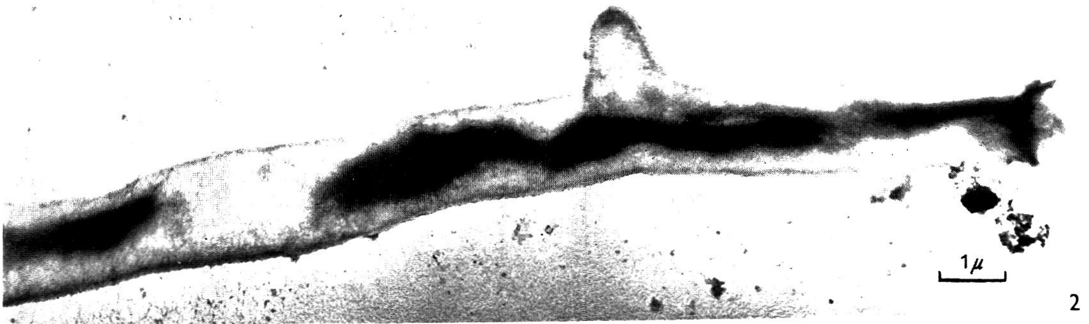
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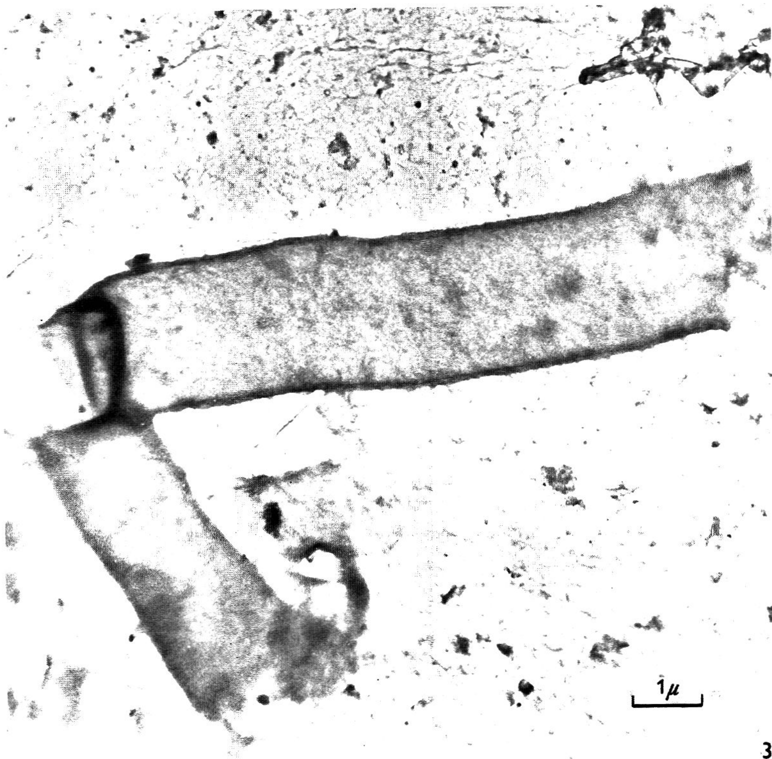
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Effect of Thiobenzoate on Cytology of *Candida albicans*

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(*Received* 11 *March* 1964)

SUMMARY

Cytological changes in *Candida albicans*, associated with exposure to the antimicrobial agent thiobenzoate, were investigated with the electron microscope. After incubation of proliferating cells for 2–3 hr with the inhibitor, the nucleus displayed a lessened electron density. Following this, gaps appeared in the nuclear membrane and canaliculi emanated toward the periphery of the cell. Complete loss of cytoplasmic organization appeared to be the terminal event. These changes were shown to differ from those incurred during autolysis, induced either by substrate deprivation by washing, or by a combination of substrate deprivation and accumulation of metabolic products in an ageing culture.

INTRODUCTION

The sulphur-containing antifungal peptide Ro 2-7758 (Hoffman-LaRoche) has been shown to inhibit the microbial sulphate reduction system (Gale, Kendall & Welch, 1963) and to induce a marked thickening of the cell wall of *Mucor corymbifera* (Gale, 1963*a*). However, the discrepancies between the *in vitro* and *in vivo* activities of Ro 2-7758, along with its limited antimicrobial spectrum, make conclusions tenuous in attempting to correlate biochemical and cytological data regarding its mechanism of action. As a result of those observations, a group of sulphur-containing synthetic aromatic compounds was investigated to determine whether certain other sulphur-containing compounds inhibit the microbial sulphate reduction system and, if so, whether these compounds inhibit microbial growth. Certain pharmacological properties of thiobenzoic acid and its *o*-fluoro- and *m*-fluoro-derivatives have been reported (Gale, Kendall & Bernheim, 1964). These compounds are initially fungistatic to *Candida albicans*, with death of the cells occurring some hours after the fungistatic action is manifest. Evidence was presented which implicates inhibition of the microbial sulphate reduction system as the fundamental mechanism of antimicrobial action.

The present paper reports on cytological alterations which occurred in *Candida albicans* in the presence of thiobenzoate.

METHODS

The strain of *Candida albicans* used and the methods of growth and fixation were as described previously (Gale, 1963*b*) except that uranyl nitrate was used as a postfixative according to the method of Vitols, North & Linnane (1961). Thio-

benzoic acid (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) was neutralized as described earlier (Gale *et al.* 1964) and added to a 16 hr Sabouraud broth culture of the test organism to final concentration 1000 μg./ml. (Bacto-Sabouraud liquid medium, Difco Laboratories, Detroit, Mich., U.S.A.). Samples were taken at intervals for fixation, following which they were embedded in methacrylate (a 30/80 mixture of ethyl + butyl monomers) containing 0·2% uranyl nitrate (Ward, 1958) and polymerized at 60°. Sections were cut with glass knives on a Porter–Blum microtome and examined in an RCA EMU 3-F electron microscope, using 50 kV. accelerating voltage through a 30 μ objective aperture.

RESULTS

Control cells of *Candida albicans* were comparable to those described in an earlier report (Gale, 1963*b*), and similar to the preparations of *Saccharomyces cerevisiae* described by Vitols *et al.* (1961), with the exception that large vacuoles with well-defined limiting membranes were seen only rarely. A comparison with the earlier report (Gale, 1963*b*) clearly shows the advantages of using uranyl nitrate as a post-fixative following permanganate fixation of yeast-like organisms which are not well preserved with osmium tetraoxide (Gale, 1963*b*; Vitols *et al.* 1961). A typical specimen is shown in Pl. 1, fig. 1, displaying the cell wall of varying electron density, the invaginating cytoplasmic membrane, mitochondria with cristae, a nucleus with a typical unit membrane, and an area suggestive of a small vacuole. The granular nucleus shows areas of lesser electron density as was also described in *S. cerevisiae* (Vitols *et al.* 1961).

Proliferating cells to which thiobenzoate was added remained virtually indistinguishable from control cells for approximately 2 hr. Since the fungistatic action of this compound is manifest immediately rather than after a latent period (Gale *et al.* 1964), it must be concluded that the arrest of growth *per se* is unaccompanied by a resolvable cytological lesion. After further incubation for 2–3 hr, however, aberrations in cell structure became evident in a large percentage of the cells, progress of which is shown in Pl. 1, 2, figs. 2–4. The initial perceptible change was a lessened electron density of the nucleus. Following this there appeared gaps in the nuclear membrane, from which canaliculi emanated toward the periphery of the cell. A lessened electron density of mitochondria was noted frequently. Upon extension of the canaliculi to the periphery there was apparently some loss of integrity of the cell wall; the complete absence of cell wall material in some areas is probably an artifact of fixation, since it seems unlikely that an altered spheroplast in broth culture would remain in a cell wall with such a defect. Cytoplasm of thiobenzoate-treated cells seemed to have a greater electron density, but no marked changes were noted in granularity. A compromised cell wall would perhaps facilitate passage of uranyl ions into the cytoplasm to confer the enhanced density.

Even in the samples which were fixed after incubation for 3 hr with thiobenzoate, a small percentage of the cells retained all appearances of normal control cells. Previous work with thiobenzoate (Gale *et al.* 1964) showed that death of 100% of the cell population in a proliferating culture of *Candida albicans* did not occur until between 10 and 12 hr after addition of the inhibitor to a final concentration of 1000 μg./ml. Although quantitative plate counts were not made at intervals in that

study, it seems reasonable to assume that time of death occurred with the usual standard distribution within the population. Since fungistasis induced by thiobenzoate was immediate in onset (Gale *et al.* 1964), an attempt was made to determine whether changes observed in thiobenzoate-treated cells were in any way similar to changes occurring in autolysing cells.

Autolysis may be induced in two ways: washed, non-proliferating cells may be incubated in a non-nutrient medium; or a cell population in a nutrient medium may be incubated for a time beyond the point at which population density is maximal. In the former case, cessation of growth and ultimate cell deterioration is due primarily to substrate depletion through washing. In the latter case, depletion of substrate and accumulation of metabolic end products presumably are both contributory factors. Consequently, the cytological changes which accompany autolysis induced in each manner were investigated.

Samples for fixation were taken aseptically at intervals from a growing culture of *Candida albicans* during a period of 120 hr; the growth curve of the culture as shown by optical density measurements levelled off after incubation for about 40 hr. A typical autolysing cell is shown in Pl. 2, fig. 5. The initial change consisted of loss of nuclear and mitochondrial membranes. Nuclear material remained cytologically distinguishable for a time, however, being detectable by a slight difference in granularity as compared with the surrounding cytoplasm. The event of cell disintegration was undoubtedly a rapid one, since no cells were observed which displayed a stage intermediate between that just described and that shown in Pl. 2, fig. 4. The cytological events of autolysis in an ageing culture thus appeared different from those which occurred during treatment with thiobenzoate.

A 16 hr culture of *Candida albicans* was washed three times with 0·1M-sodium potassium phosphate buffer (pH 7·4) to remove nutrient materials. After suspending the sedimented pellet of cells to the original volume in buffer, the suspension was returned to the incubator (37°) and samples removed at intervals for fixation. Changes were not observed until about 4 hr after washing and were not as consistent as those observed in the ageing autolysing culture. The most frequently observed forms were cells in which cytoplasm had shrunk markedly from the cell wall. Nuclei and mitochondria were usually intact until the event of complete cell disintegration occurred (Pl. 2, fig. 6). In spite of the greater variety of forms observed, it was clear that autolysis which occurred under substrate deprivation did not bear any resemblance to initial cytological changes which accompany thiobenzoate treatment.

DISCUSSION

Evidence presented here and in a preceding paper (Gale *et al.* 1964) indicates that the antimicrobial action of thiobenzoate against *Candida albicans* consists of at least two components. First of these is a fungistatic action manifest by cessation of replication of cells of an actively growing culture immediately upon addition of inhibitor. This action is unaccompanied by any observable cytological lesion and can be annulled for a time by washing the cells with a large volume of water and plating on fresh medium. The second component, irreversible cell damage, is time dependent and occurs only in proliferating cells; cells previously washed and then exposed to thiobenzoate in a nutrient-free medium were not killed within a 12 hr

period (Gale *et al.* 1964). The initial fungistasis may be explained on a nutritional deprivation induced by the inhibitor, that is, a block in metabolism of some compound(s) required for normal growth. Inhibition of the microbial sulphate reduction system, previously suggested as the primary mechanism of action of thiobenzoate and its *o*-fluoro- and *m*-fluoro-derivatives (Gale *et al.* 1964), is one way in which such a nutritional deprivation could be imposed. In intact cells the fungistatic mechanism is obviously reversible, as shown by survival of cells after removal of the inhibitor by washing.

The terminal cytological manifestation of cell damage due to thiobenzoate is apparently a lytic phenomenon, as evidenced by the similarity in appearance of drug-treated and autolysing cells after complete loss of cytoplasmic organization. Events leading to lysis, however, appear to be quite different in the two cases. Whereas initial autolytic changes in an ageing culture consist of loss of mitochondrial and nuclear membranes, and autolysing washed cells showed principally retraction of cytoplasm with a maintenance of membranes, changes induced by thiobenzoate primarily originate in the nucleus. If the hypothesis of mechanism of action of thiobenzoate on the sulphate reduction system is substantiated, the relationship of sulphur metabolism to nuclear organization remains to be clarified.

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EXPLANATION OF PLATES

The following abbreviations are used: CW, cell wall; CM, cytoplasmic membrane; N, nucleus; M, mitochondrion; NM, nuclear membrane; V, vacuole. The line in each figure indicates 1·0 μ.

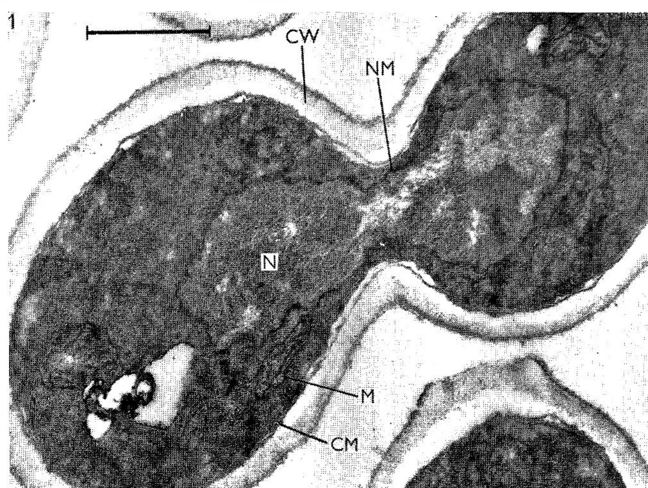
Figs. 2–4. Cells of *Candida albicans* after exposure to 1000 μg. thiobenzoate/ml., in Sabouraud broth.

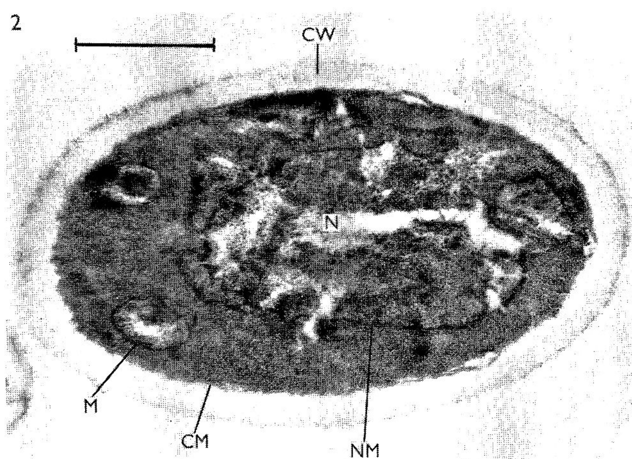
PLATE 1

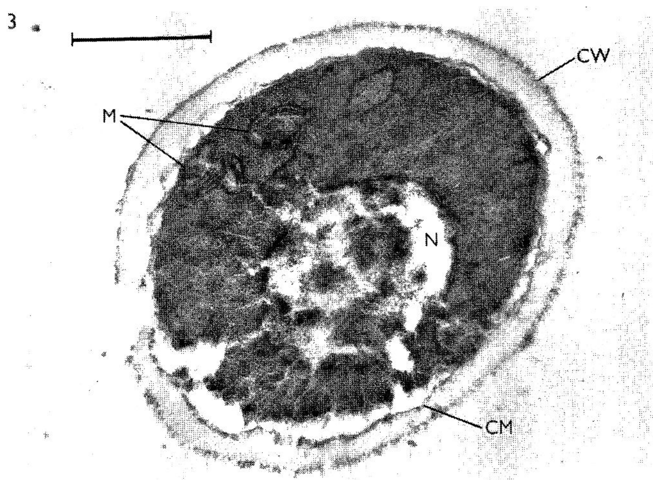
Fig. 1. Control cell of *Candida albicans* undergoing division. × 26,000.

Fig. 2. After 2 hr exposure, nuclear integrity is lost and gaps appear in the nuclear membrane. × 30,000.

Fig. 3. After 3 hr exposure, canaliculi radiate from the nucleus to the periphery of the cell. Cytoplasm has partially retracted while the cytoplasmic membrane appears to remain associated with the cell wall. × 30,000.







G. R. GALE AND H. H. McLAIN

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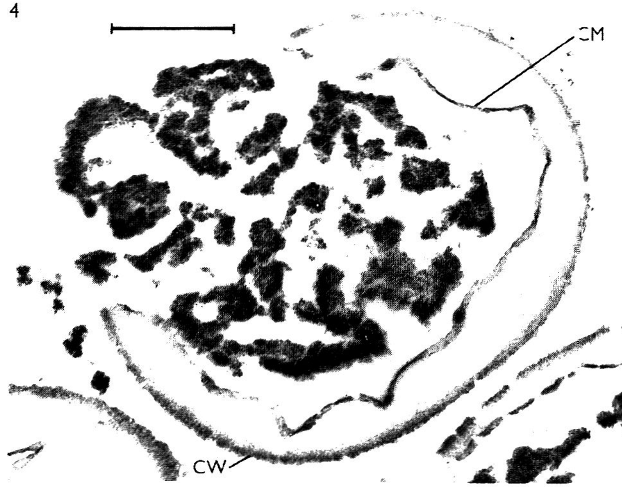

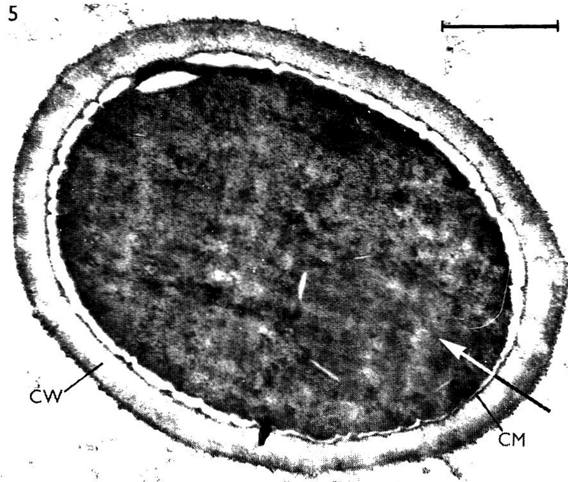

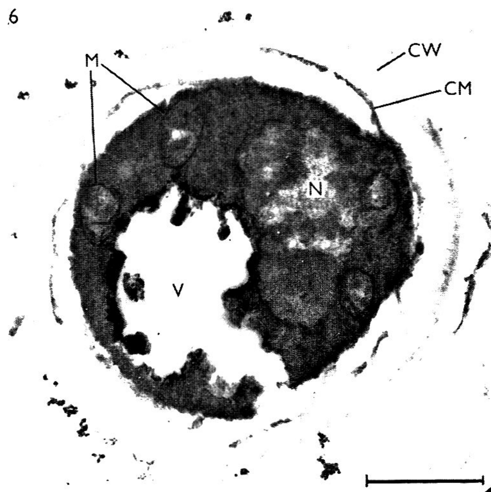

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PLATE 2

Fig. 4. After 4 hr exposure, cytoplasm is completely disorganized and no intracellular organelles can be differentiated. ×26,000.

Fig. 5. Cell of *Candida albicans* from a 66 hr culture in Sabouraud broth. The nuclear membrane cannot be detected, but the nucleus can still be differentiated by a slight difference in granularity (arrow). ×25,000.

Fig. 6. Cell of *Candida albicans* after exposure to a non-nutritional environment for 4 hr. Pre-autolytic changes consist of cytoplasmic retraction with disruption of the cytoplasmic membrane. The nucleus and mitochondria are intact. Large vacuoles were seen frequently under these conditions. ×25,000.

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With 1 *plate*
Printed in Great Britain

Some Properties of Cocoa Swollen-Shoot Virus

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(*Received* 16 *March* 1964)

SUMMARY

Partially purified preparations of the Kofi Pare isolate of cocoa swollen-shoot virus (CSSV) were usually inactivated after 10 min. at 50°, but not after 10 min. at 45°. Infectivity of freshly made preparations was greatly lessened by diluting 1/10, and lost at 1/100. The infectivity of preparations increased after storage for 24 hr; after 96 hr infectivity was retained at 0–4° but soon lost at 25°. The virus survived freezing *in vitro* and, with some loss of infectivity, freezing in leaves and storage in leaves dried over aluminium oxide. Infective material was precipitated from dilute extracts by half saturation with ammonium sulphate at 25°; it appeared to be equally stable over the range pH 6 to pH 8. Preparations of the symptomatologically distinct Kofi Pare, Mampong, Dawa, Nsaba and Bosomuoso isolates all contained similar rod-shaped particles of size about 121 × 28 mμ.

INTRODUCTION

The availability of infective preparations of cocoa swollen-shoot virus (Brunt & Kenten, 1963) has made it possible to examine the properties of the virus *in vitro*. We report here some of the properties of partially purified preparations of the Kofi Pare isolate, and the electron microscopy of this and four other symptomatologically distinct isolates.

METHODS

The cocoa swollen-shoot virus (CSSV) isolates from Kofi Pare, Bosomuoso, Mampong and Nsaba in Ghana were derived from cultures described previously (Brunt & Kenten, 1963); an isolate from Dawa (Eastern Region, Ghana) was also used. The Kofi Pare isolate, which is indistinguishable from Posnette's (1947) swollen-shoot virus strain A or virulent New Juaben strain, was used throughout, and the others for electron microscopy only.

The viruses were extracted from the leaves of infected cocoa seedlings and partially purified by methods similar to those described previously (Brunt & Kenten, 1963). Twenty-five g. infected first-flush cocoa leaves were ground in a Waring Blendor for 1 min. in 500 ml. of a solution at pH 8·0–8·2 containing 0·05 M-disodium hydrogen phosphate, 0·05 M-thioglycollic (mercaptoacetic) acid, 0·005 M-sodium diethyldithiocarbamate and 1 or 2% (w/v) hide powder (Hopkin & Williams

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Ltd.). This hide powder was first thoroughly dispersed in the solution by running the Waring Blendor at full speed for 3 min. before adding the leaves. The suspension was squeezed by hand through cotton cloth and clarified by centrifugation at 8000 *g* for 5–10 min. The virus was then sedimented by centrifuging the supernatant fluid at 75,000 *g* for 2 hr. The pellets were usually dispersed in a solution at pH 8·0 containing 0·01 M-disodium hydrogen phosphate and 0·01 M-thioglycollic acid, using about 1/35 of the volume of the original extract.

Because the original difficulties in transmitting CSSV by sap inoculation (Brunt & Kenten, 1960, 1962*b*) might have been caused by instability of the virus, at first all extracts were made as quickly as possible, with chilled apparatus and solutions. However, later extractions were made at room temperature (about 25°), after it was found that such extracts were usually more infective than those made below 8°.

All infectivity tests were made by inoculating whole cocoa beans; to increase the number of infections a little 'Celite' 545 (Johns Manville Ltd.) was added to all inocula.

Electron microscopy was done at Rothamsted Experimental Station, with samples of virus preparations made and tested for infectivity in Ghana and sent to the United Kingdom by air in chilled vacuum flasks. On arrival they were clarified by centrifugation at 8000 *g* for 10 min. and the virus again sedimented from the supernatant fluid by centrifuging at 75,000 *g* for 2 hr. The greenish pellets were dispersed in 1–2 ml. of 0·1 M-ammonium acetate and used for electron microscopy after brief centrifugation at 8000 *g* to remove insoluble material. Preliminary experiments showed that satisfactory shadowcast mounts could not be made from these preparations because they still contained mucilage even after two cycles of differential centrifugation. All electron microscopy was therefore done with mounts made by a negative staining technique (Brenner & Horne, 1959). Most microscopy was done at × 20,000 magnification; some pictures were taken at × 80,000. Particle lengths were measured either from the original photographic plates by using a scale graduated in 0·5 mm. steps and a hand lens, or were estimated by measuring the image projected by a calibrated photographic enlarger.

RESULTS

Properties of the virus in vitro

The properties of the virus were determined by using preparations made from clarified cocoa leaf extracts by one cycle of high-speed centrifugation. The sedimented virus was dispersed in phosphate + thioglycollate solution of 1/35th the original volume; any further dilutions were also made in the same phosphate + thioglycollate solution. Since the leaves had originally been macerated in 20 volumes of the extracting solution, each 1 ml. of these partially purified preparations was derived from 1·75 g. fresh leaf.

Dilution end-point. When tested fresh, the infectivity of preparations was greatly diminished by diluting 1/10 and abolished by diluting 1/100. After storage for 24 hr, however, infectivity increased (see below) and infections were obtained at 1/100 (Table 1).

Stability in vitro. Preparations of virus were kept in loosely stoppered test tubes at 0–4° or 25° and tested for infectivity after 24, 48 and 96 hr. After 24 hr the

infectivity of both samples increased; on storage up to 96 hr there was little further change in the infectivity of samples stored at 0–4° but those kept at about 25° lost much of their infectivity.

Thermal inactivation. One-ml. samples of virus preparation were heated for 10 min. to various temperatures. Infectivity, seemingly unaffected by heating up to 45°, was lost at 50° or above, except for a single infection which was obtained from one sample heated to 55° (Table 2).

Table 1. *Stability of cocoa swollen-shoot virus in vitro at room temperature (about 25°) and 0–4°*

	Duration of storage (hr)						
	0	24		48		96	
		Temperature					
Dilution of preparations*		0–4°	25°	0–4°	25°	0–4°	25°
		Infectivity (infections/beans tested)†					
1/1	34/60†	42/60	45/59	35/59	29/57	35/59	5/59
1/10	7/59	22/60	21/60	17/59	15/56	6/60	1/59
1/100	0/57	2/59	2/60	0/56	3/54	2/59	0/59

* Dilutions were made after storage with a solution (pH 8) containing 0·01 M-disodium hydrogen phosphate and 0·01 M-thioglycollic acid.

† The denominator is the total number of whole cocoa beans inoculated in two experiments and the numerator the number of plants which were infected. Beans failing to germinate were omitted from the denominator.

Table 2. *Thermal inactivation of partially purified preparations of cocoa swollen-shoot virus*

		Preparations heated for 10 min.			
		45°	50°	55°	60°
Dilution of preparations*	Unheated	Infectivity (infections/beans tested)			
1/1	19/60	23/59	0/60	1/58	0/60
1/10	6/60	5/60	0/60	0/60	0/60

* Dilutions made with a solution (pH 8) containing 0·01 M-thioglycollic acid and 0·01 M-disodium hydrogen phosphate after heating 1 ml. virus samples.

Effect of freezing and desiccation. Preparations of virus from leaves which had been frozen for 24 hr or dried *in vacuo* over aluminium oxide before they were extracted were infective, but slightly less so than those made from fresh leaves (Table 3). Freezing had little effect upon the infectivity of clarified sap or the partially purified preparations.

Precipitation with ammonium sulphate. In preliminary tests (Brunt & Kenten, 1962*a*) the sparse precipitate formed when ammonium sulphate was added to clarified cocoa leaf extracts, prepared by grinding leaves in extracting fluids containing no added protein, was not infective. However, when extracts made with fluid containing added protein (2%, w/v, hide powder) were half-saturated with ammonium sulphate at 25° the bulky precipitate produced was infective. After

dialysing the resuspended precipitate against phosphate + thioglycollate solution (pH 7·5–8·0), and removing insoluble material by low-speed centrifugation, highly infective preparations were obtained by sedimenting the virus from the supernatant fluid.

Effect of pH value. Preparations were dispersed in phosphate + thioglycollate solution adjusted to pH 6, 7 and 8, and samples tested for infectivity immediately and after 24 and 48 hr. All were similarly infective (Table 4).

Table 3. *Effects of drying or of freezing infected leaves on virus*

In each experiment thirty whole cocoa beans were inoculated with each of six samples. Infected leaves were frozen for 24 hr. Shredded leaves were dried *in vacuo* for 24 hr over aluminium oxide.

	Leaves processed		
	Immediately	After freezing	After desiccation
Expt. no.	Infectivity (infections/beans tested)		
1	32/164	20/174	32/172
2	66/180	48/179	16/136
3	63/171	57/180	—
Total	161/515	125/533	48/308
Mean (%)	33·3	23·5	15·6

Table 4. *Effect of pH value on stability of partially purified virus*

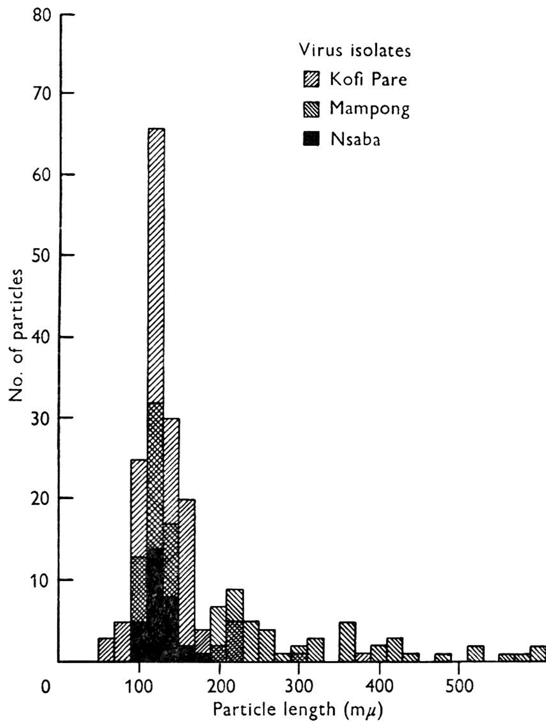
	Duration of treatment (hr)		
Suspending solution at pH	0	24	48
	Infectivity (infections/beans tested)		
6	17/40	16/40	11/30
7	22/38	24/40	13/30
8	14/39	23/40	15/30

Electron microscopy

Presence of particles. All virus preparations made from plants infected with the CSSV isolates tested contained rod-shaped particles; such particles were never found in preparations made in the same way from healthy plants. No formal correlation between particle numbers and infectivity was attempted, but more such particles were found in highly infective preparations than in less infective ones.

Morphology of the particles. The particles were straight or slightly curved, with rounded ends (Plate 1). Preparations of the isolates from Kofi Pare, Mampong and Nsaba contained enough particles to permit making histograms showing the length distribution (Fig. 1); particles of the Dawa and Bosomuoso isolates, although too few to construct histograms, gave indications of a similar length-distribution pattern. Because the distribution is skew, no valid estimate of the most common length can be made by taking the mean of all particles measured. We have overcome this difficulty by selecting a narrow range of sizes symmetrically placed about the most common length shown in the histogram, and using only particles falling within the range to estimate the most common length (Brandes & Paul, 1957).

Table 5 shows the mean value obtained for the three isolates by this method. The lengths of particles in preparations made from cocoa plants infected with the Kofi Pare, Mampong and Nsaba CSSV isolates are all similar.



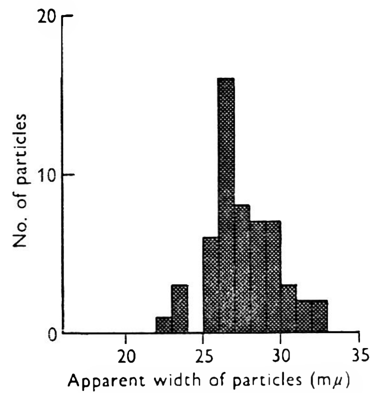


Fig. 1 Fig. 2

Fig. 1. Length distribution of particles found in virus preparations from plants infected with three isolates of cocoa swollen-shoot virus.

Fig. 2. Widths of particles seen in electron micrographs of cocoa swollen-shoot virus preparations.

Table 5. *Dimensions of virus-like particles extracted from cocoa leaves infected with three isolates of cocoa swollen-shoot virus*

Virus isolate	Number of particles measured	Particles in 91–150 mμ group (%)	Mean length of 91–150 mμ particles	Width (mμ)*
Kofi pare	102	60·8	119·6	26·7 ± 0·4
Mampong	162	82·1	122·2	29·1 ± 1·8
Nsaba	30	90·0	122·7	28·9 ± 4·0
All isolates together	294	75·9	121·0	27·9

* Particle widths estimated from micro-densitometer traces across the images.

Estimates of particle width were taken from micro-densitometer traces made across the images on the original plates. The values obtained (Fig. 2) almost certainly underestimate the true particle width, because of penetration of the phospho-

tungstate (PTA) into the outer parts of the particles; this occurs even with extremely stable viruses such as tobacco mosaic, particles of which appear to be only 12 mμ wide instead of 15 mμ when measured from shadowed mounts. The particles appear to be tubular, with their centres filled with PTA (Plate). The rounded ends are an unusual feature and we know of only one other rod-shaped virus particle (lucerne mosaic) with rounded ends (Gibbs, Nixon & Woods, 1963). Whether the rounded ends of the CSSV particles represent an essential part of their structure, or merely show that the ends of the particles are degraded, is uncertain; we incline to the latter view because a few particles in some of our better extracts have had one or both ends cut off square. No other structures could be seen in the particles, even in micrographs of good quality made at × 80,000.

Although it is not known that the particles observed in the electron microscope are those of CSSV, their size, shape, general appearance, and the fact that their presence appears to be correlated with infectivity, all suggest that they are the virus.

DISCUSSION

Cocoa swollen-shoot virus (CSSV), cocoa mottle leaf virus and strains A and B of the virus which occurs in Trinidad (Baker & Dale, 1947) are the only viruses known with certainty to be transmitted by mealybugs. It is therefore of particular interest that at least one isolate of CSSV can be studied and characterized *in vitro*. Because we have determined some of the properties of CSSV in partially purified preparations, no direct comparison with those of most other viruses is possible. However, our results clearly show that CSSV is moderately stable once it has been separated or protected from some constituents of cocoa leaves. Initial difficulties in transmitting the virus by mechanical inoculation (Brunt & Kenten, 1960, 1962*b*) therefore probably reflected the release of inhibitors of infection when cocoa leaves were macerated, rather than any intrinsic instability of the virus or an unusually small virus concentration in the leaf. Indeed preparations, which on a leaf weight basis were less than twofold concentrated, were not only infective but were so when diluted 1/10 and occasionally 1/100. No attempt was made to identify these inhibitors but, since 'vegetable tannins' occur abundantly in cocoa leaves (Holden, 1957) and inactivate other viruses (Cadman, 1959; Thresh, 1956), Brunt & Kenten (1963) suggested that these are the leaf constituents which inactivate CSSV when cocoa leaves are macerated. The protective effect of protein was explained by postulating that the 'vegetable tannins' combine with this rather than with the virus. However, fixation of tanning substances by proteins is related to the charge carried by the protein, fixation by vegetable tannins increasing with net positive charge. By contrast, fixation of aldehydes by proteins increases with net negative charge, so that at or about pH 8 it might be expected that proteins would fix aldehydes more readily than vegetable tannin. For this reason leaf aldehydes may also be important inhibitors of infection by CSSV in alkaline extracts of cocoa leaves.

Although our attempts to prepare antisera to the Kofi Pare CSSV isolate failed, now that the virus is known to be relatively stable *in vitro*, it will be worth trying to prepare antisera by using more concentrated preparations as antigens. In making such preparations the need to ultracentrifuge large volumes of clarified extracts could

0·1 μ

be avoided by making a preliminary concentration by precipitating the virus from dilute extracts with ammonium sulphate.

The technical assistance of Messrs B. M. Lagudah and J. K. Bonney is gratefully acknowledged. We also thank Dr J. W. Blencowe for much help when attempting to prepare antisera. This paper is published with the permission of the Chairman of the Managing Committee of the West African Cocoa Research Institute.

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EXPLANATION OF PLATE

Electron micrograph of particles found in preparations from plants infected with the Kofi Pare isolate of cocoa swollen-shoot virus. Negatively stained with phosphotungstate.

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The Genetic Behaviour of Colicinogenic Factor E_1

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SUMMARY

Analysis of the genetic behaviour of the colicinogenic factor E_1 ($colE_1$) in crosses involving Hfr or F^+ and F^- strains of *Escherichia coli* K12 shows: (1) this factor is transferred with very high frequency from different donor bacteria; (2) its transfer begins to take place early whatever the type of donor cell; (3) it is not linked with chromosomal markers; (4) it multiplies autonomously in the zygote at such a rate that its passage to all descendent cells is assured; (5) the non-colicinogenic character of the donor parent does not appear among recombinant or recipient cells; (6) no lethal zygosis occurs related to the transfer of a presumptive $colE_1^-$ locus.

All these facts suggest that $colE_1$ exists in an extra-chromosomal state in Hfr, F^+ and F^- bacteria.

No transfer of $colE_1$ from F^- to F^- or Hfr bacteria was observed.

INTRODUCTION

It is well known that the hereditary ability of colicinogenic bacteria to synthesize colicin can be transmitted by cell contact among strains of the family Enterobacteriaceae (Fredericq, 1954*a*, 1956; Hamon, 1956; Ozeki, Stocker & Smith 1962).

Studies on the transmission of colicinogenic factor E_1 ($colE_1$)—controlling the synthesis of colicin E_1 or ER—from F^+ to F^- strains of *Escherichia coli* K12 were carried out by Fredericq & Betz-Bareau (1953*a*, *b*). They showed that $colE_1$ was transferred efficiently to recombinants in crosses between colicinogenic (col^+)F^+ donors and non-colicinogenic (col^-)F^- recipients, and that in reciprocal crosses recombinants never acquired the non-colicinogenic character of the donor parent. From these results, and also from the lack of linkage of $colE_1$ with chromosomal markers, they considered this factor as cytoplasmic.

Moreover, the behaviour of $colE_1$ in crosses between Hfr of different types and F^- bacteria (Alfoldi, Jacob & Wollman, 1957; Alfoldi, Jacob, Wollman & Mazé, 1958) suggested that it possessed the properties assigned to episomal particles (Jacob & Wollman, 1958). Certain experimental evidence, such as lack of linkage between this factor and chromosomal markers and its transmission from the zygote to all descendent cells, led to the conclusion that it could multiply autonomously in the recipient cell at a faster rate than the chromosome.

On the other hand, the results also showed that the frequency of transfer of $colE_1$, in crosses involving different Hfr col^- strains and F^-col^- bacteria, varied with the type of Hfr employed. In reciprocal crosses, Hfr $col^- \times F^-col^+$, no transfer of the col^- character was detected and death of the zygotes was found to occur with each

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type of Hfr with a magnitude comparable to the transfer of col^+ in the corresponding reciprocal cross. The apparent correlation observed between the transfer of col^+ and the 'lethal zygosis' in both series of crosses was consistent with a chromosomal location of $colE_1$ in Hfr cells.

No indication of a chromosomal state has been found with colicinogenic factors E_2, I and V in crosses involving F^+ and different Hfr strains as donors (Nagel de Zwaig, Antón & Puig, 1962). Analysis revealed that, even though these factors were transferred with quite different frequencies ($colE_2$ and *colI* at low frequency and *colV* with maximum efficiency), each of them was transferred with similar frequencies from different types of donor cells (F^+ or Hfr), it was not linked to chromosomal markers, and in no case did the col^- character of the donor appear among recombinants.

The number of the F^- bacteria decreased in some matings when the number of the Hfr bacteria greatly exceeded that of the recipient, suggesting some type of lethal zygosis. This lethality could not be related to transfer of the $col+$ or the col^- character since it appeared also in control crosses. Subsequently, this effect was confirmed and further analysed by Clowes (1963) in his research on $colE_1$.

Studies on the genetic behaviour of colicinogenic factors E_2, I and V have been now extended, under the same conditions, to $colE_1$. Evidence is given which demonstrates the autonomous state of this factor in strains of *Escherichia coli* K12, in agreement with the conclusions reached simultaneously by Clowes (1963).

METHODS

Media. The minimal medium (*m.m.*) employed was that described by Davis & Mingioli (1950). Salts, glucose and agar were autoclaved separately and mixed before pouring on to plates. Amino acids, when required, were added at a concentration of 80 μg./ml. and thiamine at a concentration of 5 μg./ml.

Nutrient broth and nutrient agar (nutrient broth with 1·5 %, w/v, of Bacto-Agar) and L broth and L broth agar (Lennox, 1955) were also employed.

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

Nutrient broth with 0·7 % (w/v) of Bacto-Agar was used as top agar for the plating of bacteria.

Strains. Bacterial strains mentioned in this paper are listed in Table 1. They were obtained from Dr E. L. Wollman. (For detailed references of these strains see Nagel de Zwaig *et al.* 1962). Strains derived from those listed in Table 1, employed in the present study, are described in the text.

Strain 58–161 was used as a sensitive indicator for colicin E_1.

The origin and direction of the Hfr strains employed and the location of some essential characters on the linkage group of *Escherichia coli* K12 are shown in Fig. 1.

Phage P_1 used in transduction experiments was kindly provided by Professor S. E. Luria.

Resistant strains. All strains employed in matings were previously made resistant to colicin E_1 to avoid distortion of results due to the lethal action of the colicin on sensitive strains.

For the isolation of these resistant cells, a colicinogenic strain was stabbed on an agar plate and incubated for 24 hr. The colonies thus developed were sterilized by

Table 1. *Bacterial strains*

Name	Relevant characteristics
E. coli K 12	
58	F^+ *met*⁻ *str-s* $colE_1^+$
112	F^+ *cys*⁻ *his*⁻ *str-s*
Hfr H	*thi*⁻ *str-s*
Hfr 4	*thr*⁻ *leu*⁻ *thi*⁻ *str-s*
Hfr 2	*met*⁻ *str-s*
Hfr AT IIIA	*prot. str-s*
58-161	F^+ *met*⁻ *str-r*
PA 309	F^- *thr*⁻ *leu*⁻ *thi*⁻ *try*⁻ *arg*⁻ *str-r*
C600	F^- *thr*⁻ *leu*⁻ *thi*⁻ *str-r*

The following abbreviations are used: arginine: *arg*; cysteine: *cys*; galactose: *gal*; histidine: *his*; lactose: *lac*; leucine: *leu*; methionine: *met*; thiamine: *thi*; threonine: *thr*; tryptophan: *try*; valine: *val*; resistance to streptomycin: *str-r*; sensitivity to streptomycin: *str-s*; prototrophic: *prot.*
Requirement for a given amino acid or vitamin is indicated by the minus sign.

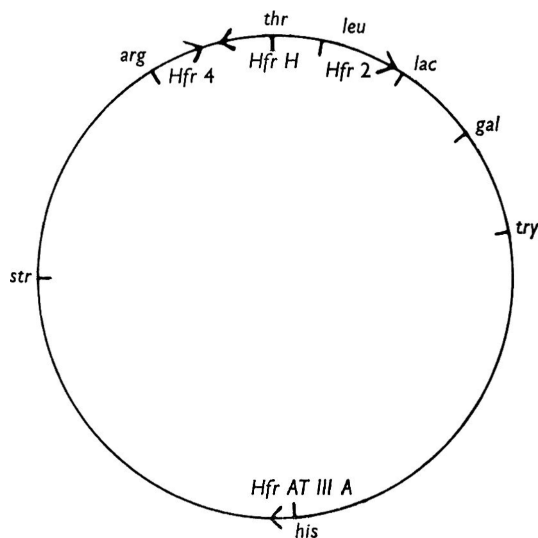

Fig. 1. Linkage map of *Escherichia coli* K 12 (Jacob & Wollman, 1961) with indication of the origin and direction of the Hfr employed. Symbols are specified under Table 1.

chloroform vapour, and melted top agar seeded with the sensitive strain poured on the surface of the plate (Fredericq, 1958). Resistant colonies were isolated from the halos formed by the action of the colicin on the sensitive strain.

Transfer of colicinogenic factor E_1. Factor $colE_1$ was first transferred from strain 58 $colE_1^+$ to strain PA 309/E_1 by: (*a*) *Mixed culture*, of both strains, incubated overnight in nutrient broth at 37°. Samples of the mixture were diluted in buffer and plated on nutrient agar plates containing streptomycin, where only strain PA 309 could grow. Col^+ colonies were detected by the halos formed on an indicator strain using the double-agar layer method (Fredericq, 1954). (*b*) *By transduction* with phage P_1 prepared by lytic growth on 58 $colE_1^+$. Col^+ colonies were also detected by the double-agar layer technique.

$ColE_1$ was transferred from PA 309 to strain 112 F^+ in mixed culture, as explained

above, and from the latter strain to different Hfr strains, by the same procedure. When $colE_1$ had to be transferred to Hfr or F^+ strains, cultures in the stationary phase of growth were employed in order to make them phenotypically F^- (Lederberg, Cavalli & Lederberg, 1952).

Matings. Details of $Hfr \times F^-$ or $F^+ \times F^-$ crosses, interrupted matings and detection of chromosomal recombinants (those receiving the capacity to synthesize an amino acid from the donor parent and the *str-r* character from the recipient) and of col^+ *str-r* cells (which combine the col^+ character of the donor and the *str-r* one of the recipient) are described in a previous communication (Nagel de Zwaig *et al.* 1962).

The frequency of chromosomal recombinants or transfer of col^+ is expressed as the ratio of the number of recombinants or col^+ *str-r* cells, respectively, to 100 initial bacteria of the minority parental strain.

Unless otherwise stated, mating mixtures were sampled and plated for recombinants and *col*+ *str-r* cells 90 min. after mixing the strains.

When the kinetics of recombinant formation was followed during a long period of time, the mating mixture was diluted 1/10 in fresh nutrient broth every 100 min. to maintain the strains in the logarithmic phase of growth.

To measure the frequency of *col*+ colonies among chromosomal recombinants or recipients, individual colonies growing on the original plates were transferred to the surface of plates containing the same selective medium; colicinogenic bacteria were then detected by the agar layer method.

For the selection of chromosomal recombinants in $F^- \times F^-$ crosses, the mixture of strains was centrifuged and resuspended in buffer before plating.

When studying the transfer of $colE_1$ from F^- to Hfr cells, platings were done on *m.m.* supplemented with the corresponding amino acids and valine to contraselect the valine-sensitive F^- strain (Manten & Rowley, 1953).

Transduction. For the preparation of stock of phage P_1 and for transduction experiments, the techniques indicated by Lennox (1955) and Arber (1960) were followed.

RESULTS

Crosses between colicinogenic donors and non-colicinogenic recipients

Transfer of colicinogeny

Frequency of transfer. The frequency of transfer of $colE_1$ was studied in Hfr $colE_1^+ \times F^- colE_1^-$ crosses involving different types of Hfr strains, and $F^+colE_1^+ \times F^- colE_1^-$ crosses, all performed in the proportion of 1 donor to 20 recipient cells.

In each cross the frequency of recombinants for an early Hfr marker was determined in order to verify its efficiency as donor.

Table 2 shows that $colE_1$ is not only transferred from F^+ and different Hfr bacteria with similar frequencies but also that its frequency of transfer greatly exceeds 100 % of input of donor cells, at 90 min. after the beginning of mating.

No differences in the transfer of col^+ were found in matings involving strains made colicinogenic by transduction or by mixed culture (See Methods).

Kinetics of transfer. The time of entry of $colE_1$ in the recipient cells was determined in matings interrupted by a Waring Blendor. In this way it was established that $colE_1$ begins to appear among recipient cells within 5 min. of the onset of mating in

Table 2. *Frequency of transfer of* $colE_1$ *in crosses between Hfr* $colE_1^+$ *or* F^+ $colE_1^+$ *and* PA 309 $colE_1^-$

Donor strains	Frequency of transfer of $colE_1$ (in %)
Hfr H $colE_1^+$	780
Hfr 4 $colE_1^-$	1000
Hfr 2 $colE_1^+$	600
Hfr AT IIIA $colE_1^+$	900
112 (F^+) $colE_1^+$	600

Crosses indicated were performed in the proportion of 1 donor to 20 recipient cells. The frequency of transfer was measured at 90 min. after mixing the parental strain and expresses the ratio (in %) of the viable count of recipients becoming col^+ to the initial viable count of donor strains.

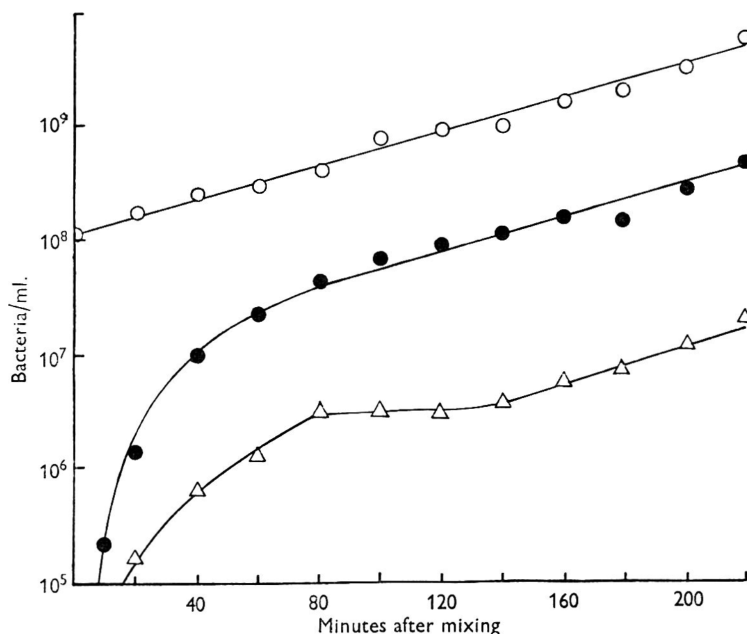

Fig. 2. The kinetics of transfer of $colE_1^+$ and $thr^+ leu^+$ in Hfr H $colE_1^+$ × PA 309 $colE_1^-$ cross, performed in the proportion of 1 donor to 20 recipient cells. Samples were taken at different times, treated in a Waring Blendor, diluted, and plated on selective media. A sample of the mating mixture was diluted 1/10 in fresh nutrient broth every 100 min. to maintain it in the logarithmic phase of growth. △, $thr^+leu^+str\text{-}r$ recombinants; ●, $col^+str\text{-}r$ bacteria; ○, $str\text{-}r$ (F^-) bacteria.

crosses using the donor strains Hfr H $colE_1^+$, Hfr 4 $colE_1^+$, Hfr AT IIIA $colE_1^+$ and 112 F^+ $colE_1^+$.

The kinetics of transfer of $colE_1$ from Hfr H $colE_1^+$ strain to PA 309 $colE_1^-$ was followed in interrupted matings using 1 donor to 20 recipient cells and was compared with the kinetics of formation of thr^+ leu^+ $str\text{-}r$ recombinants (Fig. 2).

From Fig. 2, it is clear that: (*a*) chromosomal recombinants rapidly increase in number soon after mating; later, this increase diminishes gradually attaining at about 80 min. a 'plateau' which extends till 120–140 min. After 140 min. recombinants begin to divide at the same rate as other F^- (*str-r*) cells. (*b*) The slope of the

curve for col^+ *str-r* cells also shows an initial rapid increase till about 80–100 min. Then, the slope of the curve diminishes and becomes parallel to that for other F^- cells.

At 120 min. after the beginning of mating frequencies of transfer of col^+ of 800–1000 % were attained, in contrast to frequencies of about 40–50 % measured for chromosomal recombinants.

Kinetics of transfer of col^+ similar to the one shown in Fig. 2 were observed in crosses involving Hfr 2 $colE_1^+$ and 112 F^+ $colE_1^+$ strains as donors.

When matings were performed in the proportion of 1 donor to 1 recipient cell, the differences between the kinetics of transfer of col^+ and the formation of chromosomal recombinants become more evident, since under these conditions the plateau of recombinants was attained much earlier.

Formation of recombinants

Colicinogeny among recombinants. Different types of recombinants from Hfr $colE_1^+ \times F^- colE_1^-$ crosses were selected and their col^+ or col^- character determined. As can be seen in Table 3, $colE_1$ appeared in almost the same proportion among recombinants corresponding to proximal or distal markers of any of the different Hfr strains employed. These results show that $colE_1$ is not linked to chromosomal markers localized on different sites on the linkage group of *E. coli* K12.

Table 3. *Percentage of* $colE_1^+$ *among recombinants of crosses between Hfr* $colE_1^+$ *and* PA309 $colE_1^-$

Donor strains	Recombinants			
	thr^+ leu^- *str-r*	try^- *str-r*	his^- *str-r*	arg^+ *str-r*
Hfr H $colE_1^+$	65	67	64	68
Hfr 4 $colE_1^+$	—	73	57	58
Hfr 2 $colE_1^+$	57	68	75	59
Hfr AT IIIA $colE_1^+$	79	86	74	77

One hundred or more recombinants of each type of recombinant were analysed. All crosses were performed in the proportion of 1 donor to 20 recipient cells.

The order of transfer of the Hfr markers is: Hfr H: *thr leu try his arg*; Hfr 4: *arg his try*; Hfr 2: *leu thr arg his try*; Hfr AT IIIA: *his try leu thr arg*.

The effect of colicinogeny on the frequency of recombinants. The frequency of recombinants for an early Hfr marker was determined in $col^+ \times col^-$ crosses and in the corresponding control $col^+ \times col^+$ crosses. As shown in Table 4, no significant variation or decrease in fertility becomes apparent when the frequency of recombination in both types of crosses was compared.

Crosses between non-colicinogenic donors and colicinogenic recipients

Absence of transfer of the non-colicinogenic character to recipients or recombinants

Hfr $colE_1^- \times F^- colE_1^+$ crosses involving different Hfr strains were performed in order to determine the transfer of the non-colicinogenic character.

In crosses performed in the proportion of 20 donors to 1 recipient cell, no col^- bacteria were detected among the *str-r* resulting zygotes analysed (approximately 1000); nor was the col^- character of the donor parent observed among 500

recombinants from the same crosses using either 1 to 20 or 20 to 1 donor to recipient cells.

Non-appearance of the *col*$^-$ character among recipients or recombinants cannot be explained by assuming death of zygotes which receive this character. As shown in Table 5, no decrease in number of the F$^-$ cells was observed at 90 min. after the onset of mating in crosses using 20 donors per recipient, except when the strain Hfr H was involved. In this case the number of F$^-$ cells decreased but without any relation to the transfer of *col*$^-$, since it also occurred with similar frequency in *col*$^-$ × *col*$^-$ control crosses.

Table 4. *Comparison of fertility in Hfr colE*$_1^+$ × *F*$^-$ *colE*$_1^-$ *and Hfr colE*$_1^+$ × *F*$^-$ *colE*$_1^+$ *crosses*

Donors	Type of recombinant selected	Recipient strain	
		PA 309 *colE*$_1^-$	PA 309 *colE*$_1^+$
Hfr H *colE*$_1^+$	*thr*$^+$ *leu*$^+$ *str-r*	48	52
Hfr 4 *colE*$_1^+$	*arg*$^+$ *str-r*	27	25
Hfr 2 *colE*$_1^+$	*thr*$^+$ *leu*$^+$ *str-r*	12	18

Crosses were performed in the proportion of 1 donor to 20 recipients. The frequency of recombination corresponds to 90 min. after the onset of mating.

Table 5. *Variation in number of F*$^-$ *bacteria determined at* 90 *min. after mixing in Hfr colE*$_1^-$ × PA 309 *colE*$_1^+$ *and Hfr colE*$_1^-$ × PA 309 *colE*$_1^-$ *crosses*

Donor cells	Time in min. after the onset of mating	Recipient cells	
		PA 309 *colE*$_1^-$	PA 309 *colE*$_1^+$
Hfr H *colE*$_1^-$	0	$1·4 \times 10^7$	$1·6 \times 10^6$
	90	5×10^6	5×10^6
Hfr 4 *colE*$_1^-$	0	$1·4 \times 10^7$	1×10^7
	90	$3·2 \times 10^7$	3×10^7
Hfr 2 *colE*$_1^-$	0	1×10^7	1×10^7
	90	$1·8 \times 10^7$	$1·7 \times 10^7$

All non-colicinogenic strains were previously made resistant to colicin E_1. Crosses were performed with a ratio of 20 Hfr to 1 F$^-$. Recipient cells were selected on streptomycin nutrient agar.

As far as this effect is concerned no differences were observed among crosses involving F$^-$ strains made colicinogenic by transduction or conjugation.

As in *col*$^+$ × *col*$^-$ crosses, no decrease in fertility occurred in reciprocal *col*$^-$ × *col*$^+$: it can be seen in Table 5 that similar frequencies of recombination are registered in *col*$^-$ × *col*$^+$ and *col*$^-$ × *col*$^-$ crosses.

Absence of transfer of col$^-$ *from the recipient to the donor*

Matings between an F$^-$ strain *thr*$^+$*leu*$^+$*arg*$^+$*val-s colE*$_1^+$ derived from PA 309 and Hfr H *colE*$_1^-$ *val-r* bacteria were performed using a 1:1 ratio of donor to recipient cells to determine if *colE*$_1$ could be transferred from the F$^-$ to the Hfr cell. Although under these conditions, transfer of chromosome from the Hfr to the F$^-$ cell took place, no *col*$^-$ bacteria were detected among about 5000 colonies growing on minimal

medium supplemented with thiamine and valine, which selects the Hfr non-colicinogenic strain.

This result shows that although conjugation bridges which permit the passage of genetic material are actually formed, $colE_1$ is not transferred from the F^- to the Hfr cell, or at least not with a magnitude comparable to that of its transfer in the opposite direction, that is, from the Hfr to the F^- cell.

Absence of transfer of $colE_1$ from colicinogenic F^- to non-colicinogenic F^- bacteria

Logarithmic cultures of an F^- strain, $thr^+leu^+arg^+colE_1{}^+$ derived from PA 309 and the F^- strain C600 $colE_1{}^-$ were mixed together in the proportions of 1:20 and 20:1, during 120 min.

No recombination between chromosomal markers of both strains took place as shown by the fact that no $thr^+leu^+try^+his^+$ recombinants appeared on the corresponding selective medium. Under these conditions no col^+ cells were detected among the large number of colonies which developed on a medium permitting only growth of the non-colicinogenic F^- strain. This indicates that no transfer of $colE_1$ from F^- to F^- strain occurs.

Table 6. *Comparison of fertility in Hfr $colE_1{}^-$ × F^- $colE_1{}^+$ and Hfr $colE_1{}^-$ × F^- $colE_1{}^-$ crosses*

Donors	Type of recombinant selected	Recipient strain	
		PA 309 $colE_1{}^+$	PA 309 $colE_1{}^-$
Hfr H $colE_1{}^-$	thr^+ leu^+ str-r	50	38
Hfr 4 $colE_1{}^-$	arg^+ str-r	17	23
Hfr 2 $colE_1{}^-$	thr^+ leu^+ str-r	20	12

Crosses were performed in the proportion of 1 donor to 20 recipients. The frequency of recombination was measured at 90 min. after the onset of mating.

DISCUSSION AND CONCLUSIONS

Analysis of crosses between Hfr, or F^-, and F^- strains of *Escherichia coli* K12 involving colicinogenic factor E_1 ($colE_1$) indicates that:

(1) $ColE_1$ is transferred very efficiently and with similar frequencies from F+ cells, which transfer the chromosome at low frequency, and from different Hfr cells, which transfer it at high frequency and with different origins and polarities.

(2) It begins to be transferred by these different donor strains within the first 5 min. of mating.

(3) It shows no linkage to chromosomal markers localized on different sites on the linkage map of *Escherichia coli* K12.

(4) The non-colicinogenic character of the donor parent is not transferred to the recipient or to recombinants.

All these facts can be explained on the basis of an extra-chromosomal state of $colE_1$ in F^+ and Hfr bacteria.

Evidence from kinetic experiments also suggests the autonomous state of $colE_1$. Kinetics of transfer of $colE_1$ from Hfr or F^+ strains shows that this factor is transferred with maximum efficiency during the first 80 min. of conjugation; later on, the

number of $col^+ str\text{-}r$ cells, in contrast to chromosomal recombinants, continue increasing at the same rate as other F^- cells. It is known that after a chromosomal marker is transferred to a zygote it must become integrated in the recipient genome in order to reduplicate. Only when the integration is completed, that is at about 120–140 min. after the onset of mating, chromosomal recombinants increase in number by cell division (Wollman, Jacob & Hayes, 1956). The fact that $col^+ str\text{-}r$ do not present a plateau suggests that $colE_1$ is capable of multiplying autonomously in the zygote and at such a rate that enables its passage to all daughter cells of the zygote.

This result agrees with the conclusion of Alfoldi *et al.* (1958) based on the analysis by micromanipulation of individual zygotes which received $colE_1$ factor.

The kinetics of transfer obtained with colicinogenic factor V (Nagel de Zwaig *et al.* 1962) are comparable to those presented here.

Similar conclusions as to the cytoplasmic nature of $colE_1$ were reached by Fredericq & Betz-Bareau (1953*a,b*) and Clowes (1963). The results of Alfoldi *et al.* (1957, 1958), although leading to a different conclusion, are not in disagreement with those presented here. Their observations on the apparent correlation between the transfer of col^+ and lethal zygosis, on which the hypothesis of the episomal nature of $colE_1$ was mainly based, might have been purely coincidental. Similar lethal effects as those described by them are now known to occur to a variable extent in matings involving certain strains, when the number of the donor bacteria greatly exceeds that of the female ones, but without having any relation to the transfer of a presumptive col^- locus (Nagel de Zwaig *et al.* 1962; Clowes, 1963).

That the efficient transfer of $colE_1$ does not interfere with chromosomal transfer or integration in *Escherichia coli* K 12 is shown by the fact that similar frequencies of recombination are observed in crosses involving the col^+ character and in controls. This is not the case with colicinogenic factors B or K (Fredericq & Betz-Bareau, 1956; Puig, 1963), whose presence in the donor cell drastically reduces the frequency of recombinant formation.

Results showing that colicinogenic factors, such as $colE_1$ or *colV*, $colE_2$ and *colI* (Nagel de Zwaig *et al.* 1962) and *colB* (Puig, 1963), can be transferred from Hfr cells as efficiently as from F^+ ones do not support the view suggested by Fisher (1962) that a mechanical obstruction of the conjugation canal by the chromosome might prevent the passage of all non-chromosomal material.

No transfer of $colE_1$ from F^- to F^- cells was observed, in agreement with previous observations by Fredericq (1954*b*). Neither was it found to be transferred, at least at high frequency, from F^- to Hfr cells. These results suggest that the presence of the *F* factor in a bacterial cell promotes the efficient transfer of $colE_1$ not only by determining the formation of conjugation bridges, but also by conferring on it a polarity of transfer of the same direction as that of the chromosome.

These observations on the lack of transfer of $colE_1$ among F^- cells might not apply to all colicinogenic factors; studies on the transfer of *colI* and *colB* factors in *Salmonella typhimurium* (Ozeki *et al.* 1962) indicate that they may act as fertility factors, since their presence in the cell promotes not only their own transfer, but also that of other colicinogenic factors and, in the case of *colI*, even that of the chromosome (Ozeki & Howarth, 1961; Clowes, 1961), probably by enabling the cell to conjugate. Preliminary results with *colV* and *colI* factors in *Escherichia coli* K 12

(Nagel de Zwaig, unpublished) suggest a similar situation as far as the transfer among F^- cells.

The conclusions obtained from this investigation regarding the state of $colE_1$ agree closely with those based on the genetic analysis of $colE_2$, *colI*, *colV* (Nagel de Zwaig *et al.* 1962) and *colB* (Puig, 1963). Since all the evidence so far obtained is consistent in pointing to the non-chromosomal state of these particles in *Escherichia coli* K12, they should be considered as plasmids (Lederberg, 1952).

Nevertheless, some colicinogenic factors might become fixed on the chromosome under certain conditions. One may speculate that chromosomal attachment would be more likely for colicinogenic factors resembling episomes, for which chromosomal location is well established. In this connexion, the study of colicinogenic factors having *F*-like functions or interacting with F particles, such as *colI* (Ozeki & Howarth, 1961; Clowes, 1961), *colB* (Ozeki *et al.* 1962; Fredericq, 1963; Puig, 1963) and *colV-colI* (Nagel de Zwaig, 1963) seems more promising. According to these assumptions, the isolation of an Hfr strain carrying *colB* fixed on the distal end of the chromosome as a result of a close association with the *F* factor has been reported by Fredericq (1963), though this type of evidence has not so far been obtained with a similar system (Puig, 1963).

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Nucleases of Mycoplasma

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SUMMARY

Nuclease activity was observed in several saprophytic and parasitic Mycoplasma organisms; the nucleases of *Mycoplasma laidlawii* were studied in detail. Nuclease activity of this organism was highest at the early logarithmic phase of growth, and was found mainly in the soluble fraction of the organisms. Anion-exchange chromatography of the proteins of the soluble fraction separated ribonuclease from deoxyribonuclease activity. Each enzyme had an alkaline pH optimum, required magnesium ions for activity, and degraded native and heat-denatured nucleic acids. *M. laidlawii* RNase degraded RNA-core. RNA inhibited the degradation of DNA by *M. laidlawii* deoxyribonuclease. The implications of these findings with respect to the effects of RNA and DNA on growth of *M. laidlawii* are discussed.

INTRODUCTION

Nucleic acid precursor requirements of several Mycoplasma organisms can be satisfied by a mixture of nucleosides (Razin, 1962; Razin & Cohen, 1963), or by undegraded RNA and DNA (Razin & Knight, 1960). The fact that mycoplasmas use RNA and DNA for growth led to the assumption that these organisms possess nuclease activity (Razin & Knight, 1960). However, no information was then available about the presence of ribonucleases and deoxyribonucleases in Mycoplasma, apart from a short report by Plackett (1957) who found ribonuclease activity in extracts of *Mycoplasma mycoides* var. *mycoides*. The present investigation indicates widespread nuclease activity in Mycoplasma, thus explaining the results obtained in previous nutritional studies.

METHODS

Organisms. Mycoplasma laidlawii strain A (PG 8), *M. laidlawii* strain B (PG 9), *M. bovigenitalium* (PG 11), *M. mycoides* var. *mycoides* (PG 1), *M. agalactiae* (PG 2) and *M. neurolyticum* (PG 28) were obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). *Mycoplasma mycoides* var. *capri* was obtained from Dr E. Klieneberger-Nobel (The Lister Institute of Preventive Medicine, London). *Mycoplasma gallisepticum* was provided by Mrs Ruth Bernstein (Faculty of Agriculture, The Hebrew University, Rehovoth, Israel). *Mycoplasma laidlawii* (oral strain) was isolated in our laboratory from the human oral cavity. This strain was serologically related to *M. laidlawii* strain A (M. Argaman, unpublished). Most of the work described here was done on the three *M. laidlawii* strains.

Suspensions of organisms. The organisms were grown in a modified liquid Edward medium (Razin, 1963). Fifty ml. of a young culture of the organisms in this medium were inoculated into 500 ml. medium in 1 l. Roux flasks. The flasks were incubated at 37° for 24–48 hr in an inclined position, to improve aeration. The organisms were harvested by centrifugation at 9000 *g* for 10 min., washed twice in 0·25 M-NaCl and resuspended in this solution. The amount of organism in suspension was expressed as mg. cell protein/ml. suspension. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Cell-free extracts. Cell-free extracts of *Mycoplasma laidlawii* strains and *M. bovigenitalium* were prepared by osmotic lysis of organisms (Razin, 1963). The saline-washed packed organisms (1–2 g. wet weight) were suspended in de-ionized water (about 300 ml.) and incubated at 37° for 20–30 min. to effect complete lysis (Razin, to be published). The suspension was then centrifuged at 34,000 *g* for 40 min. and the supernatant fluid separated from the sedimented membranes and kept at −20° until used. For separation of the ribosomal fraction tris buffer (0·1 M, pH 7·4) and $MgCl_2.6H_2O$ (0·01 M) were added to the suspension of disrupted organisms. Membranes were removed by centrifugation at 34,000 *g* for 40 min.; the supernatant fluid was placed in a Spinco ultracentrifuge and the ribosomes sedimented at 150,000 *g* for 90 min. *Mycoplasma gallisepticum*, which is relatively resistant to osmotic lysis (Razin, 1963), was disrupted in an M.S.E. ultrasonic disintegrator (60 watt). Seven ml. of a heavy suspension of washed organisms in 0·01 M-phosphate buffer (pH 7·5) were treated at 1·5 amp. for 2·5 min. Cell debris was removed by centrifugation at 34,000 *g* for 40 min.; the supernatant fluid was kept at −20° until used.

Chemicals. Ribonucleic acid (Na salt from yeast) obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.) was purified according to Frisch-Niggemeyer & Reddi (1957). The purified material was dialysed, with constant stirring, for 24 hr under repeated changes of de-ionized water. Deoxyribonucleic acid (Na salt from thymus; L. Light and Co.) was dissolved in de-ionized water and centrifuged to remove insoluble material. The DNA solution was then dialysed, with constant stirring, for 5 hr under repeated changes of de-ionized water. Solutions of purified RNA and DNA (10 mg./ml.) were kept at −20° until used. Highly polymerized deoxyribonucleic acid (from salmon sperm) was obtained from Mann Research Laboratories Inc. (New York 6, N.Y., U.S.A.). Pancreatic ribonuclease (RNase, 5x cryst.) was the product of the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Ribonucleic acid-core and bis-(*p*-nitrophenyl)phosphate were obtained from the Sigma Chemical Co. (St Louis 18, Mo., U.S.A.). DEAE-Sephadex A-50 (medium) was purchased from Pharmacia (Uppsala, Sweden).

Assay of nuclease activity. For qualitative assay of nuclease activity the method of Jeffries, Holtman & Guse (1957) was used. Various amounts of RNA or DNA were added to molten Edward medium agar. The final concentration of nucleic acids in the medium ranged between 0·5 and 7 mg./ml. The medium was poured into Petri dishes and drops of young broth cultures of test organism placed on the surface of the solidified agar. After incubation at 37° for 5 days the plates were flooded with N-HCl. The presence of nuclease activity was indicated by a clear zone in and around the growth region. Areas without nucleic acid hydrolysis became cloudy.

For quantitative measurement of nuclease activity the method described by Eaves & Jeffries (1963) was used. The typical reaction mixture was composed of 3 ml. 0·4 M-tris buffer (pH 8·8) containing 0·04 M-$MgCl_2.6H_2O$; 2·4 ml. 0·25 M-NaCl or de-ionized water; 4·2 ml. washed suspension of organisms or cell-free extract; 1·2 ml. RNA or DNA solution (10 mg./ml.); 1·2 ml. of thallium acetate solution (5 mg./ml.) was added to prevent microbial growth. The proportion of the components of the reaction mixture was kept constant when the total volume of the mixture was decreased. Experiments with washed organism or cell-free extracts were made in 100 ml. Erlenmeyer flasks which were shaken in a 37° water bath. The nuclease activity of fractions eluted from the Sephadex column was assayed in test tubes incubated statically at 37°. Two ml. samples of reaction mixture were removed at various times, and the enzyme reaction stopped by adding 0·5 ml. of 0·75% (w/v) uranyl acetate in 25% perchloric acid. The test tubes were shaken, placed for 10 min. in an ice bath and centrifuged at 10,000 ***g*** for 10 min. at 5°. The supernatant fluids were separated and 0·5 ml. of each was diluted with 3·5 ml. de-ionized water; the extinction at 260 mμ was measured against de-ionized water in a 'Unicam SP 500' spectrophotometer. Corrections were made for the extinction of controls containing all components of the reaction mixture save washed organisms or cell-free extracts, and for mixtures without RNA and DNA. Nuclease activity was expressed either by the net increase in extinction at 260 mμ during a given period of time, or by units calculated by dividing the increase in extinction at 260 mμ by mg. protein/ml. the reaction mixture.

Proteinase and phosphodiesterase activity in fractions eluted from the Sephadex column were tested as described by Eaves & Jeffries (1963). Casein served as substrate for proteinase assay and bis(*p*-nitrophenyl)-phosphate for phosphodiesterase assay.

Anion-exchange chromatography. This technique was used to separate RNase from DNase in extracts of *Mycoplasma laidlawii*. The crude cell-free extract was adjusted to pH 5·5 with N-HCl, and ammonium sulphate was added to 0·8 saturation (561 g./l.). The solution was left overnight at 4° and the resulting precipitate collected by centrifugation at 25,000 ***g*** for 1 hr at 5°. The precipitate was dissolved in 0·02 M-tris buffer (pH 8·1) and dialysed with constant stirring for 4 hr in the cold, against the same buffer. The buffer was changed several times during this dialysis. A granular precipitate which appeared in the dialysis bag was separated by centrifugation and discarded. The clear protein solution was then chromatographed on a DEAE-Sephadex A-50 (medium) column. Fractions were collected by a fraction collector and tested for RNase and DNase activity.

RESULTS

Occurrence of nuclease activity in various Mycoplasma strains

The qualitative method for detecting nuclease activity described by Jeffries *et al.* (1957) yielded positive results with all the mycoplasma strains tested. Clear zones appeared in and around the regions of growth on Edward medium agar supplemented with RNA or DNA, after flooding with N-HCl. Both DNA and RNA were degraded by suspensions of washed organisms of all Mycoplasma strains tested (Table 1).

Localization of nuclease activity in Mycoplasma organisms

Cell-free extracts obtained by osmotic lysis of all three *Mycoplasma laidlawii* strains and *M. bovigenitalium* degraded RNA and DNA at a higher rate than washed organisms, as measured in units of enzyme activity. Nuclease activity, however, was found also in the washed cell-membranes (Fig. 1). Separation of the ribosomal fraction of the cell-free extract by ultracentrifugation showed the nucleolytic activity to reside mainly in the soluble fraction (Table 2).

Table 1. *Nuclease activity of various Mycoplasma strains*

Reaction mixtures were composed as described under Methods. Suspensions of organisms contained 3 mg. protein/ml. Results were read after incubation for 8 hr at 37°.

Organism	Nuclease activity (increase of extinction at 260 mμ) RNA	DNA
Mycoplasma laidlawii (strain A)	2·450	1·560
M. laidlawii (strain B)	1·720	1·080
M. laidlawii (oral strain)	1·540	0·705
M. bovigenitalium	2·990	2·680
M. gallisepticum	2·650	2·100
M. agalactiae	1·040	1·740
M. mycoides var. *mycoides*	0·403	0·395
M. mycoides var. *capri*	0·348	0·350
M. neurolyticum	0·310	0·207

Table 2. *RNA and DNA degradation by ribosomal and soluble fractions obtained from Mycoplasma laidlawii (oral strain) cell-free extract*

	Substrate	Nuclease activity* (units)
Ribosomal fraction	RNA	0·82
	DNA	1·91
Soluble fraction	RNA	2·96
	DNA	4·90

* Results read after incubation for 5 hr at 37°.

An extract prepared by disrupting *Mycoplasma gallisepticum* with high-energy sound was also very active in degrading RNA and DNA. The nucleolytic activity of the extracts of *M. laidlawii* strains did not significantly diminish after storage for 8 months at −20°. The enzymic activity of the extracts was, moreover, little affected by repeated freezing and thawing.

Relation of nuclease activity to the age of the culture

The nuclease activity of washed organisms and extracts of *Mycoplasma laidlawii* was highest in organisms harvested at the early logarithmic phase of growth. Activity towards RNA declined steeply with the ageing of the culture. The decline was less pronounced with DNA as substrate (Fig. 2). Growth of *M. laidlawii* in

Edward medium containing 0·5% (w/v) glucose was characterized by a very steep decline in viable count after reaching a peak of 3–5 × 10^9 viable particles/ml. at the end of the logarithmic period. This peak was usually reached after incubation for 16–18 hr. at 37°. The cultures were practically sterile after 96–120 hr.

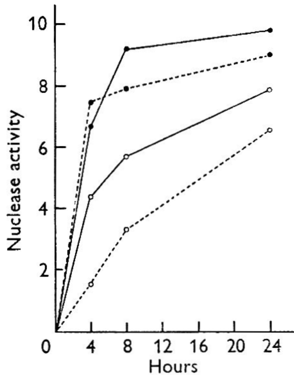

Fig. 1

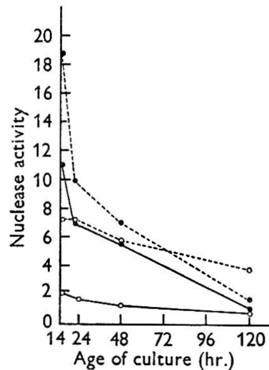

Fig. 2

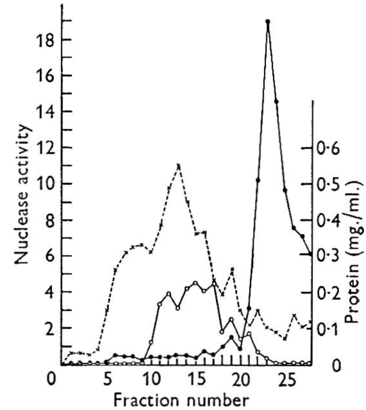

Fig. 3

Fig. 1. Degradation of RNA and DNA by cell membranes and extract of *Mycoplasma laidlawii* (oral strain). RNA (●); DNA (○). - - -, cell membranes; —, cell extract.

Fig. 2. Degradation of RNA and DNA by washed organisms and extracts of *Mycoplasma laidlawii* (oral strain) harvested at various growth phases. RNA (●); DNA (○). —, washed organisms; - - -, cell extracts. Results read after incubation for 7 hr at 37°.

Fig. 3. RNA and DNA degradation by fractions obtained by anion-exchange chromatography of *Mycoplasma laidlawii* (oral strain) proteins. Two ml. containing 26·4 mg. *M. laidlawii* proteins were placed on a DEAE-Sephadex A-50 (medium) column (1·5 × 18 cm.), equilibrated with 0·02 M-tris buffer (pH 8·1). Elution was performed by successive addition of 20 ml. samples of NaCl solutions of increasing molarity (0·1; 0·2; 0·3; 0·4; 0·5; 0·6 M-NaCl in 0·02 M-tris buffer, pH 8·1). Flow rate was about 30 ml./hr and fraction volume 4 ml. Nuclease activity of the fractions was determined after incubation for 5 hr at 37°. RNA (●); DNA (○); protein (×).

Separation of RNase from DNase activity in extracts of Mycoplasma laidlawii

Preliminary experiments with cell-free extracts of the three *Mycoplasma laidlawii* strains indicated that similar conditions were required for the degradation of RNA and DNA; degradation of both substrates was optimal at about pH 8·8. Ethylenediaminetetraacetic acid in a concentration as low as 0·0125 M, or prolonged dialysis of the extracts, completely abolished their nuclease activity, which was restored by magnesium ions. Both RNA and DNA degradation were similarly affected by heating the extracts. It therefore became plausible that *M. laidlawii* extracts contain a phosphodiesterase responsible for the degradation of both RNA and DNA. To test this the proteins of the cell-free *M. laidlawii* extract (oral strain) were concentrated by precipitation with ammonium sulphate and chromatographed on a DEAE-Sephadex A-50 column. This chromatography, however, separated RNase from DNase activity (Fig. 3), thus disproving the hypotheses that RNA and DNA were degraded by a single enzyme; however, by this method complete separation of the two enzymes was not achieved. Fractions 10–19, carrying most of the DNase activity, were combined and used for testing the DNase properties, and

fractions 21–28 were used for testing RNase properties. No detectable proteinase or phosphodiesterase activity was found in these fractions.

Properties of the DNase and RNase from Mycoplasma laidlawii (oral strain)

Optimal temperature. The combined fractions containing DNase activity and RNase activity were each tested for DNA and RNA degradation, respectively, at various incubation temperatures. The optimal temperature for DNase activity was 37°; that for RNase was 40° (Fig. 4).

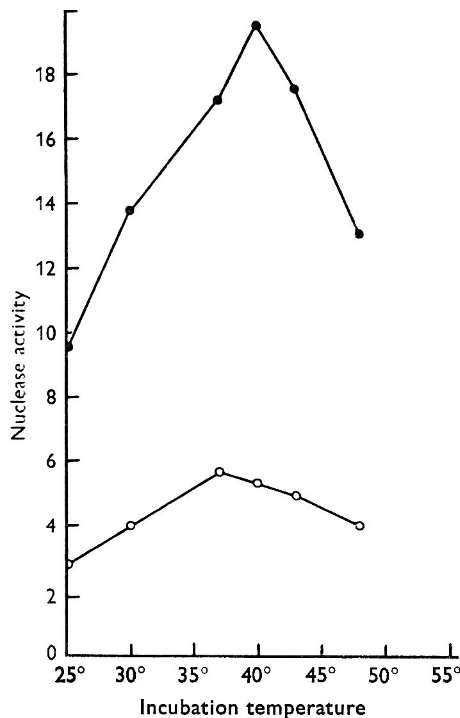

Fig. 4

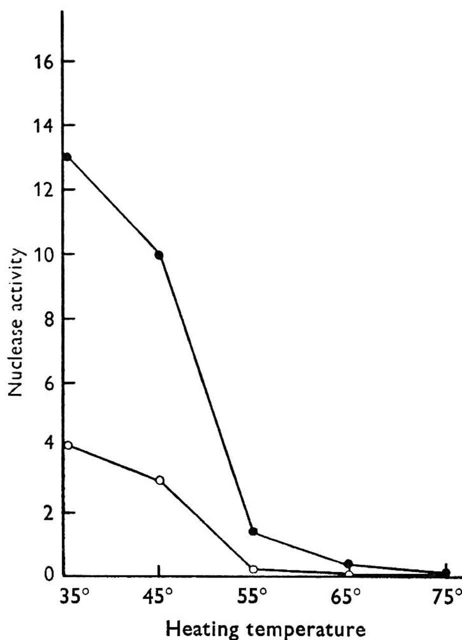

Fig. 5

Fig. 4. Optimal temperature for activity of the DNase and RNase of *Mycoplasma laidlawii* (oral strain). DNase fraction (○); RNase fraction (●). Results read after incubation for 7 hr.

Fig. 5. Heat inactivation of *Mycoplasma laidlawii* (oral strain) DNase and RNase. The enzyme solutions were heated for 10 min. at the temperatures indicated. DNase fraction (○); RNase fraction (●). Results read after incubation for 7 hr at 37°.

Heat stability. The DNase and RNase fractions were heated for 10 min. at various temperatures. No significant difference was found in the heat stability of the two enzymes; both were practically inactivated by heating at 65° for 10 min. (Fig. 5).

Optimal pH value. The optimal pH for both DNase and RNase activity was about pH 8·8 (Fig. 6).

Magnesium requirement. Omission of magnesium from the reaction mixture completely abolished DNase and RNase activity. The optimal concentration of magnesium for the activity of both enzymes was found to be 0·01 M (Fig. 7).

Manganese ions were only slightly active or inactive in replacing magnesium, calcium ions were inactive.

Degradation of heat-denatured DNA and RNA. DNA (highly polymerized) and RNA were heated in boiling water for 10 min. and rapidly cooled in an ice bath to bring about permanent denaturation. The results summarized in Table 3 show that

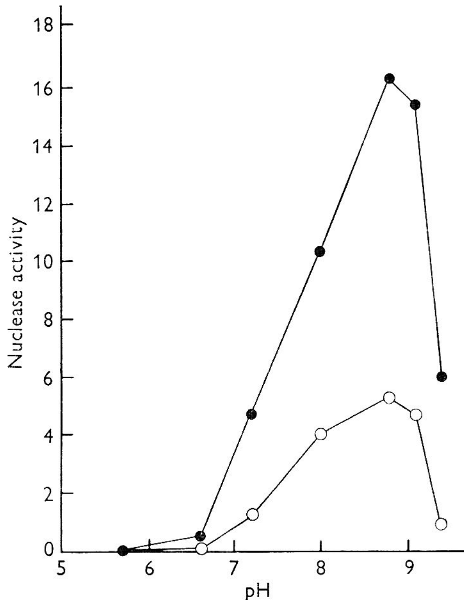

Fig. 6

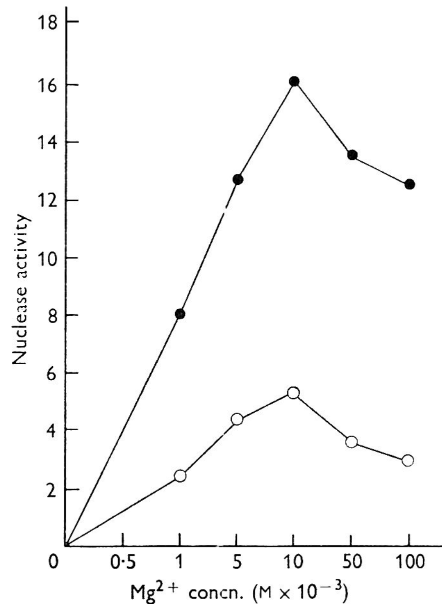

Fig. 7

Fig. 6. Effect of pH value on *Mycoplasma laidlawii* (oral strain) DNase and RNase activity. Reaction mixtures contained 0·1 M of phosphate buffer (pH 5·7; 6·6), tris buffer (pH 7·2; 8·0; 8·8; 9·1) or carbonate buffer (pH 9·4). DNase fraction (○); RNase fraction (●). Results read after incubation for 8 hr at 37°.

Fig. 7. Effect of magnesium concentration on the activity of *Mycoplasma laidlawii* (oral strain) DNase and RNase. Reaction mixtures contained various concentrations of $MgCl_2.6H_2O$ as indicated. DNase fraction (○); RNase fraction (●). Results read after incubation for 7 hr at 37°.

Table 3. *The activity of Mycoplasma laidlawii (oral strain) DNase and RNase on native and heat-denatured DNA and RNA and on RNA-core*

Concentration of all substrates 1 mg./ml. reaction mixture. Results read after incubation for 7 hr at 37°.

Enzyme	Substrate	Nuclease activity (units)
DNase fraction	Native highly polymerized DNA	13·6
	Denatured highly polymerized DNA	12·2
	Native regular DNA	4·2
RNase fraction	Native RNA	16·5
	Denatured RNA	16·2
	RNA-core	16·8
Pancreatic RNase	Native RNA	44·7
	RNA-core	1·2

heat denaturation of DNA and RNA did not affect their sensitivity to hydrolysis by the nucleases of *Mycoplasma laidlawii*. It seems noteworthy that the amount of acid-soluble products liberated by degradation of the high-polymerized DNA by *M. laidlawii* DNase was much greater than those produced by degradation of the regular commercial DNA.

Degradation of RNA-core. Table 3 shows that *Mycoplasma laidlawii* RNase degraded RNA-core. Pancreatic RNase was essentially non-active on this substrate, proving its purity.

Inhibition of Mycoplasma laidlawii DNase activity by RNA. RNA inhibited the degradation of DNA by *M. laidlawii* DNase, and this inhibitory effect increased with increasing concentration of RNA in the reaction mixture (Table 4).

Table 4. *Inhibition of Mycoplasma laidlawii (oral strain) DNase by RNA*

Concentration of DNA in reaction mixture 1 mg./ml. Results read after incubation for 7 hr at 37°.

RNA in reaction mixture (mg./ml.)	DNase activity (units)
0	6·4
1	4·6
5	3·0
10	1·3

DISCUSSION

All Mycoplasma strains examined in the present work were capable of hydrolysing both RNA and DNA. Hence, nucleolytic activity seems to be of wide occurrence in this group of micro-organisms. The comparison of the nucleolytic activity of the various Mycoplasma strains (Table 1) is somewhat inadequate in that the organisms of the different strains were not always harvested at the same phase of growth. In the case of *Mycoplasma laidlawii* nuclease activity greatly depended on the time at which the organisms were harvested; this may also be true of other Mycoplasma strains. The nucleolytic activity of *M. laidlawii* was highest at the logarithmic phase of growth and decreased steeply afterwards, paralleling the rapid death of the organisms. It is possible that the nucleases are released from the dead organisms into the surrounding medium like many other bacterial enzymes (Barker & Cannon, 1960).

In spite of the very similar conditions required for the hydrolysis of RNA and DNA by crude extracts of *Mycoplasma laidlawii*, the nucleic acids were degraded by two different enzymes. This conclusion is based mainly on the separation of RNase from DNase activity by anion-exchange chromatography of the cell-extract proteins. The enzymes separated on the DEAE-Sephadex column, called ribonuclease and deoxyribonuclease, had several properties in common. Both had the same pH optimum (8·8) and both required magnesium ions for activity. The requirement of *M. laidlawii* RNase for magnesium is quite outstanding. Whereas a requirement for magnesium, or other divalent cations, is very common for deoxyribonucleases, it is most uncommon for ribonucleases, which are frequently inhibited by divalent cations (Anfinsen & White, 1961). The ribonuclease of

M. mycoides var. *mycoides* was inhibited by magnesium (Plackett, 1957). The high pH optimum for both *M. laidlawii* nucleases is also remarkable. Most RNases from animal and microbial cells have a pH optimum of 7–8 while these from plant cells have an acid pH optimum (Anfinsen & White, 1961). Phosphodiesterases of microbial and animal origin which degrade both RNA and DNA resemble the *M. laidlawii* nucleases in having an alkaline pH optimum (8–9) and a requirement for divalent cations (Cunningham, 1959; Stevens & Hilmoe, 1960; Khorana, 1961; Healy, Stollar, Simon & Levine, 1963; Eaves & Jeffries, 1963). However, the specificity shown by the enzymes of *M. laidlawii* to RNA or DNA, and their inability to hydrolyse bis(*p*-nitrophenyl) phosphate, prevents their inclusion in the phosphodiesterase group. *Mycoplasma laidlawii* RNase cannot be related to the polynucleotide phosphorylases, which also have an alkaline pH optimum and a requirement for magnesium ions (Grunberg-Manago, 1961) because it degraded RNA in the absence of phosphate ions, which are essential for polynucleotide phosphorylase activity. *Mycoplasma laidlawii* RNase differs from pancreatic RNase in its ability to hydrolyse the portion of RNA (RNA-core) which is resistant to the action of the latter.

Of biological interest is the inhibition of *Mycoplasma laidlawii* DNase by RNA. RNA has been reported to inhibit DNA degradation by DNases of streptococci (Bernheimer & Ruffier, 1951), *Pseudomonas fluorescens* (Catlin & Cunningham, 1958) and *Escherichia coli* (Elson, 1959; Lehman, Roussos & Pratt, 1962*a*, *b*). Our special interest in this phenomenon harks back to previous nutritional studies, where high concentrations were found to inhibit growth of *M. laidlawii* in a partially defined medium (Razin & Knight, 1960) and this growth inhibition by RNA was relieved by adding more DNA to the medium. Razin & Knight (1960) suggested that the degradation of DNA by Mycoplasma is a prerequisite for its utilization, and that high RNA concentrations inhibit this enzymic degradation. Indirect evidence for this assumption was the annulment of RNA inhibition by the substitution of DNA by thymidine in the growth medium. The results obtained in the present study furnish more direct evidence for this explanation of growth inhibition by RNA in Mycoplasma.

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