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Contents

Part 1 *issued* January 1969

Part 2 *issued* February 1969

Contents

Part 3 *issued* March 1969

Contents

THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this be necessary, and not to recapitulation.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

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Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in *J. gen. Microbiol.* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

Nomenclature and Description of Micro-organisms. The author's preference in naming micro-organisms is at present accepted provided that the designation is unambiguous and conforms with international rules of nomenclature; if desired, synonyms may be added in brackets when a name is first mentioned. Names of bacteria must conform with the Bacteriological Code of the International Committee on Bacteriological Nomenclature and the opinions issued by this International Committee (*Bacteriological Code* (1958), edited by the Editorial Board of the International Committee on Bacteriological Nomenclature and published by the Iowa State College Press, Ames, Iowa, U.S.A.). Names of algae and fungi must conform with the International Rules of Botanical Nomenclature which are considered and revised at each International Botanical Congress (published by the International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952)). Names of protozoa must conform with the International Code of Zoological Nomenclature adopted by the XVth International Congress of Zoology (published for the International Commission on Zoological Nomenclature by the International Trust for Zoological Nomenclature, London (1961)). The 1913 rules will be found in C. M. Wenyon, *Protozoology*, (1926), vol. 2 (London: Baillière Tindall and Cox). One or two small changes have been made to these rules at later International Congresses.

Useful commentaries on the taxonomy of micro-organisms will be found in the articles of the Twelfth Symposium of the Society for General Microbiology, *Microbial Classification*, edited by G. C. Ainsworth and P. H. A. Sneath (Cambridge University Press).

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

Bergey's Manual of Determinative Bacteriology, 7th ed. (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Baillière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Generic Characteristics*, (1959). Baltimore, Ma., U.S.A.: The Williams and Wilkins Company.

S. T. Cowan & K. J. Steel, *Manual for the Identification of Medical Bacteria*, (1965). Cambridge University Press.

Ainsworth & Bisby's Dictionary of the Fungi, 5th ed. (1961). Kew: Commonwealth Mycological Institute.

List of Common British Plant Diseases, 4th ed. (1944), compiled by the Plant Pathology Committee of the British Mycological Society. Cambridge University Press.

Medical Research Council: Memorandum No. 23. 3rd ed. (1967). *Nomenclature of Fungi Pathogenic to Man and Animals*. London: H.M.S.O.

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

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The Spores of *Eremothecium ashbyii*

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Division of Applied Microbiology, Royal Institute of Technology, Stockholm 70, Sweden

(*Accepted for publication* 19 *August* 1968)

SUMMARY

Pure suspensions of spores of *Eremothecium ashbyii* were prepared by filtration through glass wool; the filtrates contained several million spores/ml. Germinating spores showed antibiotic effects. The efficiency of plating decreased with increasing concentrations of spores but increased when the spores were washed. The results of inactivation by ultraviolet light indicated that the spores were haploid. Inositol deficiency was lethal to the spores. These findings indicate that it should be possible to use *E. ashbyii* in genetic work, but the lack of knowledge about the life-cycle is a serious obstacle.

INTRODUCTION

Eremothecium ashbyii produces large quantities of riboflavin and is of industrial importance for this reason. The life-cycle and morphology of *E. ashbyii* has been described by Guillermond (1935, 1936). The organism grows into quite large masses of mycelium and after 3 to 5 days some 20 sickle-shaped spores are formed in the sporangium which bursts and liberates the spores which are found in large numbers in mature cultures. The significance of the spores is uncertain and no sexual reproduction has been demonstrated but, as Krneta-Jordi (1962) pointed out, this may be due to the fact that only one isolate has been studied and that *E. ashbyii* may be heterothallic. Krneta-Jordi made an extensive cytological and physiological study but she remarked that it was impossible to get pure spore cultures. This was a serious obstacle since it was difficult to get a standard inoculum. In the present paper it will be shown that pure spore suspensions can easily be obtained; experiments with these spore suspensions are reported.

METHODS

Organism. Eremothecium ashbyii OUT6022 was used; it was obtained from The Department of Fermentation Technology, Faculty of Engineering, Osaka University, Osaka, Japan.

Media. The basal defined medium (Ea) was as described by Osman & Soliman (1963). Solid Ea-medium was made by adding 2% agar. In some experiments wort agar (8%, w/v, beer wort + 2%, w/v, agar) was used.

Dilutions and washings were made with 0·9% (w/v) NaCl solution (saline).

Preparation of spore suspensions. Mature cultures were filtered through a sterile 1 cm thick layer of glass wool. The spores passed through but mycelia and hyphae were retained. The filtrates were in some cases used directly (or after dilution); in

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many experiments the spore suspensions were centrifuged, the spores washed twice with saline and then resuspended in saline (these suspensions are denoted as washed).

Total counts were made microscopically by using the Bürker counting chamber with a depth of 0·1 mm.

Viable counts of spores. Samples (0·1 ml.) of a suitable dilution were spread on Ea agar or wort agar plates which were incubated at 28°. Colonies were counted after 2 to 3 days.

Efficiency of plating is defined as the ratio between the number of colonies formed and the number of spores plated (as calculated from the total count).

Cultivation in liquid medium. Volumes (50 ml.) of the Ea medium were introduced into 300 ml. Erlenmeyer flasks which were inoculated with spores and incubated on a rotary shaker at 28°.

Ultraviolet (u.v.) treatment. The samples that were to be u.v.-irradiated were washed twice and resuspended in saline. Samples (10 to 20 ml.) of suspension were transferred to a 10 cm. diam. glass Petri dish and irradiated by a 10 W. Hg lamp (giving about 1000 ergs/mm^2 per min. at wavelength 2537 Å at the distance used). Dilutions were spread in the dark on Ea medium agar and incubated at 28°.

Treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Washed spores were suspended in 0·2 M-acetate buffer (pH 5·0, Megnet, 1965). NTG (1·5 mg./ml.) was dissolved in the same buffer. One ml. of spore suspension (about 2×10^6 spores/ml.) and 2 ml. NTG solution were mixed (Megnet, 1965) and the whole incubated at 28°. Samples were filtered through Millipore filters and the spores were washed with acetate buffer and then resuspended and spread on Ea medium agar.

RESULTS

Preparation of pure spore suspensions

When *Eremothecium ashbyii* is grown in a liquid medium mycelia are formed that are easily seen by the naked eye. After a few days hyphal tips are transformed to spore cells which later excrete 10 to 20 sickle-shaped spores each. At this stage large amounts of riboflavin are produced, giving a yellow colour to the medium. Finally, there is a mixture of spores, small hyphae and mycelia of various sizes (Krneta-Jordi, 1962). It proved to be very easy to separate the spores from the other components by filtration through a layer of glass wool about 1 cm. thick. The filtrate so obtained contained virtually no other forms but spores at a concentration of several million/ml. as counted microscopically. Krneta-Jordi (1962) studied the germination of the spores in hanging drops, but since she was unable to prepare pure spore suspensions this made it impossible to study the physiology of spore germination. The spore suspension prepared by filtration as described were well suited for further studies.

Germination of spores

Pure spore suspensions were prepared by filtration through glass wool as described. When these suspensions were diluted and plated the number of colonies formed was much less than the total spore count. The efficiency of plating increased with increasing dilution, indicating the presence of some antibiotic substance in the medium where the spores were formed (Fig. 1). This conclusion was further strengthened when it was found that the efficiency of plating increased when the spores were centrifuged

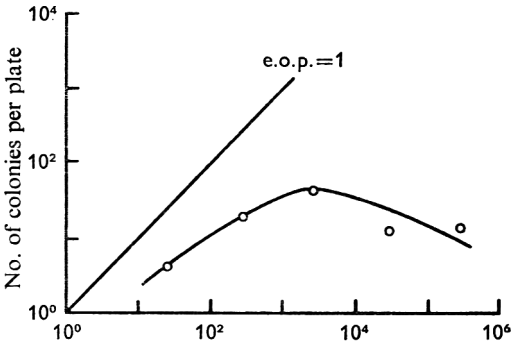

Fig. 1

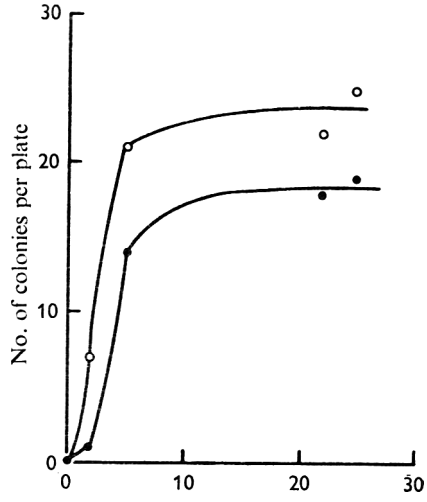

Fig. 2

Fig. 1. *Eremothecium ashbyii*. Viable count in spore suspensions as a function of the concentration of spores. Dilutions were made in saline.

Fig. 2. *Eremothecium ashbyii*. Colonies formed on wort agar after incubation in liquid Ea medium for the time indicated on the abscissa. Total count (microscope) showed that 350 (○) and 35 (●) spores were spread per plate.

Table 1. *Efficiency of plating of spores of Eremothecium ashbyii*

The spore suspension contained $2{\cdot}6 \times 10^6$ spores/ml. by direct count microscopically.

	Efficiency of plating (colony count)	
Dilution	Filtrate	Washed spores
None	$5{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-3}$
1/100	$1{\cdot}6 \times 10^{-2}$	$4{\cdot}2 \times 10^{-4}$

and washed with saline before plating (Table 1). The germination on Ea medium agar was always rather inefficient. The spores did not germinate on wort agar but only on the Ea medium agar; the reason for this is not known. However, wort agar could be used as culture medium when the spores had been pregrown in liquid Ea medium (Fig. 2).

The antagonistic effect of the growing mycelia on the germination of spores was apparent when liquid Ea medium was inoculated with inocula of various sizes (washed spores). After incubation for 30 hr viable (colony) counts showed that the frequency of germination increased with decreasing size of inoculum (Fig. 3). At greater sizes of inoculum the viable count decreased. The findings of Fig. 3 were also verified by microscopic examination. After incubation for 30 hr, most of the spores had not germinated and formed small mycelia when the inoculum was large (Fig. 3) but almost all spores had germinated at the lowest concentration tested. Growth was obtained even when the number of spores in a flask was less than 10.

Growth curve

The spores were formed after 3 to 4 days. The growth process can be followed by viable count, but this method does not say anything of the increase in cell mass for a mycelial organism. Sporulation was easily shown since the viable counts gave typical one-step growth curves (Fig. 4). However, the efficiency of plating was low: the total count of spores was $2{\cdot}00 \times 10^6$ per ml. in the experiment shown in Fig. 4. Since sporulation and excretion of riboflavin occurred simultaneously it was easy to know when a culture contained fair amounts of spores.

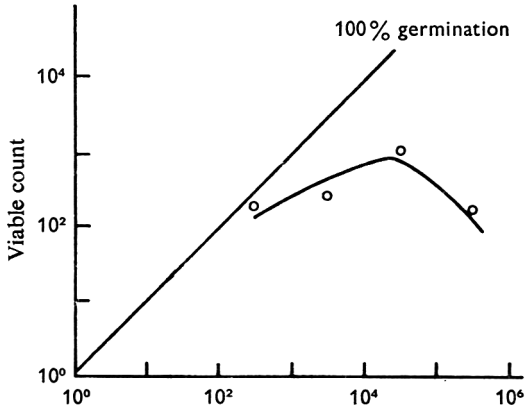

Fig. 3

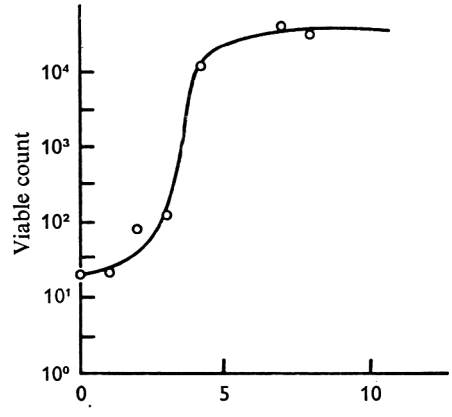

Fig. 4

Fig. 3. *Eremothecium ashbyii*. Germination of spores in liquid Ea medium. The spores were incubated for 30 hr before plating on Ea medium agar.

Fig. 4. *Eremothecium ashbyii*. Growth curve (viable count) for a culture starting from a small inoculum.

Effect of various treatments of spores

Ultraviolet irradiation. Spores were susceptible to killing by u.v. irradiation (Fig. 5), which was more efficient with spores which had started to germinate than with resting spores. An apparent increase in death-rate was found already after 2 hr of pre-growth in liquid Ea medium, the effect was fully developed after 4 hr. The germinating spores were rather sensitive to u.v. irradiation; about 400 ergs/mm.^{2} was sufficient to decrease survival to 0·1 % as compared to about 2000 ergs/mm.^{2} for haploid *Saccharomyces cerevisiae* treated in the same apparatus (Nordström, 1964). Since the efficiency of plating was dependent on the spore concentration it was meaningless to follow the survival curve to lower values. However, the curves seemed to be linear. U.v. irradiation also had a marked effect on the germination of the spores in liquid Ea medium (Fig. 6).

Treatment with NTG. The spores were killed by NTG; incubation in acetate buffer with 1 mg. NTG/ml. for 3 hr decreased the viable spore count to about 1 % of the initial value.

Inositol-less death. 2×10^5 spores/ml. were incubated in liquid Ea medium with and without inositol (Table 2). The viable count increased about 10 times in Ea medium + inositol but decreased 50 times in Ea medium without inositol.

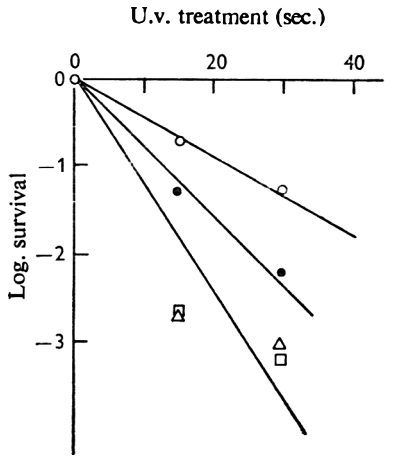

Fig. 5

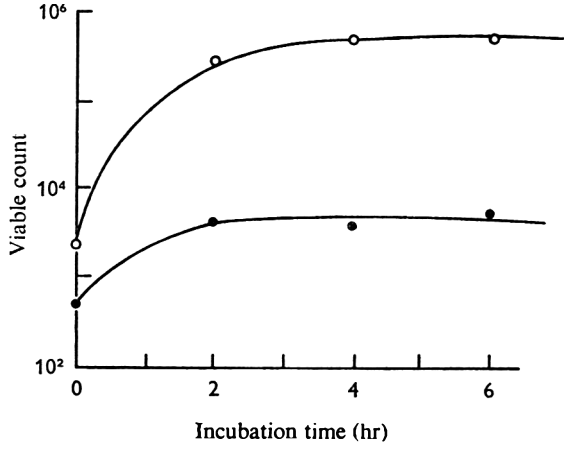

Fig. 6

Fig. 5. *Eremothecium ashbyii.* Effect of pre-growth in liquid Ea medium on survival of u.v.-irradiation. The spores were grown in liquid Ea medium for 0 (○), 2 (●), 4 (△) and 26 (□) hr.

Fig. 6. *Eremothecium ashbyii.* Effect of u.v.-irradiation on germination of spores. The liquid Ea medium was inoculated with untreated spores (○) and the same concentration of spores u.v.-irradiated for 15 sec (●) (about 250 ergs/mm^2.). Viable counts were made at intervals. The number of spores added initially was $1{\cdot}79 \times 10^6$/ml.

Table 2. *Effect of inositol deficiency on survival of Eremothecium ashbyii spores*

Viable counts were read at 0 and 5 days of incubation of spores in the Ea medium, with and without inositol.

	Viable count	
Medium	Initial	5 days
+Inositol	$5{\cdot}2 \times 10^4$	$7{\cdot}0 \times 10^5$
−Inositol		$1{\cdot}0 \times 10^3$

DISCUSSION

Jinks (1952) and Griggs (1952) studied the back mutation assay method used in microbial genetics and reported that large inocula lead to a lower efficiency of plating than smaller inocula because of competition for limited resources in the medium. However, Kølmark & Westergaard (1952) showed that this does not occur on media containing excess nutrients. In such back-mutation studies the growth of a small number of prototrophs is repressed by the presence of a large number of auxotrophs. In the present paper competition for nutrients may be one reason for the low efficiency of plating at large inocula but it cannot be the main reason since *Saccharomyces cerevisiae* can form heavy lawns of colonies on the media used. Furthermore, the efficiency of plating increased when the spores were washed before plating (Table 1). Thus the presence of a substance that represses spore germination seems to be reasonable. It would be a selective advantage for an organism to prevent the spores from germination close to large masses of mycelium.

From the present results it can be concluded that at least some conditions needed for a genetic study of *Eremothecium ashbyii* are present. It is possible to isolate single cells (spores). However, nothing is known about the nuclear state of the spores, whether they are haploid or diploid, etc.; Fig. 5 may suggest a haploid status since the u.v.-survival curves seem to be linear. This evidence, however, is rather weak since the result of Fig. 5 may be greatly influenced by the low efficiency of plating and by the dilution effect (Fig. 1). Krneta-Jordi (1962) showed that the spores are uninucleate. A serious obstacle is the lack of knowledge of the life-cycle of *E. ashbyii*. The systematic position of this genus, and, thus, the genetic significance of the spores are very uncertain (Lodder & Kreger-van Rij, 1952). They may or may not be formed meiotically, etc. Guillermond (1935) has proposed that Eremothecium can be related to either Spermophthora or Dipodascus. In the former case Eremothecium should have lost the ability to conjugate and the spores should be vegetative spores. In the latter case, Eremothecium should have lost its sexual differentiation. In both cases, the organism would be difficult to use in genetic work. However, other possibilities may also be open. The results of u.v. irradiation showed that the spores were uninucleate and haploid (one-hit curves). Thus, it should be possible to use *E. ashbyii* in mutation work. However, it cannot be a good organism for genetic work since the efficiency of plating is low at high spore concentrations on plates, and is even lower when the spores are not washed before plating. This makes it rather laborious to use. One prerequisite for trying crosses with an organism is to have genetic markers, preferably auxotrophic markers. Since inositol deficiency was lethal in the complete medium it may be possible to select auxotrophic mutants; inositol deficiency has been shown to be useful in other fungal systems (Megnet, 1964; Lester & Gross, 1959). Minoura (1952) showed that inositol is a growth factor of *E. ashbyii*, but it has to be shown that auxotrophs of Eremothecium really can be rescued from inositol-less death.

This work was supported by the Swedish Council for Applied Research. The skilful technical assistance of Mrs Kerstin Ekengren-Jansson is gratefully acknowledged.

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With 2 plates
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Growth of *Plasmodiophora brassicae* in Host Callus

By D. S. INGRAM
Department of Botany, University of Glasgow

(*Accepted for publication* 20 *August* 1968)

SUMMARY

Explants of young tumour tissue from several Brassica species infected with *Plasmodiophora brassicae* plasmodia gave rapid callus growth on medium containing 2,4-dichlorophenoxyacetic acid and coconut milk. Degeneration of the callus followed as resting spores were formed. The resting spores germinated *in situ* and primary plasmodia and zoosporangia developed in some cells, then secondary plasmodia reappeared and normal callus growth was resumed; thereafter all stages of the parasite life-cycle were present in active cells. This situation has been maintaned for over 13 months by 8-weekly transfer of calluses to new medium. Callus clones containing only vegetative plasmodia were established on a coconut-milk + 2, 4-dichlorophenoxyacetic acid medium. Sporogenesis did not occur readily in these clones unless callus was transferred to a kinetin + α-naphthylacetic acid medium or to other media which did not contain 2,4-dichlorophenoxyacetic acid. Stages of the *P. brassicae* life-cycle found in callus appeared similar to those in intact hosts, while zoosporangia were formed in the root hairs of *Sinapis alba* organ cultures infected with callus-produced resting spores. It was concluded that on suitable media *P. brassicae* may be maintained in complete balance with host callus.

INTRODUCTION

Tissue cultures offer convenient substrates for the growth of obligate parasites under controlled and sterile environmental conditions, making precise physiological investigations possible. The first report of the dual culture of an obligate parasite and its host was by Morel (1944), who grew *Plasmopara viticola* in callus cultures of vine. Since that time, with the exception of the work of Hotson & Cutter (1951) and Cutter (1959; 1960) involving the juniper + *Gymnosporangium juniperi-virginianae* and the *Arisaema triphyllum* + *Uromyces ari-triphylli* combinations, there have been few reports of the successful establishment and long-term maintenance of obligate parasites in host tissue cultures. Hotson & Cutter (1951) were able to establish their cultures by transferring explants of surface-sterilized juniper gall tissue, infested with *Gymnosporangium juniperi-virginianae*, to callus medium. A similar procedure was adopted by Strandberg, Williams & Yukawa (1966) in the first report of the culture of *Plasmodiophora brassicae* in callus of *Brassica oleracea* var. *capitata*, Badger Shipper. However, meristematic regions of infected calluses had to be transferred each week on a medium containing α-naphthylacetic acid + kinetin. No apparent difference was found between *P. brassicae* grown in intact hosts and in callus, and callus tissues, like intact host tissue, reacted to the parasite by cell and nuclear hypertrophy and increased

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starch content. These observations were further substantiated by electron microscope studies (Williams & Yukawa, 1967).

The present study, begun before publication of the work of Strandberg *et al.* (1966), confirms their findings for growth of infected Brassica callus on a kinetin + α-naphthylacetic acid medium, and also indicates that *Plasmodiophora brassicae* may be grown for long periods in complete balance with host callus on a coconut milk + 2,4,-dichlorophenoxyacetic acid medium.

METHODS

Organisms. Infected plants of *Brassica rapa*, Golden Ball turnip and Balmoral turnip, *B. napobrassica*, Wilhelmsburger swede, *B. napus* var. *annua*, Giant English rape, *B. oleracea* var. *capitata*, Early Drumhead cabbage, and *B. chinensis*, Wong Bok, were obtained by transplanting 10-day seedlings into John Innes potting compost containing approximately 1×10^8 *Plasmodiophora brassicae* resting spores/g. The strain of the parasite used has arbitrarily been denoted S and has been shown to be pathogenic to a wide range of Crucifers (I. J. McEvoy, personal communication). Plants harvested after 4 to 6 weeks of growth under greenhouse conditions had normally developed large clubs. Healthy plants were grown in sterile John Innes potting compost in the greenhouse.

The callus culture medium consisted of tobacco medium salts and micronutrients (Hildebrandt, 1962). Ferric tartrate was omitted and replaced with $FeSO_4.7H_2O$ (0·0398 g./l.) and Na_2EDTA (0·0532 g./l.). This medium was supplemented with calcium pantothenate (2·5 mg./l.), thiamine hydrochloride (0·1 mg./l.), glycine (3·0 mg./l.), α-naphthylacetic acid (NAA, 0·1 mg./l.), 2,4-dichlorophenoxyacetic acid (2,4-D, 6·0 mg./l.) and coconut milk (150 ml./l.), and was solidified with 6·0 g. Davis agar/l. (coconut milk medium). In a chemically defined medium (the kinetin medium) 2,4-D and coconut milk were omitted, the NAA content was raised to 0·5 mg./l. and kinetin was added at 1·0 mg./l. (P. H. Williams, personal communication). In some experiments the kinetin medium was further supplemented with extra vitamins and micronutrients (pyridoxine HCl 0·1 mg./l.; nicotinic acid 0·5 mg./l.; mes-oinositol 0·2 mg./1.; biotin 4·0 μg./1.; $CuSO_4.5H_2O$ 0·02 mg./1.; H_2MoO_4 0·017 mg./l.) or with an amino acid mixture based on the analysis of the amino acids of coconut milk of Tulecke, Weinstein, Rutner & Laurencot (1961). All tissue culture media were adjusted to pH 5·5 with NaOH or HCl and were then autoclaved at 115° for 10 min.

Callus cultures were always incubated at 26° in a culture room lit continuously with a single 100 W. tungsten lamp.

Infected cultures were initiated from *Plasmodiophora brassicae* clubs from 4- to 6-week-old plants: pieces of club approximately 1·0 cm. diameter and 2·0 cm. long were washed in water, immersed in 95 % (v/v) ethanol in water for 1 min., then in 1 % (w/v) $HgCl_2$ in water for 4 min. After four washes in sterile distilled water the clubs were trimmed to remove dead tissues contaminated with mercuric chloride, and were cut into 1·5 mm^3 to 2·0 mm^3 explants which were placed on 15 ml. medium contained in 25 × 150 mm. Pyrex glass tubes and incubated at 26°. A similar procedure was used for the initiation of callus cultures from organs of healthy plants grown for 4 to 6 weeks in sterile soil.

When callus cultures had become established, those which were to be transferred

to fresh medium every 7 to 14 days were always grown singly on 15 ml. medium contained in 25 × 150 mm. Pyrex glass tubes, while those which were to be transferred every 8 weeks were grown on 30 ml. medium contained in 100 ml. Pyrex glass Erlenmeyer flasks (3 calluses/flask). The growth of established, infected callus tissue of *Brassica rapa*, Golden Ball, on different media was compared by using 15 ml. batches of medium contained in 150 × 25 mm. glass tubes. These were inoculated singly with 1·5 mm^3 explants of actively-growing callus tissue, previously maintained for 12 months on coconut-milk medium, and maintained for 1 week before beginning the test on medium without growth factors. The growth of the calluses was assessed visually and by fresh and dry weights after being transferred each week for 5 weeks on the test media.

Root organ cultures were established from sterile seedlings of *Sinapis alba* in a liquid medium composed of salts, micronutrients, vitamins and sucrose (Street & Henshaw, 1966) supplemented with *meso-inositol* (50·0 mg./l.), kinetin (0·00125 mg./l.) and α-naphthylacetic acid (0·0001 mg./l.). This medium was adjusted to pH 4·8 with NaOH or HCl and autoclaved at 115° for 10 min. Sterile seedlings were grown as follows: seeds of *S. alba* were washed in 95% (v/v) ethanol in water for 1 min., immersed in 5% (w/v) filtered calcium hypochlorite solution for 7 min. and washed three times in sterile distilled water; they were then germinated in the dark at 26° on potato + glucose agar. After 3 days of growth, 0·5 cm. root-tip segments were removed from the seedlings and placed in 20 ml. medium contained in 100 ml. Pyrex glass Erlenmeyer flasks. Further incubation of the cultures was at 26° in the dark.

Organ cultures were infected with *Plasmodiophora brassicae* by placing 2 cm. root tip segments in 25 ml. Erlenmeyer flasks, containing 5 ml. of medium, and inoculating with callus-produced resting spores suspended in organ culture medium (see above). (The final concentration of spores in the flasks was about 1×10^6/ml.). Resting spores were extracted from calluses under completely sterile conditions: pieces of callus tissue were placed in a small quantity of organ culture medium in a test-tube, frozen at −20° for 30 min. and then, after thawing at room temperatures, were homogenized with a glass rod. The resulting suspension was filtered through muslin to remove debris and added to the culture flasks after spores had been counted with a haemocytometer.

Histological techniques. Calluses were removed from the culture medium and fixed in 3% glutaraldehyde in 0·1 M-phosphate buffer (0·994 g. Na_2HPO_4 + 0·4083 g. KH_2PO_4, all in 100 ml. water at pH 7·2) for 3·5 hr. They were then washed for two periods of 1 hr in the buffer, fixed in 1% osmic acid in buffer for 1 hr, and finally given two further 1 hr washes in buffer. All procedures were done under vacuum at 4°. Calluses to be examined were embedded in wax and 8 μ sections stained with Delafield's haematoxylin (Ingram, 1967).

Stages of the *Plasmodiophora brassicae* life-cycle were identified by reference to: (*a*) sections of club tissue from intact plants; (*b*) whole roots of intact Brassica seedlings infected with the root hair stages of the parasite according to the method of Channon, Flint & Hinton (1964); (*c*) illustrations prepared by Karling (1942).

RESULTS

Initiation of Plasmodiophora brassicae infected callus cultures

When explants of 4-week club tissue from the Brassica species listed above were placed on the coconut-milk medium and incubated at 26°, a phase of rapid callus growth was normally initiated after 4 to 6 days which continued until at 4 weeks the fresh weight of calluses was between 60 and 140 mg. However, after 4 to 6 weeks of incubation cultures began to show signs of decline: the tissues became brown and unhealthy and growth virtually ceased. Continued incubation of such degenerate calluses led, after a further 3 to 4 weeks, to renewed and healthy tissue growth which arose from isolated points on the callus mass. Growth of the new callus continued without further interruption, explants being transferred to fresh medium every 8 weeks (Table 1). In contrast, explants of healthy tissue from roots and hypocotyls of the Brassica species being investigated after 4 to 6 days initiated callus growth which proliferated without check on the coconut-milk medium.

Table 1. *Development of callus, containing Plasmodiophora brassicae from explants of Brassica rapa, Balmoral club, on the coconut-milk medium*

Approx time on the medium (days)	Quality of callus growth	Stage of *P. brassicae* life-cycle present in the callus
7–27	Vigorous growth	Advanced vegetative plasmodia
28–42	Callus became unhealthy and growth ceased	Advanced and cleaved plasmodia and resting spores
43–56	Callus not growing	Resting spores and primary plasmodia and zoosporangia
57–63	Renewal of vigorous healthy growth	Resting spores, primary plasmodia and zoosporangia and young, vegetative plasmodia
> 64	Vigorous healthy growth continuing without check	All stages of the parasite life-cycle

Histological investigations of sample infected calluses of *Brassica rapa*, Balmoral and Golden Ball, and *Brassica oleracea* var. *capitata*, Drumhead, were made at the time of callus initiation, at intervals up to 9 weeks afterwards and, in the case of Golden Ball, at 10 and 14 months afterwards. Because the variation in the rate of growth of individual calluses was slight it was possible to trace the complete pattern of host and parasite development during the early stages of infected tissue-culture growth. The interrupted nature of these early stages of growth was found to correspond with clearly defined steps in the development of the parasite within the tissues.

At the time of tissue culture initiation clubs, which were to be used as a source of explants, contained numerous cells infected with *Plasmodiophora brassicae*. The infected cells were usually hypertrophied, with enlarged nuclei and nucleoli, and contained an abundance of starch. Several types of parasite plasmodia were present, the most numerous being at an advanced vegetative stage with vacuoles, many nuclei and an abundance of lipid droplets. Resting spores had not normally formed in the cells at this stage.

The pattern of development of callus from club tissues was essentially the same in all three species investigated. During the first 4 weeks of callus growth cell division took place throughout the explants and a large body of new tissue was produced. However, division of hypertrophied, infected cells was not evident, and newly-formed callus cells did not contain the parasite. Stages of sporogenesis of *Plasmodiophora brassicae* plasmodia (i.e. cleavage and formation of resting spores) became evident in infected cells of the inoculum after about 4 weeks of incubation, when a rapid decline in callus vigour was recorded. At the same time the walls of infected cells became brown and did not take up stain. At approximately 6 weeks all plasmodia had undergone sporogenesis, and resting spores were the only part of the parasite life-cycle represented in the tissue cultures.

After about 8 weeks of incubation when callus proliferation was renewed, it was noted that many enlarged, brown-walled cells, which had obviously once contained resting spores, were partially or completely empty, the place of the spores being occupied by amorphous debris. Within such cells, or close to them, stages of the *Plasmodiophora brassicae* life-cycle normally found in the root hairs of intact plants (Karling, 1942) were noted. These infected cells did not contain starch, and frequently appeared to be dead. The parasite consisted either of small primary plasmodia with 1 or 2 nuclei and no lipid, or clusters of primary zoosporangia. The latter (Pl. 1, fig. 1, 2) often contained from 3 to 16 incipient zoospores. Primary plasmodia and zoosporangia in callus closely resembled similar structures observed in the root hairs of intact infected Brassica seedlings.

Following the appearance of zoosporangia, secondary plasmodia soon developed in adjacent newly-formed cells. The appearance of secondary plasmodia (Pl. 1, fig. 3, 4) coincided with renewed callus growth, which then continued unchecked. Many of the secondary plasmodia developed to form resting spores, and subsequent sections of calluses revealed all stages of the *Plasmodiophora brassicae* life-cycle in the tissues. Even after 14 months, Golden Ball calluses, which had been transferred on 30 ml. coconut-milk medium contained in 100 ml. Erlenmeyer flasks every 8 weeks and in which no part of the original clubroot inoculum remained, contained representatives of each stage of the parasite life-cycle. Vegetative plasmodia were usually in the most recently-formed cells of small meristematic centres scattered through the calluses, while other stages, including resting spores and primary zoosporangia, were in the cells of the older parts of the callus. Infected callus cells resembled infected cells of intact hosts in frequently being hypertrophied, in usually possessing enlarged nuclei, and in containing considerably more starch than uninfected cells. Indeed, healthy calluses, unlike infected calluses, contained very little starch. The parasite was anatomically indistinguishable from the parasite observed in intact hosts.

Control of the parasite life-cycle in tissue culture

By selection of callus tissues and frequent transfer to fresh coconut-milk medium it was possible to establish clones of *Brassica rapa*, Golden Ball, callus containing only vegetative plasmodia. During the establishment of these clones from club tissue the parasite went through the stages of sporogenesis and resting spore germination already described. As soon as new callus growth containing vegetative plasmodia formed on the inoculum it was removed to fresh medium. Thereafter, only meristematic tissues were transferred to 15 ml. fresh medium contained in 25 × 150 mm. Pyrex glass tubes

every 7 to 14 days. The parasite present in calluses maintained in this way (Pl. 1, fig. 3, 4) did not appear to develop beyond the vegetative plasmodial stage and resting spores were never formed. Despite this, new tissues always contained plasmodia in the cells.

Table 2. *Mean fresh weights of two replicate calluses, and stages of callus and parasite development when Brassica rapa, Golden Ball, tissues, infected with Plasmodiophora brassicae, were maintained without transfer on 15 ml. and 60 ml. batches of the coconut-milk medium*

Time on the medium (weeks)	Fresh weight (mg.) of calluses on:		Callus and *P. brassicae* development on:	
	15 ml. medium	60 ml. medium	15 ml. medium	60 ml. medium
2	83·70	78·50	A*	A*
4	165·50	156·75	A	A
6	311·80	308·00	B	A
8	529·75	667·90	C	A
10	453·50	922·75	D	B
12	473·15	1140·26	D	C
16	400·50	1049·50	D	D

* A = healthy callus growth with vegetative plasmodia; B = callus senescing at centre, plasmodia vegetative; C = callus senescent and containing a few spore-filled cells (medium becoming dehydrated); D = callus completely dead and medium showing extreme signs of dehydration.

Resting spores were not formed readily by *Plasmodiophora brassicae* maintained in a vegetative state in Golden Ball callus for 12 months. Table 2 illustrates the results of a test where 2 mm^3 explants of Golden Ball callus, containing *P. brassicae* maintained in a vegetative state for 12 months, were transferred singly to 15 ml. batches of coconut-milk medium contained in 25 × 150 mm. test-tubes, or to 60 ml. batches of coconut-milk medium contained in 40 × 200 mm. test-tubes and incubated without further transfer. Two sample calluses were removed from each treatment at intervals, weighed and examined by freehand section for the presence of resting spores. Initially calluses grew well on both volumes of medium, with senescence at the centre of the tissue masses becoming evident after 4 to 6 weeks. By 8 weeks the 15 ml. batches of medium had started to dry out and calluses senesced rapidly. The 60 ml. batches of medium did not show extreme signs of dehydration until 10 or 12 weeks, but when they did, calluses senesced rapidly. Resting spores had developed after about 8 weeks in a few cells of calluses maintained on 15 ml. batches of medium, although young vegetative plasmodia were still abundant in the tissues. When senescence took place the parasite died with the tissues and no more resting spores were formed. The situation was essentially the same in calluses maintained on the 60 ml. batches of medium, although senescence and the appearance of a few resting spore cells did not take place for approximately 12 weeks. None of the calluses ever contained a large number of cells filled with resting spores.

However, resting spores did develop after 4 to 5 weeks in most of the infected cells of Golden Ball calluses previously transferred weekly on the coconut-milk + 2,4-D medium, when these were maintained without transfer on medium containing no growth factors or on medium supplemented with coconut milk alone. Resting spores

did not develop readily in Golden Ball calluses maintained without transfer on medium containing 2,4-D alone.

Growth of Plasmodiophora brassicae infected callus on a kinetin + α-naphthylacetic acid medium

Calluses infected with *Plasmodiophora brassicae* were established on the kinetin medium from 4- to 6-week-old clubs of *Brassica rapa*, Golden Ball, and *B. napobrassica* Wilhelmsburger. Early stages of callus growth and parasite development were similar to those described for establishment of infected callus on the coconut-milk medium. However, infected tissues did not normally grow as well on the kinetin medium as on the coconut-milk medium: callus growth was slower and normally looked brown and unhealthy. Furthermore, it was not possible to maintain callus containing all stages of the *P. brassicae* life-cycle for long periods on the kinetin medium. Unless meristematic tissues containing only vegetative plasmodia were rigorously selected and transferred to fresh medium every 7 to 14 days the parasite rapidly underwent sporogenesis, cell walls became lignified and the callus mass died after about 4 weeks. Sporogenesis took place rapidly even in *B. napobrassica* calluses, where *P. brassicae* had been kept in a vegetative state for over a year by weekly transfer, when the calluses were left untransferred for 4 weeks on the kinetin medium.

Table 3. *Mean fresh and dry weights of five replicate calluses after 5 weeks of growth from inocula of infected Brassica rapa, Golden Ball, callus tissue on the coconut-milk medium and on the kinetin medium, unsupplemented and supplemented with extra vitamins and trace elements and with an amino acid mixture*

(1) Extra vitamins: pyridoxine HCl (0·1 mg./l.), nicotinic acid (0·5 mg./l.), mesoinositol (0·2 mg./l.), biotin (4·0 μg./l.). (2) Extra trace elements: $CuSO_4.5H_2O$ (0·02 mg./l.), H_2MoO4 (0·017 mg./l.). (3) Amino acid mixture: based on an analysis of the amino acids of coconut milk (Tulecke *et al.* 1961).

Medium	Weight of callus (mg.)	
	Fresh	Dry
Coconut-milk medium	291·94 ± 31·37	24·88 ± 2·77
Kinetin medium	175·94 ± 22·11*	19·84 ± 2·94
Kinetin medium ± extra vitamins and trace elements	162·66 ± 12·80*	18·00 ± 1·37
Kinetin medium ± extra vitamins + trace elements + amino acids	182·08 ± 37·85*	20·98 ± 4·44

* These calluses contained many cells filled with resting spores at the end of the experiment.

The growth of infected calluses could not be improved markedly, nor could sporogenesis of the parasite be delayed, by supplementing the kinetin medium with an extra vitamin and micronutrient mixture (pyridoxine HCl; nicotinic acid; meso-inositol; biotin; $CuSO_4.5H_2O$; H_2MoO_4) or with an amino acid mixture based on an analysis of the amino acids of coconut milk (Tulecke *et al.* 1961). Plate 2, fig. 6 and Table 3 refer to a growth test where explants of infected *Brassica rapa*, Golden Ball, callus were incubated for 5 weeks on the coconut-milk medium and on the kinetin medium, unsupplemented and supplemented with the extra vitamins and micronutrients mixture and with the amino acid mixture.

Infection of root organ cultures

When 2·0 cm. tip segments from root organ cultures of *Sinapis alba* were infected with 1×10^6 resting spores of *Plasmodiophora brassicae* (obtained from infected calluses grown on the kinetin medium) primary plasmodia and zoosporangia developed in the root hairs after 4 to 9 days. Only a small proportion (less than 1 %) of the root hairs were infected, but the plasmodia and zoosporangia, when examined microscopically in unstained preparations using bright-field or phase-contrast illumination, appeared identical to similar structures formed in the root hairs of *S. alba* seedlings infected with resting spores from intact plants according to the method of Channon *et al.* (1964). Clubs did not develop on organ cultures, even after 10 weeks of incubation.

DISCUSSION

Maintenance of both host and parasite in an active state is an important problem in growing obligate parasites in tissue culture. Hotson & Cutter (1951) and Cutter (1959) overcame this problem and were able to grow clones of Juniper callus infected with *Gymnosporangium juniperi-virginianae* for several years on artificial medium. Other workers (Constabel, 1957; Rossetti & Morel, 1958; Maheshwari, Hildebrandt & Allen, 1968), however, have been less successful with rust fungi, and the author (unpublished data) found that *Uromyces fabae* did not colonize callus formed on infected segments of *Vicia faba* stem, although sporulation did take place on those segments. *Plasmodiophora brassicae* has been grown in callus of *Brassica oleracea* var. *capitata* over an extended period (Strandberg *et al.* 1966), but active infected tissues had to be transferred on a kinetin + α-naphthylacetic acid medium every week. The present study indicates that *P. brassicae*, either in a vegetative plasmodial form or going through its whole life-cycle, may be maintained in complete balance with host callus for long periods on suitable media.

It is interesting that *Plasmodiophora brassicae* went through all stages of its life-cycle during callus culture initiation, and that the various stages of the life-cycle were reflected in the quality of growth of the callus tissues. Sporulation of *P. brassicae* plasmodia soon after transfer of tissue explants to the callus medium was probably inevitable, since the plasmodia in the clubs used as a source of inoculum were at such an advanced stage of development. Since hypertrophied infected cells of the inoculum did not divide, callus tissues produced initially were not infected with vegetative parasite plasmodia, and became infected only after resting spores had germinated *in situ* and primary plasmodia and zoosporangia had been formed. In intact hosts, *in situ* germination of resting spores is unlikely, and in callus some constituent of the tissue culture medium, such as auxin or EDTA, may have been responsible for inducing the process. Both the sequence of events during callus initiation and the behaviour of established infected calluses when left untransferred on the kinetin medium suggested that sporulation of *P. brassicae* was inhibitory to callus proliferation, even though many cells in the tissue mass did not contain the parasite. However, *P. brassicae* continued its life-cycle in established *Brassica rapa*, Golden Ball, callus for nearly 2 years, during which time no inhibition of tissue growth was noted, possibly because the ratio of plasmodia undergoing sporogenesis to the total number of infected cells in such calluses was very small.

Clones of *Brassica rapa*, Golden Ball, callus containing only vegetative plasmodia were established by selection and frequent transfer on the 2,4-D + coconut-milk medium. Sporogenesis did not occur readily in such clones once they had become well established, even when the tissues were allowed to senesce. Sporogenesis was induced, however, when the tissues were transferred to other media which did not contain 2,4-D, suggesting that the auxin status of culture media may be important in the initiation of sporulation of *Plasmodiophora brassicae* in callus.

Histological investigations of calluses infected with *Plasmodiophora brassicae* indicated that both the parasite and host-cell reactions to infection in tissue culture are the same as in intact hosts, thus confirming the observations of Strandberg *et al.* (1966).

Brassica seedlings have been infected with callus-produced resting spores (P. H. Williams, personal communication), and further evidence for the viability of resting spores formed in callus is provided by the demonstration of their ability to infect root hairs of *Sinapis alba* root organ cultures. Smith (1956) showed that *Plasmodiophora brassicae* resting spores germinated better in non-sterile soil than in sterile soil, an effect which could be reproduced by treating spores with macerating enzymes in sterile conditions. A similar phenomenon was demonstrated by Mosse (1962), where certain bacteria or macerating enzymes were necessary for infection of sterile host plants by Endogone species. It is possible that the reasons why only a small proportion of the root hairs of *S. alba* organ cultures became infected after inoculation with callus-produced resting spores may be explained in similar terms. However, no effort was made to wash resting spores used for root organ culture inoculation and some inhibitor of germination produced in the calluses might have been carried over in the inoculum. Despite these possibilities, resting spores did germinate within calluses, as discussed above. The fact that club formation did not occur in organ cultures may indicate a fault in technique, for Smith (1956) has reported growth of *P. brassica* in excised root cultures.

Hotson & Cutter (1951) reported that *Gymnosporangium juniperi-virginianae* grew out of host calluses onto the culture media; this result could not be achieved by other workers (Constabel, 1957; Rossetti & Morel, 1958). Although a large number of calluses infected with *Plasmodiophora brassicae* were maintained during the present study, no evidence was found that the parasite ever grew out of the tissues. Even when infected callus tissues were grown in liquid suspension culture (unpublished data), *P. brassicae* was never detected free in the culture medium. However, it is significant that primary plasmodia and zoosporangia formed in calluses were frequently seen to be in cells which were dead, indicating a lesser degree of dependence on the host cells than secondary plasmodia, which were always associated with living cytoplasm. This possibility is supported by observations that many Crucifers and non-Crucifers, though not susceptible to the secondary stages of the *P. brassicae* life-cycle, are susceptible to the primary (root hair) stages (Köle & Philipsen, 1956).

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EXPLANATION OF PLATES

PLATE 1

Stages of the *Plasmodiophora brassicae* life cycle from tissue cultures grown on the Coconut milk medium.

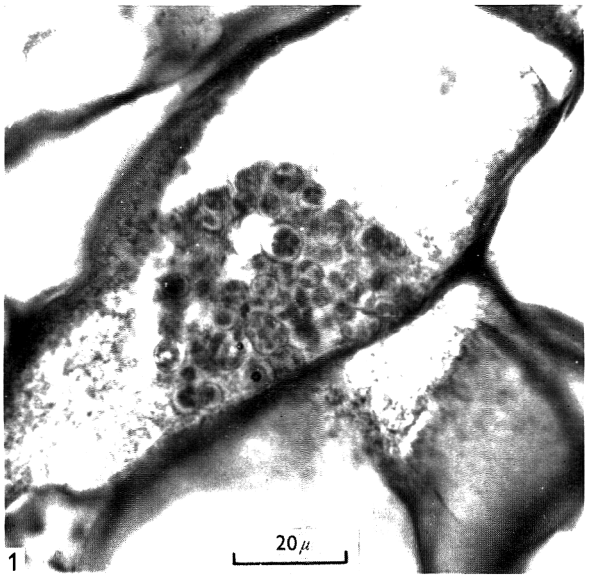
Fig. 1 and 2. Primary zoosporangia, with incipient zoospores, in cells which had previously contained resting spores, formed during callus culture initiation from *Brassica rapa*, Balmoral, club tissues. (Stained with Delafield's haematoxylin.)

Fig. 3 and 4. Vegetative (secondary) plasmodia released from thick hand sections of 12-month-old *Brassica rapa*, Golden Ball, callus. (Unstained preparation.)

PLATE 2

Fig. 5. Resting spores of *Plasmodiophora brassicae* formed in *Brassica rapa*, Balmoral, callus grown on medium without growth factors. (Unstained preparation.)

Fig. 6. Five replicate examples each of *Brassica rapa*, Golden Ball, callus after 5 weeks of growth on (A) coconut-milk medium, (B) kinetin medium, (C) kinetin medium + extra vitamins and micronutrients, (D) kinetin medium + extra vitamins + micronutrients + an amino acid mixture.



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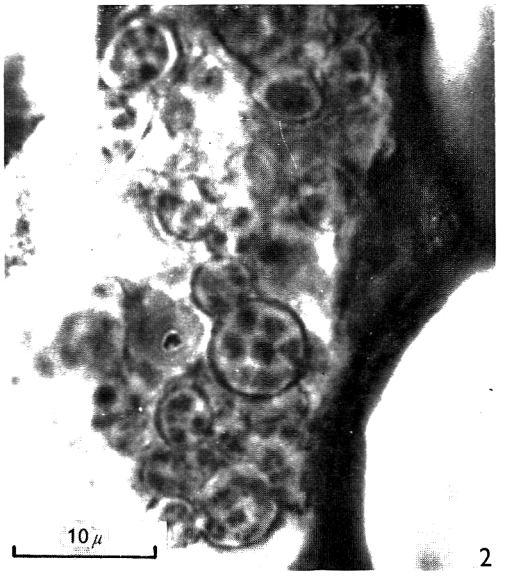

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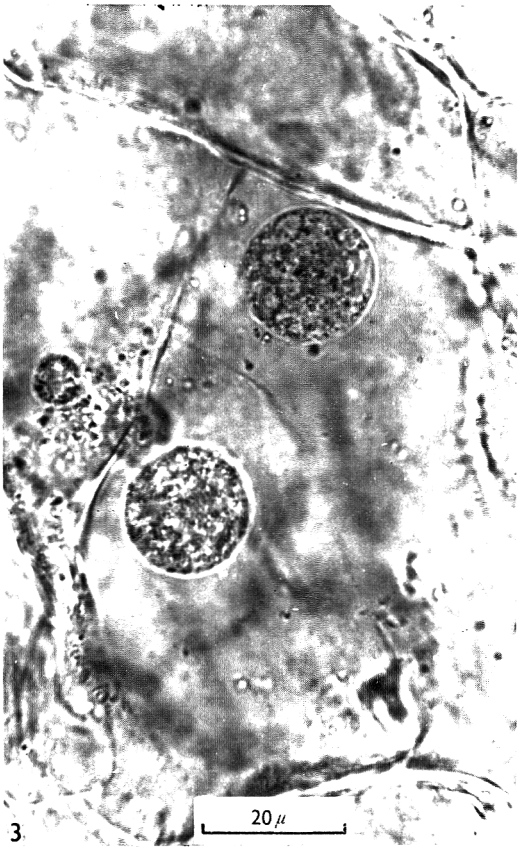

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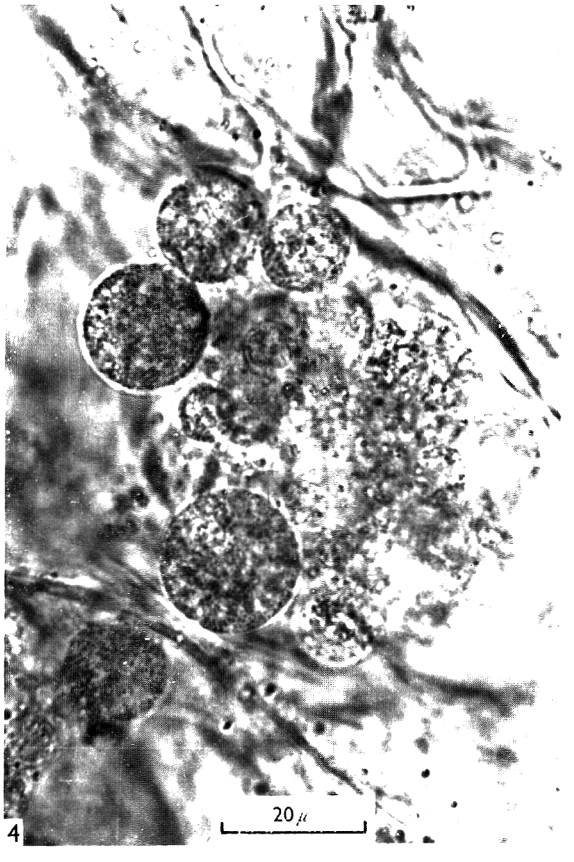

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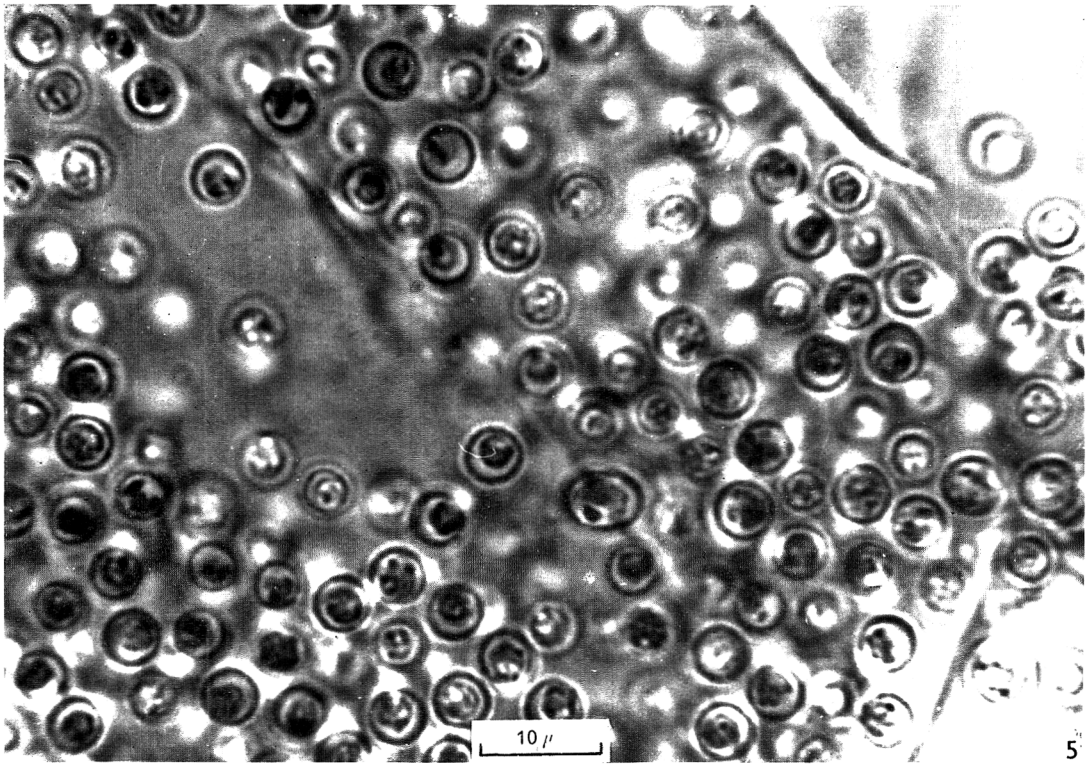

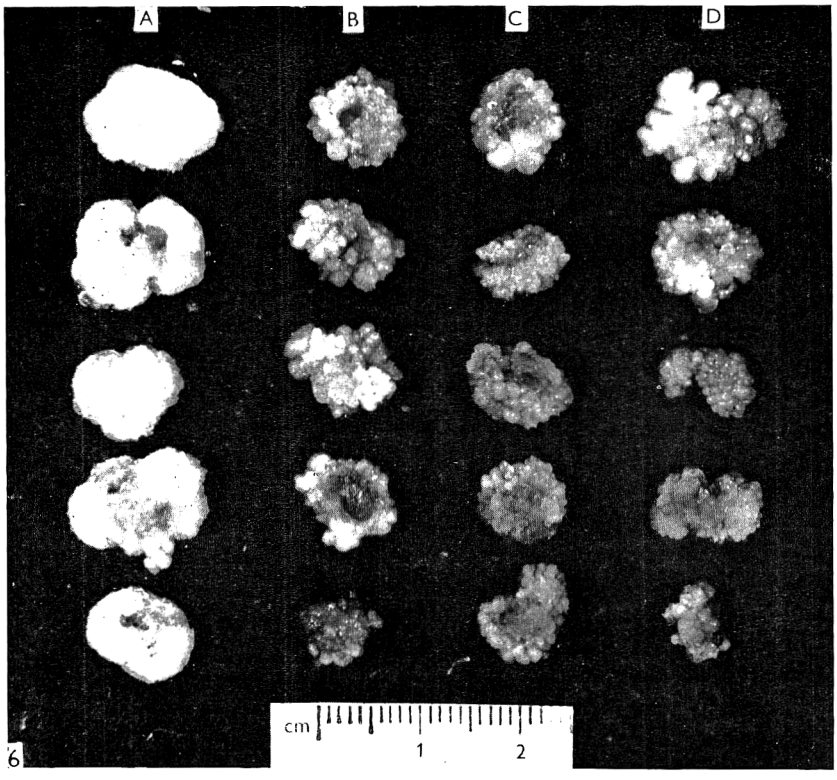

D. S. INGRAM

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Some Biological Effects of Volatile Metabolites from Cultures of *Saccharomyces cerevisiae* Meyen ex Hansen

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SUMMARY

The gas mixtures from cultures of *Saccharomyces cerevisiae* inhibited growth and sporulation of *Aspergillus niger* and germination of seeds of *Lepidium sativum*. In test conditions seven volatile organic metabolites in the culture gases were identified by gas–liquid chromatography (GLC) as acetaldehyde, ethyl acetate, ethanol, *n*-propanol, isobutanol, and a mixture of isopentanols (1 part 2-methyl-butan-1-ol to 2 parts 3-methyl-butan-1-ol); changes in the concentrations of CO_2 and O_2 were also measured. Pure samples of each of these components were tested at the concentrations found in the culture gases in order to identify the inhibitory substances.

Inhibition of growth of *Aspergillus niger* could be produced by these culture gas concentrations of acetaldehyde and of ethanol. Inhibition by this concentration of CO_2 was just significant in these tests. The effect on sporulation could be produced by the CO_2, but not by these concentrations of any of the other identified components.

The effect of *Lepidium sativum* seed germination could be produced by these culture gas concentrations of ethanol and of 3-methyl-butan-1-ol to a lesser extent. Slight effects were also observed with a lowered O_2 concentration and with a raised CO_2 concentration but not with the other constituents.

INTRODUCTION

Dick & Hutchinson (1966) reported that gases from cultures of *Saccharomyces cerevisiae* inhibited the growth and/or sporulation of several species of fungi, and Hutchinson (1967) reported that they inhibited spore germination and prothallial growth of *Pteridium aquilinum*. Lösel (1964) summarized previous work on the biological effects of volatile fungal products. Robinson & Park (1966) and Robinson, Park & Garrett (1968) identified acetaldehyde as one of the volatile products from cultures of *Fusarium oxysporum* and of *Rhizopus stolonifer* which can inhibit fungal spore germination; they also identified ethanol amongst the nine otherwise unidentified peaks which they saw in gas–liquid chromatograms of samples from these cultures, but they found that it did not inhibit spore germination in their tests. They state that none of the volatile germination inhibitors affected the growth of hyphae 'to any marked degree' in their tests. Norrman (1968) examined the effects of vapours from aqueous solutions of a variety of organic compounds on the morphology of *Pestalotia rhododendri*. He found that spore production was stimulated by many compounds in the order of activity alcohols > esters > acids > aldehydes > ketones, and that high vapour concentrations of many of these substances often inhibited linear growth of

the colony. We now report on the analysis of the gas mixtures produced by *S. cerevisiae* in defined conditions, and on the identification of the active components.

METHODS

Analysis of the gases above yeast cultures

Cultures of *Saccharomyces cerevisiae* Meyen ex Hansen (Glasgow University Collection I, referred to as 'yeast' throughout this article) were prepared by spreading a suspension of cells over the surface of 200 ml. of 5% malt agar (50 g. Oxoid malt extract + 20 g. Oxoid agar in 1 l. of deionized water) in each of a series of 1 l. Roux bottles. The cultures were then incubated at 30° in the dark for 48 h. Five ml, 1 ml. and 0·5 ml. were then withdrawn by syringe through the cotton-wool plug for analysis of organic volatiles, O_2 and CO_2 respectively. Similar samples were also taken from the assemblies used to examine the biological effects of these gases (described below).

The samples were analysed in an Aerograph Model 204 gas–liquid chromatograph, using a flame ionisation detector for organic volatiles and a micro cross-section detector for CO_2 and O_2, under the operating conditions shown in Table 1.

Table 1. *Operating conditions used for chromatographic analysis of yeast culture gases*

Column	Dimensions	Material	Packing	Temp. (°C)	Carrier gas and flow rate (ml./min.)	Hydrogen flow rate (ml./min.)
A	10 ft × ¼ in	S.S.*	Molecular sieve 5A 30/60 mesh	65	He 22	—
B	14 in × ¼ in	Copper	Davison 08 grade silica gel 30/60 mesh	50	He 30	—
C	5 ft × ⅛ in	S.S.	20% Carbowax 1500	65	N_2 25	25
D	6 ft × ⅛ in	S.S.	15% Dinonyl phthalate	60	N_2 30	30
E	6 ft × ⅛ in	S.S.	20% 1,2,3-Tris-(2-cyanoethoxy) propane	50	N_2 30	25
F	6 ft × ¼ in	Copper	25% glycerol	60	N_2 30	25

60/80 mesh Chromosorb W was used as support in columns C–F.
* Stainless steel.

The constituents of the culture gases were identified by comparison with retention times of authentic pure samples and by syringe reactions (Hoff & Feit, 1964). The concentration of each identified metabolite was estimated by comparing the height of the peaks in representative samples with those of known concentration of authentic pure samples in parallel tests.

Examination of the effects of culture gases on Aspergillus niger

A suspension of conidia of *Aspergillus niger* van Tieghem (Glasgow University Collection I) was made in 2% malt agar (20 g. Oxoid malt extract + 20 g. Oxoid agar in 1 l. deionized water), at approximately 45°, poured immediately into a Petri dish and incubated for 18 h. at 24°.

A series of 1 l. Roux bottles each containing 200 ml. of 2% malt agar were then each inoculated centrally with a 4 × 2 mm. disc cut from this suspension, the same

suspension being used for all cultures in any one experiment. This inoculation method produced a single symmetrical colony in each vessel. All cultures were incubated at 24° in a low light intensity. The effects of complete yeast culture gases were examined by joining *Aspergillus niger* cultures and yeast cultures by means of a glass T-piece passing through a rubber bung in the neck of each Roux bottle. Gas samples were taken by a syringe through a serum cap closing the lateral arm of the T-piece. Control cultures were paired with Roux bottles containing uninoculated 5% malt agar. The effects of identified organic components of the yeast-culture gas mixture were examined by placing samples of authentic pure compounds (purity verified by GLC) on filter-paper strips in the necks of the Roux bottles, and replacing the cotton-wool plug by a serum cap. The weight of each sample was that required to give an initial vapour pressure of the metabolite in the sealed vessel equal to the highest found at any stage during the incubation of yeast cultures with *A. niger*, or with cress seedlings. Untreated filter papers and serum caps were added to the control cultures at the same time.

Changes in O_2 and in CO_2 content during each experiment in the sealed assemblies were measured using a micro cross-section detector and column A (O_2) or column B (CO_2). Atmospheres with comparable O_2 and CO_2 concentrations to the final ones were made by partially evacuating freshly inoculated Roux bottles and replacing the air by N_2 (for reduced O_2) or by authentic CO_2. Obviously by this method the N_2 content was increased and the initial CO_2 content was decreased in proportion to changes in the O_2 content. It is thought that these changes can be disregarded at this stage, particularly as the reduction of CO_2 concentration would be counteracted very quickly by respiration.

After 8 days incubation, linear growth of a colony was assessed by taking the mean of two diameters at right angles to each other, and any gross effects on sporulation were estimated by eye.

Examination of the effects of culture gases on germination of cress seeds and on seedling growth

Seeds of *Lepidium sativum* L. orient (cultivar Dobbies 'white curled' 1964, referred to as cress throughout this article) were sterilized by shaking in aqueous $HgCl_2$ (0·01 %, w/v) for 1 min, followed by three changes of deionized water. Twenty sterilized seeds were placed in each of a series of 1 l. Roux bottles containing 200 ml. 0·5% Oxoid agar in 1 l. deionized water. These were then paired with yeast cultures, control bottles, or received authentic single substances by the methods described above. All cultures were held at 22–26° under continuous illumination from G.E.C. 'Warm White' fluorescent tubes.

After 5 days incubation a seed was recorded as germinated when it had produced a radicle as long as the diameter of the seed. Gross differences in the appearance of seedlings were noted by eye.

RESULTS

The only metabolites found in samples in sufficient concentration for identification were CO_2, acetaldehyde, ethyl acetate, ethanol, *n*-propanol, isobutanol, and isopentanols. Oxygen concentrations below atmospheric were also noted. They were all found in samples both from pure yeast cultures and from the paired assemblies during biological activity tests.

Figure 1 shows the chromatogram of a typical sample taken from a pure yeast culture after 2 days growth.

These are mostly common respiratory metabolites and their production is likely to be affected by many environmental factors. It was therefore deliberately decided to restrict the investigation to the study of their production and effects in the sealed assemblies used for the biological activity tests.

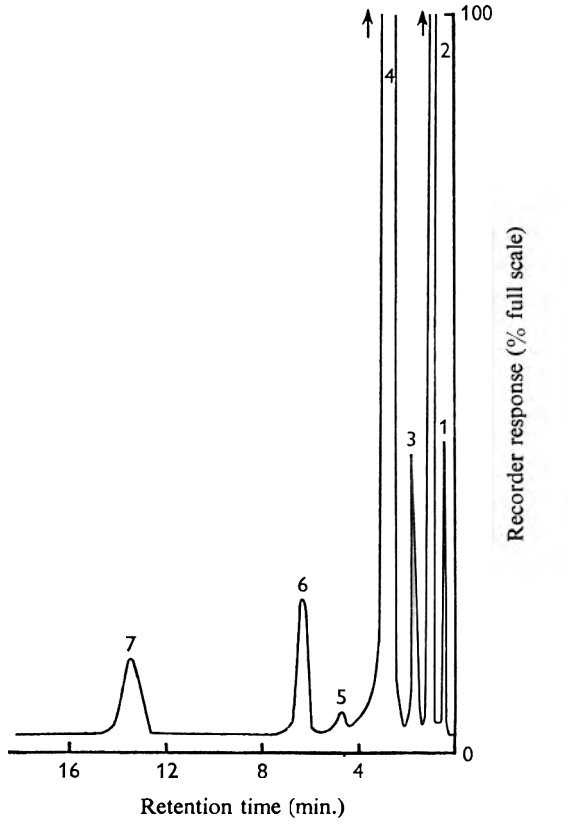

Fig. 1. Typical chromatogram of *Saccharomyces cerevisiae* volatiles on column C (Table 1) after 2 days growth. (1) Injection effect, (2) acetaldehyde, (3) ethyl acetate, (4) ethanol, (5) *n*-propanol, (6) isobutanol, (7) isopentanols.

Table 2. *Volatile organic compounds identified in gases above Saccharomyces cerevisiae cultures*

Constituent	Range of maximum concentration (μg./l. of air)
Acetaldehyde	260–320
Ethyl acetate	13–15
Ethanol	800–1000
n-Propanol	6–8
Isobutanol	12–16
2-Methyl-butan-1-ol	4–6
3-Methyl-butan-1-ol	8–10

Table 2 shows the range of maximum concentrations of each metabolite measured in these tests. Figure 2 shows the changes in concentration of each measured in a representative test with *Aspergillus niger*. The changes followed this pattern consistently within the different ranges of concentration of each metabolite found in the three experiments in which this was followed.

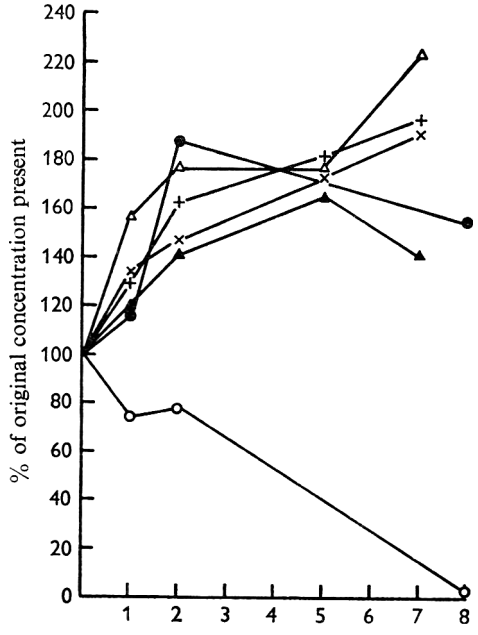

Fig. 2

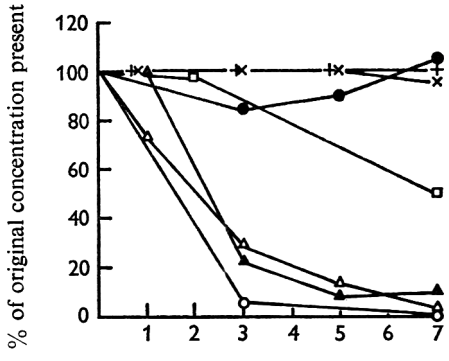

Fig. 3

Fig. 2. Changes in concentration of identified volatile metabolites in test assemblies of *Aspergillus niger* and yeast, expressed as % of concentration of each present immediately after the cultures were paired. Acetaldehyde, ○——○; ethyl acetate △——△; ethanol, ●——●; *n*-propanol ▲——▲; isobutanol, ×——×; 2-methyl-butan-1-ol+3-methyl-butan-1-ol, +——+.

Fig. 3. Changes in concentration of authentic pure compounds in tests with *Aspergillis niger*, expressed as percentage of concentration present at the start of the tests. The initial concentration of each was equal to the highest found at any stage in the other activity tests. Symbols on graph as in Fig. 2, with oxygen, □——□. Concentrations in μg./l. of air: acetaldehyde, 320; ethyl acetate, 15;ethanol, 1000; *n*-propanol, 8; isobutanol, 16; 2-methyl-butan-1-ol+3-methyl-butan-1-ol, 16.

The O_2 content of the sealed assemblies containing yeast and *Aspergillus niger* decreased from approximately 13% (v/v) immediately after pairing to approximately 8% (v/v), and the CO_2 content increased from approximately 15% (v/v) to approximately 25% (v/v).

Figure 3 shows the initial concentration of authentic pure compounds in the synthetic gas/air mixtures, and the changes in this concentration during a representative test of the effect of the mixture on *Aspergillus niger*. Figure 4 compares the effects of the total culture gases and of the synthetic air/gas mixtures on the size of the diameter of *A. niger* colonies after 8 days growth. Each experiment involved the measurement and comparison of at least three cultures in each treatment with at least three controls and in each case at least three replicate experiments have given similar results.

The diameters are reduced in the presence of complete culture gases and in the assemblies with an initial concentration of 320 μg./l. acetaldehyde, or of 1000 μg./l. of ethanol, or of 25% (v/v) CO_2. These concentrations are the maximum found in any tests of yeast culture gases; since acetaldehyde concentration falls off rapidly while that of ethanol varies relatively slightly (Fig. 3), then it appears that both can contribute to the effects of complete culture gases; it seems likely that CO_2 may also contribute to the effects of total culture gases in some conditions, although in these tests the concentration found only slightly exceeded the minimum inhibitory one (between 20% (v/v) and 25% (v/v)). Sporulation, assessed by eye, was consistently

Fig. 4. Effects of yeast culture gases on diameters of colonies of *Aspergillus niger*. At least three replicates were used in each experiment. The vertical axis represents the mean diameters of test colonies expressed as a percentage of the mean diameter of the controls. ↔ = least significant difference at 5% level. Each section on the horizontal axis represents a group of experiments using, from left to right, complete culture gases, 10% O_2, 20% CO_2, 25% CO_2, acetaldehyde, ethyl acetate, ethanol, *n*-propanol, isobutanol, and 2-methyl-butan-1-ol + 3-methyl-butan-1-ol.

reduced by about half by the presence of complete culture gases, or of 25% (v/v) initial CO_2 concentration, and by about one-third in the presence of 20% initial CO_2 concentration. It seems likely therefore that CO_2 may contribute to the effects. No other identified compounds have been shown to have any significant biological effect on *Aspergillus niger* in the concentrations tested. The possibility that ethyl acetate and/or *n*-propanol can contribute to the effects of the total culture gases has not been eliminated, however, as their concentrations fall off rapidly in the tests of authentic pure compounds. Our reasons for deliberately deciding not to investigate this possibility further are given in the discussion below.

The O_2 content of the sealed assemblies containing yeast and cress seeds decreased from approximately 17% (v/v) immediately after pairing to approximately 10% (v/v), and the CO_2 content increased from approximately 7% (v/v) to approximately 20% (v/v). The range of concentrations of other identified metabolites found in any test is shown in Table 2, and the changes in concentration measured during a representative test are shown in Fig. 5. Figure 6 shows the initial concentration of pure compound in the synthetic gas–air mixture and the changes in this concentration during a representative test. Figure 7 shows the effects of total culture gases and of pure compounds on seed

germination. Each experiment involved the measurement and comparison of at least four cultures in each treatment with at least four controls, and at least three replicate experiments have given similar results.

Germination is significantly reduced in the presence of complete culture gases, in assemblies with 10% (v/v) initial O_2, and in those with an initial concentration of 1000 μg./l. ethanol or of 10 μg./l. 3-methyl-butan-1-ol. Since this is the maximum

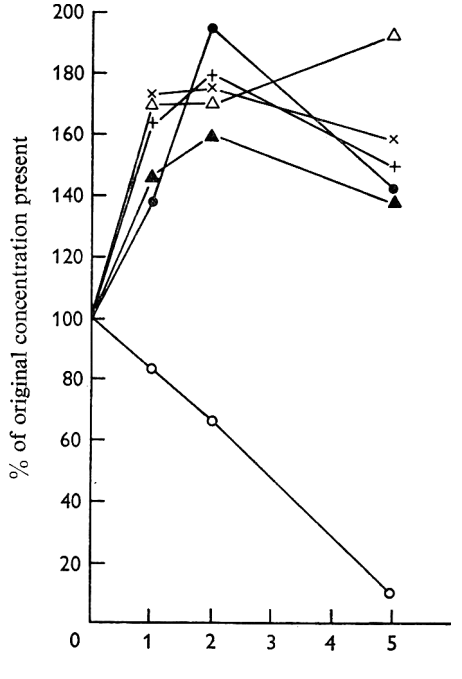

Fig. 5

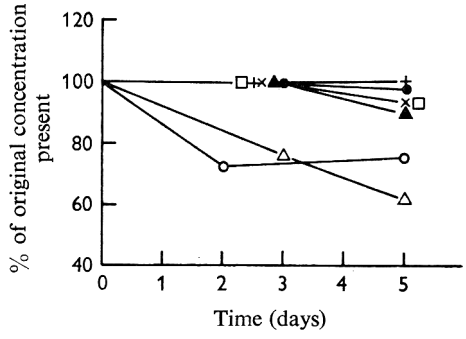

Fig. 6

Fig. 5. Changes in concentration of identified volatile metabolites in test assemblies of yeast and cress seeds, expressed as percentage of concentration present at start of test. Symbols on graph as in Fig. 2.

Fig. 6. Changes in concentration of authentic pure compounds in tests with cress seeds, expressed as percentage of concentration present at the start of the test. The initial concentration of each was equal to the highest found at any time in other activity tests. Symbols on graph as in Fig. 2. Concentrations in μg./l. of air: acetaldehyde, 320; ethyl acetate, 15; ethanol, 1000; *n*-propanol, 8; isobutanol, 12; 2-methyl-butan-1-ol, 6; 3-methyl-butan-1-ol, 10.

concentration found in any tests of yeast culture gases, and since the ethanol and 2-methyl-butan-1-ol concentrations vary only slightly (Figure 6), then it appears that both these can contribute to the effects of the complete gases. The significant reduction of germination in experiments with acetaldehyde at 500 μg./l. of air (i.e. $\times$ 1·5 the highest concentration above yeast cultures) and in those using isobutanol at 30 μg./l. of air (i.e. twice the yeast culture concentration) suggest that these might have an effect in some conditions.

The evidence that the concentration of acetaldehyde fell off rapidly during all these tests suggests, however, that it does not contribute to the effect in these conditions.

The possibility that ethyl acetate can contribute to the effect of the total culture gases has not been entirely eliminated, as its concentration falls off by 40% during the test of the authentic pure compound. Our reasons for deliberately deciding not to investigate this further are given in the discussion below.

The few seedlings which grew in cultures with complete culture gases or with ethanol, and those which grew with CO_2 (20%, v/v) were severely stunted and chlorotic; in most of these cultures growth was restricted to formation of a radicle a few millimetres long. They were less stunted and less chlorotic in cultures with acetaldehyde. The few seedlings which grew in the presence of 3-methyl-butan-1-ol were slightly stunted but similar in colour to those in the controls and to those in cultures with ethyl acetate, *n*-propanol and isobutanol.

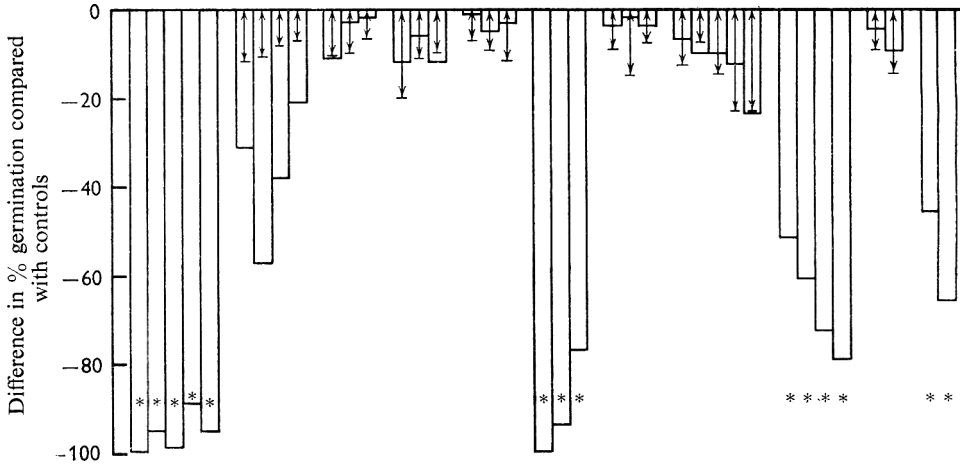

Fig. 7. Effects of yeast culture gases on germination of cress seeds. At least three replicates were used in each experiment. The vertical axis represents the mean germination of test cultures expressed as a percentage of the mean germination of the controls. ↔ = least significant difference at 5% level. * = Differences clearly significant by empirical observation. Each section on the horizontal axis represents a group of experiments using, from left to right, complete culture gases, 10% O_2, 20% CO_2, acetaldehyde, ethyl acetate, ethanol, *n*-propanol, isobutanol, 2-methyl-butan-1-ol + 3-methyl-butan-1-ol, 2-methyl-butan-1-ol, and 3-methyl-butan-1-ol.

DISCUSSION

No unusual metabolites have been found in the yeast culture gases; their biological effects can be due to the production of a number of common respiratory metabolites which may be effective singly or as parts of a mixture. The results contrast with those of the study of *Fomes annosus* (Glen, Hutchinson & McCorkindale, 1966), which showed that the biological activity of this fungus is due to the production of a single unusual metabolite (hexa-1,3,5-triyne); the effects on hyphal growth of *A. niger* extend the work of Robinson *et al.* (1966, 1968), who report that acetaldehyde and ethanol had no such effects on the species which they examined in these conditions. Only a limited comparison can be made with the results of Norrman (1968), owing to differences in concentrations and conditions tested. The differences in response to ethanol may be related to the fact that the maximum concentrations he tested were less than one-fifth of those found in the yeast/Aspergillus assemblies and used in our tests of

authentic pure ethanol. In his tests with *Pestalotia rhododendri* acetaldehyde, *n*-propanol, and isobutanol stimulated sporulation, isobutanol and *n*-propanol inhibited linear growth, all at the concentrations similar to those at which they showed no appreciable effect on *Aspergillus niger* colonies in our tests of authentic pure compounds. He found that 0·1 % 'iso-amyl alcohol' inhibited sporulation; it is not, however, possible to compare this result with our tests, as he does not specify whether this is a mixture of 2-methyl-butan-1-ol and 3-methyl-butan-1-ol or pure 3-methyl-butan-1-ol. Neither *P. rhododendri* nor *A. niger* were apparently affected by similar concentrations of ethyl acetate.

The composition of the gas mixtures of these common metabolites from yeast will vary greatly in different cultural conditions and hence any detailed analysis of one condition would have very limited general biological application. At the present time it does not seem justifiable to continue the analysis of such obviously complex possible interactions and of the possible patterns in the cumulative effects of mixtures. The report is therefore published as an illustration of a complex interaction of a mixture of common respiratory metabolites which may have obvious ecological importance in some conditions—for example, in the ecology of soil micro-organisms.

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The Motility of Some Clostridium Species

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(*Accepted for publication* 22 *August* 1968)

SUMMARY

The capillary tube method is found to be better than the hanging drop method for examining the motility of cultures of clostridia in different media. In the capillary tube, *Clostridium butyricum* and *C. sporogenes* moved away from air and oxygen gas-phases at the meniscus. Reasons for the failure to show this negative aerotaxis in *C. septicum* are discussed. The motility of *C. sporogenes* was activated by L-arginine.

INTRODUCTION

By comparison with the extensive literature devoted to the study of factors which affect the motility of aerobic bacteria, the motility of anaerobes has seldom been mentioned. The present editions of standard bacteriological textbooks which mention methods for demonstrating the motility of anaerobes state that the capillary tube is superior to either the hanging drop or plain slide and coverslip methods (Willis, 1960; *Topley and Wilson's Principles*, 1964; Cruickshank, 1965); no comparative study appears to have been published. Beijerinck (1893) reported that in his slide and coverslip preparations anaerobic bacteria formed densely populated central zones and less densely populated peripheral zones and that motility persisted longer in the central zones, where he assumed that the oxygen tension was less. Smith (1960) stated that the motility of *Clostridium septicum* persisted longer in the presence of oxygen than did that of *C. oedematiens*. Aerotaxis was first recorded by Engelmann (1881) who described the aggregation of '*Bacillus termino*' in the neighbourhood of an oxygen source and used this organism as an indicator of oxygen production by photosynthetic mechanisms of plants, isolated plant cells, algae and chloroplasts. Further reports by Engelmann (1882*a*, *b*, 1894) mentioned a variety of bacteria on which oxygen had a chemotactic effect. Jennings & Crosby (1901) studied the reactions of *Spirillum volutans* and *S. undula* near illuminated algae: away from the algae the organisms moved randomly and with no indication that their direction was influenced by the focus of oxygen production; organisms entering the zone near the algae were unable to leave, for whenever they reached the edge of the zone their direction of motion was reversed. This indirect experimental system was used also by Baracchini & Sherris (1959) to show that *C. histolyticum*, *C. septicum* and *C. sporogenes* moved away from an oxygen source. There appears to be no other recorded attempt to show negative aerotaxis by obligate anaerobic organisms; this subject seemed worthy of examination.

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METHODS

Culture of organisms. The three Clostridium strains used were obtained from the Manchester University Collection of Bacteria (MUCOB). They were *C. sporogenes* (MUCOB 383), *C. septicum* (MUCOB 158) and *C. butyricum* (MUCOB 162). Each organism was maintained by subculture on horse-blood agar in Petri dishes incubated anaerobically (McIntosh and Fildes jar), with duplicate plates incubated aerobically to test for contamination by aerobes. Liquid medium was inoculated from a 1-day colony on a blood agar plate. Solid and liquid cultures were incubated at 36° in a McIntosh and Fildes jar.

Preparation of samples of cultures for microscopical examination. Flat capillary tubes, at least 100 mm. long, were prepared from glass tubing by a modification of the method of Wright & Colebrook (1921). These were filled by capillary action from the deepest parts of the liquid culture to be examined. An air space was left at the end not immersed in the culture, and this end occluded by the forefinger while the other end was sealed in a Bunsen flame. The tube was then reversed and the air space sealed in the flame. Oxygen or nitrogen was passed from a cylinder with a reducing valve through a wash bottle containing water and then through a very fine capillary tube which was introduced into an opened end of a capillary tube containing a culture; the gas was allowed to play continuously on the meniscus of the culture. Measurements of distances relative to the meniscus were made with the vernier scale on the microscope stage.

Staining. Flagella were stained by a modification of Gray's method (Preston & Maitland, 1952).

RESULTS

Methods of examining for motility: comparison of hanging-drop and capillary-tube methods

Organisms grown in liquid medium. Clostridia grown anaerobically for 20 hr at 36° in four different media were examined for motility by the hanging-drop and capillary-tube methods. An attempt was made to assess the proportion of motile organisms in each preparation; the results are recorded in Table 1. Motility was affected by the medium in which the organism was grown, and a medium suitable for showing active motility of one strain was not necessarily satisfactory for another. The motility of *Clostridium butyricum* was seen best in digest broth; Robertson cooked meat medium was better for *C. septicum* and *C. sporogenes*. In general the motility of these three organisms was more active and persisted longer in a capillary tube than in a hanging drop, even when the latter preparations were sealed with petroleum jelly.

The character of the motility in the three organisms differed. *Clostridium butyricum* showed a variety of kinds, the commonest being a cork-screw motion in which the leading end of a bacillus described smaller rotational movements than the trailing end. Other bacilli showed rapid jerky movements, whilst some progressed steadily in straight lines. *C. butyricum* showed less frequent changes of direction than *C. septicum* or *C. sporogenes*. The slow undulating movement of *C. septicum* most nearly resembled the classical description of motility in the genus *Clostridium*, which is said to be 'slow and stately'. *Clostridium sporogenes* showed rapid jerky movements with frequent changes in direction.

Organisms grown on solid medium. Cultures of the three organisms were grown anaerobically for 20 hr at 36° on blood agar and the growth was suspended in water, Sorensen's phosphate buffer (pH 7), peptone water or digest broth. Each suspension was non-motile by both capillary tube and hanging drop techniques. But by staining it was shown that all three species had produced flagella on the solid medium. No difference in the morphology of the flagella was seen as between bacteria grown on solid medium and those grown in liquid medium. Perhaps the loss of motility resulted from exposure to air during removal of the cultures from the anaerobic jar and transfer of the surface growth to a suspending medium. No attempt was made to do these operations in an oxygen-free atmosphere.

Table 1. *Motility of Clostridium species grown in four different culture media: comparison of hanging drop and capillary tube methods of examination*

Samples of cultures grown anaerobically for 20 hr at 36° were examined by the hanging drop (HD) and capillary tube (CT) techniques at various times (*t*) after sampling. The proportion of motile bacilli seen in each preparation was graded: −, no motile bacilli seen; ±, motile bacilli constituted less than 5 % of the population; +, 5 to 15 % of bacilli motile; + +, 15 to 40 % motile; + + +, 40 to 100 % motile.

		C. butyricum				*C. septicum*				*C. sporogenes*			
t (min.)	...	0	15	30	60	0	15	30	60	0	15	30	60
Robertson cooked meat medium	HD	+	±	−	−	+ +	+ +	−	−	+ + +	+ +	+ +	+
	CT	+	+	+	+	+ +	+ +	+ +	+ +	+ + +	+ + +	+ + +	+ + +
Digest broth	HD	+ +	+	−	−	−	−	−	−	+ +	+ +	+ +	−
	CT	+ − +	+ + +	+ + +	+ + +	±	±	±	±	+ +	+ +	+ +	+ +
Peptone water*	HD	−	−	−	−	−	−	−	−	−	−	−	−
	CT	−	−	−	−	±	±	±	±	−	−	−	−
Nutrient broth + thioglycollic acid (0·1 %, w/v)	HD	−	−	−	−	+	+	+	±	+ +	+ +	+	+
	CT	−	−	−	−	+	+	+	+	+ +	+	+	+ +

* The organisms grew very poorly in this medium.

Influence of incubation temperature on motility

Triplicate cultures of *Clostridium butyricum* in digest broth, and of *C. septicum* and *C. sporogenes* in Robertson cooked meat medium, were incubated for 20 hr at 36°, 30° and 25°. They were examined for motility by the capillary tube method. No significant difference in the type or duration of motility of the cultures grown at the three temperatures was observed.

Examination of the motility of three clostridia by the capillary tube method

The previous experiments showed that, as far as motility was concerned, the best results were obtained when the capillary tube method was used for the examination of digest broth cultures of *Clostridium butyricum*, and Robertson cooked meat cultures of *C. septicum* and *C. sporogenes*, incubated at 36°. These conditions were used for the examination of these organisms, in cultures incubated for 18 hr.

Clostridium butyricum. The motility of this organism was different in different culture media. Sometimes it was sluggish, although usually more than 50 % of the organ-

isms were motile; on other occasions it was much more active. In preparations showing sluggish motility the bacilli lost their motility first in the region near the meniscus and this zone of non-motile bacilli gradually extended along the capillary tube (see Table 2). All motility throughout the tube ceased within 48 hr, and the final sediment

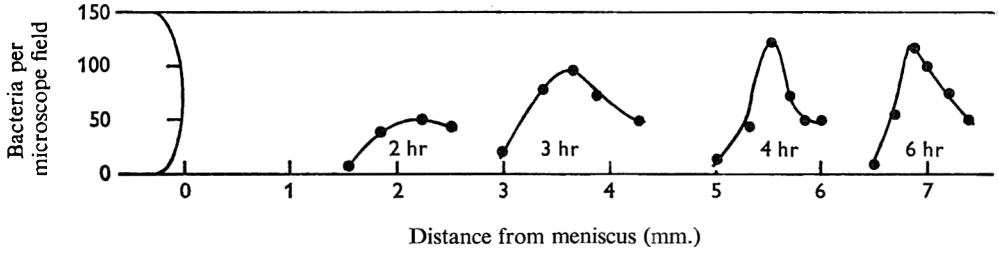

Fig. 1. Diagrammatic representation of the formation and movement of a dense band of *Clostridium butyricum* in a capillary tube, due to negative aerotaxis.

Table 2. *Progressive loss of motility of Clostridium butyricum when exposed to air*

A sample from a culture of *C. butyricum*, grown anaerobically for 18 hr in digest broth at 36°, was transferred to a capillary tube and examined microscopically. Motility ceased near the gas/liquid meniscus; the distance to which this zone of non-motile organisms extended from the meniscus was measured at intervals (t) after transfer of the culture to the tube. Two experiments were made (A, B).

	Distance (mm) from meniscus to nearest motile organisms	
Time (t) (min)	Expt. A.	Expt. B.
60	3·8	4·1
140	4·6	5·3
200	5·0	5·6
230	5·4	5·9
320	6·0	6·2
380	6·3	6·7
1440	7·1	8·0
2880	7·2	7·5

of bacilli appeared uniform throughout the tube. With cultures showing more active motility, a similar progressive loss of motility was observed, beginning near the gas/liquid meniscus and proceeding along the capillary. The time relationships were like those shown in Table 2, but now a dense band of motile organisms could be seen between the zones of motile and non-motile organisms. This band became progressively more dense and more easily recognizable, but of no greater width, as it receded from the meniscus. An attempt to give some idea of the size and density of this band, and its speed of travel, was made by counting at intervals the number of organisms visible in the field of the microscope (see Fig. 1).

Clostridium septicum. The motility of this organism was always very low. Cultures never showed more than 40% of motile bacilli, usually only about 15%. Loss of motility occurred first in the region of the gas/liquid meniscus and gradually extended along the tube. In a typical series of observations, the zone of non-motile bacilli extended 2 mm. from the meniscus at 1 hr, 4 to 5 mm. at 4 hr, and 8 to 9 mm. at 8 hr; farther along the tube, no progressive extension of the zone of non-motile bacilli was

seen, but all the organisms became non-motile by 24 hr. The sedimented organisms were then uniformly distributed along the tube.

Clostridium sporogenes. The motile organisms appeared to recede from the gas/liquid meniscus. Within 2 hr a band of increased concentration of organisms was seen which gradually moved from the meniscus until at 6 hr it became stationary about 2·5 mm. from it. The width of the band was about 0·7 mm., the edge nearer the meniscus being very sharply defined. Between this well-defined edge and the meniscus, there were only very few sedimented bacilli, the difference in concentration between this region and the remainder of the tube being very obvious. Motility throughout the tube ceased within 48 hr.

Table 3. *Movement of Clostridium butyricum and C. sporogenes away from oxygen, in capillary tube*

Time (min.)	Distance (mm.) from meniscus to dense band of organisms		
	C. butyricum		*C. sporogenes*
	Expt. 1	Expt. 2	
20	2·4	2·5	—
45	5·4	5·7	1·3
60	6·1	6·2	—
85	7·2	7·0	4·5
105	7·9	7·8	—
120	—	—	5·7
140	8·7	8·7	—
170	9·3	9·1	6·7
200	9·9	9·7	6·9
230	10·6	10·0	—
260	10·9	10·4	6·9
305	11·7	11·1	6·9
365	12·4	11·9	—
1440	13·2	—	—

Influence of oxygen on motility

Clostridium butyricum. When the gas-space near the meniscus was continuously flushed with oxygen, a dense band of organisms developed as it had done when the space was filled with air. The band was thinner and better defined, and it moved more quickly and to a greater distance than when the gas was air. As the band moved, the organisms between it and the gas/liquid meniscus became non-motile. The band persisted for some hours but gradually became less distinct and dispersed after about 24 hr. The results of duplicate experiments are shown in Table 3.

Clostridium septicum. The result with oxygen was similar to that with air except that the loss of motility progressed more rapidly and extended 8 to 9 mm. from the meniscus after only 3 hr. A further extension of the zone of non-motile bacilli was not obvious; motility ceased throughout the tube within 24 to 48 hr. At no time was a band of increased concentration of bacilli seen; the bacilli appeared to sediment uniformly throughout the tube.

Clostridium sporogenes. As with *C. butyricum*, loss of motility occurred more rapidly and extended farther when oxygen replaced air at the gas/liquid meniscus. A band of bacilli became visible within 45 min., moved rapidly from the meniscus and became

stationary at 6·9 mm. from the meniscus after about 3 hr (Table 3). Beyond the band, motility persisted for about 24 hr, after which the bacilli sedimented throughout the tube. The sediment was less dense in the region extending 6·9 mm. from the meniscus than in the rest of the tube.

Influence of nitrogen on motility

When the gas space in the capillary was flushed with a stream of nitrogen, all three species remained uniformly motile. Progressive loss of motility extending gradually from the meniscus was not observed nor was a dense band of organisms formed. Motility persisted throughout the tube for several hours and then became uniformly sluggish and ceased after about 48 hr.

Influence of medium in which organisms were suspended

Motility of cultures of *Clostridium butyricum* and *C. sporogenes* in digest broth, and of *C. septicum* in Robertson cooked meat medium, was observed at 20 hr; the cultures were then centrifuged at 2500 rev./min. for 10 min. In separate experiments the deposits were washed once or three times in glass-distilled water, peptone water or digest broth. After re-suspension in the same media, they were examined for motility by the capillary tube technique. In no case were any motile bacilli of *C. butyricum* or *C. septicum* seen; but even after washing three times, *C. sporogenes* was still motile, for more than 8 hr in peptone water and in digest broth, but only for 10 min. in distilled water. Stained preparations of each organism, before and after washing, showed no morphological change in the flagella. It seems likely that the loss of motility of *C. butyricum* and *C. septicum* on washing was a reflexion of extreme sensitivity to oxygen. Each organism remained motile after centrifugation when resuspended in the supernatant fluid, but when this was removed and the bacilli were exposed to air they became non-motile.

Table 4. *Duration of motility of Clostridium sporogenes related to initial concentration of arginine*

Concentration of arginine (μM) ...	100	50	25	12·5	6·25
Duration of motility after organisms in water control had become non-motile (min.)	81	40	22	13	4

Effect of amino acids. Since a washed suspension of *Clostridium sporogenes* showed motile organisms much longer in peptone water or digest broth than in distilled water, it seemed likely that motility might depend on the presence of amino acids. *C. sporogenes* was therefore incubated anaerobically for 20 hr in digest broth, washed three times in distilled water, the third centrifuged deposit was re-suspended in a few drops of distilled water and a drop of this suspension added to 0·5 ml. of a 10 mM solution of each of the following amino acids: L-arginine, L-asparagine, L-aspartic acid, DL-glutamine, glycine, DL-methionine, DL-serine, DL-threonine, DL-tryptophane; as a control, a drop of the suspension was added to 0·5 ml. distilled water. Motility persisted for only 10 to 15 min. in the water-control and in eight of the nine amino acids, but with L-arginine the organisms remained motile for more than 8 hr.

Concentration of arginine. One drop of a washed suspension of *Clostridium sporogenes*, prepared as described above, was added to 0·5 ml. of a solution of arginine at concentrations of 100 to 6 μM. The duration of motility, persisting after organisms in a water-control had become non-motile, was proportional to the concentration of arginine in the solution, over the range tested (Table 4).

DISCUSSION

The motility of each of the organisms studied (*Clostridium butyricum*, *C. septicum*, *C. sporogenes*) was inhibited by air, so that the hanging-drop method of examination was not very satisfactory except for the least oxygen-sensitive of the three, namely *C. sporogenes*. By the capillary tube method, the relative sensitivities of the three species could be examined in more detail. All three became non-motile near the gas/liquid meniscus, the loss of motility occurring most rapidly and extending farthest from the meniscus with *C. septicum*, and being least rapid and least extensive with *C. sporogenes*. The active component of the air appeared to be oxygen, since when air was replaced with nitrogen no inhibition of motility occurred near the meniscus, but when air was replaced with oxygen, motility at a given distance from the meniscus ceased more rapidly than with air and the organisms became non-motile for a greater distance from the meniscus. Moreover there was evidence, with *C. sporogenes* and *C. butyricum*, of negative aerotaxis—movement of organisms away from the air/liquid interface. A dense band of organisms developed between the non-motile bacilli near the meniscus and the motile bacilli in the rest of the tube, and this band gradually moved farther away from the meniscus. The concentration of organisms between meniscus and band was less than that on the other side of the band, suggesting that the motile members of the bacterial population were escaping from the adverse effect of a high concentration of oxygen by moving away from the meniscus. The fact that the band of *C. butyricum* moved farther than that of *C. sporogenes* suggested that it was less tolerant of oxygen. *C. septicum* showed neither a zone near the meniscus where the density of organisms was less than that in the rest of the tube nor a dense band of organisms. Perhaps this organism does not have a negative aerotactic response; or perhaps its motility was so slow that it was overwhelmed by the increasing oxygen concentration as air diffused inwards from the meniscus, and the organism was rendered non-motile before it could escape from the inhibitory zone.

Clostridium sporogenes remained motile for a short period after washing and resuspending in distilled water, probably because of residual traces of essential metabolites from the culture medium. This motility was prolonged by L-arginine, but not by any of eight other amino acids tested. Sherris, Preston & Shoesmith (1957) showed that arginine alone, of 20 amino acids they tested, prolonged the motility of a pseudomonad under relatively anaerobic conditions; and it is possible that the motility of *C. sporogenes* too is dependent on energy liberated by the metabolism of arginine, used directly or indirectly through the mediation of energy-rich phosphate bonds, as suggested by Sherris *et al.* (1957) for their pseudomonad. This involves the assumption that adenosine triphosphate (ATP) is the immediate source of energy. However, whilst De Robertis & Peluffo (1951) claimed that ATP enhanced the motility of *Proteus vulgaris*, and De Robertis & Franchi (1952) reported that bacterial flagella contracted in the presence of ATP, Barlow & Blum (1952) found no ATPase activity

in flagellar preparations, nor did addition of ATP to a suspension of flagella cause any change in the shape or in the state of aggregation of the flagella. Another mechanism by which energy might be provided for the motility of *C. sporogenes* is through a Stickland reaction in which arginine served as a hydrogen acceptor (Woods, 1936). A second amino acid would be required as a reductant, and this might be difficult to reconcile with the observation that arginine added alone was effective. However, it is possible that an amino acid present in the cell pool, or a product of arginine metabolism, might act as reductant.

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Effect of Selected Antibiotics and Other Inhibitors on Competence Development in *Haemophilus influenzae*

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SUMMARY

The effect of streptomycin, penicillin, polymyxin, chloramphenicol, novobiocin, erythromycin, D-cycloserine, 8-azaguanine, and 6-azauracil on competence development in *Haemophilus influenzae* was studied.

D-cycloserine and 6-azauracil had no inhibitory effects on either competence development or cell viability in concentrations that were inhibitory to growth. Penicillin and polymyxin reduced cell viability and competence development proportionately thereby maintaining a high percentage of transformation in the treated cultures. Chloramphenicol, novobiocin, 8-azaguanine, erythromycin, and streptomycin inhibited markedly competence development with little or no effect on cell viability.

INTRODUCTION

The term 'competence' describes a specific physiological state attained by certain bacterial populations (Marmur, Falkow & Mandel, 1963) that permits them to react with deoxyribonucleic acid (DNA), and this reaction between competent cells and DNA may result in a permanent genetic change or transformation.

In *Haemophilus influenzae* populations the attainment of the competent state has been shown to be dependent on protein synthesis since chloramphenicol prevented its development (Goodgal, 1958; Stuy, 1962; Leidy, Jaffee & Alexander, 1962). The results presented here substantiate and extend these observations to other inhibitors of protein biosynthesis, namely, 8-azaguanine, novobiocin, erythromycin, and streptomycin.

The inhibition of competence development and transformation by sodium metaperiodate has been reported previously (Ranhand & Lichstein, 1966).

METHODS

Organism and cultural conditions. The organism used was *Haemophilus influenzae* strain RD described by Goodgal & Herriott (1961). The trypticase growth (phase-1) medium and cultural conditions employed have been described previously (Ranhand & Lichstein, 1966; Ranhand & Herriott, 1966).

Competence development and transformation. Competence was developed in the medium described by Spencer & Herriott (1965) modified with 0·05 % acid hydrolysed

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casein (Ranhand & Lichstein, 1966). Transformation to streptomycin resistance was determined by standard procedures (Ranhand & Lichstein, 1966).

Transforming deoxyribonucleic acid (DNA). Transforming DNA was prepared according to the method of Goodgal & Herriott (1961) from *H. influenzae* organisms that were resistant to about 2 mg. streptomycin per ml.

Antibiotics and other inhibitors. The antibiotics and inhibitors used were as follows: streptomycin sulphate, sodium penicillin G, polymyxin B sulphate, D-cycloserine, chloramphenicol, sodium novobiocin, erythromycin gluceptate, 8-azaguanine, and 6-azauracil.

D-cycloserine was prepared in phosphate buffer, pH 8 (Curtiss, Berg & Charamella, 1965). Chloramphenicol was dissolved in 40% ethanol at 2 mg. per ml. and diluted appropriately with distilled water. 8-Azaguanine was dissolved in 0·04 M-NaOH at 2 mg./ml. and diluted appropriately in phosphate buffer, pH 6·7. All other stock solutions were prepared in distilled water. All solutions were stored at 5–7° and were used within 1 week after preparation.

Inhibition of competence development. Haemophilus influenzae cells were grown in the trypticase medium and diluted into the competence development medium with or without inhibitor. The cells were shaken at 37° for 90 min., at which time transforming DNA was added for 30 min. At the end of the 30 min. period the cells were diluted and plated according to standard procedures (Ranhand & Lichstein, 1966).

RESULTS AND DISCUSSION

Effect of D-cycloserine, 6-azauracil, penicillin, and polymyxin on competence development

Since competence most likely involves the cell surface (Tomasz & Beiser, 1965), agents that affect cell-wall biosynthesis and membrane integrity were tested for their ability in preventing competence development (Table 1).

D-Cycloserine, an inhibitor of D-alanyl-D-alanine synthetase and DL-alanine racemase (Strominger, Threnn & Scott, 1959), and 6-azauracil, a potential inhibitor of cell-wall biosynthesis (Otsuji & Takagi, 1959) had little or no influence on either viability or competence development yet both inhibited growth at concentrations greater than 5 and 13 μg. per ml., respectively. These results suggest that biochemical processes in *Haemophilus influenzae* that lead to the competent state require neither D-alanyl-D-alanine synthetase, nor DL-alanine racemase, nor both, nor reactions that require pyrimidine nucleotides as biosynthetic carrier molecules (Strominger & Smith, 1959). However, since these agents were added to organisms during the competence development period (phase-2), the negative results obtained here do not preclude their function during growth (phase-1).

Penicillin, an inhibitor of cell wall biosynthesis (Strominger, Kazuo, Matsuhashi & Tipper, 1967), and polymyxin, a membrane destroying agent (Newton, 1956), inhibited both viability and competence development equally (Table 1). Since organisms in competent cultures are at least 75% competent (Goodgal & Herriott, 1961) or comprise a lower but constant percentage of the population (Thomas, 1955) this dual inactivation implies that both competent and non-competent organisms are affected to the same extent. Since sublethal concentrations of penicillin and polymyxin did not influence competence development, their direct effect on the process, if any, could

not be evaluated. In these experiments, no attempt was made to protect potential spheroplasts from lysis.

The killing of both transformable and non-transformable *H. influenzae* in the competent population by penicillin differs from the result obtained by Nester (1964) with competent *Bacillus subtilis* populations. In this latter case, only the non-competent population appeared to be killed.

Table 1. *Effect of* D*-cycloserine, 6-azauracil, penicillin G, and polymyxin B on competence development*

Inhibitor (μg./ml.)	Transformations per ml. (T) ($\times 10^{-7}$)	Viable cells (v.c.) per ml. ($\times 10^{-9}$)	Survival (%) T/ml.	Survival (%) V.C./ml.	Transformation frequency (%)*
		D-cycloserine			
0	2·1	1·7	(100)	(100)	1·2
5	1·9	1·8	91	106	1·1
10	3·2	1·5	152	88	2·1
15	2·2	1·7	105	100	1·3
20	4·3	2·0	205	118	2·1
25	2·3	1·8	110	106	1·3
		6-Azauracil			
0	4·6	1·7	(100)	(100)	2·7
12·5	4·6	1·4	100	82	3·1
25	3·5	1·1	76	65	3·2
50	2·6	1·0	54	59	2·6
75	3·0	1·4	65	82	2·2
100	2·8	1·4	61	82	2·0
		Penicillin G			
0	1·8	2·4	(100)	(100)	0·75
0·1	1·6	1·7	89	71	0·94
0·2	0·6	0·8	33	33	0·75
0·4	0·8	0·8	45	33	1·00
0·6	0·4	0·8	22	33	0·50
		Polymyxin B			
0	3·1	1·9	(100)	(100)	1·7
0·5	3·4	1·6	110	84	2·2
1·0	2·6	1·4	84	75	1·9
1·5	1·3	0·6	42	32	2·1
2·0	1·1	0·5	36	24	2·4
2·5	0·5	0·2	16	10	2·6
3·0	0·06	0·05	2	3	1·2

* Transformation frequency equals the number of transformations per ml. × 100 divided by the number of viable cells per ml.

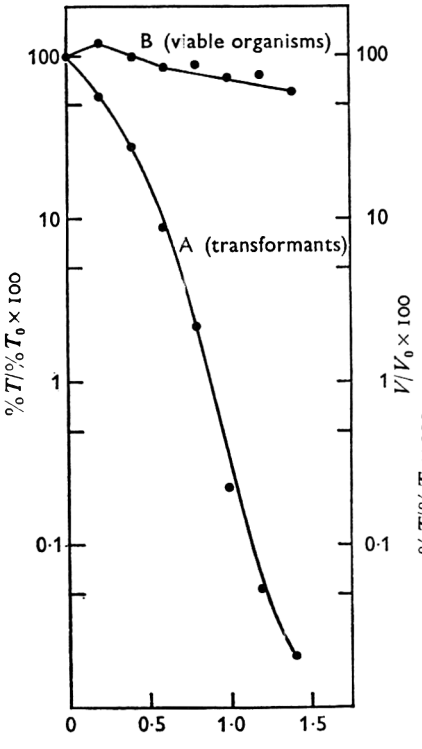
Effect of chloramphenicol and 8-azaguanine on competence development

The sensitivity of competence development to chloramphenicol is shown in Fig. 1. At chloramphenicol concentrations that reduced the number of competent organisms to about 10% of control values (0·7 μg./ml.), about 85% of the organisms remained viable; at concentrations where competence was reduced to less than 1% of the control values (1·0 μg./ml.), about 50% of the organisms remained viable. These data show that competence development is more sensitive to the action of chloramphenicol than is viability.

When 8-azaguanine was used to inhibit competence development (Fig. 2) there was no apparent loss in viability at concentrations that reduced the level of competence to about 5% of control values (3·0 μg./ml.).

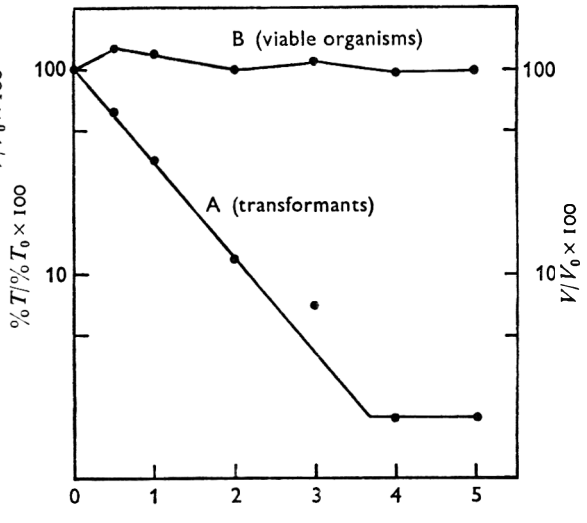
Effect of erythromycin, streptomycin, and novobiocin on competence development

Since chloramphenicol and 8-azaguanine inhibited competence development it was also of interest to see if other inhibitors of protein biosynthesis acted in a similar fashion. Therefore, the effects of erythromycin, novobiocin, and streptomycin were examined.



Chloramphenicol concentration (μg./ml.)

Fig. 1



8-Azaguanine concentration (μg./ml.)

Fig. 2

Fig. 1. Inhibition of competence development by chloramphenicol. Curve A (transformants) = percentage transformation of treated cultures (T) divided by percentage transformation of untreated control (T_0) × 100. Curve B (viable organisms) = viable organisms per ml. of treated cultures (V) divided by viable cells per ml. of untreated control (V_0) × 100. T_0 = $4{\cdot}3 \times 10^7$ per ml.; V_0 = $1{\cdot}6 \times 10^9$ per ml.

Fig. 2. Inhibition of competence development by 8-azaguanine. Curves A (transformants) and B (viable organisms) have the same notation as described in the legend to Fig. 1. T_0 = $2{\cdot}6 \times 10^7$ per ml.; V_0 = $1{\cdot}6 \times 10^9$ per ml.

Erythromycin inhibited competence development in a very complex manner yet had no effect on viability at concentrations that reduced transformation to 1% of control values (1·0 μg./ml.) (Fig. 3). Novobiocin (0·1 to 0·6 μg./ml.) and streptomycin (1 to 4 μg./ml.) (Figs. 4, 5) likewise inactivated competence development in a complex

manner, but viability was also reduced to about 50 % of the control values. The complexity of these inactivation curves may reflect the modes of action of these agents (Davis & Feingold, 1962; Taubman, Jones, Young & Corcoran, 1966; Brock, 1966; Wishnow, Strominger, Birge & Threnn, 1965) or heterogeneity in the populations

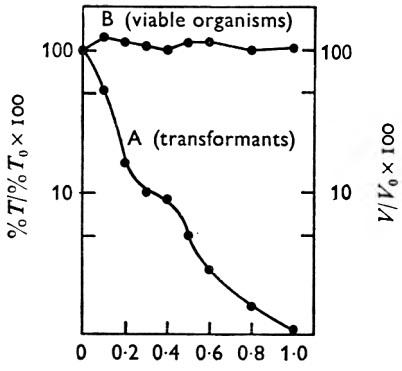


Fig. 3

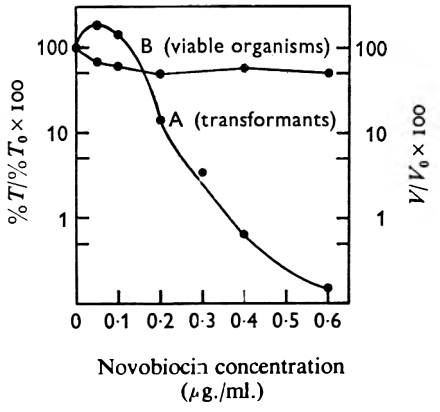


Fig. 4

Fig. 3. Inhibition of competence development by erythromycin. Curves A (transformants) and B (viable organisms) have the same notation as described in the legend to Fig. 1. $T_0 = 4{\cdot}6 \times 10^7$ per ml.; $V_0 = 1{\cdot}4 \times 10^9$ per ml.

Fig. 4. Inhibition of competence development by novobiocin. Curves A (transformants) and B (viable organisms) have the same notation as described in the legend to Fig. 1. $T_0 = 3{\cdot}1 \times 10^7$ per ml.; $V_0 = 1{\cdot}9 \times 10^9$ per ml.

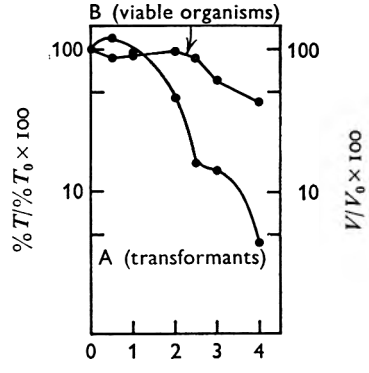


Fig. 5

Fig. 5. Inhibition of competence development by streptomycin. Curves A (transformants) and B (viable organisms) have the same notation as described in the legend to Fig. 1. $T_0 = 4{\cdot}3 \times 10^7$ per ml.; $V_0 = 2{\cdot}0 \times 10^9$ per ml.

tested. Moreover, since the effect of these antibiotics was similar, and since streptomycin can precipitate DNA (Cohen, 1947), it was thought that these compounds were inactivating the transforming DNA. However, the results given in Table 2 show that this was not the case; these antibiotics were acting on some cellular processes.

The data presented here, at least for the prevention of competence development produced by chloramphenicol, implies that the attainment of the competent state by *Haemophilus influenzae* organisms may be the result of the induction of an enzyme or protein (Sypherd, Strauss & Treffers, 1962; Weber & DeMoss, 1966). It is believed that the function of this protein is to activate or modify pre-existing DNA binding sites that are produced by the metabolism of inosine and lactate during phase-1 (Ranhand & Lichstein, 1966).

Table 2. *Effect of various antibiotics on transforming DNA**

Antibiotic (μg/ml.)	Transformations per ml. ($\times 10^{-6}$)	Viable cells per ml. ($\times 10^{-9}$)	Transformation frequency (%)†
None	3·6	2·5	0·14
Erythromycin			
0·25	3·7	2·7	0·14
0·6	3·4	2·2	0·16
Streptomycin			
3	3·4	2·4	0·14
6	3·7	2·4	0·16
Novobiocin			
0·3	3·6	2·4	0·15
0·6	3·6	2·6	0·14

* *H. influenzae* organisms were grown in the trypticase medium and made competent as already described (Ranhand & Lichstein, 1966). Transforming DNA (1·1 μg./ml.) was pre-incubated in the competence development medium with or without the antibiotics as indicated above for 30 min. at 37° prior to its addition to competent cells. The final DNA concentration added to competent organisms was 0·06 μg./ml.

† Transformation frequency equals the number of transformed organisms per ml. × 100 divided by the number of viable organisms per ml.

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Antigenic Properties of Mycoplasma Organisms and Membranes

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SUMMARY

The antigenic properties of the soluble and membrane-bound proteins of Mycoplasma organisms were studied by a variety of gel-diffusion techniques. The strains tested belonged to several distinct serological groups, showing no cross-reactions. One group consisted of the *Mycoplasma laidlawii* strains and the other of caprine and bovine strains, which were closely related to *Mycoplasma mycoides* var. *mycoides*. The avian strains tested were serologically heterogenous. After Mycoplasma membranes were solubilized by sodium dodecyl sulphate (SDS) the antigenic properties of their membrane proteins were analysed. In immunodiffusion tests they showed a serological specificity similar to the soluble cell proteins. This specificity was not found in lipids of *M. laidlawii* membranes extracted with chloroform + methanol. In gel diffusion tests the hydrophobic protein fraction isolated from *M. laidlawii* membranes by detergent action and $(NH_4)_2SO_4$ precipitation reacted with antiserum to whole organisms, was antigenic in rabbits and there produced an antiserum which reacted with it, but not with the soluble cell proteins or membrane material solubilized in SDS.

INTRODUCTION

Biochemical and cultural properties usually are insufficient for the identification and classification of organisms of the order Mycoplasmatales. Serological properties have therefore been widely used for these purposes (Edward *et al.* 1967). Although a variety of serological reactions are successfully used for the identification of mycoplasmas, little is known about the cell components responsible for these reactions. Some progress has been made in the characterization of Mycoplasma antigens. Lipids, apparently located in the cell membrane, were found to be responsible for the complement-fixing ability of *M. pneumoniae* (Kenny & Grayston, 1965; Prescott, Sobeslavsky, Caldes & Chanock, 1966; Sobeslavsky, Prescott, James & Chanock, 1966; Lemcke, Marmion & Plackett, 1967; Marmion, Plackett & Lemcke, 1967). The extracted lipids, when combined with protein became highly immunogenic, stimulating high values for complement-fixing and growth-inhibiting antibodies. In contrast, the serological activity of the lipid fraction of *M. mycoides* var. *mycoides* was limited. Complement-fixing activity and precipitin reactions could be attributed to the galactan produced and excreted by this organism (Marmion *et al.* 1967). Like *M. pneumoniae*, *M. fermentans* was also found to possess specific lipid antigens, while the lipid extracted from *M. hominis* and *M. pulmonis* cross-reacted and had little serological activity. The major antigens in the last three Mycoplasma species appear to be protein in nature (Deeb & Kenny, 1967; Kenny, 1967; Lemcke & Hollingdale, 1968).

Little has been done so far to characterize the serological properties of the Mycoplasma proteins. To the best of our knowledge all the work done so far has been concerned with the soluble cell proteins released by cell disruption. The insoluble cell proteins, mainly located in the cell membrane, have not so far been studied because of the difficulties involved in their solubilization, which is essential for their biophysical and serological characterization. Nevertheless, there is little doubt that membrane proteins, being surface antigens, play a crucial role in immunogenesis against Mycoplasma infections. Some indications that antigens present in the cell membrane combine at this site with antibody to inhibit growth of mycoplasmas were recently published (Williams & Taylor-Robinson, 1967; Lemcke & Hollingdale, 1968).

The cell proteins, the synthesis of which is strictly dictated by the cell genome, might be expected to contribute the most specific cell antigens, and in fact the electrophoretic patterns of Mycoplasma cell proteins have been shown to be species-specific and highly reliable tools for the identification and classification of mycoplasmas (Razin & Rottem, 1967). The serological characterication of mycoplasma proteins is likely to help in putting the classification of these organisms on a sounder basis.

Table 1. *Designation of Mycoplasma strains and antisera*

Type or species	Strain	Recovered from	Obtained from	Antiserum
M. laidlawii	A(PG 8)	Sewage	D. G. ff. Edward*	aA
	B(PG 9)	Sewage	D. G. ff. Edward*	aB
	OR	Human oral cavity	Isolated in our laboratory	aOR
M. mycoides	PG 1	Cattle	D. G. ff. Edward*	aPG 1
var. *mycoides*	V5	Cattle	A. W. Rodwell†	—
M. mycoides	PG 3	Goat	D. G. ff. Edward*	aPG 3
var. *capri*	pp. goat	Goat	E. Klieneberger-Nobel‡	app
Mycoplasma sp.	14	Goat	M. E. Tourtellotte§	—
	Y	Goat	A. W. Rodwell†	—
M. gallisepticum	A 5969	Chicken	M. E. Tourtellotte§	a 59
	S6	Chicken	H. E. Adler‖	—
M. gallinarum	J	Chicken	P. F. Smith¶	—
Mycoplasma sp.	R	Chicken	R. Bernstein-Ziv**	aR

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‡ The Lister Institute of Preventive Medicine, London, S.W. 1., England.
§ University of Connecticut, Storrs, U.S.A.
‖ School of Veterinary Medicine, University of California, Davis, U.S.A.
¶ University of South Dakota, Vermillion, U.S.A.
** Faculty of Agriculture, The Hebrew University, Rehovoth, Israel.

METHODS

Mycoplasma strains. The designation and source of the Mycoplasma strains examined are given in Table 1.

Media and growth conditions. A modified liquid Edward medium (Razin, 1963) was used. This medium was modified to contain 5% (v/v) of pooled inactivated human serum for the propagation of organisms utilized as immunizing antigens. The medium used for growing organisms to obtain antigens for analysis contained 2% (v/v) Difco-PPLO serum fraction instead of the human serum. One litre Roux bottles

containing 500 ml. medium, or 2 to 4 litre Erlenmeyer flasks half filled with medium, were inoculated either with a 1 to 2% inoculum of an 18 to 24 hr culture of the *Mycoplasma laidlawii* strains, or with a 5 to 10% inoculum of a 20 to 36 hr culture of all the other strains. Cultures were incubated statically at 37° under aerobic conditions. The organisms were harvested by centrifugation at 12,000*g* for 10 min., washed twice and resuspended in 0·25 M-NaCl.

Preparation of antigens for rabbit immunization. Washed organisms were resuspended in 0·25 M-NaCl to a protein concentration of 20 mg./ml. as determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Thiomersalate in final concentration of 10^{-4} (w/v) was added to prevent bacterial growth, and the suspension kept at 4° until used. One volume of this suspension was added to an equal volume of Freund's complete adjuvant (Difco) and homogenized in the M.S.E. ultrasonic disintegrator (60 W., 20 kc.). The ultrasonic treatment was done in ice for 1 min. periods, with intervals to avoid heating. The white viscous emulsion obtained after treatment for 5 to 10 min. was transferred into an hypodermic syringe by means of a spatula and kept at 4° during the immunizing period.

Immunization of rabbits. Young rabbits (1·5 to 2 kg.) received two 0·5 ml. intramuscular injections of the antigen + adjuvant mixture into the hind leg at 1 week's interval. After a week rest 0·5 ml. of a suspension disrupted ultrasonically for 1 min., without adjuvant, was injected in the same manner, followed by a second injection of the same material 3 days later. The rabbits were bled 7 to 10 days after the last injection and the serum was separated. Six weeks after bleeding the rabbits received two 0·5 ml. intramuscular booster injections of the cell suspension at an interval of 3 days. Rabbits were bled again on the 14th day after the last injection. The antisera collected were divided into 2 ml. portions and stored at −20°. The list of antisera prepared is given in Table 1.

Preparation of antigens for analysis

Whole organism suspensions. Organisms grown in the PPLO-serum fraction supplemented medium were washed twice and resuspended in 0·25 M-NaCl to a concentration equiv. 20 mg. cell protein/ml.

Soluble cell proteins. The soluble cell proteins of the *Mycoplasma laidlawii* strains were liberated from the organisms by osmotic lysis (Razin, 1963). Washed packed organisms (about 100 mg. cell protein) were resuspended in 30 ml. of de-ionized water and incubated in a 37° bath for 30 min. Membranes and cell debris were sedimented at 35,000*g* for 30 min. at 10°, the supernatant fluid separated and again centrifuged as above. The concentration of protein in the supernatant fluid obtained was about 2 mg./ml. The protein concentration was increased to 10 mg./ml. by perevaporation. Being more resistant to osmotic lysis, the other mycoplasmas were disrupted by ultrasonic treatment to liberate the soluble proteins. Suspensions of organisms in 0·25 M-NaCl were treated at 0° in the disintegrator for 10 to 15 1-min. periods, with 2 min. intervals to avoid heating. The soluble cell fraction was separated and concentrated as described above.

Solubilized cell membranes. Cell membranes obtained after osmotic lysis or ultrasonic disruption of the organisms were washed 4 to 5 times and resuspended in de-ionized water to a concentration of 20 mg. membrane protein/ml. The membrane suspension was mixed with an equal volume of 1% (w/v) sodium dodecyl sulphate

(SDS) (Razin, Morowitz & Terry, 1965). The suspension cleared within 5 min. of incubation at room temperature. A minute amount of insoluble material was removed by centrifugation at 37,000*g* for 10 min.

Defatted membrane protein. Lipid was removed from the membranes by butanol extraction at 0° according to Rodwell, Razin, Rottem & Argaman (1967). The hydrophobic protein fraction obtained was dissolved by SDS (1 mg. detergent/mg. protein).

Hydrophobic membrane protein. This protein fraction was isolated by ammonium sulphate salting out of membranes solubilized in a mixture of SDS and sodium deoxycholate (Rodwell *et al.* 1967).

Methods for antigenic analysis

Agglutination of whole cell. This was carried out either on glass slides at room temperature or in test-tubes at 52° according to Bailey *et al.* (1961).

Double diffusion in agar. The test was done according to Ouchterlony (1964). Noble agar 1% (w/v) was prepared in veronal+HCl buffer (pH = 8·2; ionic strength 0·05 Grabar, 1964). Microscope slides (50 × 75 mm.) were covered with 5 ml. melted agar. Peripheral holes of 3 mm. diameter and a central hole of 5 mm. diameter were cut in the agar and were filled with 0·03 to 0·04 ml. of antiserum or antigen solution. Optimal precipitation lines were detected after 24 to 48 hr incubation at 37° in a damp chamber. The agar slides were washed for 48 hr in 0·9% (w/v) NaCl and then covered with filter paper and dried overnight at 37°. The dried slides were immersed for 20 min. in 0·5% (w/v) aqueous nigrosin solution containing 5% (w/v) trichloroacetic acid and 5% (w/v) sulphosalicylic acid (Flechner & Olitzki, 1966). Decolorization was accomplished by several washings of the slides in 2% acetic acid. For staining of proteins and lipids, the technique of Uriel (1964) was used. The slides were stained for 60 min. in a Sudan Black 10B solution, decolorized in 50% (v/v) ethanol in water, and then stained for 30 min. in Azocarmine solution, and decolorized in 2% acetic acid. Lipids stained dark-blue and proteins red.

Microelectrophoresis and microimmunoelectrophoresis according to Scheidegger (1965). For microelectrophoresis, agar-covered slides were connected by filter-paper bridges to the reservoirs containing veronal+HCl buffer (pH 8·2). The cell protein solutions to be analysed were introduced into the 3 mm. diameter holes cut in the agar across the centre of the slide. Current (5 V/cm.) was applied for 60 min. For immunoelectrophoresis two trenches, 2 mm. wide, were cut in parallel to the protein pattern at a 5 mm. distance from the sample hole. Antisera were introduced into the trenches and results were read after incubation at 37° for 48 hr in a damp chamber. Precipitation lines were detected either by direct illumination or after staining with nigrosin.

Transmigration. The method described by Calliford (1964) was used with a slight modification. Two parallel rows of 3 mm. holes were cut at 1 cm. distance in an agar-covered slide. The antigen solutions (0·03 to 0·04 ml.) were put in one row, and the antisera (0·03 to 0·04 ml.) in the other row. The agar-covered slides were connected to the buffer reservoirs as described for microimmunoelectrophoresis. The antisera row was on the anode side. An electric current (5 V/cm.) was applied for 60 min. Antigen antibody precipitates were formed in the interspace between the rows.

Immunoelectrophoresis on cellulose acetate strips was done according to Flechner & Olitzki (1966) using tris+EDTA+boric acid buffer (pH 8·9; Aronsson & Grönwall, 1957).

Polyacrylamide-gel electrophoresis and immunoelectrophoresis. The gel system consisted of 7·5% Cyanogum 41 (a mixture of acrylamide, 95%, and *N,N'*-methylene-bisacrylamide, 5%, supplied by E-C Apparatus Co. Philadelphia, Pennsylvania, U.S.A.) in 0·25 M-tris+HCl buffer (pH 8·3). Polymerization was induced by adding 0·05 ml. *N,N,N',N'*-tetramethylethylenediamine and 25 mg. ammonium persulphate to 10 ml of Cyanogum solution. Samples (50 to 100 μl. containing 150 to 300 μg. protein) were put on top of the gels and mixed with 50 μl. of a 40% (w/v) sucrose solution in the electrophoresis buffer (tris+EDTA+boric acid, pH 8·9, diluted 1/10 in de-ionized water). The tubes were then filled with the dilute buffer and electrophoresis done at room temperature for 2 hr at a constant current of 5 mA/tube, with the lower electrode as anode. The gels were stained for 30 min. in 1% (w/v) Amido Black 10B in 7% (v/v) acetic acid. Destaining was done electrolytically in 7% acetic acid (Razin, 1968). For immunodiffusion, protein samples were run in triplicate. One of the gels was stained to locate the protein bands. Another gel was cut along its long axis, and the two halves were laid on an agar-covered slide, on both sides of a trench filled with antiserum. The third gel was cut transversally into 5 mm. slices which were put sequentially between two trenches filled with antiserum. The slides were incubated at 37° for 48 to 72 hr and precipitation lines were detected in diffuse light or after staining with nigrosin.

RESULTS

Agglutination of whole Mycoplasma organisms by antisera

The simple slide-agglutination technique separated the Mycoplasma strains into several distinct groups. One group included the *Mycoplasma laidlawii* strains and the other the bovine and caprine strains. The avian mycoplasmas were serologically heterogeneous; thus the *M. gallisepticum* strains and the avian strain J were not agglutinated by antiserum against strain R, and vice versa. The results obtained by the tube-agglutination technique confirmed those obtained by slide agglutination (Table 2).

Table 2. *Tube agglutination titre of whole Mycoplasmas in 0·25 M-NaCl*

	Antisera					
Organism	aA	aB	aOR	app	aPG I	aR
M. laidlawii A	16–32*	2	8	0	0	0
M. laidlawii B	2	16	2	0	0	0
M. laidlawii OR	4–8	2	8	0	0	0
M. mycoides var. *mycoides* PG I	0	0	0	4–8	256	0
M. mycoides var. *capri* pp. goat	0	0	0	32–64	8	0
M. mycoides var. *capri* PG 3	0	0	0	16	8	0
Mycoplasma sp. avian strain R	0	0	0	0	0	128

* Reciprocals of the highest antiserum dilution yielding a positive reaction.

Gel-diffusion precipitin reactions with soluble cell proteins

Transmigration. This technique permitted the rapid determination of the serological relationships of the soluble cell proteins of the various Mycoplasma strains (Fig. 1). The results corresponded well with those obtained by agglutination of whole organisms. No cross-reactions were evident between the *Mycoplasma laidlawii* and the bovine, caprine or avian strains. On the other hand Fig. 1 shows common antigens within the serological groups. Thus *M. laidlawii* strains B and OR reacted with the

antiserum prepared against *M. laidlawii* strain A, and *M. mycoides* var. *mycoides* reacted with the antiserum prepared against *M. mycoides* var. *capri*. No cross-reactions were seen between *M. gallisepticum* and the other avian strains.

Agar double diffusion. This method gave a more detailed picture of the serological relationships between the mycoplasmas. The results corresponded well with those

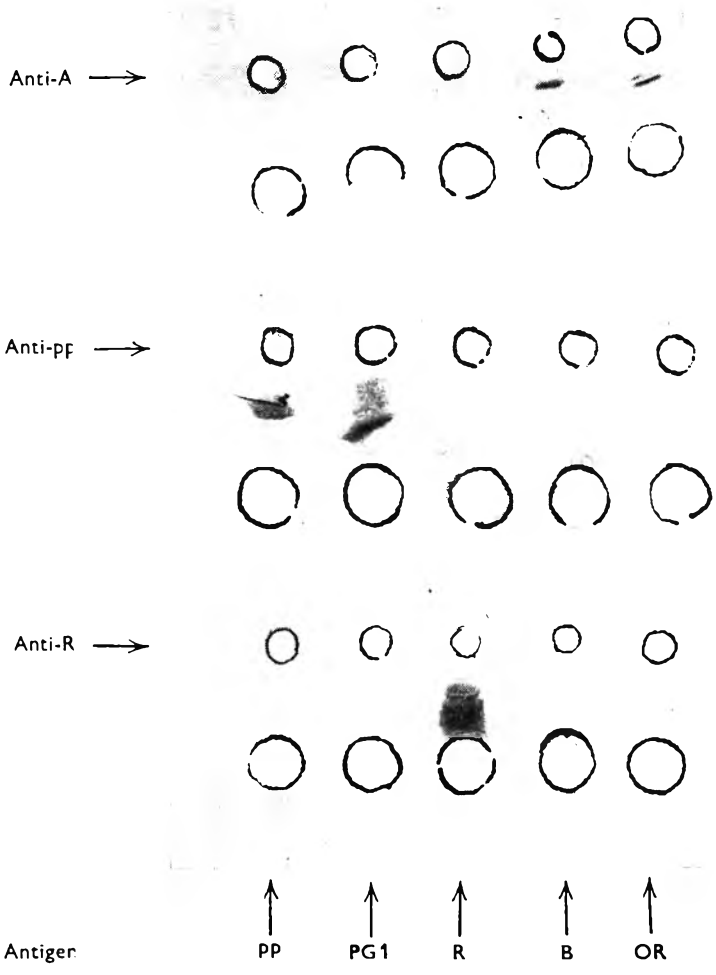

Fig. 1. Precipitation lines formed by transmigration of soluble cell proteins of various mycoplasmas against specific antisera. Antigens analysed (large holes) were obtained from *Mycoplasma mycoides* var. *capri*, pp. goat, *M. mycoides* var. *mycoides* PG 1, *Mycoplasma* sp. avian strain R, *M. laidlawii* strains B and OR.

obtained by the rapid transmigration technique. Table 3 and Fig. 2 show the different serological groups and the close serological interrelationships of strains within the groups. In order to check the specificity of the precipitation lines, all antigens used were tested against normal rabbit serum; no precipitation lines were formed with any of the antigens. Furthermore, all our antisera were tested for preciptation with the growth medium used to prepare the antigens for analysis, and the results were all negative.

Microimmunoelectrophoresis. Optimal conditions for electrophoresis were determined by testing the solubility and electromigration of the soluble cell proteins of the various mycoplasmas at pH values ranging from 2·3 to 11·5. The soluble cell proteins remained soluble at values above pH 4·5; at this pH precipitation began and was complete at about pH 3·5. Only the proteins of the *Mycoplasma gallisepticum* strains began to precipitate at pH 2·3. Optimal conditions for electromigration of the soluble

Table 3. *Mycoplasma gel-diffusion precipitin reactions of soluble cell proteins*

Soluble proteins of:	Antiserum aA	aB	aOR	app	aPG 1	aPG 3	a 59	aR
M. laidlawii A	*4**	2	3	—	—	—	—	—
M. laidlawii B	*3–4*	*3–4*	3	—	—	—	—	—
M. laidlawii OR	3	3	*3–4*	—	—	—	—	—
M. mycoides var. *mycoides* PG 1	—	—	—	3	*4–5*	ND	—	—
M. mycoides var. *mycoides* V5	—	—	—	3	3	3	—	—
M. mycoides var. *capri*, pp. goat	—	—	—	*5–6*	3–4	2–3	—	—
M. mycoides var. *capri* PG 3	—	—	—	2–3	3	*3–4*	—	—
Mycoplasma sp. strain Y	—	—	—	2–3	3	3	—	—
Mycoplasma sp. strain 14	—	—	—	3–4	2–3	2–3	—	—
M. gallisepticum A 5969	—	—	—	—	—	—	*4*	—
M. gallisepticum S 6	—	—	—	—	—	—	2–3	—
Mycoplasma sp. avian strain R	—	—	—	—	—	—	—	*4–5*
M. gallinarum strain J	—	—	—	—	—	—	—	—

* Maximum number of precipitation lines is given. Precipitation lines caused by homologous antisera are shown in italic.
ND, not done; —, no precipitation lines.

proteins were therefore tested in the range of 6·0 to 9·0, with several buffers. The best protein migration and separation was achieved in veronal + HCl buffer (Grabar, 1964) at pH 8·3. At this pH value most of the proteins migrated towards the anode, except those of *M. gallisepticum* which migrated toward the cathode. Figure 3 shows the serological cross-reactions between the *M. laidlawii* strains by the microimmunoelectrophoresis technique. All antigens migrated towards the anode, while in *M. mycoides* var. *capri*, analysed under the same conditions, some antigens migrated towards the cathode. The bovine, caprine and avian strains also revealed more precipitation lines than the *M. laidlawii* strains.

Immunoelectrophoresis on cellulose acetate strips. Electrophoresis of the soluble cell proteins of all the mycoplasmas excep, *Mycoplasma laidlawii* separated them into 6 to 8 fractions on the cellulose acetate strips. The soluble proteins of *M. laidlawii* did not separate well by this technique. Immunoprecipitation lines were formed under the cellulose acetate strips, but were fewer and weaker than those obtained by agar microimmunoelectrophoresis, and were shared by all protein fractions.

Polyacrylamide-gel immunoelectrophoresis. Electrophoresis of the soluble proteins separated them into several diffuse fractions. Sectioned gels transferred to agar-covered slides for diffusion against antisera, showed similar precipitation lines as were obtained by agar immunoelectrophoresis, i.e. the precipitation lines ran along most of the electrophoretic pattern (Fig. 4).

Antigenicity of the cell membrane

Whole membranes. Solubilization of the membranes is a prerequisite for their immunological analysis by immunodiffusion techniques. The detergent sodium dodecyl sulphate (SDS) was found to be most efficient in solubilizing mycoplasma membranes (Razin *et al.* 1965). However, in the concentration used for membrane solubilization SDS precipitated some of the rabbit serum proteins. The antisera tested were therefore

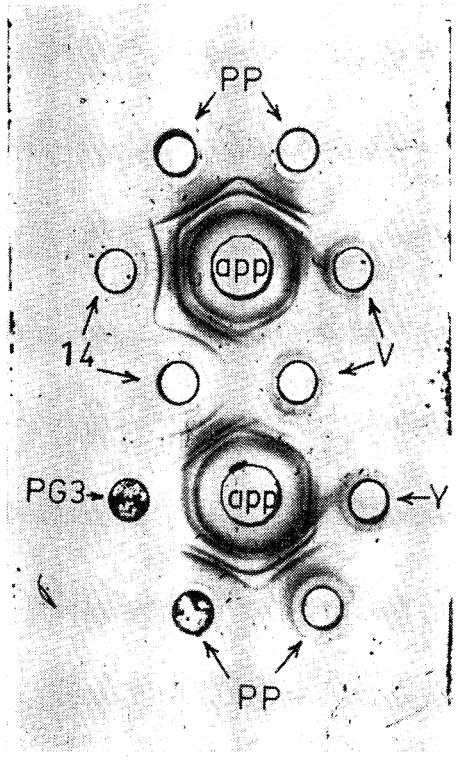

Fig. 2

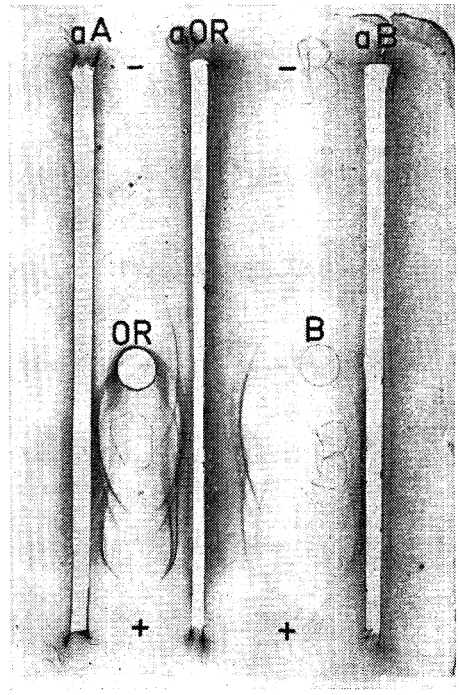

Fig. 3

Fig. 2. Antigenic relationship of the soluble cell proteins of caprine and bovine mycoplasmas. Central holes contain antiserum to *Mycoplasma mycoides* var. *capri* pp. goat (app). Peripheral holes contain soluble cell proteins of *M. mycoides* var. *capri* pp. goat (PP), *Mycoplasma* sp. strain Y, *M mycoides* var. *capri* PG 3, *M. mycoides* var. *mycoides* V 5 (V), *Mycoplasma* sp. strain 14.

Fig. 3. Antigenic relationship of the soluble cell proteins of *Mycoplasma laidlawii* strains shown by microimmunoelectrophoresis. Holes contain soluble cell proteins of *M. laidlawii* strains OR and strain B. Trenches contain antiserum to *M. laidlawii* strains A (aA), *M. laidlawii* strain OR (aOR), and *M. laidlawii* strain B (aB).

pretreated with 1% (w/v) SDS at 37° for 60 min. and the precipitate formed was removed by centrifugation. No further precipitate was formed on a longer incubation time, or by addition of more SDS to the treated sera. This treatment did not cause any detectable loss of antiserum potency (see also Jaquet, Bloom & Cebra, 1964). Table 4 shows that the solubilized Mycoplasma membranes formed between 1 to 3 precipitation lines with the homologous antiserum to whole organisms, indicating that the mem-

branes retained their antigenic group specificity as shown by whole cells (Table 2), and soluble cell proteins (Table 3). Reactions with SDS-treated normal rabbit serum, or with SDS alone, were always negative. Membranes of *M. laidlawii* solubilized in

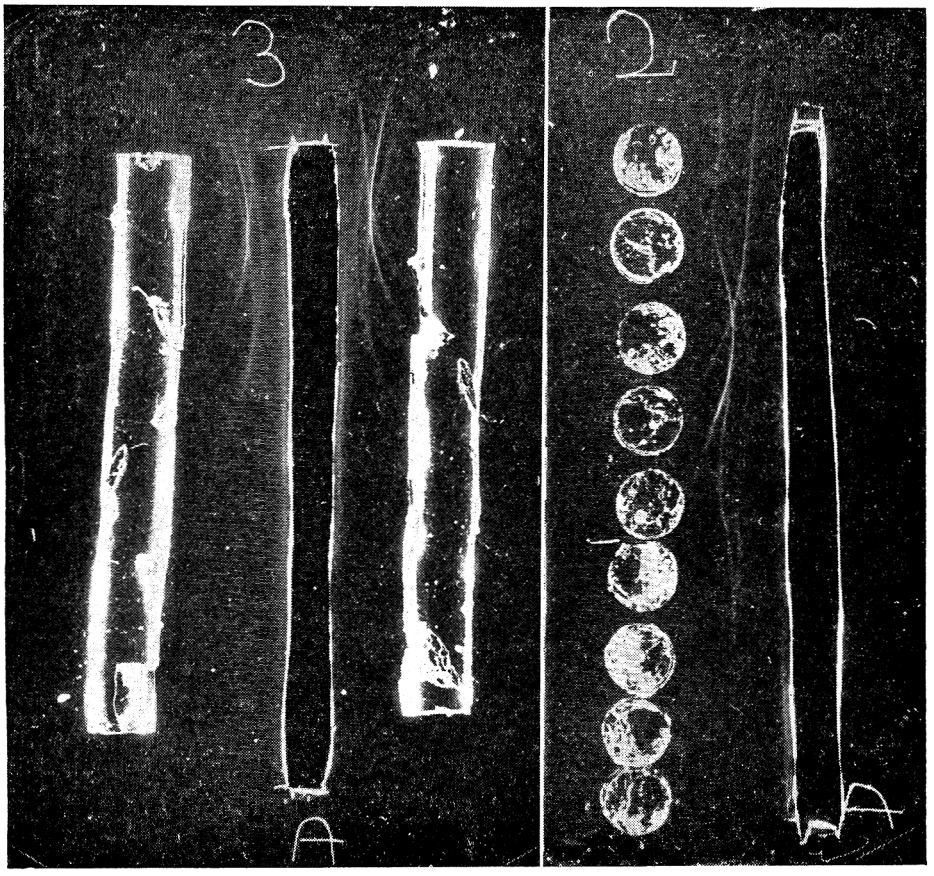

Fig. 4. Polyacrylamide-gel immunoelectrophoresis of soluble cell proteins of *Mycoplasma laidlawii* strain OR. The trenches were filled with antiserum to *M. laidlawii* strain A. The gel to the left was sectioned after electrophoresis along its long axis into two halves, while the gel to the right was sectioned transversally.

Table 4. *Agar double-diffusion reactions of SDS-solubilized mycoplasma membranes with antisera to whole cells*

Membranes of	Antiserum: aA	aPG I	app	a59	aR
	Maximum no. of precipitation lines				
M. laidlawii OR	3	—	—	—	—
M. mycoides var. *mycoides* PG I	—	2	2	—	—
M. mycoides var. *capri*, pp. goat	—	1	2	—	—
M. gallisepticum A 5969	—	—	—	2	—
Mycoplasma sp. avian strain R	—	—	—	—	2

—, No precipitation lines.

SDS were also subjected to polyacrylamide gel electrophoresis; a few protein bands were resolved. Unstained gels, run simultaneously, were sliced and tested by immunodiffusion against whole-cell antiserum. Two or three precipitation lines appeared (Fig. 5). Soluble cell proteins of several Mycoplasma strains were mixed with SDS at the same concentration used for membrane solubilization. In gel-diffusion tests the SDS-treated material exhibited similar precipitation lines as the untreated soluble proteins.

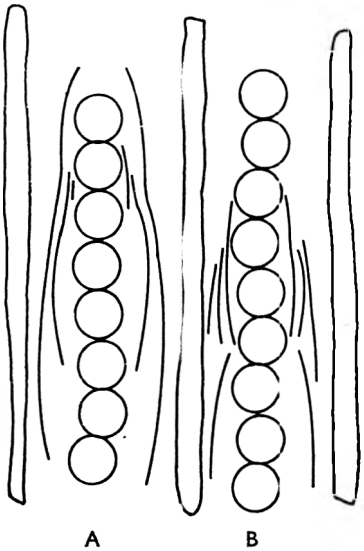

Fig. 5

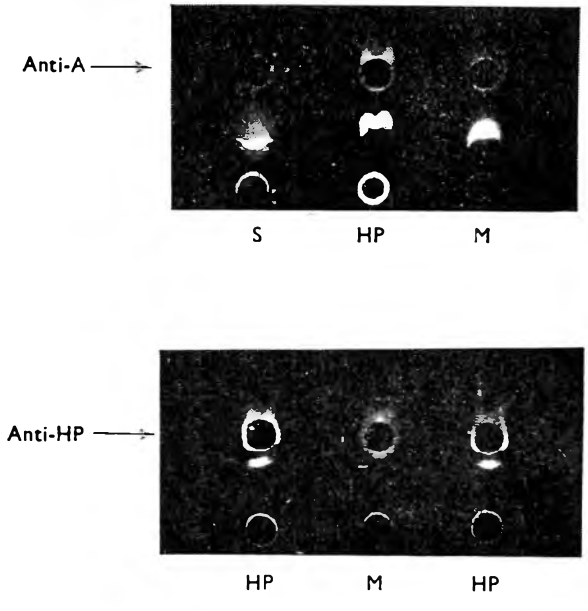

Fig. 6

Fig. 5. Polyacrylamide-gel immunoelectrophoresis of *Mycoplasma laidlawii* OR membrane proteins. The gels were sectioned transversally after electrophoresis, and the trenches were filled with antiserum to *M. laidlawii* strain A. A = membranes solubilized in SDS. B = butanol-extracted membrane proteins solubilized in SDS.

Fig. 6. Precipitation lines formed by transmigration of hydrophobic membrane protein (HP), whole membranes (M), and soluble cell proteins (S) of *Mycoplasma laidlawii* OR against antiserum to whole cells of *M. laidlawii* strain A (upper part of figure) and against antiserum to hydrophobic protein (lower part of figure). All preparations were dissolved in SDS.

Butanol-extracted membranes. Lipid-free butanol-extracted membrane proteins solubilized in SDS were electrophorized in alkaline polyacrylamide gels. As with whole membranes, the immunodiffusion of the sectioned gels revealed several precipitation lines (Fig. 5).

Hydrophobic membrane proteins. An antiserum produced in rabbits against the hydrophobic membrane protein of *Mycoplasma laidlawii* was tested by transmigration against the hydrophobic protein fraction, whole membranes, and soluble cell protein solubilized in SDS. The same antigens were simultaneously tested against an antiserum to whole organisms. Figure 6 shows that the hydrophobic protein fraction reacted with both its homologous and whole-organism antiserum. However, membrane proteins and soluble cell proteins (not shown in Fig. 6) reacted only with the whole-organism antiserum.

Membrane lipids. The staining method suggested by Uriel (1964) was used to detect protein and lipid components in the precipitation lines. The precipitation lines formed by soluble cell proteins, treated or untreated with SDS and tested against SDS-treated antisera, did not stain for lipid; a heavy precipitation line obtained with solubilized membranes did stain intensively. Membrane lipids extracted from *Mycoplasma laidlawii* according to Folch, Lees & Sloane-Stanley (1957) were solubilized in tris + HCl + EDTA buffer (pH 7·7) containing 1% (w/v) SDS. The lipid solution tested by gel diffusion against the SDS treated antisera to the various mycoplasma groups reacted by forming Sudan Black-positive precipitation lines with several of the antisera and showed none of the *M. laidlawii* group antigenic specificity.

DISCUSSION

Most of the mycoplasma strains included in the present study fell into two distinct serological groups: one included the *Mycoplasma laidlawii* strains and the other the bovine and caprine strains. The avian mycoplasma studied were serologically heterogeneous. The close serological relationship between *M. laidlawii* strains A and B is well known. Although originally these strains were separated on serological grounds (Laidlaw & Elford, 1936) they were indistinguishable by the complement-fixation test (Lemcke, 1964). Nevertheless, Clyde (1964) noticed that the growth of the two strains was suppressed by both their homologous and heterologous antisera, but that the inhibition zones produced by the heterologous antisera were only half as wide as those produced by the homologous antiserum. This finding has been confirmed by us (unpublished). The electrophoretic patterns of membrane proteins of the two strains were similar, but not identical (Rottem & Razin, 1967). Nucleic acid homology tests showed about 70% homology between the DNA of both strains (McGee, Rogul & Wittler, 1967). All the evidence, with that of the present study, indicates that *M. laidlawii* A and B strains are closely related but not identical. The present serological data also substantiate the findings by the electrophoretic technique that the *M. laidlawii* strain OR, isolated from the human oral cavity, is related to or identical with *M. laidlawii* strain A (Rottem & Razin, 1967).

The bovine and caprine mycoplasma strains included in our study, form a closely related group. The caprine strains resemble each other in their heavy growth and tendency to lyse and form sticky precipitates when the cultures age. When cell proteins of the caprine and bovine strains were subjected to polyacrylamide-gel electrophoresis very similar electrophoretic patterns were revealed (Razin, 1968). Lemcke (1965) by using the gel precipitin technique showed that *Mycoplasma mycoides* var. *mycoides* and *M. mycoides* var. *capri* (pp. goat) have certain antigenic components in common. All these results speak in favour of regarding the caprine strains tested as varieties of *Mycoplasma mycoides.*

The few avian strains included in our study did not form a serological group. The *Mycoplasma gallisepticum* A 5969 and S6 strains showed antigenic similarity. The electrophoretic patterns of their membrane proteins were almost identical (Rottem & Razin, 1967). However, these strains had no antigenic components in common with the two other avian strains J and R, which were also clearly differentiated by the electrophoretic technique (Rottem & Razin, 1967; Razin, 1968).

The present study provides some information on the electrophoretic properties of

the soluble cell proteins of mycoplasmas. The proteins of the *Mycoplasma laidlawii* strains consistently showed a higher electrophoretic homogeneity than the proteins of the other strains, as shown by the non-separation of protein fractions by electrophoresis on cellulose acetate strips and by the few protein bands appearing upon electrophoresis of the *M. laidlawii* soluble proteins in alkaline polyacrylamide gels. In microimmunoelectrophoresis tests at pH 8·3, all the soluble *M. laidlawii* proteins moved towards the anode, resulting in poor separation and formation of a few precipitation lines. The soluble cell proteins of the other mycoplasmas showed more electrophoretic heterogeneity. Thus even at pH 8·3 part of the proteins of *M. mycoides* var. *capri* moved towards the cathode, leading to better separation, and the identification of more precipitation lines. The migration of most of the *M. gallisepticum* soluble proteins to the cathode at pH 8·3 indicates their highly basic nature.

The solubilization of Mycoplasma cell membranes by sodium dodecyl sulphate (SDS) permitted the antigenic analysis of membrane proteins. SDS did not destroy the antigenic activity of at least part of the membrane proteins, as shown by immunodiffusion tests. Precipitin reactions done with solubilized membranes revealed the same serological group-specificity as shown by the soluble cell proteins, although fewer precipitation lines were produced. Staining with Sudan Black indicated that membrane lipids also took part in the precipitin reaction, either as such or in association with protein. *Mycoplasma laidlawii* membrane lipids extracted with chloroform + methanol formed a Sudan Black-stained precipitation line when tested against antiserum to *M. laidlawii*. However, a similar precipitation line was also formed with heterologous antisera, indicating that *M. laidlawii* lipids do not possess the antigenic specificity shown by membrane proteins. Kenny (1967) showed that lipids extracted from *M. hominis*, *M. arthritidis* and *M. pulmonis* cross-reacted in complement-fixation tests, while lipids of *M. pneumoniae* and *M. fermentans* were serologically species-specific. The possibility of protein denaturation by SDS should be considered. There is little doubt that at the concentration used for membrane solubilization, the SDS causes conformational changes at least in some membrane proteins (Shapiro, Vinuela & Maizel, 1967). Thus, the adenosine triphosphatase activity of Mycoplasma membranes was lost during solubilization by SDS (Rottem & Razin, 1966). However, SDS did not affect the NADH oxidase activity of the solubilized membranes (Razin *et al.* 1965) indicating a different degree of sensitivity of different membrane proteins to the denaturating effects of SDS. An indication that SDS at the concentration used does not radically alter the antigenic properties of cell proteins is found in that SDS-treated soluble cell proteins of several mycoplasmas exhibited similar precipitation lines as untreated soluble proteins. Mommaerts (1967) showed by measurements of circular dichroism that SDS, at a concentration similar to that used by us, did not significantly change the conformation of proteins of sarcotubular membranes. Treatment by SDS did not destroy the immunogenic activity of at least part of the mycoplasmal membrane proteins (the hydrophobic protein fraction). However, the antiserum to the protein fraction did not react with the soluble cell proteins, or with whole membranes solubilized with SDS. This might indicate a change in the antigenic properties of the hydrophobic membrane proteins during their isolation by SDS action. It may be concluded that in spite of possible artifacts arising from conformational changes in some membrane proteins, solubilization of Mycoplasma membranes by detergents may allow the further antigenic analysis of membrane proteins.

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Genetic Determination of the Antigenic Specificity of Flagellar Protein in Salmonella

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SUMMARY

Using non-flagellate(*fla*$^-$) mutants of gn-complex antigenic derivatives of a stable phase-1 strain of *Salmonella abortusequi*, the positions of the antigenic specificity-determining sections on the genetic map of *H1*, a structural gene for flagellar protein, were located by establishing a system for selecting intra-*H1* recombinants. Before the recombination experiments, a factor analysis of the gn-complex antigens of the strains was made by cross-absorption tests: nine factors were detected.

P22 phage-mediated transductions were carried out between different pairs of *H1*-linked *fla*$^-$ mutants, whose *H1*-alleles were different and whose *fla*$^-$ sites were on opposite sides of *H1*. The *fla*$^+$ transductants, present as swarms in semi-solid medium, were isolated and the composition of their flagellar antigens examined with specific antisera. Among 1366 *fla*$^+$ transductants obtained, 26 clones were shown to have 'recombinant antigens' carrying some factors of one or both of the parental-type antigens. With several 'recombinant antigens', some new specificities with weak antigenicity were detected. The flagellar protein, 'flagellin', of an 'antigen recombinant' was proved to be a recombinant of the two parental flagellins by finger-printing analysis of tryptic digests.

On the assumption that these 'antigen recombinants' resulted from a single cross-over within *H1*, the relative positions of the antigenic specificity-determining sections within donor and recipient *H1* with regard to the cross-over point were obtained for individual 'antigen recombinants'. Summarizing the relative positions thus inferred, it was shown that each of the antigenic specificity-determining sections maps as a unit; and that as a whole they form a linear array within *H1*. Sections specifying some tryptic peptides of flagellins were also mapped within *H1*.

INTRODUCTION

Salmonella flagella exhibit a wide variety of antigenic specificities (Kauffmann, 1964). Many Salmonella species manifest two alternative types of flagellar antigen: one is called phase-1 and the other phase-2 (Andrews, 1922). Genetical analysis by transduction shows that the phase-1 and phase-2 specificities are determined by allelic genes at two loci, *H1* and *H2* (Lederberg & Edwards, 1953). Chemically, a Salmonella flagellum is a polymer of a single protein, flagellin, the molecular weight of which is 36,000 to 40,000 (Kerridge, Horne & Glauert, 1962; Asakura, Eguchi & Iino, 1964; McDonough, 1965). The genes *H1* and *H2* are therefore the structural genes specifying the primary structures of the flagellins of the respective phases (Iino & Lederberg, 1964).

Immunological tests show that the flagellar antigen is a complex composed of

several antigenic factors. That is, one flagellin molecule carries several distinct antigenic determinants. The so-called gn-complex antigens, a group of phase-1 antigens possessing the factor g in common, have been analysed antigenically in detail, as shown in the Kauffman–White scheme (Kauffmann, 1964). After reviewing the compositions of naturally occurring gn-complex antigens, Iino (1959) proposed that the antigenic specificities are determined by several sections in the *H* gene, each corresponding to a different antigenic specificity. According to this hypothesis, intragenic recombination between different alleles of *H1* or *H2* will result in the production of a recombinant antigen carrying specificities derived from both parents, unless the specificity of each antigen is altered by conformational changes in the flagellin molecule resulting from recombination. Analysis of such recombinant antigens should therefore disclose the arrangement of antigen-determining sections within *H* genes. Some evidences of intragenic recombination between the *H2* alleles, *H2-1,2* and *H2-e,n,x* (Iino, 1960), and between some mutant alleles of *H1-i* (Joys & Stocker, 1963) has already been obtained, but the ordering of specificity-determining sections has not previously been attempted.

In the present study, an attempt has been made to map the antigenic specificity-determining sections in *H1* by intragenic recombination between different alleles of *H1-g...*

METHODS

Bacteria. Salmonella dublin NCTC4197 (1,9,12; g,p:-), *S. derby* NCTC1729 (1, 4, 5, 12; g,f:-), and *S. enteritidis* NCTC4196 (1,9,12; g,m:-) were received from Dr Joan Taylor (Central Public Health Laboratory, London). *Salmonella* sp. strain SJ6 (?; g,t:-) is a stock strain of this Institute. These naturally occurring strains are heterogeneous in various characters, for example, in their somatic antigens, phage sensitivity, etc. For the present study, four artificial strains were prepared by transferring the alleles of *H1-g...* by P22 phage-mediated transduction from the above four naturally occurring strains to a single recipient strain SJ241 (4, 12; a: (e,n,x)), a phase-1 stable strain of *S. abortusequi*. Their strain numbers and some characteristics are shown in Table 1. The artificial strains are thought to have the same genetic background except *H1* and its vicinity. Since they are stable in phase-1 and have identical somatic antigens, only phase-1 antigens are indicated here when describing their antigenic formulae. All of them are *nml*$^+$.

Non-flagellate (*fla*$^-$) mutants were selected from strains TR6, TR16 and TR17 by phage M8, a mutant of phage χ. This phage attacks motile Salmonella organisms except for some specific serotypes including gn-complex antigenic Salmonella, but cannot attack non-flagellate (*fla*$^-$) or paralysed (*mot*$^-$) cells (Meynell, 1961). Phage M8 is a host-range mutant able to attack motile salmonellas with gn-complex antigens (Sasaki, 1962; Yamaguchi, 1968). The method used here for selecting *fla*$^-$ mutants was that described by Joys & Stocker (1965) for isolating *fla*$^-$ mutants from *S. typhimurium* LT2 with the χ-phage. The *fla*$^-$ mutants used in the present study are listed in Table 2. They are stable and their mutation sites are very closely linked to *H1*. Complementation tests with the mutants of the already known *fla* cistrons showed that TR16-*fla-227* and TR17-*fla-251* carry deletions in the *flaA* cistron and that TR6-*fla-373* has a deletion extending over the *flaA*, *B*, and *D* cistrons. The other three mutants, TR6-*fla-351*, TR16-*fla-204*, and TR17-*fla-263*, do not complement each other but com-

plement with every *fla*⁻ mutant of the known *fla* cistrons. The possibly new cistron represented by these three mutants is tentatively described as *flaL* in this paper. Reciprocal transductions were made between each of these mutants and a *fla*⁻ mutant of SJ241 (*flaA*⁻ *H1-a*), and the flagellar antigen types of the *fla*⁺ transductants examined. From the ratios of the transductants carrying the donor-type antigen to those carrying the recipient-type antigen in every cross, it was shown that the *flaA* cistron and the *flaL* cistron are on the opposite sides of *H1*. Details of the mapping of these *fla*⁻ sites will be published elsewhere.

Table 1. *Derivatives of Salmonella abortusequi SJ241, given H1-g...-alleles from gn-complex antigenic Salmonella by transduction*

Strain no.	O antigens	H antigens		Sensitivity to phage		*nml**	Donor of *Hl-g...*
		phase 1	phase 2	P22	M8		
TR6	4,12	g,t	(e,n,x)†	s‡	s	+	*Salmonella* sp. SJ6
TR11	4,12	g,p	(e,n,x)	s	s	+	*S. dublin* NCTC4197
TR16	4,12	f,g	(e,n,x)	s	s	+	*S. derby* NCTC1729
TR17	4,12	g,m	(e,n,x)	s	s	+	*S. enteritidis* NCTC4196

* The *nml* character was determined by the presence or absence of ε-*N*-methyllysine in acid-hydrolysed flagellin examined in the Hitachi automatic amino acid analyser.
†(): unexpressed antigen type.
‡ s: 'sensitive' to the indicated phage.

Table 2. *Properties of non-flagellate mutants of Salmonella*

Frequency of co-transduction with *H1*: percentage of *fla*⁺ clones with the phase-1 antigen of donor in the total *fla*⁺ transductants produced in the P22 phage-mediated transduction from *Salmonella abortusequi* SJ241 (a: (e,n,x)) to the *fla*⁻ mutant. About 500 *fla*⁺ transductants were examined for each strain.

Complementation group: examined by trail formation in the P22 phage-mediated transduction from the *fla*⁻ mutant to standard mutants of the known *fla* cistrons derived from *S. typhimurium* TM2 (Iino & Enomoto, 1966). The sequence of the *fla* cistrons is *his...flaB-flaD-flaA-H1-flaL...motA,B...trp*.

Strain	Reversion	Frequency of co-transduction with *H1* (%)	Complementation group	Original strain
TR6-*fla*-351	—	89	L	TR6
TR6-*fla*-373	—	87	A, B, D	TR6
TR16-*fla*-204	—	96	L	TR16
TR16-*fla*-227	—	88	A	TR16
TR17-*fla*-251	—	54	A	TR17
TR17-*fla*-263	—	90	L	TR17

Media. Nutrient broth was composed of 1% (w/v) peptone and 1% (w/v) meat extract, adjusted to pH 7·2. Nutrient agar was prepared by the addition of 1·5% (w/v) agar to nutrient broth. Semi-solid medium (Edwards & Bruner, 1942; Stocker, Zinder & Lederberg, 1953) was prepared by adding 0·35% (w/v) agar and 8% (w/v) gelatin to the nutrient broth. All cultures were incubated at 37°.

Immunization procedure. Since the content of flagellar antigen of a bacterium is roughly proportional to its motility, the bacteria used for immunization, agglutination or absorption were first passed through semi-solid medium. Overnight broth cultures inoculated with highly motile organisms were centrifuged and the pellet suspended in

formalinized saline (formaldehyde concentration: 0·5%) to a final concentration of about 5×10^8 bacteria/ml. Rabbits were immunized by one intraperitoneal and five successive intravenous injections of a total of 6 ml. of bacterial suspension at 3-day intervals, and bled 1 week after the final injection. The antisera were processed according to Edwards & Ewing (1955).

Absorption of antisera. Highly motile organisms of an absorbing strain were grown in nutrient broth overnight, and used to inoculate trays ($20 \times 30 \times 1{\cdot}8$ cm.) each containing 200 ml. nutrient agar. After incubation for 24 hr, the bacteria were harvested in formalinized saline (formaldehyde concentration: 0·5%), and the suspension kept at room temperature overnight. The bacteria were then centrifuged, resuspended in sterile saline and kept at 0°. This suspension was centrifuged before use and the pellet mixed with the antiserum to be absorbed in the proportion of about 0·5 g. wet weight organisms to 1·0 ml. serum. After 2 hr at 37° the mixture was centrifuged. If this once-absorbed serum still agglutinated the absorbing organisms, this process was repeated until no activity with the absorbing organisms remained.

Titration of antisera. The titres of antisera were determined by tube agglutination of two-fold serial dilutions incubated at 52° for 2 hr and expressed as the reciprocal or its logarithm (to base 2) of the highest dilution showing activity.

Transduction between fla⁻ mutants. Phage P22 (Zinder & Lederberg, 1952) was used as transducing phage throughout. The phage was propagated by the soft agar-layer method (Adams, 1959) on a donor strain, and the lysate mixed with broth culture of the recipient (about 5×10^8 cells/ml.) at a m.o.i. of 5 to 10. After 10 min. at 37°, the mixture was diluted 10^{-1} and 10^{-2} with nutrient broth, and the dilutions brushed in line on semi-solid medium. The *fla*⁺ transductants were selected as swarms after incubation for 15 hr.

Preparation of flagellin. Broth cultures of highly motile bacteria were inoculated on nutrient agar in trays. After incubation for 24 hr at 37°, the bacteria were harvested in saline, washed, resuspended in distilled water, and de-flagellated by shaking (about 750 strokes/min., 25 mm. amplitude). The suspension was centrifuged at 3000 rev./min. for 40 min. The supernatant, containing the flagella, was then centrifuged at 30,000 rev./min. in a Spinco Model L ultracentrifuge for 45 min. The deposited flagella were disintegrated into monomers of flagellin by treatment with 0·01 N-HCl for 30 min. at room temperature. The solution was then centrifuged at 30,000 rev./min. for 45 min. to remove turbidity, and the supernatant dialysed for 5 hr against three changes of distilled water and lyophilized *in vacuo*. No further preparation was undertaken since the flagellin preparation thus obtained showed only one peak and one band in column chromatography using DEAE-cellulose and electrophoresis, respectively (Enomoto & Iino, 1962).

Tryptic peptide maps. Lyophilized flagellin was dissolved in 0·05 M-phosphate buffer (pH 8·0) at 2%, denaturated by heating at 90° for 5 min., and digested with trypsin (2%) at 25° for 24 hr. A sample of the digest was applied to a sheet (40×30 cm.) of Tōyō no. 50 filter paper and subjected to electrophoresis in pyridine + acetic acid + water (135 + 15 + 1 by vol.) at pH 6·65 for 3·5 hr at 15 V/cm. After drying, the paper was subjected twice to ascending chromatography with *n*-butyl alcohol + acetic acid + water (3 + 1 + 1 by vol.) and the peptide spots developed with ninhydrin dissolved in acetone (0·25%, w/v).

RESULTS

Further analysis of factor compositions of gn-complex antigens

Although the compositions of the gn-complex antigens have been analysed in some detail, it has often been recognized that the factor g may be further subdivided into several factors (e.g. Edwards & Ewing, 1955). Thus, an analysis of the gn-complex antigens was carried out with the four gn-complex antigenic strains, TR6 (g,t), TRI1 (g,p), TRI6 (f,g), TRI7 (g,m), by cross-absorption.

Table 3. *Cross-absorption-agglutination test of anti-gn-sera*

		Activity of absorbed antiserum on			
Antiserum	Absorbed with	TR6	TRI1	TRI6	TRI7
Anti-TR6	TRI1	+	−	+	+
	TRI6	+	+	−	+
	TRI7	+	−	+	−
	TRI1 and TRI6	+	−	−	+
	TRI1 and TRI7	+	−	+	−
	TRI6 and TRI7	+	−	−	−
Anti-TRI1	TR6	−	+	+	+
	TRI6	+	+	−	+
	TRI7	−	+	−	−
	TR6 and TRI6	−	+	−	+
	TR6 and TRI7	−	+	−	−
	TRI6 and TRI7	−	+	−	−
Anti-TRI6	TR6	−	+	+	+
	TRI1	+	−	+	−
	TRI7	+	−	+	−
	TR6 and TRI1	−	−	+	−
	TR6 and TRI7	−	−	+	−
	TRI1 and TRI7	+	−	+	−
Anti-TRI7	TR6	−	+	+	+
	TRI1	+	−	−	+
	TRI6	+	+	−	+
	TR6 and TRI1	−	−	−	+
	TR6 and TRI6	−	+	−	+
	TRI1 and TRI6	+	−	−	+

+, Agglutination occurred; −, no agglutination occurred.

Several rabbits were immunized with TR6, TRI1, TRI6 and TRI7, respectively. Antisera of titre of 12,800 to 25,600 against the homologous antigens were obtained. These were fully absorbed with organisms of one or two non-homologous test strains, and the residual cross-reactivities examined by slide agglutination (Table 3). Since the somatic (o) antigens are common to all the strains used, the activities remaining after absorption are those for the factor(s) present on the flagellar antigen of the immunizing strain but absent from that of the absorbing strain. The results indicated the presence of at least nine factors in the gn-complex antigens examined. Four were specific to the respective strains and were therefore regarded, respectively, as factors t, p, f, and m in the Kauffmann–White scheme. The other five factors common to two or three strains were regarded as parts of a complex of factors, which has so far been given the symbol g; they will be described as g_1, g_2, g_3, g_4 and g_5, respectively. The factor

compositions of the four strains are shown in Table 4. There remained the possibility that besides these nine factors a factor or factors common to all the four antigens exists, which would have been overlooked in the present cross-absorption test.

Table 4. *Factors present in the gn-complex antigens*

(g_0): a factor or a complex of factors common to all these gn-complex antigens which would not be detected in the present cross-absorption test.

Strain	Factor composition									
TR6	(g_0)	g_1	g_2	g_3	.	.	t	.	.	.
TR11	(g_0)	g_1	.	.	g_4	g_5	.	p	.	.
TR16	(g_0)	.	.	g_3	g_4	.	.	.	f	.
TR17	(g_0)	g_1	g_2	.	g_4	g_5	.	.	.	m

Table 5. *Anti-factor sera used for detecting the factors of flagellar antigens of fla⁺ recombinants from different pairs of fla⁻ mutants*

Factor to be detected	Anti-factor(s) serum used	Preparation of the anti-factor(s) serum: Serum	Preparation of the anti-factor(s) serum: Absorbed with
	(*a*) For *fla*⁺ transductants between *fla*⁻ mutants of TR6 and TR16		
g_1	Anti-g_1,g_5,p	Anti-TR11	TR16
g_2	Anti-g_2,m	Anti-TR17	TR11
g_4	Anti-g_4,g_5,p	Anti-TR11	TR6
t	Anti-t	Anti-TR6	TR16 and TR17
f	Anti-f	Anti-TR16	TR6 and TR11
	(*b*) For *fla*⁺ transductants between *fla*⁻ mutants of TR6 and TR17		
g_3	Anti-g_3,f	Anti-TR16	TR17
g_4	Anti-g_4,f	Anti-TR16	TR6
g_5	Anti-g_5,p	Anti-TR11	TR6 and TR16
t	Anti-t	Anti-TR6	TR16 and TR17
m	Anti-m	Anti-TR17	TR6 and TR11
	(*c*) For *fla*⁺ transductants between *fla*⁻ mutants of TR16 and TR17		
g_1	Anti-g_1,g_5,p	Anti-TR11	TR16
	Or anti-g_1,g_2,t	Anti-TR6	TR16
g_2	Anti-g_2,t	Anti-TR6	TR11 and TR16
g_3	Anti-g_3,t	Anti-TR6	TR17
g_5	Anti-g_5,p	Anti-TR11	TR6 and TR16
f	Anti-f	Anti-TR16	TR6 and TR17
m	Anti-m	Anti-TR17	TR6 and TR11

Recombination within H1

The system for detecting recombinants was as follows. P22 phage-mediated transduction was done between a pair of non-flagellate (*fla*⁻) mutants whose *H1*-alleles differed; whose *fla*⁻ sites were cotransduced with *H1* in high frequency; and were on the opposite sides of *H1*: that is, between *fla*⁻ mutants *fla-a* (-*fla-a*⁻ - *H1-A-fla-b*⁺-) and *fla-b* (-*fla-a*⁺ - *H1-B-fla-b*⁻-). The *fla*⁺ transductants developing as swarms in semi-solid medium were isolated and their antigenic compositions examined. The occurrence of an *fla*⁺ transductant in this case required at least one cross-over in the region between the two *fla*⁻ sites surrounding *H1*. Thus, the closer the *fla*⁻ sites to *H1*, the larger should be the relative frequency of intra-*H1* recombinants among the *fla*⁺ transductants.

Table 6. *Factors present in the flagellar antigens of fla⁺ recombinants from different pairs of fla⁻ mutants*

Factor composition of flagellar antigen: examined by slide agglutination with anti-factor sera listed in Table 5.

A, B, D and L in parentheses: complementation groups of *fla*⁻ mutants.

Transduction		*fla*⁺ transductant							
Donor	Recipient	No. of clones	Factor composition of flagellar antigen						Strain no. of 'antigen type recombinant'
			g_1	g_2	g_4	f	t		
(*a*) The *fla*⁺ transductants between TR6- and TR16-*fla*⁻ mutants									
TR6-*fla-373* (A,B,D)	TR16-*fla-204* (L)	63	+	+	−	−	+	(TR6-type)	.
		32	−	−	+	+	−	(TR16-type)	.
		3	−	−	+	−	+	(Rec.-type I)	SJ1740 to SJ1742
		1	−	−	−	−	+	(Rec.-type II)	SJ1743
TR16-*fla-204* (L)	TR6-*fla-373* (A,B,D)	85	+	+	−	−	+	(TR6-type)	.
		15	−	−	+	+	−	(TR16-type)	.
TR6-*fla-351* (L)	TR16-*fla-227* (A)	28	+	+	−	−	+	(TR6-type)	.
		68	−	−	+	+	−	(TR16-type)	.
		3	+	+	+	+	−	(Rec.-type III)	SJ1744 to SJ1746
TR16-*fla-227* (A)	TR6-*fla-351* (L)	35	+	+	−	−	+	(TR6-type)	.
		64	−	−	+	+	−	(TR16-type)	.
		1	−	+	+	+	−	(Rec.-type IV)	SJ1747
(*b*) The *fla*⁺ transductants between TR6- and TR17-*fla*⁻ mutants									
			g_3	g_4	g_5	m	t		
TR6-*fla-373* (A,B,D)	TR17-*fla-263* (L)	56	+	−	−	−	+	(TR6-type)	.
		44	−	+	+	+	−	(TR17-type)	.
TR17-*fla-263* (L)	TR6-*fla-373* (A,B,D)	88	+	−	−	−	+	(TR6-type)	.
		52	−	+	+	+	−	(TR17-type)	.
TR6-*fla-351* (L)	TR17-*fla-251* (A)	17	+	−	−	−	+	(TR6-type)	.
		91	−	+	+	+	−	(TR17-type)	.
TR17-*fla-251* (A)	TR6-*fla-351* (L)	23	+	−	−	−	+	(TR6-type)	.
		98	−	+	+	+	−	(TR17-type)	.
		1	−	−	+	+	+	(Rec.-type V)	SJ1748
(*c*) The *fla*⁺ transductants between TR16- and TR17-*fla*⁻ mutants									
			g_1	g_2	g_3	g_5	f	m	
TR16-*fla-227* (A)	TR17-*fla-263* (L)	74	−	−	+	−	+	−	(TR16-type)	.
		56		+	−	+	−	+	(TR17-type)	.
		7	+	+	+	−	+	−	(Rec.-type VI)	SJ1749 to SJ1755
TR17-*fla-263* (L)	TR16-*fla-227* (A)	92	−	−	+	−	+	−	(TR16-type)	.
		35		+	−	+	−	+	(TR17-type)	.
		5	+	+	+	−	+	−	(Rec.-type VII)	SJ1756 to SJ1760
		1	−	+	+	−	+	−	(Rec.-type VIII)	SJ1761
TR16-*fla-204* (L)	TR17-*fla-251* (A)	11	−	−	+	−	+	−	(TR16-type)	.
		71		+	−	+	−	+	(TR17-type)	.
		2	−	−	−	+	−	+	(Rec.-type IX)	SJ1762 and SJ1763
TR17-*fla-251* (A)	TR16-*fla-204* (L)	22	−	−	+	−	+	−	(TR16-type)	.
		120		+	−	+	−	+	(TR17-type)	.
		1	−	−	−	+	−	+	(Rec.-type X)	SJ1764
		1	+	−	−	+	−	+	(Rec.-type XI)	SJ1765

Transductions were made in the combinations mentioned above, using two *H1*-linked *fla*⁻ mutants each of TR6, TR16 and TR17, listed in Table 2; the *fla*⁻ sites of the two were on the opposite sides of *H1*. The antigenic compositions of the *fla*⁺ transductants obtained were examined by slide agglutination tests with anti-factor(s) sera (Table 5). Since not all the antisera for single factors were available, antisera for a complex of factors were sometimes used. For example, anti-g_1,g_5,p serum was used to detect factor g_1 of the transductants formed in the cross between *fla*⁻ mutants of TR6 (g_1,g_2,g_3,t) and TR16 (g_3,g_4,f), because factors g_5 and p were absent from both of the parental type antigens. As another example, anti-g_1,g_5,p and anti-g_1,g_2,t sera were used to detect factor g_1 of transductants obtained from crosses between *fla*⁻ mutants of TR16 (g_3,g_4,f) and TR17 (g_1,g_2,g_4,g_5,m). The former was used for the detection of g_1 in clones lacking factor g_5, and the latter for g_1 in those lacking factor g_2.

The majority (1340 clones) of the total 1366 *fla*⁺ transductants had either donor- or recipient-type antigens, while the remaining 26 had new combinations of antigens (Table 6). For example, four types of transductant clones were obtained in transducing from TR6-*fla-373* (*H1-g*$_1$,*g*$_2$,*g*$_3$,*t*) to TR16-*fla-204* (*H1-g*$_3$,*g*$_4$,*f*) as shown in Table 6 (*a*). Among them, the first and second were the donor- and the recipient-types, respectively, and the remaining two were new types; the one carried factor t of the donor together with g_4 of the recipient, and the other carried only t of the donor, among the factors tested. All in all, eleven such new types (recombinant-type I to XI in Table 6) were found in the present experiment. Among them, seven types had some factors of both of the donor- and the recipient-type antigens, and the other four had only part of the factors of one of the parental type antigen. On the supposition that these new type clones arose by intragenic recombination between alleles of *H1-g*.., they were designated as 'antigen recombinants', and the new type antigens as 'recombinant antigens.'

Serological nature of 'recombinant antigens'

One strain of each of the eleven types of recombinant was examined for the antigenic reactivity of their individual factors and also for the existence of antigenic factors absent from the parental type antigens. Individual factors were examined by measuring the titres of anti-factor(s) sera with organisms of the test strains. These sera were those used to detect 'antigen recombinants' (Table 5). As shown in Table 7, the reactivity of individual factors of the 'recombinant antigens' was almost equal to that of the corresponding factors of the parental type antigens.

A rabbit was immunized with actively motile cells of each 'antigen recombinant' to be tested. This antiserum was then repeatedly absorbed with a mixture of the parental strains. Since the 'antigen recombinants' used as immunogens had been lysogenized with P22 phage, which is known to cause formation of somatic antigen 1 by the host organism (Zinder, 1957), the absorbing bacteria were first lysogenized with the phage. After every absorption, residual activity was titrated with the homologous antigen by tube agglutination (Table 8). Antisera, except those against SJ1740, SJ1747 and SJ1749, were easily inactivated by several absorptions; that is, there was no factor on these 'recombinant antigens' other than those present on the parental type antigens. Weak activities remained against SJ1740, SJ1747 and SJ1749 after full absorption. These fully absorbed antisera did not agglutinate any antigen used here other than their respective

homologous antigens. Also they did not agglutinate salmonellas with the following flagellar antigens: a; b; e, n, x; i; 1, 2; g, m, s; m, t; g, m, q; g, s, t; g, q.

Although some 'recombinant antigens' had a unique antigenic pattern, the results obtained here indicate that of the 'recombinant antigens', at least seven types were almost complete mosaics, made up partly of donor-type antigen and partly of recipient-type antigen. The other six types may also be regarded as molecular mosaics, assuming that an active part of the antigen of one parent antigen had been replaced by an inactive part from the other parent.

Table 7. *Antigenic reactivity of individual factors of the flagellar antigens in the 'antigen recombinants'*

Titres of the anti-factor(s) antisera on the indicated antigens were determined by tube agglutination and expressed as log to base 2.

Antigen	Log titre to base 2 of anti-factor(s) serum (Factor to be examined)				
	Anti-g_1,g_5,p (g_1)	Anti-g_2,m (g_2)	Anti-g_4,g_5,p (g_4)	Anti-f (f)	Anti-t (t)
(a) 'Antigen type recombinants' between TR6- and TR16-*fla*⁻ mutants					
TR6 } (Parental type)	5	7	< 0	< 0	11
TR16 } (Parental type)	< 0	< 0	9	9	< 0
SJ1740 (Rec.-type I)	< 0	< 0	7	< 0	11
SJ1743 (Rec.-type II)	< 0	< 0	< 0	< 0	11
SJ1744 (Rec.-type III)	5	7	8	9	< 0
SJ1747 (Rec.-type IV)	< 0	7	9	8	< 0
	Anti-g_3,f (g_3)	Anti-g_4,f (g_1)	Anti-g_5,p (g_5)	Anti-m (m)	Anti-t (t)
(b) 'Antigen type recombinants' between TR6- and TR17-*fla*⁻ mutants					
TR6 } (Parental type)	7	< 0	< 0	< 0	13
TR17 } (Parental type)	< 0	6	8	9	< 0
SJ1748 (Rec.-type V)	< 0	< 0	7	9	13
	Anti-g_1,g_5,p (g_1)	Anti-g_1, g_2,t(g_1)	Anti-g_2,t (g_2)	Anti-g_3,t (g_3)	Anti-g_5,p (g_5)	Anti-f (f)	Anti-m (m)
(c) 'Antigen type recombinants' between TR16- and TR17-*fla*⁻ mutants							
TR16 } (Parental type)	< 0	< 0	< 0	10	< 0	10	< 0
TR17 } (Parental type)	nt	nt	10	< 0	8	< 0	10
SJ1749 (Rec.-type VI)	4	nt	10	9	< 0	9	< 0
SJ1756 (Rec.-type VII)	5	nt	10	10	< 0	9	< 0
SJ1761 (Rec.-type VIII)	< 0	nt	9	10	< 0	10	< 0
SJ1762 (Rec.-type IX)	nt	< 0	< 0	< 0	8	< 0	10
SJ1764 (Rec.-type X)	nt	< 0	< 0	< 0	8	< 0	10
SJ1765 (Rec.-type XI)	nt	5	< 0	< 0	8	< 0	10

nt = not tested

Tryptic peptide maps of flagellins of 'antigen recombinants' and parental-type strains

To show that the 'antigen recombinants' had resulted from recombination within *H1*, flagellins were isolated from the parental strains and from one strain of each of the eleven types of 'antigen recombinants', and their tryptic digests analysed by the finger-printing technique.

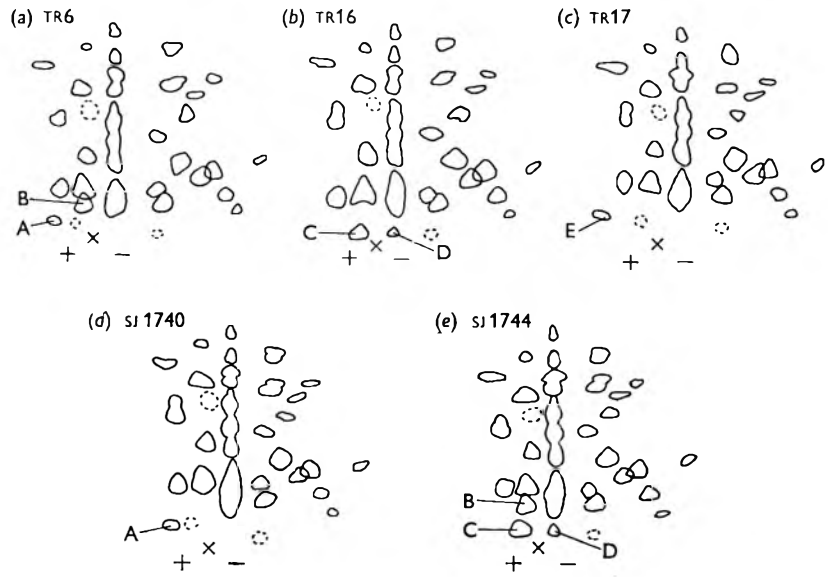

Fig. 1. Tracing of the tryptic peptide maps of flagellins of TR6, TR16 and TR17, gn-complex antigenic derivatives of a phase-1 stable strain of *Salmonella abortusequi*, and SJ1740 and SJ1744, 'ant.gen recombinants' between TR6 and TR16. Electrophoresis at pH 6·7 followed by twice of ascending chromatography with *N*-butanol + acetic acid + water (3 + 1 + 1).

Table 8. *Log titres (to base 2) of antisera against 'recombinant antigens' after every absorption with their parental type antigens*

Antiserum was absorbed with the mixture of 0·5 g (in wet weight) of each absorbing strains per 1·0 ml. serum at one time. Organisms lysogenized with P22 phage were used for absorption. After every absorption, the remaining activity was tested on the homologous antigens by tube agglutination and expressed as log titre to base 2.

Serum	Absorbed with	Time of absorption				
		0	1	2	3	4
Anti-SJ1740 (Rec.-type I)	TR6 and TR16	15	8	4	3	3
Anti-SJ1743 (Rec.-type II)	TR6 and TR16	14	4	1	< 0	.
Anti-SJ1744 (Rec.-type III)	TR6 and TR16	14	4	1	< 0	.
Anti-SJ1747 (Rec.-type IV)	TR6 and TR16	15	9	5	4	4
Anti-SJ1748 (Rec.-type V)	TR6 and TR17	14	5	1	< 0	.
Anti-SJ1749 (Rec.-type VI)	TR16 and TR17	15	7	3	2	2
Anti-SJ1756 (Rec.-type VII)	TR16 and TR17	14	7	1	< 0	.
Anti-SJ1761 (Rec.-type VIII)	TR16 and TR17	15	7	1	< 0	.
Anti-SJ1762 (Rec.-type IX)	TR16 and TR17	14	5	1	< 0	.
Anti-SJ1764 (Rec.-type X)	TR16 and TR17	15	6	1	< 0	.
Anti-SJ1765 (Rec.-type XI)	TR16 and TR17	15	6	1	< 0	.

Tryptic peptide maps of flagellins of the three parental type strains and two of the 'antigen recombinants' are shown in Fig. 1. The approximate number of spots was 35 to 36 for every specimen and almost all the spots, except a few, seemed to be common to all parental and recombinant-type strains. Five spots in all were distinguishable among the maps of the parental-type strains: two spots (A, B in Fig. 1) were specific for TR6, two (C, D) for TR16, and one (E) for TR17. These different spots

were found in various combinations on the maps of the 'antigen recombinants', and no other spot except those found on the maps of the parental-type strains was detected. For example, three spots were found besides the common spots on the map of SJ1744, a recombinant of TR6 and TR16: one was the same as B specific for TR6 and the other two were the same as C and D specific for TR16, respectively. This proves that flagellin of SJ1744 was a mosaic, made up of parts of the flagellins of TR6 and TR16. The distribution of the spots A–E among the maps of the 'antigen recombinants' is shown in Table 9. It must be kept in mind that there must have been other different peptides which were indistinguishable in the finger-printing maps of the present experiment.

Table 9. *Distribution of the differing spots on the peptide maps of flagellins of the 'antigen recombinants'*

Recombinant between *fla*⁻ mutants of	Strain	Differing spots*				
		A	B	C	D	E
	(Parental type) TR6	+	+	.	.	.
	(Parental type) TR16	.	.	+	+	.
	(Parental type) TR17	.	.	.	.	+
TR6 and TR16	SJ1740	+	.	.	.	.
	SJ1743	+	+	.	.	.
	SJ1744	.	+	+	+	.
	SJ1747	.	.	+	+	.
TR6 and TR17	SJ1748	+	+	.	.	.
TR16 and TR17	SJ1749	.	.	.	.	+
	SJ1756	.	.	+	+	.
	SJ1761	.	.	+	+	.
	SJ1762	.	.	.	.	+
	SJ1764	.	.	.	.	+
	SJ1765	.	.	.	.	+

* As for the differing spots A to E, refer to Fig. 1.

Mapping of antigenic specificity-determining sections and sections corresponding to some tryptic peptides of flagellins within H1

Provided that every 'antigen recombinant' resulted from a single cross-over within *H1*, their origins were inferred from their antigenic factor compositions (Table 6) and their tryptic peptide compositions (Table 9). The results are illustrated in Fig. 2 where *flaA* and *flaL* are placed on the left and right of *H1*, respectively. The italicized symbols g_1, g_2, *A*, *etc.* in the figure represent sections corresponding to the respective factors or peptides. From these inferred origins, an attempt was made to map antigenic specificity-determining sections and sections for the differing peptides within *H1*.

First, the 'antigen recombinants' between the *fla*⁻ mutants of TR6 and TR16 illustrated in Fig. 2 (*a*) will be considered. In SJ1747, g_2 is on the left and separated by the presumed cross-over point from the other sections g_1, g_4, *f*, *t*, *A*, *B*, *C* and *D*. In SJ1743, not only g_2 but also g_1 is separated from the other sections. Furthermore, g_2, g_1 and *B*, and g_2, g_1, *B*, and g_4 are separated from the other groups in SJ1744 and SJ1740, respectively. These suggest the following arrangement of the sections.

$$-flaA-\overbrace{g_2-g_1-B-g_4-(f,\ t,\ A,\ C,\ D)}^{H1}-flaL-$$

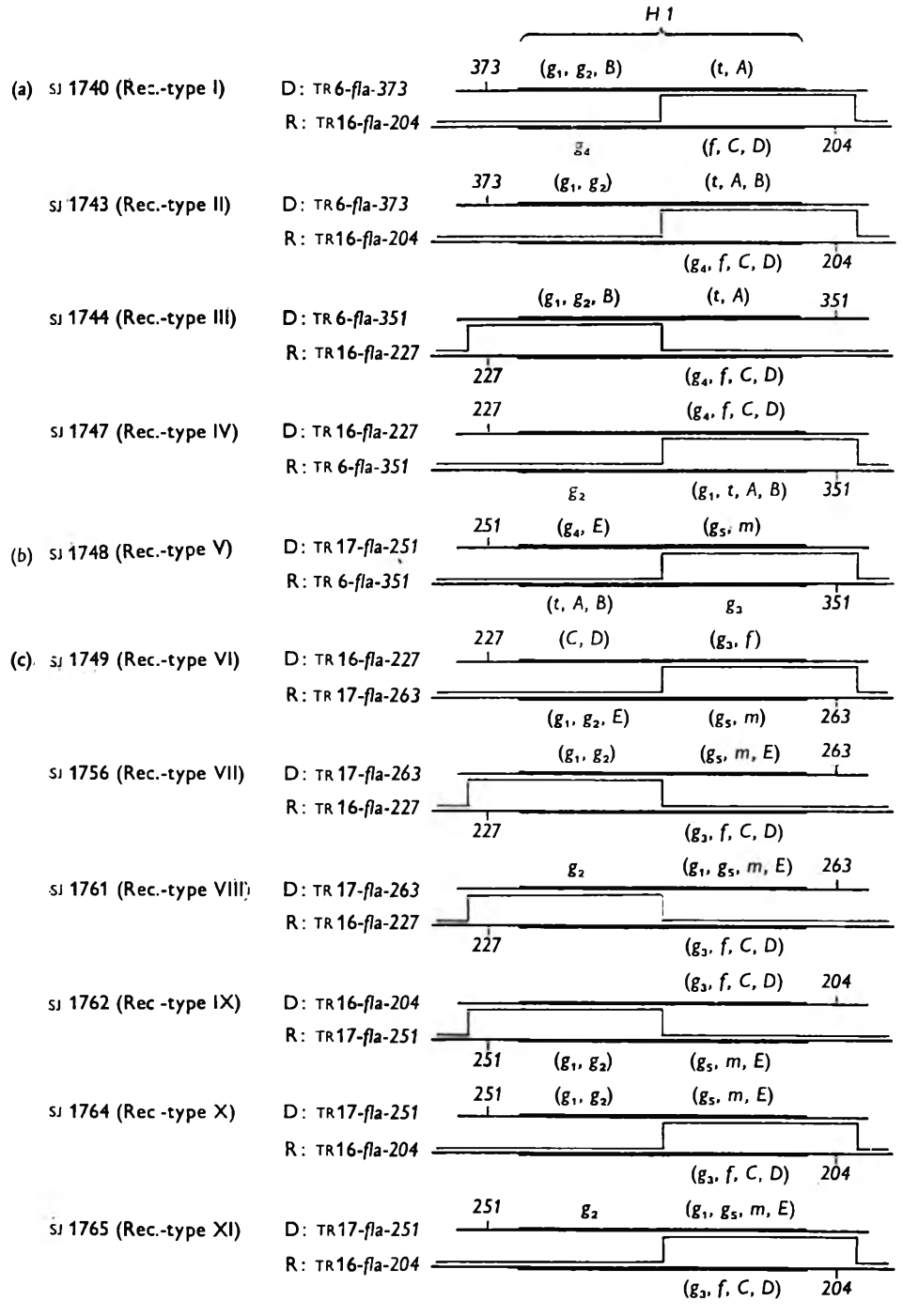

Fig. 2. Inferred origines of the 'antigen recombinants' through a single intra-*H1* recombination in P22 phage-mediated transduction. (*a*) Recombinants between *fla*⁻ mutants of TR6 and TR16; (*b*) between those of TR6 and TR17, and (*c*) between those of TR16 and TR17. D, donor; R, recipient. g_1, g_2, *etc.*, antigenic specificity-determining sections. *A*, *B*, *etc.*, sections in *H1* for the peptides A to E of the tryptic digests of flagellins (refer to Fig. 1).

Furthermore, SJ1748 (Fig. 2*b*), the only one 'antigen recombinant' obtained between *fla*⁻ mutants of TR6 and TR17, suggests the arrangement

$$-flaA-(g_4,\ t,\ A,\ B,\ E)-(g_3,\ g_5,\ m)-flaL-$$

In SJ1761 and SJ1765, the 'antigen recombinants' between *fla*⁻ mutants of TR16 and TR17 (Fig. 2*c*), g_2 is on the left separated from the other sections g_1, g_3, g_5, f, m, C, D, and E; and in SJ1715, SJ1762 and SJ1764, g_2 and g_1 are separated from others to the left. In SJ1749, g_2, g_1, C, D, and E are separated to the left. Thus, the following order is obtained:

$$-flaA-g_2-g_1-(C,\ D,\ E)-(g_3,\ g_5,\ f,\ m)-flaL-$$

On considering the results as a whole the order is that shown in Fig. 3. No contradiction has been found for any of the 'antigen recombinants' considered.

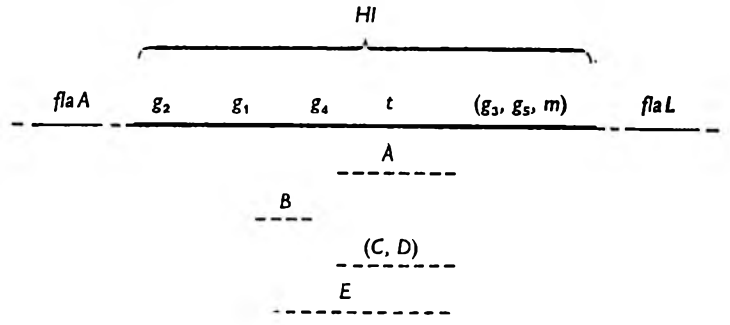

Fig. 3. Arrangement of the antigenic specificity-determining sections and locations of the sections for some tryptic peptides of flagellins within *H1*. Section *f* is somewhere to the right of g_4, *C*, *D*, and *E*. The orders of the sections in parentheses are not yet determined. *A*, *B*, *C*, *D*, and *E* are somewhere on the respective regions shown by dotted lines.

An attempt to assign antigenic specificities to the specific peptides

It is to be expected that a section specifying a tryptic peptide covering the whole or a part of an antigenic determinant of flagellin will behave in intra-*H1* recombination always in company with the antigenic specificity-determining section corresponding to that determinant. From this point of view, the sections *A* to *E* were examined for their association with any one of the antigenic specificity-determining sections by checking the data presented in Fig. 2. The section *A* was always associated with section *t* so far as the present study shows. The other sections *B*, *C*, *D*, and *E* were not associated with any one of the antigenic specificity-determining sections considered here.

DISCUSSION

Salmonella mutants with altered flagellar antigens may arise through mutation in the *H* gene (Iino & Mitani, 1964; Joys & Stocker, 1966; Yamaguchi & Iino, 1966). On occasion the serological character of some flagellar antigens is also modified by a gene, *nml* (Dr B. A. D. Stocker, personal communication), whose product, probably

an enzyme, is thought to methylate some lysine residues of flagellin to ε-*N*-methyllysine (NML) residues (Stocker, McDonough & Ambler, 1961; Kerridge, 1966). We must therefore consider the possibility of mutation in either *H1* or *nml* as well as of recombination within *H1* as the origin of the 'antigen recombinants' described here. Against this is the behaviour of mutants with altered flagellar antigens selected by cultivation of cells in semi-solid medium containing anti-flagellar antiserum. In this way, we isolated sixteen antigen type mutants from a gn-complex antigenic strain (Yamaguchi & Iino, 1966). The frequency of their occurrence was 10^{-6} to 10^{-7}/inoculated organism. In each case, a factor or several factors had been only slightly altered, so retaining cross-reactivity with the original factor or factors. A mutant that had lost one or more factors and/or had gained one or more rather strong ones, such as the 'antigen recombinants', was not found. The possibility may therefore be excluded that the 'antigen recombinants' occurring here at the high frequency of 2×10^{-2} (26 clones/1366 *fla*$^+$ transductants) had arisen through mutation.

On the assumption that each 'antigen recombinant' arose by a single cross-over within *H1*, mapping within *H1* was performed. All the serological data and the results of finger-printing so far obtained are consistent with this assumption, indicating that each of the antigenic specificity-determining sections locates as a unit and that as a whole they are arranged linearly within *H1*.

The present study was made possible by the following facts: (1) *fla*$^-$ mutants whose mutations are closely linked to *H1* and at either side of it were obtained; (2) as the result of further analysis of the factor compositions of the antigens used, many kinds of anti-factor(s) sera became available; (3) antigenic factors on the 'recombinant antigens' retained almost the same serological character as that of the corresponding factors on the parental type antigens.

In the Kauffmann–White scheme (Kauffman, 1964), nine factors of the gn-complex antigens were described: f, g, m, p, q, s, t, u, and z_{51}. In the present study, it has been shown that the factor g is a complex composed of two or more of at least 5 factors, g_1 to g_5. It must be mentioned here that, if these gn-complex antigens are modified by the presence or absence of ε-*N*-methyllysine (NML), namely *nml*$^+$ or *nml*$^-$, the analysis of the gn-complex antigens without NML may give somewhat differing results from the present one.

The presence of chromosomal sections marked with the respective antigenic specificities of flagellin within *H1* has been shown in the present study, but this does not necessarily mean that the specificity of an antigenic determinant of flagellin depends exclusively on the amino acid sequence or the primary structure specified by the corresponding section. It has been reported that ribonuclease oxidized by performic acid, a process which markedly alters its conformation without any change in its primary structure, does not cross-react serologically with native ribonuclease (Brown, 1962). It has also been reported that in Salmonella, several antigenic factors present on the wild-type flagellin may change simultaneously as a result of only one presumptive amino acid replacement (Joys & Stocker, 1966; Yamaguchi & Iino, 1966). Therefore, the amino acid sequence determined by an antigenic specificity-determining section manifests the specific antigenicity only when it has a specific tertiary structure in the whole conformation of the flagellin molecule.

Although some new specificities with weak antigenicity were detected on several 'recombinant antigens', all the 'recombinant antigens' obtained here are almost

perfect mosaics of the parental antigens in their serological nature. This implies that the tertiary structure of individual antigenic determinants on the recombinant flagellins had suffered little or no change by recombination. This may also be expected from the strong resemblance between the tryptic peptide maps of the parental flagellins. Experiments to obtain 'recombinant antigens' between the non-cross-reacting antigens a and gn-complex were carried out in parallel with the present study, but were unsuccessful. One of the reasons may be that since a and gn-complex flagellins differ considerably in their amino acid composition (McDonough, 1965), recombinant flagellins, if produced, might be so abnormal in their conformation that they could not form motile flagella.

In globular proteins like flagellin, the folding of the polypeptide chains brings into apposition amino acids remote from each other in the primary structure. Some antigenic determinants may therefore include amino acids from two or more points along the chain. For such a determinant, the antigenic specificity-determining section should locate as two or more units within the structural gene. Although it was shown that each of the antigenic specificity-determining sections considered in the present study is located as a unit within *H1*, this does not exclude the above possibility.

Many attempts have been made to define the structural features of antigenic determinants of globular proteins by isolating and characterizing fragments of the whole molecule which retain the ability to combine with specific antiserum. In the present study, an attempt was made to assign antigenic specificities of flagellin to some specific tryptic peptides, on the assumption that the tryptic peptide corresponding to an antigenic determinant on the flagellin molecule always corresponds to the antigenic specificity manifested by the determinant in intra-*H1* recombination. Though the data are as yet insufficient for complete assignment, the present results are sufficient to suggest that more peptide analysis will make this approach possible.

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A Comparison of the Polygalacturonases Produced *in vivo* and *in vitro* by *Penicillium expansum* Thom.

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SUMMARY

Polygalacturonase (PG) was produced by *Penicillium expansum* in rotted apples and in liquid culture medium containing pectin; acetone precipitates of the enzyme were prepared from both sources. The PG extracted from apples (*in vivo* PG) behaved as an endo-PG and degraded sodium polypectate to tetragalacturonic acid. PG from culture filtrates (*in vitro* PG) before purification had quite different properties, the final hydrolysis product being monogalacturonic acid. PG from both sources was purified by ion-exchange chromatography. The properties of some purified PG preparations were quite different from those of the crude material. None of the purified *in vitro* PG released monogalacturonic acid from sodium polypectate, whilst some preparations from both sources had identical endo-PG properties after purification. Changes in the properties of endo-PG produced *in vivo* and *in vitro* were apparent during purification and a series of distinct endo-PG's were obtained which yielded unresolved oligomers, tri- or tetragalacturonic acid as final hydrolysis products. It is suggested that the changes in behaviour of the various PG preparations during purification were brought about by changes in the molecular configuration of the enzyme, and that such changes could account for some of the variability between enzymes produced *in vivo* and *in vitro*.

INTRODUCTION

Pectic glycosidases (poly α-1,4-galacturonide glycano hydrolase E.C. 3.2.1.15) have been classified according to their substrate specificity and the position of the bonds which they hydrolyse. Thus polygalacturonases (PG) preferentially attack pectic acid whilst polymethylgalacturonases (PMG) preferentially attack pectins of high methoxyl content. Endo-PG and endo-PMG were further defined as hydrolysing the α-1,4-glycosidic bonds in the substrate at random, whilst exo-PG and exo-PMG hydrolyse the terminal link (Demain & Phaff, 1957). Poly α-1,4-D-galacturonide lyases (E.C. 4.2.99.3) have been similarly classified (Bateman & Millar, 1966). It has been inferred that pectic enzymes produced by the pathogen are involved in the development of plant disease in a variety of host/pathogen combinations (Husain & Kelman, 1959), particularly for soft rots of fleshy organs. Difficulties in assessing the individual role of these enzymes are primarily due to problems of extraction from host tissue, resulting

from inactivation by oxidised host polyphenols (Akinrefon, 1967), and this has frequently led to the use of *in vitro* produced enzymes in experimental studies. Differences between enzymes produced *in vivo* and *in vitro* have been frequently demonstrated (Bateman & Millar, 1966). *Botrytis cinerea* produces different types of enzymes in culture media at various pH values (Damle, 1952) and yet different enzymes on onion leaves (Hancock, Millar & Lorbeer, 1964). *Penicillium expansum*, which is responsible for considerable wastage in stored apple fruit, produced endo-polygalacturonase *in vivo* and endo-polymethyl-galacturonase *in vitro* where pectin was the sole carbon source (Cole & Wood, 1961). Oxidation of polyphenolic materials does not occur to any great extent in *P. expansum* rotted apples, and extracts have relatively high degrees of pectic enzyme activity (Cole & Wood, 1961). By using various separation techniques, particularly ion-exchange chromatography, isolation and purification of pectic enzyme mixtures produced simultaneously by some fungi has been achieved (Rexova-Benkova & Slezarik, 1966; McClendon & Kreisher, 1963; Endo, 1963), and the presence of distinct types of endo-PG demonstrated (McClendon & Kreisher, 1963; Endo, 1963). The formation and dissociation of enzyme complexes during ion-exchange chromatography has also been recorded (Swinburne & Corden, 1967). The objective of the present work was to separate the various PGs produced *in vivo* and *in vitro* by *Penicillium expansum* and to compare their properties.

METHODS

The isolate of *Penicillium expansum* used in this work came from a naturally rotted Bramley's Seedling apple obtained from Co. Armagh, Northern Ireland, and was maintained on 2% malt agar.

PGs produced *in vitro* were obtained from shake cultures grown in a modified Richards medium containing (g./l.): 4, $MgSO_4.7H_2O$; 5·0, NH_4NO_3; 2·5, KH_2PO_4; 0·02, $ZnSO_4$; 0·02, $FeCl_3.6H_2O$; 10, pectin N.F. (Sunkist Inc.); in tap water. The pH value of the medium increased from 4·2 to 4·7 after 6 days' incubation at 25°. The organism was removed by centrifugation and discarded. Toluene (0·1 %, v/v) was added to the supernatant fluid as preservative and the supernatant fluid dialysed against running tap water for 18 hr. At each stage samples of the enzyme mixture were plated for sterility. Several methods for the precipitation of the PG from the dialysate were evaluated. The method finally adopted was to add slowly an equal volume of acetone at −20° to the dialysate at 0°. The mixture was allowed to stand for 15 min., the precipitate removed by centrifugation and redissolved in a minimum volume of water and stored at −20°. Approximately 83% of the PG, as determined viscometrically, was recovered.

Pectic enzymes produced *in vivo* were obtained from rotted cortical tissue removed from Bramley's Seedling apples held 15 days at 25° following artificial inoculation with *Penicillium expansum* by a cork borer method (Granger & Horne, 1924). The rotted tissue was first frozen to aid maceration and 2 kg. samples homogenized with 2 l. 0·5 N-NaCl containing 5% (w/v) polyvinylpyrrolidone (General Aniline and Film Co., Polyclar AT; Akinrefon, 1967). The homogenate was pressed through muslin and filtered through Whatman no. 1 paper until a clear solution was obtained. After adding toluene (0·1 %, v/v) the filtrate was dialysed against running tap water for 18 hr. To obtain a suitably flocculent precipitate of protein from the dialysate with

acetone it was found necessary first to add NaCl (0·2 g./100 ml.) followed by 2 vol. acetone. After the precipitate had been redissolved in water, viscometric assay indicated that 88% of the original PG had been recovered.

Enzyme+substrate reaction mixtures contained 0·5% (w/v) sodium polypectate (Sunkist), 10% (v/v) enzyme sample in 0·1 M-sodium acetate buffer (pH 5·0) with 0·001% (v/v) Merthiolate (Eli Lilly and Co.) as a preservative. Controls contained boiled enzyme or an equivalent volume of water. For viscosity decrease assays 8 ml. samples of the reaction mixture previously equilibrated to 37·5° were rapidly transferred to Cannon-Fenske viscometers (size 200) and the time taken (t) for the relative viscosity to fall to 50% (ηR_{50}) of the initial value determined by the method of Wood (1955). Units of PG activity were calculated as $1000/t$. Release of reducing groups in various enzyme+substrate reaction mixtures incubated at 37·5° were determined colorimetrically with dinitrosalicylic acid reagent (Miller, 1959) and calculated as μg. galacturonic acid/ml. The percentage of galacturonic acid anhydride units in the sample of sodium polypectate used throughout these studies was estimated with carbazole reagent (Cole & Wood, 1961) to be 63·3% (w/w). The quantity of galacturonic acid that would be present in the enzyme+substrate reaction mixture when the hydrolysis was complete was calculated from this, and the hydrolysis at any stage was calculated as % of the galacturonic acid equivalent found by reducing-group methods of this quantity. For determinations of % hydrolysis at ηR_{50} viscometric and reducing-group assays were made simultaneously on the same reaction mixture.

Hydrolytic products in the reaction mixtures were identified chromatographically (Young & Corden, 1964). Reference spots of authentic monogalacturonic acid were included on each paper. Other oligomers were identified by their R_F values, and the identification was checked by calculating the Rm value, where $R\text{m} = \log([1/R_F]-1)$ (Bate-Smith & Westall, 1950). A linear relationship between Rm and the number of galacturonic acid residues in each oligomer to pentagalacturonic acid was found.

Molecular exclusion chromatography of various PG preparations were done on columns of Sephadex G-200 (Pharmacia Ltd.) equilibrated with 0·1 M-sodium acetate buffer (pH 5·0). A column 42 × 2·5 cm. was prepared and fitted with a water jacket which enabled separations to be carried out at 10°. Samples containing PG were brought to approximately 5% (w/v) sucrose to increase their density and 3 ml. samples were layered by syringe pipette on top of the column beneath the eluant head. The column was eluted with the buffer at a rate of approximately 10 ml./hr and 4·4 ml. fractions collected.

Ion-exchange chromatography of the various PG preparations were done on columns of either diethylaminoethylcellulose (DEAE-cellulose; Whatman D.E. 52) or carboxymethylcellulose (CM-cellulose; Whatman CM. 52). After precycling the exchangers according to the manufacturers' instructions, DEAE cellulose was equilibrated with tris-HCl buffer (pH 7·0; $I = 0{\cdot}05$) and CM cellulose was equilibrated with 0·01 M-sodium acetate buffer (pH 5·0). Columns 45 to 50 cm. × 2 cm. were prepared from each of the exchangers, and 10 ml. samples containing PG applied. Eluant was delivered, at room temperature, to the column by a peristaltic pump at a flow rate of approximately 80 ml./hr and consisted of buffer with a linear gradient of sodium chloride concentration. Fractions of 10·8 ml. were collected.

PG in the fractions collected from the various columns was assayed by a cup-plate method (Dingle, Reid & Solomons, 1953). A dilution series of the enzyme applied to

the column was included on each plate, enabling the relative % PG concentration of each fraction and the over-all recovery to be estimated. The presence of sodium chloride in enzyme preparations did not alter the size of the halos they produced in this assay.

Fractions within individual peaks of PG eluted from the various columns were bulked and concentrated to approximately the volume of the original sample by dialysis against Carbowax 20 M. in Visking tubing at 2°. The concentrates were then stored at −20°.

Protein concentrations were estimated by determining the extinction at 260 and 280 mμ (Beckman D.B.-G.) and reference to the data of Warburg & Christian (1942).

RESULTS

Properties of the crude in vivo and in vitro PG preparations

The crude *in vitro* PG contained 0·10 unit PG/μg. protein and the *in vivo* preparation 0·36 unit/μg. protein, as determined viscometrically with sodium polypectate as substrate. When pectin was used as the substrate the viscometric activities of both preparations were approximately 5% of these values, indicating that PMG was not present in either.

No enzymes of the transeliminase type were detected by the micromethod of Ayers, Papavizas & Diem (1966) in either preparation incubated with pectin or sodium polypectate at pH 5·0 or 8·0.

Table 1. *The hydrolysis of sodium polypectate by crude in vivo and in vitro PG of Penicillium expansum and the nature of the hydrolytic products*

	Crude *in vivo* PG							Crude *in vitro* PG						
		Hydrolytic products*							Hydrolytic products*					
Time (hr)	% Hydrolysis	1	2	3	4	5	FR	% hydrolysis	1	2	3	4	5	FR
1	40·6	−	−	+	+	++	+	64·9	+	±	±	+	+	+
4	50·8	±	−	+	++	+	+	75·1	+++	−	−	−	−	±
8	50·1	.	.	.	.	.	.	78·2	.	.	.	.	.	.
10	.	+	−	+	++	±	+	.	+++	−	−	−	−	−
24	52·0	+	−	+	+++	−	−	92·2	+++	−	−	−	−	−

* Columns 1 to 5 mono- to penta-galacturonic acid; FR, unresolved oligomers; −, none detected; ±, trace; ++, relative concentration.

The hydrolysis of sodium polypectate by both enzyme preparations was followed over a 24 hr period, and the nature of the breakdown products was determined (Table 1). Monogalacturonic acid was the principal hydrolytic product released by the *in vitro* PG and virtually the only compound detectable after 4 hr incubation. The hydrolysis by the *in vitro* PG had reached 92% after 24 hr and would probably have been completed in a longer time. With the *in vivo* PG, however, only slight increase in hydrolysis occurred after 4 hr incubation when approximately half the available substrate bonds had been hydrolysed. Monogalacturonic acid in small quantities was detected at the later stages of hydrolysis, but the principal breakdown product was tetragalacturonic acid, with a small amount of trigalacturonic acid found at all stages (Table 1).

The difference between the crude *in vitro* and *in vivo* PG was further illustrated by

the values of the % hydrolysis of sodium polypectate at ηR_{50} for both enzymes (Table 4). Thus crude *in vitro* PG hydrolysed more than three times as many bonds as the crude *in vivo* PG to achieve the same reduction in viscosity.

Single peaks of PG were separated from both crude *in vivo* and *in vitro* preparations on Sephadex G. 200 (Fig. 1). The elution volumes of PG from both sources were almost identical, the average of duplicate experiments being 154 ml. for *in vitro* PG and 156 ml. for *in vivo* PG. Approximately quantitative recovery of PG was obtained from both preparations. Non-enzyme protein was eluted immediately following the void volume of the column and after the PG peaks from both crude preparations (Fig. 1). Rechromatography of the PG within each peak on Sephadex G. 200 resulted in elution at the same volume for each.

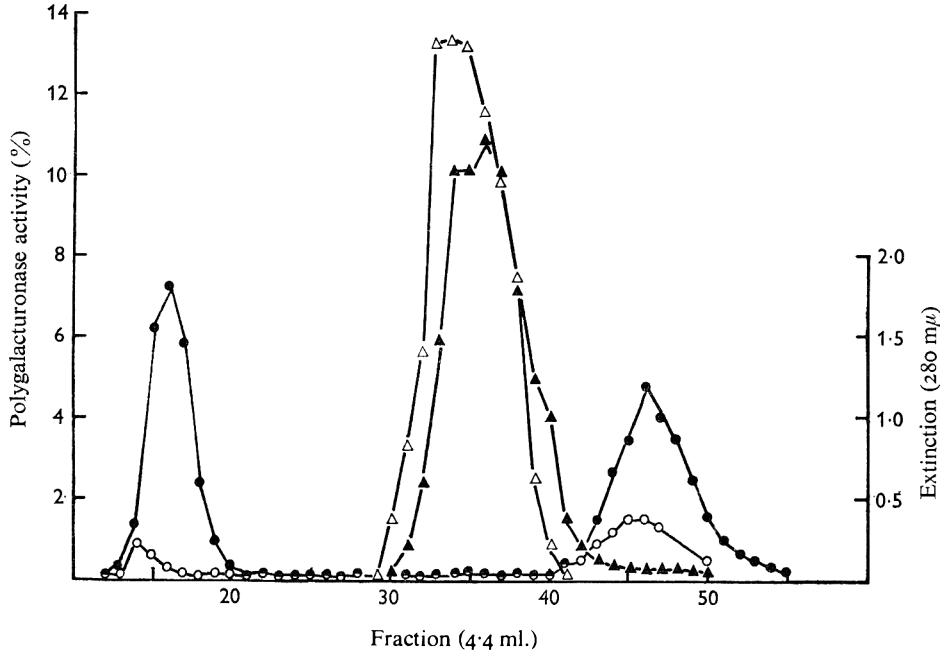

Fig. 1. Elution of PG produced *in vitro* and *in vivo* by *Penicillium expansum* from a column of Sephadex G. 200. PG activity is expressed as a % of the sample applied; —▲—, *in vivo* PG; —△—, *in vitro* PG. Extinction values at 280 mμ of fractions shown as —●— for *in vivo* preparation, —○— for *in vitro* preparation.

DEAE-cellulose chromatography of crude in vivo and in vitro PG

None of the PG in the *in vivo* preparation was retained by DEAE-cellulose, but was eluted in a single peak with the void volume (Fig. 2). Most of the protein was adsorbed and eluted as a single peak after the PG. In three separate experiments 74–75% of the *in vivo* PG applied to the columns was recovered, representing about a 200-fold purification. Likewise the PG in the crude *in vitro* preparations passed through DEAE-cellulose columns with the void volume and was eluted as a single peak whilst most of the protein was adsorbed and eluted at a higher salt concentration (Fig. 2). Recovery of *in vitro* PG from these columns was somewhat variable, the average of three experiments being 101%, representing a threefold purification.

After concentrating the preparations eluted from DEAE-cellulose of *in vitro* and *in vivo* PG, henceforward referred to as *in vitro* and *in vivo* DEAE-PG, respectively, to their original volume, they were separately applied to the Sephadex G. 200 column. The *in vitro* DEAE-PG was eluted in a single peak with an elution volume of 154 ml., which was identical to that of the crude *in vitro* PG. The *in vivo* DEAE-PG was similarly eluted as a single peak with an elution volume of approximately 157 ml. which corresponded closely to that obtained for the crude enzyme. The % hydrolysis with time of sodium polypectate by *in vitro* and *in vivo* DEAE-PG was followed, the nature of the hydrolytic products determined, and the value of the percentage hydrolysis at ηR_{50} was obtained (Table 2).

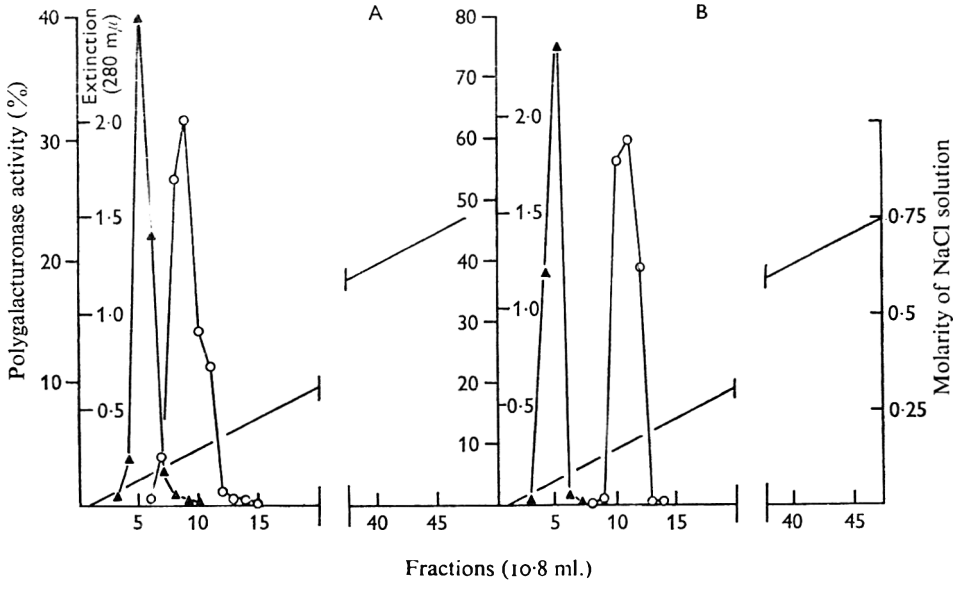

Fig. 2. Purification of PG produced *in vitro* and *in vivo* by *Penicillium expansum* on columns of DEAE-cellulose eluted with Tris-HCl buffer pH 7·0 and a linear gradient of sodium chloride. A. *in vivo* preparation; B, *in vitro* preparation. —▲—, PG activity expressed as a % of the sample applied to the column; —○—, extinction value at 280 mμ. Approximate salt concentrations shown by solid line.

Table 2. *The hydrolysis of sodium polypectate by in vivo and in vitro DEAE-PG of Penicillium expansum and the nature of the hydrolysis products*

	In vivo DEAE-PG							*In vitro* DEAE-PG						
		Hydrolytic products*							Hydrolytic products*					
Time (hr)	% Hydrolysis	1	2	3	4	5	FR	% Hydrolysis	1	2	3	4	5	FR
1	45·9	−	±	±	±	±	+++	40·1	−	−	−	−	−	+++
4	55·4	−	±	±	+	+	++	52·1	−	−	±	±	+	++
10	60·0	−	±	±	++	++	+	.	.	.	.	.	.	.
24	63·0	−	±	+	+++	±	±	60·6	−	−	±	++	++	+

* Columns 1 to 5 mono- to penta-galacturonic acid; FR, unresolved oligomers; −, no detected; ±, trace ++, relative concentration.

The final % hydrolysis of the substrate achieved by the *in vivo* DEAE-PG after 24 hr was higher than with the crude *in vivo* PG, although the principal hydrolytic products that accumulated, namely tetra- with a little tri-galacturonic acid, were the same for both preparations. Monogalacturonic acid was not detected at any stage of the hydrolysis, although a trace of digalacturonic acid was present throughout. There was no significant difference between the values of the % hydrolysis at the point where 50% viscosity loss occurred for crude *in vivo* PG and for *in vivo* DEAE-PG (Table 4).

The properties of the *in vitro* DEAE-PG were however quite different from those of the original crude material. The degree of hydrolysis by *in vitro* DEAE-PG after 24 hr was very much less than that obtained with the crude *in vitro* PG (Table 2) and the principal hydrolytic products were tetra- and penta-galacturonic acids with a small quantity of trigalacturonic acid; monogalacturonic acid was not detected at any stage. This difference was also reflected in the % hydrolysis at ηR_{50}, the value for the *in vitro* DEAE-PG was less than half that of the crude *in vitro* PG (Table 4). This value was also significantly different from that obtained with crude *in vivo* PG and the *in vivo* DEAE-PG in spite of the similarity between the hydrolytic products released by these three enzyme preparations (Table 4).

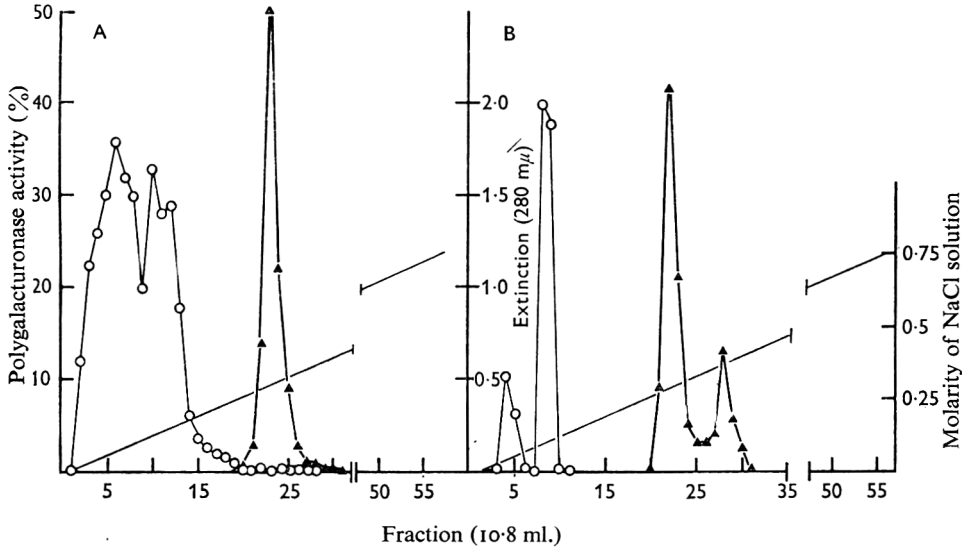

Fig. 3. Purification of PG produced *in vivo* and *in vitro* by *Penicillium expansum* on columns of CM-cellulose eluted with 0·01 M-sodium acetate (pH 5·0) and a linear gradient of sodium chloride. A, *in vivo* preparation; B, *in vitro* preparation. —▲—, PG activity expressed as a % of the sample applied to the column; —○—, extinction values at 280 mμ. Approximate salt concentration shown as a solid line.

CM-cellulose chromatography of crude in vivo and in vitro PG

All the PG in the crude *in vivo* enzyme preparation was adsorbed on CM-cellulose columns, and eluted as a single peak by approximately 0·3 M-NaCl, whilst most of the other protein was eluted at or near the void volume of the column (Fig. 3). In three separate experiments an average of 94% of the crude *in vivo* PG applied to these columns was recovered, representing an approximately 100-fold purification after concentration to approximately the original volume (referred to as *in vivo* CM-PG).

All the PG in the crude *in vitro* enzyme preparation was also adsorbed on CM-cellulose, but was eluted in two separate peaks (Fig. 3). The first peak was eluted by approximately 0·3 M-NaCl and contained an average of 75% of the PG recovered, whilst the second peak containing an average of 25% of the recovered PG was eluted by 0·35 M-NaCl. The recovery of crude *in vitro* PG applied to such columns was consistently high, the average of four separate experiments being 118%. Most of the inactive protein in the crude *in vitro* PG preparation was eluted at low salt concentrations (Fig. 3). The fractions within each peak were concentrated to approximately the volume of the original sample. The PG in the first peak, referred to as *in vitro* CMI-PG, represented an eightfold purification, whilst the PG in the second peak, referred to as *in vitro* CMII-PG, represented a fourfold purification of the original crude material.

The *in vivo* CM-PG was eluted from the Sephadex G. 200 column as a single peak in a volume of approximately 145 ml., which was less than the elution volume of the original crude enzyme. Similarly, the *in vitro* CMI-PG was eluted as a single peak in 141 ml., which was less than the crude enzyme, whilst *in vitro* CMII-PG was eluted as a somewhat broad peak in a volume of between 158 and 167 ml.

Table 3. *The hydrolysis of sodium polypectate in vivo CM-PG and in vitro CMI-PG*

	In vivo CM-PG							*In vitro* CMI-PG						
Time (hr)	% Hydrolysis	Hydrolytic products* 1	2	3	4	5	FR	% Hydrolysis	Hydrolytic products* 1	2	3	4	5	FR
1	55·3	.	.	.	.	.	.	.	−	−	−	−	−	+++
4	56·3	−	±	±	+	++	+	37·8	.	.	.	.	.	.
10	57·6	−	+	++	+	±	−	.	.	.	.	.	.	.
24	71·0	−	+	+++	±	−	−	68·0	+	±	±	++	−	+

* Columns 1 to 5 mono- to penta-galacturonic acid; FR, unresolved oligomers; −, none detected; ±, trace; ++, relative concentration.

The *in vivo* CM-PG achieved a considerably higher % hydrolysis of sodium polypectate in 24 hr (Table 3) than did the crude *in vivo* PG (Table 1). Penta- and tetragalacturonic acid were the main hydrolytic products detected after 4 hr incubation, but at all subsequent stages of the hydrolysis only trigalacturonic remained in quantity, with some digalacturonic acid, whereas only traces of tetragalacturonic acid could be detected (Table 3). The percentage hydrolysis of sodium polypectate at ηR_{50} was twice that obtained with the crude *in vivo* PG and this value was also significantly different from both crude *in vitro* PG and *in vitro* DEAE-PG (Table 4).

Although the *in vitro* CMI-PG hydrolysed sodium polypectate more completely than the *in vitro* DEAE-PG (Table 2) the final % of hydrolysis was still considerably less than had been achieved by the crude *in vitro* PG. Tetragalacturonic acid was the principal hydrolytic product after 24 hr incubation. In contrast with *in vitro* DEAE-PG, monogalacturonic acid was present after 24 hr. The value for the % hydrolysis of sodium polypectate at ηR_{50} by *in vitro* CMI-PG was not significantly different from those obtained with *in vitro* DEAE-PG or *in vivo* DEAE-PG (Table 4).

The *in vitro* CMII-PG preparation had relatively low activity and consequently it was necessary to follow the hydrolysis of sodium polypectate over 48 hr. No hydrolytic products were detected until after 24 hr of incubation, when 37·9% hydrolysis

had occurred, but only unresolved oligomers were detected. During the next 24 hr only a slight increase in hydrolysis to 43% was obtained, and no further products were detected chromatographically. The value for the % hydrolysis at ηR_{50} was the lowest obtained and significantly different from all other preparations (Table 4).

Table 4. *The percentage hydrolysis of sodium polypectate by various enzyme preparations at the point where half the initial relative substrate viscosity was lost*

Source	Preparation	% hydrolysis	Fiducial limits (5%)
In vivo	Crude precipitate	1·7	±0·38
	DEAE preparation	1·9	±0·71
	CM preparation	4·2	±0·32
	DEAE/CM preparation	2·0	±0·08
In vitro	Crude precipitate	6·1	±1·18
	DEAE preparation	2·8	±0·16
	CMI preparation	1·8	±0·38
	CMII preparation	0·34	±0·81
	DEAE/CM preparation	1·8	±0·10

Rechromatography of in vivo and in vitro DEAE-PG on CM cellulose

After concentration to approximately their original volume, 10 ml. of *in vivo* and *in vitro* DEAE-PG were separately applied to CM-cellulose columns which were eluted with a linear gradient of NaCl. All the PG in the *in vivo* DEAE preparation was adsorbed on these columns and was eluted as a single peak by approximately 0·3 M-NaCl, corresponding exactly to the crude *in vivo* PG. Approximately 105% of the *in vivo* DEAE-PG applied to CM cellulose columns was recovered (referred to as *in vivo* DEAE/CM-PG).

The PG in the *in vitro* DEAE preparation was also adsorbed on CM cellulose, and was similarly eluted as a single peak by approximately 0·3 M-NaCl. No PG peak corresponding to *in vitro* CMIIPG was obtained. In two experiments 72 and 82% of the *in vitro* DEAE-PG applied to CM cellulose columns was recovered (referred to as *in vitro* DEAE/CM-PG). After reconcentrating both the *in vivo* and *in vitro* DEAE/CM-PG preparations to their original volumes they were separately applied to the Sephadex G. 200 column. The *in vivo* DEAE/CM-PG was eluted in a single peak in a volume of approximately 150 ml., which was only slightly less than that obtained for the crude *in vivo* PG. *In vitro* DEAE/CM-PG was also eluted as a single peak with an elution volume identical to the original crude material, namely 154 ml. There were no significant differences in values for % hydrolysis at ηR_{50} between either of the DEAE/CM preparations nor were they different from *in vitro* CMI-PG, *in vivo* DEAE-PG or crude *in vivo* PG (Table 4).

A comparison of cup-plate and reducing-group assays

Because the crude *in vitro* PG rapidly liberated monogalacturonic acid from sodium polypectate the presence of an exo-PG was suspected, but no exo-PG activity was observed in any of the purified preparations. The possibility that exo-PG was present in enzyme fractions but had escaped detection by cup-plate assay was therefore investigated. Fractions eluted from a CM-cellulose column following the application of crude *in vitro* PG were assayed both by the cup-plate method and by determining the

reducing groups released from 1 ml. of 0·5% (w/v) sodium polypectate in 1 hr at 35·5° by 0·1 ml. of each enzyme fraction. Cup-plate assay showed PG activity in two peaks as usual; 70% was recovered in the first peak (*in vitro* CMIPG) and approximately 33% was recovered in the second (*in vitro* CMII-PG). Only those fractions shown to contain PG by cup-plate assay liberated reducing groups from sodium polypectate. Reducing-group assay also showed that 70% of the applied PG was recovered in the first peak. However, the fractions in the second peak only contained approximately 3% of the applied PG, giving an over-all recovery of 73%. Thus the activity of *in vitro* CMII-PG relative to the crude *in vitro* PG as measured by reducing-group assay was only one tenth the cup-plate assay value. In a further comparison the activity of *in vitro* CMIIPG relative to the crude *in vitro* PG was found to be 3·3 and 5·3% by cup-plate and viscometric assays, respectively but only 0·3% by measurement of the rate of release of reducing groups.

DISCUSSION

The differences between the crude pectic enzymes produced *in vivo* and *in vitro* by the isolate of *Penicillium expansum* used in these studies were in the chemical bond hydrolysed rather than in the substrate attacked. Cole & Wood (1961), however, found that other isolates of *P. expansum*, produced PMG *in vitro* and PG *in vivo*. Differences in bond specificity for these enzymes is similar to those found between PG produced *in vivo* and *in vitro* by *Rhizoctonia solani* (Bateman, 1963); thus crude *in vivo P. expansum* PG did not degrade sodium polypectate further than tetragalacturonic acid, whilst crude *in vitro* PG completely hydrolysed the substrate to monogalacturonic acid. It is doubtful whether all or even part of the PG activity observed in the crude *in vitro* preparation can be attributed to exo-PG. Liberation of monogalacturonic acid at an early stage in hydrolysis has been taken to be evidence for the presence of exo-PG (e.g. Hancock *et al.* 1964). However % hydrolysis at ηR_{50} for crude *in vitro* PG of *P. expansum* was considerably lower than for exo-PG produced by *Coniothyrium diplodiella* with pectic acid as the substrate (Endo, 1964). Whilst the value obtained would obviously depend on the initial chain length of the substrate, the figure of 6·1% is probably too low for exo-PG alone. This value could represent the net result of the combined action of exo- and endo-PG; but the inability to distinguish more than one enzyme by molecular exclusion-chromatography of the crude material and the absence of a distinct exo-PG by ion exchange chromatography suggests that only one PG was present. From thermal inactivation studies Bateman (1963) also concluded that, *in vitro*, *R. solani* produced only one PG, whose properties closely resembled the crude *in vitro* PG of this study. No difficulties of classification pertain to the crude *in vivo* PG where all the observed activity can be attributed to endo-PG which did not significantly degrade the substrate beyond tetragalacturonic acid. A similar endo-PG has been described for yeasts (Luh & Phaff, 1954).

One of the most interesting features of this study was that purification of both crude *in vitro* and crude *in vivo* PG of *Penicillium expansum* sometimes yielded enzymes whose properties did not correspond with the original material. Thus none of the ion-exchange cellulose preparations of *in vitro* PG liberated significant quantities of a monogalacturonic acid. The high recovery values obtained indicate that no PG was lost during purification, for example by inactivation of exo-PG in the original crude material. Consistently high recoveries of PG from *Fusarium oxysporum* f. sp. *lyco-*

persici applied as a crude culture filtrate to DEAE-cellulose columns were also obtained by T. R. Swinburne & M. E. Corden (private communication). From a comparison of the results of the various PG assay methods it was apparent that the cup-plate technique was directly comparable with viscometric assay, but not necessarily with reducing-group assay. The cup-plate assay is relatively more sensitive to endo-PG than to exo-PG, and, moreover, enzymes with low percentage hydrolysis at ηR_{50}, such as *in vitro* CMII-PG, produce proportionately larger halos than endo-PGs, such as *in vitro* CMI-PG, which hydrolyse the substrate more completely. This suggests that the differences between crude *in vitro* PG and *in vitro* DEAE-PG and CMI-PG might be due to changes in the properties of the enzyme molecule. If such changes resulted in an over-all increase in the viscometric activity the recovery values on a cup-plate assay would be high. The stability of PG at the pH value of the DEAE-cellulose columns is relatively low (Bateman, 1963) and some inactivation of *in vivo* PG may have taken place. If *in vitro* PG were also inactivated at this pH value then the over-all recovery on DEAE-cellulose might have been even higher if this effect could have been avoided. With one exception, all changes in properties of enzyme preparations resulted in a decrease in % hydrolysis at ηR_{50}. The exception to this was *in vivo* CM-PG, where a marked increase was obtained, and the final hydrolytic product was also changed from tetra- to tri-galacturonic acid. The somewhat low recovery values obtained for *in vivo* PG on CM-cellulose may be related to enzyme inactivation, but the lower sensitivity of the cup-plate assay with such enzymes may also have affected the results.

Most preparations of *Penicillium expansum in vivo* and *in vitro* PG had similar properties. Thus crude *in vivo*-PG, DEAE-PG, DEAE/CM-PG, *in vitro* CMI-PG and DEAE/CM-PG were virtually identical in properties and might reasonably be assumed to be identical enzymes. The remaining preparations, all with properties of endo-PG, were however quite distinct from these and from each other. The simultaneous production of distinct forms of endo-PG has been described for various fungi (McClendon & Kreisher, 1963; Endo, 1963). The three forms of endo-PG separated from bran cultures of *Coniothyrium diplodiella* also differed in the relative ability to reduce substrate viscosity in relation to the number of bonds hydrolysed (Endo, 1963). The existence of forms of endo-PG with different properties shows that some PGs are, paradoxically, 'more random' in their hydrolytic action than others, a similar situation to that described for cellulases (Nisizawa, Hashimoto & Shibata, 1963). If the hydrolysis of $\alpha 1:4$-glycosidic bonds in pectate substrates by endo-PG were truly random the percentage hydrolysis at ηR_{50} for most endo-PG preparations would be similar, since the number of bonds hydrolysed at ηR_{50} is a small proportion of the total. That this is not so is apparent from this and other studies (Endo, 1963).

The cause of the changes in enzyme properties during purification cannot be ascertained from the experiments reported here. Previous experiments with PG produced by *Fusarium oxysporum* f. *lycopersici* suggested that dissociation phenomena took place on DEAE-cellulose, as indicated by Sephadex G200 separation of the products (Swinburne & Corden, 1967). Whilst some slight differences were observed in elution volume on Sephadex G200 of *in vitro* and *in vivo* PG of *Penicillium expansum* following ion-exchange cellulose chromatography, these were much less than those observed with the Fusarium PG. The changes in property could have resulted either from alterations in the secondary or tertiary structure of the enzyme molecule as a result of the ionic conditions of separation (Steinburg & Mihalyi, 1957), or, less

probably, from the presence of substances which modified the mode of action of the enzyme. The explanation of these phenomena must await further experiments. If a range of properties can be evoked from one basic enzyme by appropriate treatments, the differences observed between *in vivo* and *in vitro* enzymes may also be attributable to different forms of the same enzyme. Bateman's (1963) work has shown that care must be taken in relating the properties of PG produced *in vitro* to the role of the enzyme in disease development *in vivo*, and this investigation suggests that care must be taken in extraction of enzymes produced *in vivo* to prevent changes from the native enzyme.

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Regulation of the Arginine Pathway in *Proteus mirabilis*

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SUMMARY

The action of the first enzyme in the arginine pathway of *Proteus mirabilis* strain 13 is controlled by feed-back inhibition and the formation of arginine-synthesizing enzymes is influenced by the concentration of the end-product. Concentrations of arginine below 10 μg./ml. induce their formation, but higher concentrations result in repression. The variations in enzyme synthesis caused by changes in the arginine content of the medium are markedly smaller than those observed in *Escherichia coli*. The carbon source affects the response of enzyme synthesis to arginine. Two mutants *argR-1* and *argR-2* which excrete arginine have higher enzyme levels than the wild type. In one of these the enzymes are repressible and in the other mutant the enzymes are de-repressed. The sites of these two mutants map closely in a locus *argR* which is linked to a *his* marker but not to any of the structural arginine genes. The de-repression of enzyme synthesis in these mutants is limited and less than in *argR* mutants of *E. coli*. The derepressed state of *argR-2* gives it a growth advantage over the wild type if arginine is withdrawn. The derepressed mutants also exhibit a changed response to feedback inhibition.

INTRODUCTION

Arginine is synthesized in *Proteus mirabilis* strain 13 by 8 enzymes which are coded by 8 structural genes (Prozesky, 1967, 1968). The arginine pathway and its genetic topography in this organism are presented in Fig. 1. The biosynthesis of arginine in

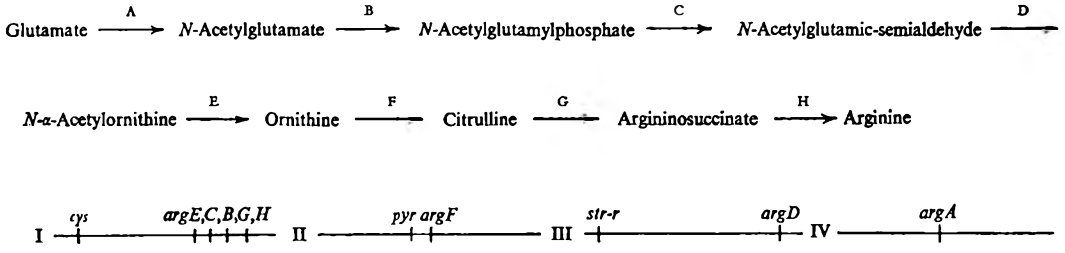

Fig. 1. The biosynthetic pathway of arginine and its genetic topography in *Proteus mirabilis* strain 13. A to H represent enzymic steps and corresponding arginine genes in the four linkage groups (I–IV, Prozesky, 1968) defined by transductions. Reference markers are *str-r* = streptomycin resistance; *pyr* = pyrimidine; *cys* = cysteine. Map not to scale.

Escherichia coli is controlled by feed-back inhibition of the first enzyme acetylglutamate synthetase (Vyas & Maas, 1963) and by pathway-wide repression of enzyme synthesis by the end-product (Vogel, 1957). The control by repression in *E. coli* strain B differs from that in strains K-12, W and C (Gorini & Gundersen, 1961; Jacoby

& Gorini, 1967). In *E. coli* B repression is mediated by an unknown mechanism related to glucose metabolism and arginine causes induction of enzyme synthesis (Gorini, Gundersen & Burger, 1961). Induction by arginine has also been described for acetyl-ornithine-δ-transaminase in a mutant of *E. coli* strain W (Vogel, Bacon & Baich, 1963) and high-level inducibility for ornithine transcarbamylase in hybrids of *E. coli* B and K-12 (Jacoby & Gorini, 1967). In *Bacillus subtilis* the repression control is similar to that in *E. coli* K-12 (Lehrer & Jones, 1962; Vogel & Vogel, 1963). Udaka (1966) examined a number of bacterial species and found that in about half of these slight repression was exerted by arginine on ornithine transcarbamylase and that in organisms which synthesize ornithine by transacetylation (Udaka & Kinoshita, 1958) feed-back inhibition is active on the second enzyme (*N*-acetyl-γ-glutamokinase) in the pathway. Vogel, Albrecht & Cocito (1961) also found repression and non-repression of arginine-synthesizing enzymes in some enterobacteria. The control of arginine synthesis thus varies in different organisms and may have taxonomic importance (Udaka, 1966; Prozesky, 1967).

Despite differences in repression control among *Escherichia coli* strains a single genetic locus determines repression of the entire pathway. This *argR* locus (Jacoby & Gorini, 1967) is active in the *trans* position and is the regulator gene of the arginine pathway (Maas & Clark, 1964). The arginine genes in *E. coli* K-12 and W are scattered around the genome except for the *argE, C, B, H* genes which are closely linked (Maas, 1961; Vogel *et al.* 1963; Glansdorff, 1965). The *Proteus mirabilis* genes show a similar grouping but the *argG* gene is included in an *argE, C, B, G, H* cluster (Fig. 1). The *argE, C, B, H* genes in *E. coli* may constitute one or more operons (Jacob, Perrin, Sanchez & Monod, 1960). Co-ordinate repression of some of their enzymes has been demonstrated (Glansdorff & Sand, 1965) and polar mutants in or near the cluster which influence more than one gene in the group have been found (Baumberg, Bacon & Vogel, 1966; Sand & Glansdorff, 1967). *ArgE* and *argH* are not regulated co-ordinately (Glansdorff & Sand, 1965; Baumberg *et al.* 1966). The regulation of arginine synthesis in *P. mirabilis* strain 13 was investigated in an attempt to contribute to the taxonomic data available for the Proteus–Providence group of bacteria (Coetzee, Smit & Prozesky, 1966) and possibly to obtain information about the operon status of the *argE, C, B, G, H* gene group.

METHODS

Media. The minimal medium was described by Prozesky (1967). Enriched with a mixture of growth factors (Novick & Maas, 1961; Prozesky, 1967) it was used as a complete defined arginine-free medium to which various concentrations of L-arginine were added as required. L-Canavanine sulphate (100 μg./ml.) was substituted for L-arginine in this medium to select for de-repressed mutants (Maas, 1961). Glycerol (0·2%, w/v) was substituted for glucose in some experiments (Gorini *et al.* 1961). Difco SS agar with streptomycin sulphate (1 mg./ml.) was used for the selection of streptomycin-resistant transductants (Prozesky, 1968).

Chemicals. Amino acids, growth factors, arginine precursors, co-enzymes and other chemicals were obtained as described (Prozesky, 1967). The L-arginine analogue L-canavanine sulphate was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A., and diethyl sulphate from British Drug Houses Ltd., Poole, England.

Bacteria. Proteus mirabilis strain 13 (Coetzee & Sacks, 1960) and mutants of this strain (Table 1) were used. The mutants *argR-1* and *argR-2* were selected on arginine-free medium with L-canavanine. Plates were treated with 0·1 ml. diethyl sulphate (Goldberg, Keng & Thorne, 1965) and inoculated with about 5×10^9 bacteria which had been washed twice in NaCl (0·9 %, w/v). Colonies which protruded above the background growth after 48 hr incubation were picked off, purified on the same medium without diethyl sulphate and examined for excretion of arginine. This was done by streaking the mutants next to mutant *argH-1* (Maas & Clark, 1964; Prozesky, 1967) on minimal medium together with wild-type controls (Prozesky, 1967). The histidineless mutant *his-7* was selected as described (Prozesky, 1967) and has not been characterized enzymatically. Streptomycin-resistant mutants of *argR-1* and *argR-2* were obtained as described by Coetzee & Sacks (1960). Cultures were maintained on nutrient agar slants at 4° and were incubated at 37°.

Table 1. *Mutants of Proteus mirabilis strain 13*

Strain	How obtained	Mutagenic agent used	Reference
13 (wild type)	—	—	Coetzee & Sacks, 1960
Mutant			
argR-1	Selection	Diethyl sulphate	See Methods
argR-2	Selection	Diethyl sulphate	
argR-1, *argB-1*	Transduction	—	
argR-2, *argB-1*	Transduction	—	
argR-1, *cysE-6*	Selection	$MnCl_2 + H_2O_2$	
argR-2, *cysE-7*	Selection	$MnCl_2 + H_2O_2$	
argR-1, *str-r-17*	Selection	—	Coetzee & Sacks, 1960
argR-2, *str-r-18*	Selection	—	
argB-1	Selection	$MnCl_2 + H_2O_2$	Prozesky, 1967
argH-1	Selection	$MnCl_2 + H_2O_2$	
his-7	Selection	$MnCl_2 + H_2O_2$	

Construction of mutants by transduction. The effect of the *argR* mutations (Table 1) on the synthesis of acetylglutamate synthetase was determined in mutants *argR-1*, *argB-1* and *argR-2*, *argB-1* because the *argB* (*N*-acetyl-γ-glutamokinase) enzyme block is a prerequisite in a mutant used for the determination of acetylglutamate synthetase in resting cell suspensions (Vyas & Maas, 1963; Prozesky, 1967). The linkage of the *argB* and *cysE* genes (Prozesky, 1968) made it possible to transduce the *argB-1* lesion (Prozesky, 1967) into the two *argR* mutants. Mutants *argR-1*, *cysE-6* and *argR-2*, *cysE-7* (Table 1) were selected from *argR-1* and *argR-2* respectively as described (Prozesky, 1967, 1968) and were characterized by Mr W. O. K. Grabow (Grabow & Smit, 1967). These two mutants were then transduced to cysteine independence with phage 34/13 prepared as described (Prozesky, de Klerk & Coetzee, 1965) on the arginineless mutant *argB-1* (Prozesky, 1968). Transductants were selected on minimal medium which contained L-ornithine monohydrochloride (100 μg./ml.). Replication to minimal medium enabled the identification of ornithine-requiring colonies which were tested for the *argB-1* marker by enzyme assay (Prozesky, 1967).

Preparation of cell-free extracts and enzyme assays. Cultures were grown and cell extracts were prepared by sonication as described (Prozesky, 1967). Protein concentrations in the extracts were determined by the method of Gornall, Bardawill & David

(1949). Acetylglutamate synthetase was measured with resting suspensions of mutant *argB-1* (wild-type activity) and in constructed mutants *argR-1*, *argB-1* and *argR-2*, *argB-1* for de-repressed mutant activity. The enzymes and their assay methods (Prozesky, 1967) are given in Table 2. *N*-Acetylglutamic-γ-semialdehyde dehydrogenase could not be determined in our strain (Prozesky, 1967) and this enzyme was omitted from this investigation. Arginase was determined according to Ramaley & Bernlohr (1965) and the ornithine produced by this enzyme was determined by the method of Chinard (1952).

Feed-back inhibition of enzyme activity. This was determined by assay of acetylglutamate synthetase (the first enzyme in the pathway) and *N*-acetyl-γ-glutamokinase (the second enzyme in the pathway) in the presence of various concentrations of L-arginine hydrochloride (Vyas & Maas, 1963; Udaka, 1966).

Table 2. *Enzymes of the arginine pathway in Proteus mirabilis strain 13*

Enzyme	E.C. no.*	Position in pathway	Reaction product measured	Reference for method
Systematic name				
AcetylCoA: L-glutamate *N*-acetyltransferase	2.3.1.1	A	*N*-Acetylglutamate	Vyas & Maas (1963)
ATP: *N*-acetylglutamate 5-phosphotransferase		B	*N*-Acetyl-γ-glutamyl hydroxamate	Baich & Vogel (1962)
L-Ornithine: 2-oxoacid aminotransferase	2.6.1.11	D	*N*-Acetyl-glutamic-γ-semialdehyde	Albrecht & Vogel (1964)
α-*N*-Acetyl-L-ornithine amidohydrolase	3.5.1.d	E	Ornithine	Vogel & Bonner (1956)
Carbamoylphosphate: L-ornithine carbamoyltransferase	2.1.3.3	F	Citrulline	Jones (1962)
L-Citrulline: L-aspartate-ligase	6.3.4.5	G	Disappearance of citrulline	Ratner (1955)
L-Argininosuccinate-arginine lyase	4.3.2.1	H	Ornithine	Baumberg, Bacon & Vogel (1965)
L-Arginine ureohydrolase	3.5.3.1	—	Ornithine	Ramaley & Bernlohr (1965)

* = E.C. no. = Enzyme Commission number.

Repression and de-repression of enzyme synthesis. Enzyme levels were determined in cultures grown in arginine-free or minimal medium which contained different concentrations of L-arginine. In some experiments glycerol was used as carbon source in the medium to determine the effect of glucose catabolism on the effect of the end-product L-arginine (Gorini *et al.* 1961).

Adaptation from L-arginine-containing to an L-arginine-free medium. The wild-type and mutant *argR-2* were grown with aeration (Prozesky, 1967) in arginine-free medium (100 ml.) enriched with L-arginine hydrochloride (10 μg./ml.) for 6 hr. The cultures were centrifuged at 12,000 *g* for 10 min. and washed twice with cold NaCl (0·9 %, w/v). The pellets were resuspended quantitatively in prewarmed arginine-free medium (100 ml.) and grown as before. The extinctions of the cultures at 580 mμ were determined (Vyas & Maas, 1963) at 30 min. intervals.

Genetic mapping of argR mutants. Lysates of phage 34/13 were prepared and transduction experiments performed as described (Prozesky *et al.* 1965; Prozesky, 1968).

Recipients were mixed with phage at a multiplicity of input of 5, and adsorbtion continued for 15 min. at 37°. The bacteria were centrifuged, resuspended in NaCl (0·9 %, w/v) and samples were plated. Appropriate controls were included (Prozesky & Coetzee, 1966; Prozesky, 1968). Direct selection for transfer of *argR* markers by L-canavanine-resistance (Jacoby & Gorini, 1967) was not possible with this strain because of poor differential toxicity of the L-arginine analogue for *argR* and *argR*$^+$ (wild-type) bacteria and the high rate of mutation to canavanine resistance (see below). The *argR* property was scored as an unselected marker by streaking next to *argH-1* on minimal medium to detect excretion of arginine. The argininеless and other auxotrophic mutants previously used to detect linked transduction of *arg* markers (Prozesky, 1968) were recipients in transduction experiments with *argR-1* and *argR-2* as donors. Prototrophic transductants were selected on minimal medium and examined for concomitant inheritance of *argR-1* and *argR-2* markers. In initial scanning experiments with the large number of auxotrophic mutants (Prozesky, 1968) 50 colonies from each cross were tested for arginine excretion. For possible co-transduction of *argR* and streptomycin-resistance (*str-r*) the wild type was used as recipient with *argR-1*, *str-r-17* and *argR-2*, *str-r-18* as donors. Selection was for *str-r* (Prozesky, 1968) and the *argR* markers were scored as above.

Table 3. *Feedback inhibition of early enzymes in the arginine pathway*

Enzyme A = acetylglutamate synthetase. Enzyme B = *N*-acetyl-γ-glutamokinase. Results are the average of two experiments. Enzymes were determined in cultures grown for 36 hr in arginine-free medium enriched with L-arginine-HCl (10 μg./ml.) Arginine was added to the incubation mixtures in various concentrations.

L-arginine-HCl in the incubation mixture (μg./ml.)	Enzyme specific activity			
	Enzyme A			Enzyme B
	13 (wild type)	*argR-1*	*argR-2*	Strain 13
0	59·9*	49·6	62·8	1·1†
5	42·5	—	—	1·0
10	39·6	33·0	58·7	1·0
50	38·2	—	—	1·1
100	36·6	—	—	1·0

* 1 unit = 1 μg./mg. dry wt bacteria/hr; † 1 unit = 1 μmole/mg. protein/hr.

RESULTS

Feed-back inhibition of enzyme activity. L-Arginine exerts strong feed-back inhibition on the first enzyme in the pathway acetylglutamate synthetase but the second enzyme (*N*-acetyl-γ-glutamokinase) is not significantly affected by the presence of the end-product (Table 3). Similar results were described by Vyas & Maas (1963) for *Escherichia coli* K-12 and by Udaka (1966) for *Proteus vulgaris*. As in the case of *E. coli* K-12 feedback inhibition is especially active at low arginine concentrations (Table 3). *ArgR-1*, *argB-1* shows more pronounced feedback inhibition of acetylglutamate than the wild-type while in *argR-2*, *argB-1* the action of the enzyme is affected less by arginine (Table 3).

Response of synthesis of arginine enzymes to L-arginine in the wild-type organism. The effect of various concentrations of the end-product on arginine enzyme synthesis is

Table 4. *The effect of* L-*arginine on enzyme synthesis in the wild type and in derepressed mutants*

Enzymes were determined in bacteria grown in arginine-free medium or minimal medium (where indicated) supplemented with none or various concentrations of L-arginine. Figures are the average of two experiments.

Arginine concentration (μg./ml.) ...	0			5			10			20			50			100		
	Specific activity* of enzymes in strains																	
Enzyme	13 (wild type)	*argR-1*	*argR-2*	13	*argR-1*	*argR-2*	13	*argR-1*	*argR-2*	13	*argR-1*	*argR-2*	13	*argR-1*	*argR-2*	13	*argR-1*	*argR-2*
Acetylglutamate synthetase	42·0†	56·2	61·4	50·5	50·8	62·0	58·4	44·4	62·6	—	—	—	53·1	39·1	60·1	45·0	32·0	56·2
N-Acetyl-γ-glutamokinase	2·0	3·6	3·4	2·8	2·7	3·5	3·5	2·1	3·6	—	—	—	2·6	1·5	3·0	1·9	1·0	2·4
N-Acetylornithine-δ-transaminase	2·8‡	3·7	3·7	2·9	3·5	3·8	3·6	3·3	3·8	—	—	—	3·2	2·8	3·4	2·8	2·4	3·1
Acetylornithinase	18·6	27·8	29·2	21·2	25·9	29·4	23·1	23·8	29·6	—	—	—	22·0	20·4	26·7	18·4	15·0	24·0
Ornithine transcarbamylase	2·4	4·0	3·0	2·8	3·7	3·3	3·2	3·3	3·7	3·0	3·1	3·5	2·4	2·4	2·9	1·5	1·0	2·0
Ornithine transcarbamylase (minimal medium)	0·7	1·0	0·9	0·8	0·9	0·9	0·8	0·8	0·9	—	—	—	0·6	0·6	0·7	0·4	0·3	0·6
Condensing enzyme	0·3	0·6	0·5	0·4	0·5	0·5	0·5	0·4	0·6	—	—	—	0·4	0·3	0·5	0·4	0·3	0·5
Argininosuccinase	0·5	1·7	1·4	0·8	1·5	1·5	1·0	1·3	1·5	1·0	1·2	1·5	0·9	0·9	1·4	0·8	0·5	1·3
Argininosuccinase (minimal medium)	0·4	0·9	0·7	0·5	0·7	0·7	0·5	0·6	0·8	—	—	—	0·5	0·5	0·7	0·5	0·4	0·6
Arginase	0·057§	0·095	0·061	—	—	—	—	—	—	—	—	—	—	—	—	0·083	0·076	0·080

* 1 unit = 1 μmole/mg. protein/hr except. † 1 unit = 1 μg./mg. dry wt bacteria/hr. ‡ 1 unit = 0·1 increase in E_{440}/mg. protein/hr. § 1 unit = 1 μmole/mg. protein/min.

shown in Table 4. The response of two of the enzymes is illustrated in Fig. 2. L-Arginine induces the enzymes until its concentration exceeds 10 μg./ml. Higher concentrations cause a progressive decline in enzyme levels. The seven enzymes are not induced or repressed co-ordinately (Ames & Garry, 1959). This is best seen in the responses of ornithine transcarbamylase and argininosuccinase (Fig. 2). The levels of the former enzyme vary more than those of the latter. This was also found in *Escherichia coli* (Vogel, 1961). There may be a co-ordinate response to arginine by *N*-acetyl-γ-glutamokinase, argininosuccinase and the condensing enzyme but the small variations in levels of these enzymes (Table 4) do not allow conclusions to be made. The induction

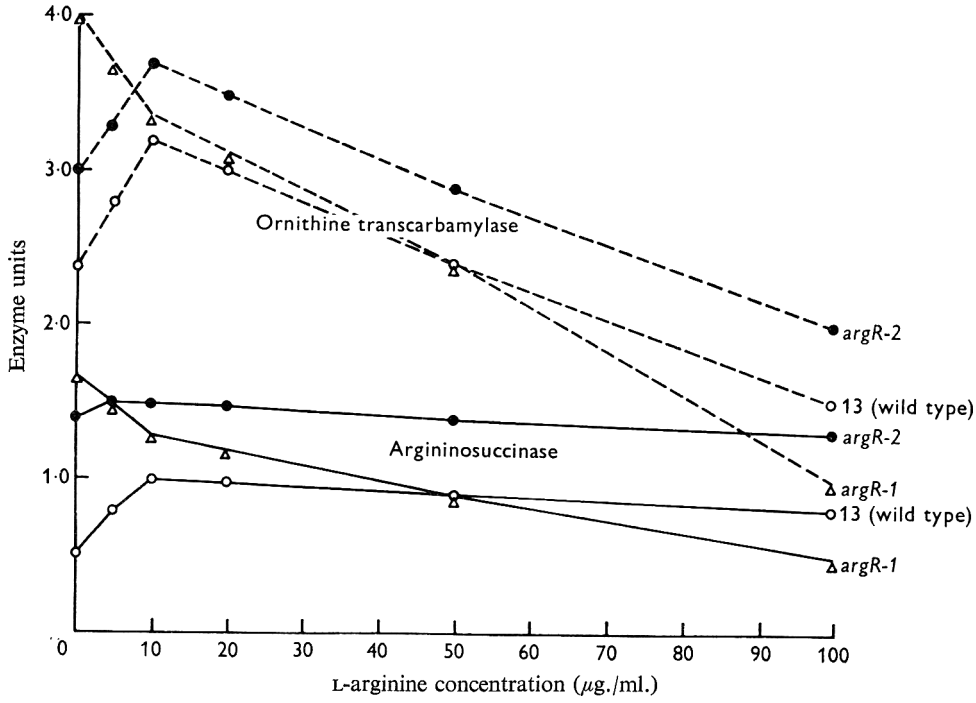

Fig. 2. The effect of arginine on the synthesis of arginino-succinase and ornithine transcarbamylase in the wild type and the *argR* mutants. Cultures were grown for 36 hr in arginine-free medium to which various concentrations of arginine were added. Enzymes were determined in extracts of disrupted cells. Solid lines: Argininosuccinase. Broken lines: Ornithine transcarbamylase. 13 (wild-type), —○—; *argR*-1, —△—; *argR*-2, —●—.

by low concentrations of arginine suggest that as in *E. coli* strain B (Gorini & Gundersen, 1961) L-arginine is an antagonist of an unknown inhibitor of the synthesis of the enzymes; possibly derived from glucose metabolism (Gorini *et al.* 1961). This was tested by repeating the assays of two of the enzymes with glycerol as carbon source (Fig. 3). The enzyme levels are slightly raised by the glycerol medium and there is less induction by arginine but also less repression at higher concentrations. Results obtained with bacteria grown in minimal medium (Table 4) show that enzyme levels are markedly lower than when arginine-free medium is used but the pattern of response to arginine is similar in the two media.

Mutants with aberrant regulation of arginine synthesis. The yield of canavanine-

resistant colonies was about 1 per 10^8 bacteria plated. Of 51 such colonies tested three excreted arginine. One of these *argR-1* showed a poor feeding response with *argH-1*. *ArgR-1* and *argR-2* were chosen for detailed investigation. Enzyme assays show (Table 4, fig. 2) that *argR-1* and *argR-2* have higher levels of arginine enzymes than the wild type, but in *argR-1* there is repression by both low and high concentrations of arginine. *ArgR-2* is less inducible and also less repressible by the end product. In glycerol medium *argR-1* and the wild type behave similarly and *argR-2* is less affected

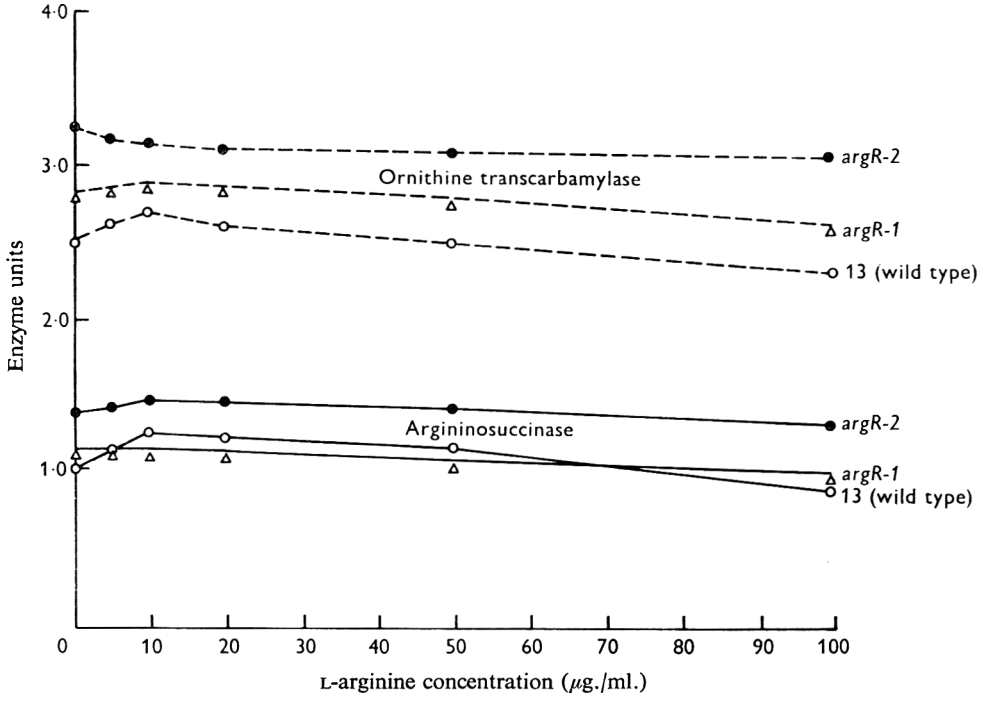

Fig. 3. Response of synthesis of argininosuccinase and ornithine transcarbamylase to variations in arginine concentration when glycerol is substituted for glucose as the medium carbon source. Procedures and designations are as in Fig. 2.

by the different carbon source (Fig. 3). The arginase levels of the wild type and the *argR* mutants show that *argR-1* and *argR-2* do not excrete arginine because of reduced breakdown of arginine by arginase. Other possible degradation reactions of arginine were not investigated.

Adaptation to the presence or absence of arginine. When *argR-2* and the wild type are switched from an arginine-containing to an arginine-free medium the resumation of growth by the former is more prompt and growth proceeds faster than that of the wild type (Fig. 4). The derepressed state of *argR-2* confers a growth advantage to it which it does not possess in the presence of L-arginine. This is probably because maximum arginine synthesis occurs in the mutant in the presence or absence of the amino acid (Gorini *et al.* 1961).

Co-transduction argR and other markers. No co-transduction of *argR-1* and *argR-2* could be demonstrated with any of the structural arginine genes (Prozesky, 1968) or with the *str-r* marker. Of the miscellaneous auxotrophic mutants tested only a number

of *his* (histidineless) mutants were rendered prototrophic by transduction with concomitant transfer of both *argR* markers. Five of the *argR* colonies from each of the crosses *argR-1* (donor) × *his-7* (recipient) and *argR-2* (donor) × *his-7* (recipient) were

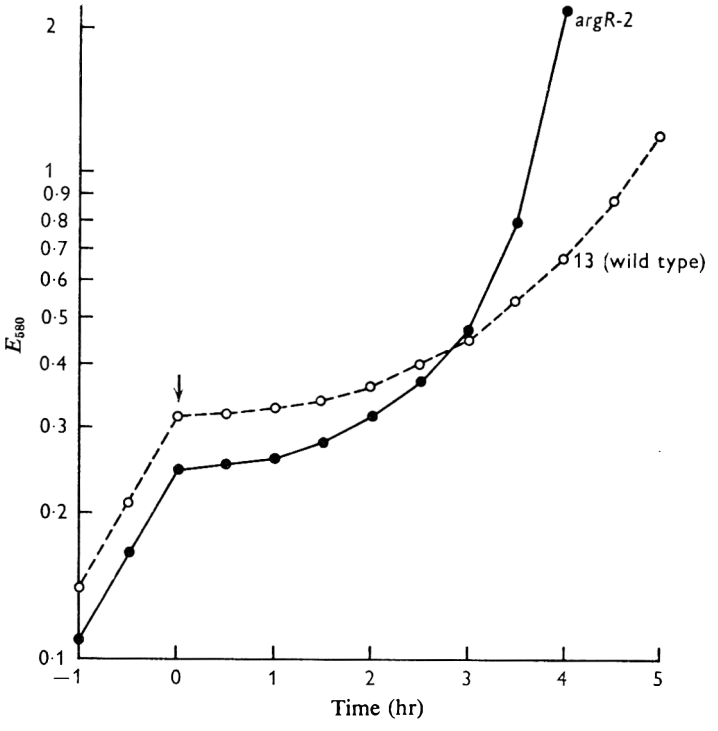

Fig. 4. Growth curves of the wild type and *argR-2* when the bacteria are switched from arginine-enriched to arginine-free medium. Cultures were grown with aeration in arginine-free medium enriched with L-arginine (10 μg./ml.) for 6 hr, washed in the cold and inoculated quantitatively in arginine-free medium (arrow). The E_{580} of samples was determined at intervals. 13 (wild type), —○—; *argR-2*, —●—.

Table 5. *Levels of ornithine transcarbamylase and argininosuccinase in argR transductants*

Cultures were grown in arginine-free medium and enzymes were then determined in cell-free extracts. OTC = ornithine transcarbamylase; AS = argininosuccinase.

Organism		OTC	AS
13 (wild type)		2·4*	0·5*
Transductant: *argR-1* × *his-7*	a	3·8	1·5
	b	3·5	1·5
	c	4·1	1·5
	d	4·0	1·6
	e	4·0	1·8
Transductant: *argR-2* × *his-7*	a	3·2	1·4
	b	3·2	1·4
	c	3·4	1·6
	d	3·0	1·2
	e	3·0	1·3

* Specific activity of enzymes: 1 unit = 1 μmole/mg. protein/hr.

picked off, purified and grown in arginine-free medium for assays of ornithine transcarbamylase and argininosuccinase. The enzyme levels in these cultures were compared with the levels obtained in the wild type (Table 5). Enzyme levels in the transductants were significantly higher. The variation in enzyme activity among different clones is unexplained.

Distance of argR-1 and argR-2 from his-7. A stable marker *his-7* (O. W. Prozesky, unpublished) was chosen as a linked reference marker for a ratio test with *argR-1* and *argR-2*. The results are presented in Table 6 and show that *argR-1* and *argR-2* are closely linked and situated in the same locus *argR* (Jacoby & Gorini, 1967).

Table 6. *A ratio test with argR mutant donors and his-7 as recipient in transduction experiments*

Donor	No. of colonies examined*	*argR*	*argR*$^+$	$\frac{argR^+}{argR+argR^+}$
argR-1	700	132	568	81·14
argR-2	700	116	584	83·43

* Selection was made for prototrophs. *ArgR* and *argR*$^+$ were scored as unselected markers by their ability or inability respectively to feed *argH-1* on minimal agar.

DISCUSSION

The feed-back inhibition of acetylglutamate synthetase by arginine confirms the finding that arginine is synthesized by the 'enterobacterial' pathway in *Proteus mirabilis* (Prozesky, 1967). The feed-back inhibition is active at low arginine concentrations as was found in *Escherichia coli* (Vyas & Maas, 1963). The finding that *argR-1* is more sensitive to feed-back inhibition than the wild type and that *argR-2* is refractive parallels the repression results and supports the hypothesis that feedback inhibition and repression are reflexions of translational control (Vogel & Vogel, 1967) and basically constitute the same process (Cline & Bock, 1966). Similar results have been obtained by Somerville & Yanofsky (1965) and Ames (1965) for tryptophan and histidine biosynthesis. Acetylglutamate synthetase is measured with resting cell suspensions (Vyas & Maas, 1963) because determination of the enzyme in cell-free extracts is not sensitive enough to reflect feed-back inhibition (Vyas & Maas, 1963). Arginine added to minimal medium without a nitrogen source during the determination may constitute a nitrogen source for *P. mirabilis* strain 13 (O. W. Prozesky, unpublished) so that an amount of repression may be reflected by the results of the feed-back inhibition experiments. The difference between the repression results (Table 2) and feed-back inhibition values (Table 3) indicate that the repression concomitantly measured is small. Support for this is that the activity of acetylglutamate synthetase measured during feed-back inhibition with the wild type is not raised by low arginine concentrations while enzyme induction is observed at low arginine concentrations in repression experiments.

The presence of arginine in the growth medium has a surprisingly small effect on synthesis of arginine enzymes (Table 2). Similarly, the differences between the enzyme levels in the wild-type and in mutants with defective regulation are not as extreme as encountered in *Escherichia coli* (Jacoby & Gorini, 1967), although the slightly raised levels of enzymes in the *Proteus mirabilis argR* mutants cause excretion of arginine.

Udaka (1966) also reported small variations in the synthesis of ornithine transcarbamylase in a number of bacterial species tested (including *P. vulgaris*) for their repression response to arginine. The dramatic repression and derepression by the end-product observed in *E. coli* strains K-12 and W (Maas, 1961; Vogel, 1961; Jacoby & Gorini, 1967) may be a feature of only some bacteria. The induction of anabolic arginine enzymes by arginine is a feature of the entire arginine pathway in *E. coli* B (Gorini & Gundersen, 1961) and has been described for acetylornithine-δ-transaminase in a mutant of *E. coli* W (Vogel *et al.* 1963).

The induction of arginine enzymes by low end-product concentrations followed by repression at higher concentrations (Fig. 2) has not been described in other bacteria. Vogel (1961) and Maas (1961) noted that arginine-synthesizing enzymes are less sensitive to repression in *Escherichia coli* when growth is suboptimal. This may explain the lower enzyme levels in minimal medium and glycerol arginine-free medium with less induction and repression obtained here (Table 4; Fig. 3). When arginine-free medium is enriched with arginine it is a complete defined medium which results in optimal growth (Novick & Maas, 1961). The induction in the wild type by low arginine concentrations may only reflect better growth of the organism. *ArgR-1* and *argR-2* overproduce arginine and have a growth advantage over the wild type in arginine-free medium (Fig. 4). The latter mutants show no or little enzyme induction by arginine, probably because their growth is optimal in the absence of supplied arginine. Sercarz & Gorini (1964) identified two functional arginine compartments in *E. coli*: endogenous arginine linked to protein synthesis and growth and exogenous arginine linked to repression. It is possible that an arginine content up to 10 μg./ml. in arginine-free medium serves to replenish the endogenous (protein-synthesizing) arginine compartment of *Proteus mirabilis* strain 13 and that higher levels cause repression. The level of 10 μg./ml. has also been established as the concentration at which arginineless mutants grow as well as the wild type (O. W. Prozesky, unpublished). This is the level at which the L-arginine permease system of *E. coli* is saturated with arginine (Maas, 1965). The low repressibility of arginine-synthesizing enzymes can be compared to the low inducibility of *E. coli* β-galactosidase observed in *P. mirabilis* F-lac and F-*lac-i* organisms (Colby & Hu, 1968). These workers concluded that the anomalous regulation of the above episome is due to the different cytoplasmic environment. The two *argR* mutants of *P. mirabilis* represent the two types of regulation-deficient mutants selected by canavanine-resistance in *E. coli* B (Maas, 1961). These are derepressed (*argR-2*) and repressible by arginine (*argR-1*). The gene locus *argR* of *P. mirabilis* is not linked to any of the structural arginine genes. The corresponding gene *argR* is linked to *argG* in *E. coli* strains (Jacoby & Gorini, 1967). The *argG* gene of *P. mirabilis* maps in a different position than in *E. coli* (Prozesky, 1968). Canavanine-resistant mutants which do not excrete arginine and are not regulatory deficient may be arginine permease (*argP*) mutants (Maas, 1965) or arginine-tRNA synthetase mutants (Hirschfield & Maas, 1967) and have not been investigated.

It is not known whether the *argR* gene of *Proteus mirabilis* is the counterpart of the *argR* genes of the *Escherichia coli* strains (Jacoby & Gorini, 1967). Like the latter genes, its effect is pathway-wide and it is not closely-linked to the structural genes. Experiments to test the effect of the *P. mirabilis argR* gene in the *trans* position (Maas & Clark, 1964) have not proved feasible in this strain due to lack of a conjugation system. The differences in the extent of control of enzymes by arginine are properties

of the *argR* genes in *E. coli* (Jacoby & Gorini, 1967). The unique behaviour of *P. mirabilis* in this respect may be a characteristic of the *argR* gene or the structural genes. This could be determined by transfer of the genes (Jacoby & Gorini, 1967) between *P. mirabilis* and *E. coli* strains. Gemski, Wohlieter & Baron (1967) have succeeded in transferring some chromosomal genes from *E. coli* K-12 to *P. mirabilis* strain WR 11. It would be interesting to establish whether *P. morganii* has the 'Proteus type' of arginine control described here or a control mechanism similar to that of *E. coli* as it resembles *E. coli* in guanine+cytosine molar content (Hill, 1966).

The small variations in enzyme levels obtained during repression and derepression and the absence of *N*-acetyl-glutamic-γ-semialdehyde dehydrogenase (enzyme C, Fig. 1) activity (Prozesky, 1967) prevented the demonstration that control of the enzymes coded by the *argE, C, B, G, H* cluster is co-ordinate (Glansdorff & Sand, 1965). No operator or promoter-type mutants (Jacob *et al.* 1960; Jacob, Ullman & Monod, 1964) were identified nor was aberrant regulation behaviour of a single enzyme encountered (Vogel *et al.* 1963; Jacoby & Gorini. 1967).

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Detection and Study of Cosynthesis of Tetracycline Antibiotics by an Agar Method

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SUMMARY

Pairs of non-tetracycline-producing mutants of *Streptomyces rimosus* or *S. aureofaciens* were grown side by side on agar. Their ability to produce antibiotic by cosynthesis was tested by placing a strip of agar cut from the combined culture on plates containing *Bacillus subtilis*. The activity was revealed as an inhibition halo formed on *B. subtilis*, opposite one or other mutant strain. The strain surrounded by the halo was considered as a converter of an intermediate product secreted by the other strain. Two types of mutants were observed: a rare type probably affecting the main pathway of antibiotic biosynthesis, and a more frequent type probably affecting some regulatory process.

INTRODUCTION

Inactive mutants (that is non-producers of antibiotic) of *Streptomyces aureofaciens* and *S. rimosus* blocked in the biosynthesis of the tetracycline molecule, have been described by McCormick, Hirsch, Sjolander & Doerschuk (1960) and by Alikhanian, Orlova, Mindlin & Zeitzeva (1961). Some pairs of inactive mutants, in mixed liquid culture, were found to produce substantial amounts of tetracycline antibiotics (McCormick *et al.* 1960). This phenomenon, which is comparable to syntrophism between complementing auxotrophic mutants, has been called 'co-operative biosynthesis' or 'cosynthesis' and it has been attributed to the conversion to tetracycline by one strain of some intermediate synthesized and secreted by the other. The two cosynthesizing strains are blocked in different biosynthetic steps, the 'converter' being blocked at an earlier step than the 'secretor'. The present paper describes a simple method of detecting cosynthesis between pairs of inactive mutants, by an agar plate test, which has the additional advantage of allowing immediate recognition of the converter and secretor member of each pair.

METHODS

The organisms. Streptomyces rimosus R6, an oxytetracycline producer, and *Streptomyces aureofaciens* A4, a chlortetracycline producer (both isolated from soil and maintained in the Culture Collection, Department of Industrial Microbiology, Faculty of Technology, Zagreb University) were used for the isolation of inactive mutants. Auxotrophic mutants (methionine (*met*) and tryptophan (*try*) requiring) of

these strains, which had retained their ability to produce antibiotic, were often used as a source of inactive mutants. *Streptomyces* sp. PLIVA 670, an inactive wild strain isolated from soil, was used in some cosynthesis experiments, together with other wild strains of various origins. *Bacillus subtilis* 3004 (Culture Collection, Department of Industrial Microbiology, Faculty of Technology, Zagreb University) was used for the bioassay of tetracyclines.

Media. The medium used to test the antibiotic production of the Streptomyces strains was: malt extract 10 g., yeast extract 4 g., glucose 4 g., agar 20 g., tap water 1000 ml., pH 6·8. The medium used for *B. subtilis* was: peptone 6 g., caseine hydrolysate 4 g., yeast extract 3 g., beef extract 1·5 g., glucose 1 g., agar 20 g., tap water 1000 ml., pH 6·6. All ingredients were Difco standard quality.

Mutagenic treatment. u.v. radiation: agitated suspensions of spores were irradiated by a 'Philips' TUV 15 germicidal lamp, to a survival rate of 1 to 0·1 %.

Nitrous acid. Spores were suspended in 0·2 M-acetate buffer at pH 4·4. A freshly prepared solution of 0·5 M-$NaNO_2$ was added to the spore suspension to give a final concentration of 0·017 M. Treatment was stopped at the required time by diluting samples into five volumes of 0·2 M-phosphate buffer pH 7·0. After 12 min. of treatment the survival rate was about 0·1 %.

Ethyleneimine and 1,3-diepoxybutane. These were added to spore suspensions in water to final concentrations of 0·1 and 1 % (v/v) respectively. Survival rates of 1 to 0·1 % were obtained after 60 min. of treatment with ethyleneimine and after 40 min. with 1,3-diepoxybutane.

Isolation of inactive mutants. Mutagen-treated suspensions of spores were diluted and spread on agar medium. Colonies picked at random were transferred to a second plate in such a way that not more than 10 well separated colonies grew on the second plate. After 7 days' incubation at 28° melted agar containing spores of *Bacillus subtilis* was poured over the plates and these were incubated overnight at 37°. Colonies not producing a halo of inhibition were considered as inactive mutants, and were re-tested in liquid medium to prove their inactivity. Inactive mutants were designated *otc* or *ctc*, according to whether derived from *Streptomyces rimosus* (oxytetracycline producer) or *S. aureofaciens* (chlortetracycline producer) respectively.

Detection of cosynthesis. Two inactive mutants to be tested were streaked on opposite halves of a plate, about 1–2 mm apart (each covering one half of the plate). The plate was incubated 4 to 7 days at 28°. A strip of agar 8 cm. × 0·5 to 0·7 cm. was cut from the plate at right angles to the line of separation between the two strains. This strip was placed on the surface of an agar plate containing the test organism (*B. subtilis*). The plate was kept for 2 hr in the refrigerator to allow diffusion of antibiotic from the strip into the plate and then incubated overnight at 37°. Any antibiotic activity was revealed as a zone of inhibition in the growth of *B. subtilis*, around one region of the strip (Pl. 1, fig. 1).

Paper chromatography. Chromatograms of pieces of agar containing active substances, placed on Whatman no. 1 paper, were run in nitromethane + benzene + pyridine (20 + 10 + 3 by vol) and examined under the ultraviolet light. Occasionally, chromatograms were also placed on agar plates containing *B. subtilis* 3004, kept for 2 hr in a refrigerator, and incubated overnight at 37°. The R_F values of products giving an inhibition halo was determined for such chromatograms. Other solvents were also used to check antibiotic substances (see Lancini & Sensi, 1964).

RESULTS

Isolation of inactive mutants

Table 1 summarizes the results obtained in a search for inactive mutants of *Streptomyces rimosus* and *S. aureofaciens*. Inactive mutants represented about 0·2 to 0·5 % of the survivors of mutagenic treatment; the most effective was ultraviolet irradiation with a survival rate of 1 to 0·1 %. Many of the inactive mutants showed a decreased ability to sporulate, or loss or increase of brown pigment production. Some of the mutants retained traces of activity: these were often the most effective in cosynthesis.

Table 1. *Isolation of inactive mutants from Streptomyces rimosus R6 and Streptomyces aureofaciens A4*

	S. rimosus R6			*S. aureofaciens* A4		
		Inactive mutants isolated			Inactive mutants isolated	
Mutagen*	No. colonies tested	no.	%	No. colonies tested	no.	%
Ultraviolet radiation	3976	22	0·55	2295	4	0·30
Nitrous acid	732	0	0	1561	3	0·19
Ethyleneimine	509	1	0·19	—	—	—
Diepoxybutane	2694	6	0·22	764	1	0·17

* Conditions of treatment as in text.

Appearance of antibiotic cosynthesis

As shown in Pl. 1, fig. 1, a clear zone of inhibition could surround a short region of the strip bearing the two combined strains. Such a halo, which did not appear if the strains had been grown on separate plates, is evidence of antibiotic cosynthesis. As a rule the inhibition occurred only on one side of the line of separation between the mutants The mutant surrounded by the halo was evidently the one which converted to tetracycline a compound, possibly a normal precursor, secreted by the other mutant. The halo-producing mutant was thus blocked in an earlier step of the biosynthesis. In the example of Pl. 1, fig. 1, an inactive mutant of *Streptomyces rimosus* (*otc* C 15) is shown to complete antibiotic biosynthesis started by a wild-type inactive *Streptomyces* sp. 670. The nature of the antibiotic produced was in some cases tested by paper chromatography of a piece of agar cut from the region of activity. In intraspecific cosynthesis, pairs of *S. rimosus* mutants always produced oxytetracycline, and *S. aureofaciens* mutants, chlortetracycline.

Grouping of inactive mutants of Streptomyces rimosus

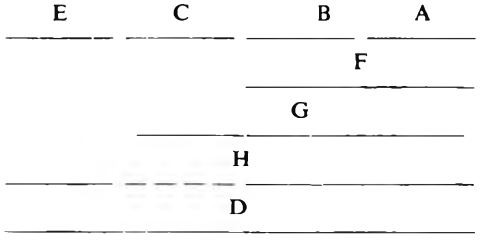
Twenty-eight inactive mutants (*otc*) of *Streptomyces rimosus*, all obtained from auxotrophic mutants, were tested for their cosynthetic ability in 756 possible pairs. They were grouped according to their pattern of complementation, into eight groups. Each of the larger group comprised inactive mutants derived from different auxotrophic parents, but inactive mutants from the same auxotrophic parent could be found in different groups. For instance, inactive mutants in group A were obtained from three auxotrophs: *met-9*, *met-18*, *try-2*, and inactive derivatives from mutant *met-18* were found in groups A, F and G. This suggests that the complementation

pattern of an auxotrophic inactive mutant was not due to its nutritional requirement. The cosynthetic interactions between mutants of different complementation groups are shown in Table 2, where the code number of the mutants belonging to the various groups is also recorded. Mutants of groups A, B, C and E (which have been named class 1) show a mutually simple complementation pattern, each mutant giving co-synthetic activity with all mutants of the other groups. More complex patterns are shown by mutants of the groups F, G, H and D (called class 2) which complement with two (F). or only one (G and H), or none (D) of the other groups of mutants (Table 2). Mutants of class 2 never act as 'converters', as is evident from Table 2, where letters corresponding to groups F, G, H and D never appear in the diagram illustrating cosynthesis between groups. Consequently members of these four groups never complement each other. In this respect group A could be placed in class 2. Class 1 groups can be arranged in order, by placing each secretor mutant after all mutants converting its product. They turn out to be in the sequence: E → C → B → A. Class 2 groups give complementation, if any, only with the first two groups of this sequence.

Table 2. *Grouping of inactive mutants of Streptomyces rimosus R6*

Attribution of mutants to complementation groups		Cosynthesis between groups*								
Mutants	Group	Groups	E	C	B	A	F	G	H	D
otc 4, 5, 12, 13, 65, 123	A	E	—	E	E	E	E	E	—	—
otc 17	B	C		—	C	C	C	—	C	—
otc 15	C	B			—	B	—	—	—	—
otc 90, 98, 104, 118	D	A				—	—	—	—	—
otc 2	E	F					—	—	—	—
otc 10, 91, 94, 111, 112, 113, 114, 117	F	G						—	—	—
otc 8, 64, 105	G	H							—	—
otc 95, 96, 119, 120	H	D								—

Complementation pattern†



* The sign — indicated no cosynthesis. Each letter indicates cosynthesis with a halo on the side of the strain of the corresponding group.

† Non-overlapping segments correspond to complementing groups.

Grouping of inactive mutants of Streptomyces aureofaciens

Ten inactive mutants of *Streptomyces aureofaciens* (*ctc*) were studied in combined cultures, all fully inactive derivatives of prototrophic strain. Cosynthesis was barely evident with many pairs and reliable evidence was obtained only after repeated tests. Some of the mutants (*ctc 8*, *ctc 9*, and *ctc* 10) had to be disregarded because of doubtful responses. The remaining six were placed in four groups, the complementation

pattern of which is represented in Table 3. Groups A, B and C belong to class 1 (see preceding section), group D to class 2.

The order of the steps controlled by mutants of class 1 was deduced by distinguishing the 'secretor' and 'converter' member of each pair. Mutants *ctc A1*, *ctcB3* and *ctc C8* were particularly considered (Pl. 1, fig. 2). The three groups could be arranged in the order C → B → A (i.e. 5 → 3 → 1).

Table 3. *Grouping of inactive mutants of Streptomyces aureofaciens A4*

Attribution of mutants to complementation groups		Cosynthesis between groups*				
Mutants	Group	Groups	A	B	C	D
ctc 1, 2, 4	A	A	—	B	C	A
ctc 3	B	B		—	C	—
ctc 5	C	C			—	—
ctc 6, 7	D	D				—
ctc 8, 9, 10	Doubtful					
Complementation pattern: †		C	B	A		
		D				

* See note* to Table 2. † See note † to Table 2.

Detection of interspecific cosynthesis. Cosynthetic activity was also evident in combinations between an inactive mutant of *Streptomyces rimosus* and an inactive mutant of *S. aureofaciens*. Two mutants of *S. rimosus* belonging to groups *otc E* and *otc B*, combined with a mutant of group *ctc A* of *S. aureofaciens*, gave a halo on the side of the *S. rimosus* mutants, which thus acted as converters. This result was expected, because gene *ctc A* is a late gene in chlortetracycline biosynthesis, while *otc B* and *otc E* are earlier genes in oxytetracycline biosynthesis.

Cosynthesis was also detected between several inactive wild Streptomyces isolates and inactive mutants of either *Streptomyces rimosus* or *S. aureofaciens*. In fact the best haloes were often observed in such interspecific combinations. The halo invariably appeared on the side of the inactive mutant, the wild-type strain thus acting as a class 2 mutant. Cosynthesis of tetracycline antibiotics in combinations of different streptomycetes with inactive mutants of tetracycline producing species has been reported by McCormick, Sjolander & Hirsch (1961).

DISCUSSION

The agar method for the study of tetracycline cosynthesis, which may possibly be of general application in the study of secondary metabolism, has two distinct advantages over liquid mixed culture: the saving of space and material and the immediate recognition of the 'secretor' and the 'converter' member of each pair of cosynthesizing strains. Cosynthesis on agar must presumably involve the diffusion of some product, possibly a normal precursor of tetracycline biosynthesis, since no contact between the organisms was established in the combined culture.

The experiments described, which were carried out primarily to develop the method, have already shown interesting results. The complementation pattern is rather similar in the two species examined. In both, two distinct classes of inactive mutants occurred.

The first class, comprising groups (genes?) *otc B*, *otc C*, and *itc E* (and perhaps *otc A*) of *Streptomyces rimosus*, and groups *ctc B* and *ctc C* (and perhaps *ctc A*) of *S. aureofaciens*, consist of groups of mutants which complement with each other, and may act in different combinations either as 'secretors' or as 'converters'. They are very likely mutants in structural genes involved in the main pathway of antibiotic biosynthesis. A second class of mutants, comprising groups *otc D*, *otc F*, *otc C* and *otc H* (and perhaps *otc A*) of *S. rimosus* and *ctc D* (and perhaps *ctc A*) of *S. aureofaciens*, consists of groups of mutants which do not complement with each other, but complement with only some (if any) of the mutants of the first class, and can only act as secretors in cosynthesis. The nature of these mutants is not clear. The possibility that they arose by multisite mutations covering several structural genes seems unlikely, mainly because of their exceedingly high frequency, as compared with that of the assumed point mutations in the structural genes. They cannot involve genes controlling non-diffusible products, because they could never act (on this assumption) as secretors. It seems more probable that mutants of the second class are not altered in genes directly involved in the main pathway of antibiotic biosynthesis, but in regulatory genes or more generally, genes controlling the onset of secondary metabolism (Bu'lock, 1965), i.e. the shift of normal metabolic channels towards the antibiotic pathway. Whatever the function of genes affected in class 2 mutants, these mutants represent the great majority of the inactive mutants (up to 90% in *S. rimosus*, if we regard the *otc A*) group as belonging to class 2). We can thus conclude that only a small fraction of inactive mutants (class 1) are actually blocked in the main antibiotic pathway and only these should be used for the study of pathways of antibiotic biosynthesis.

It is a pleasure to acknowledge the advice of Dr D. Vlašić and to thank him for many helpful discussions. We are also indebted to Miss Jasenka Korajlija for her excellent technical assistance.

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EXPLANATION OF PLATE

Fig. 1. Antibiotic cosynthesis by a pair of inactive Streptomyces strains as revealed by the agar method (central strip). The central strip was cut from a 6-day culture on an agar dish, half seeded with *S. rimosus otc C* 15 (darker part of the strip) and half seeded with wild *Streptomyces* sp. 670. The right-hand strip is from a control pure culture of *S. rimosus otc C* 15, and the left-hand strip from a control culture of *Streptomyces* sp. 670. The strips were placed on agar medium embedded with *Bacillus subtilis* and the dish was incubated overnight. An inhibition halo is evident around the central strip on the *S. rimosus* half.

Fig. 2. Cosynthesis between inactive mutants of *Streptomyces aureofaciens*: *ctc A* 1 (1), *ctc B* 3 (3), *ctc C* 5 (5). Strain 1 is always a 'converter', strain 5 always a 'secretor', strain 3 is a 'converter' with 1 and a secretor with 5.

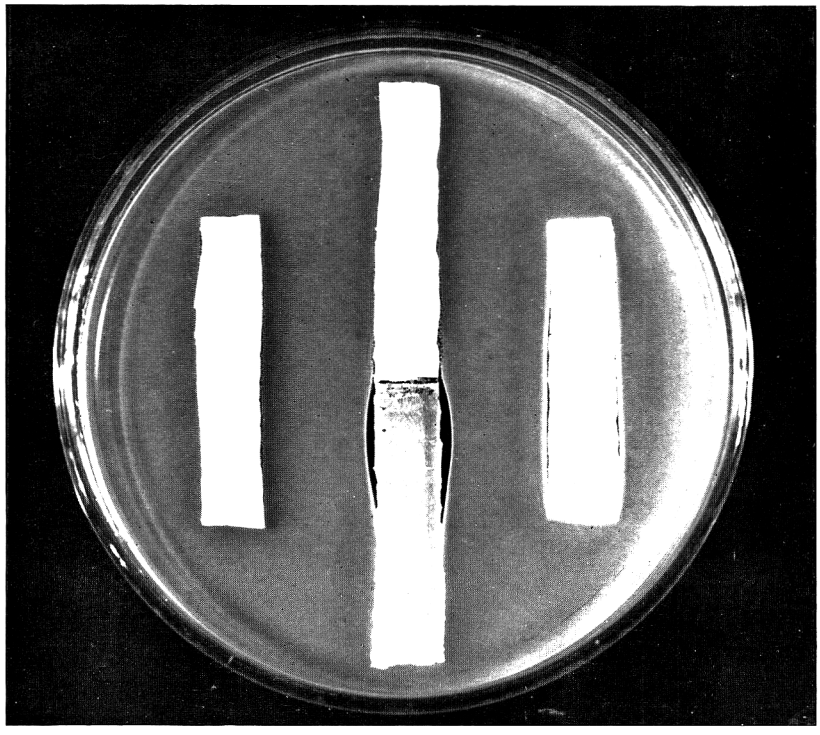

Fig. 1

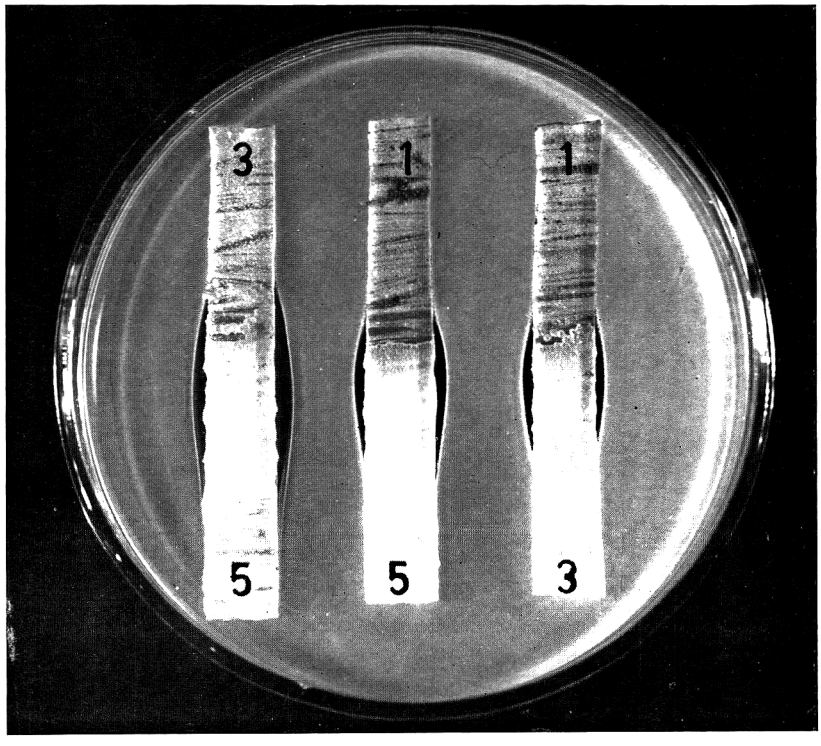

Fig. 2

(*Facing p.* 108)

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R-factor Gene Expression in Gram-negative Bacteria

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SUMMARY

Methods by which the R-factor 1818 was transferred from *Escherichia coli* K12 to different species of Gram-negative bacteria are described. Possession of 1818 resulted in the production of similar amounts of R-factor specific penicillinase per organism in *E. coli*, *Serratia marcescens*, *Alkalescens* sp. and *Aerobacter aerogenes*. The Aerobacter strain produced additionally a 'chromosomal' penicillinase which had no influence on the level of R-factor enzyme.

Two different strains of *Proteus mirabilis* containing R-factor 1818 produced only about $\frac{1}{20}$th of this penicillinase activity per bacterium Two further R-factors 7268 and TEM also specifying penicillinase were introduced into the *P. mirabilis* strains and enzyme activities per bacterium of about $\frac{1}{20}$th and $\frac{1}{100}$th, respectively, were obtained compared with those in *E. coli*. All organisms in cultures of *P. mirabilis* exhibited properties of R-factor possession and thus R-factor instability cannot explain these results. It would seem that the phenotypic expression of R-factor penicillinase genes is impaired in this species.

INTRODUCTION

It has been shown that F prime (F′) episomes can be detected in Proteus and Serratia strains due to the presence of a physically distinguishable satellite DNA fraction of Escherichia base composition (Marmur *et al.* 1961; Falkow, Wohlhieter, Citarella & Baron, 1964). Similar satellite bands have been observed when R-factors are present in Proteus and Serratia (Falkow, Wohlhieter, Citarella & Baron, 1963; Falkow, Citarella, Wohlhieter & Watanabe, 1966; Rownd, Nakaya & Nakamura, 1966). Calculations based on the relative amounts of DNA in the main and satellite bands have indicated that approximately one R-factor exists per bacterial chromosome in Escherichia and Serratia, whereas there are about ten copies per *Proteus mirabilis* chromosome (Rownd *et al.* 1966). On the other hand, Falkow *et al.* (1964) have shown that *F-lac* β-galactosidase synthesis in *P. mirabilis* is constitutive at about 25% of the level of that of the same gene on the chromosome of fully induced *Escherichia coli*. However, it could be argued that comparisons between the products of a gene on an episome with those of a chromosomal gene are not valid. Nevertheless, Colby & Hu. (1968) have extended this work to compare levels of β-galactosidase produced by episomal *lac* genes in *E. coli* and *P. mirabilis* and obtained broadly similar results. They conclude that there is an anomalous regulation in Proteus which may be attributed to the cytoplasmic environment of the F-*lac* episome.

The expression of penicillin resistance carried by R-factors is by the production of penicillinase by the cells harbouring such episomes (Anderson & Datta, 1965; Ander-

son & Lewis, 1965). This communication concerns the introduction of penicillinase specifying R-factors into *Escherichia coli*, *Serratia marcescens*, *Aerobacter aerogenes*, *Alkalescens* sp. and *Proteus mirabilis* using an *E. coli* donor. Subsequent investigations of the penicillinase levels in the resultant R-factor containing bacteria show that Escherichia, Serratia, Aerobacter and Alkalescens express a similar level of R-factor gene activity per bacterium. However, two strains of *P. mirabilis*, including that of Rownd *et al.* (1966), were found to exhibit much less R-factor gene activity per organism than the other species and with R-factor TEM the level was less than 1 %. Kontomichalou (1967) also found that *P. mirabilis* produced less R-factor penicillinase than *E. coli* but she observed much higher levels than reported here since all her R-factors including TEM produced about one-third of the *E. coli* level. These results at first sight do not agree with the work of Rownd *et al.* (1966) as one would expect that, if there are as many as more than 10 copies of the R-factor per *P. mirabilis* organism, higher enzyme levels would be found. It is possible, however, to resolve these differences by postulating extreme impairment of R-factor gene function in *P. mirabilis*.

METHODS

Bacterial strains. The strains of *Escherichia coli* K12, *Serratia marcescens*, *Aerobacter aerogenes*, *Alkalescens* sp. and *Proteus mirabilis* that were used in this study are listed in Table 1.

R-factors. All R-factors were kindly supplied by Dr Naomi Datta; they were carried by the same host strain, *Escherichia coli* K12, and are listed in Table 2.

Media. Davis & Mingioli (1950) liquid and solid minimal medium (DM) was prepared as described by Smith (1967). Oxoid Nutrient Broth no. 2 and MacConkey agar were supplied by Oxoid Ltd., Southwark Bridge Road, London, S.E. 1.

Drugs and chemicals. Streptomycin sulphate, tetracycline hydrochloride, sodium ampicillin and sodium sulphadiazine were bought commercially as sterile products. These were weighed and dissolved in sterile distilled water using aseptic procedures. Amino acids, vitamins and other reagents were purchased from L. Light and Company Limited, Colnbrook, Buckinghamshire.

Supplements. Amino acid supplements with the exception of methionine were dissolved in distilled water, adjusted to pH 7·4 with NaOH, autoclaved and added singly at a final concentration of 40 μg. of the L isomer/ml.. Nicotinic acid was used at a final concentration of 5 μg./ml. A great deal of trouble was experienced with the growth of strain K12 on minimal media and this was finally traced to heat sterilization of methionine. It was found that only freshly prepared filter-sterilized DL-methionine (50 μg./ml.) gave satisfactory viable counts with this organism as judged by comparison with growth on nutrient agar.

Table 1. *Bacterial strains*

Organism	Designation	Relevant characters	Dry weight (viable count per mg. × 10^{-9})	Source	Reference
Escherichia coli K12	K12	*met* $^{-}_{1}$ F^{-}*	4·2	W. Hayes	Meynell & Datta (1966)
E. coli K12	J6-2	*pro*$^{-}_{2}$. *his*$^{-}$. *try*$^{-}$. *lac* $^{-}$. F^{-}	4·1	W. Hayes	Clowes & Rowley (1954)
Serratia marcescens		Ap^{r} (1·0), Tc^{r} (1·0)	4·0	NCTC 1377	—
Aerobacter aerogenes	418	Ap^{r} (1·0), Tc^{r} (6×10^{-7})	4·2	J. M. T. Hamilton-Miller	Smith & Hamilton-Miller (1963)
Alkalescens sp.	V2	Sm^{r} (3×10^{-6})	2·5	R. H. Gorrill	Heptinstall & Gorrill (1955)
Proteus mirabilis	130	Sm^{r} (2×10^{-6}), Tc^{r} (1·0), *nia*$^{-}$†	5·8	NCTC 5887	—
P. mirabilis	F67	Sm^{r} (1·0), Tc^{r} (1·0) *nia*$^{-}$†	6·0	Dr F. Kauffmann	Rownd *et al.* (1966)

* This is 58.161/*sp.* which although behaving as F^{-} retains some F^{+} characteristics, see reference quoted.

† This requirement was not absolute but when added stimulated growth considerably.

met^{-}, methionine requiring; *pro*$^{-}_{2}$, proline requiring; *his*$^{-}$, histidine requiring; *try*$^{-}$, tryptophane requiring; *lac*$^{-}$, lactose non utilization; *nia*$^{-}$, nicotinic acid requiring.

Ap^{r}, ampicillin resistant; Tc^{r}, tetracycline resistanct; Sm^{r}, streptomycin resistant. The figures in parentheses indicate the frequency of resistant bacteria.

Transfer of R-factor 1818

An incubation temperature of 37° was used throughout this work. Overnight broth cultures of bacteria were used. Donor culture (0·1 ml.) was mixed with 1·0 ml. of recipient culture and 4·9 ml. of warm fresh broth and incubation continued for 6 hr. To wash the organisms 20 ml. of DM medium without glucose (DM buffer) were then added and the mixture centrifuged, the supernatant discarded and the pellet reconstituted in 6 ml. of DM buffer with the aid of a Vortex junior mixer. These steps were necessary to exhaust the mixed culture of nutrients, which could otherwise be carried over on to the selective media and allow growth of the donor strain.

Table 2.

R-factor	Species in which isolated	Resistances
1818	*S. typhimurium*	Ap Sm Su Tc
7268	*S. paratyphi B*	Ap (Sm*) Su Cm Km
TEM	*E. coli*	Ap Sm

These R-factors were originally described by Datta & Kontomichalou (1965). Subsequently 1818 and 7268 have been designated R46 and R1 respectively (Meynell & Datta 1966).

Ap, ampicillin; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline; Cm, chloramphenicol; Km, Kanamycin; *low level of resistance only.

Serial decimal dilutions of 'washed' mixed culture were made in DM buffer and 0·1 ml. amounts plated and spread with a glass spreader on selective media supplemented for the recipient only and containing antibiotic sufficient to inhibit growth of the recipient lacking the R-factor. Controls were made using donor and recipient bacteria cultured separately to ensure that: (*a*) no growth of donor bacteria could occur, and (*b*) no growth of the recipient strains could occur unless they possessed the R-factor. To investigate the inoculum size effect further controls were made using donor bacteria killed by ultraviolet irradiation in DM buffer mixed with a similar number of viable recipient bacteria. In addition plates spread with viable donor bacteria were overlaid with an Oxoid cellulose acetate filter membrane which was then spread with a similar number of viable recipient bacteria. These controls showed that at dilutions $< 10^{-2}$ 10 μg./ml. Streptomycin (Sm) or Tetracycline (Tc) was necessary to prevent growth of recipient bacteria without the R-factor and that neither Sulphonamide (Su) nor Ampicillin (Ap) could be used reliably at these dilutions. Further tests showed that significant Ap destruction had occurred after 3 days incubation of plates spread with such numbers of donor bacteria. At dilutions $> 10^{-2}$ it was found that optimum recovery of all 1818 recipients was obtained using Sm, Tc, Ap or Su at a concentration of 5 μg./ml.

In order to estimate the number of donor and recipient bacteria dilutions were also spread on MacConkey agar. It was found that colonies of all the mixtures used could be differentiated by eye this way. In addition, as a check, separate platings were made on DM media appropriately supplemented or otherwise manipulated to allow the separate growth of the donor or recipient strain. With the Proteus strains it was found that MacConkey agar was extremely inhibitory since less than 1 % formed colonies as judged by total counts. However, complete recovery of these bacteria was observed using DM agar supplemented with nicotinic acid.

Incubation of all plates was made for up to 3 days. Selected colonies were purified by three successive streakings on the same selective media before the strains were regarded as pure. In addition streakings were made on nutrient and MacConkey agar to check that no donor organisms persisted and on media containing Sm, Su, Ap or Tc to ensure that all resistances had been transferred.

Estimation of penicillinase

Cultivation and counting. Cultures were grown overnight at 37° in 50 ml. amounts of nutrient broth in 100 ml. bottles. Viable counts were made by dilution in nutrient broth at 37° and spread on nutrient agar at 37°. The Proteus strains, were counted on DM agar supplemented with nicotinic acid which prevented spreading of the colonies. In order to test whether segregation of bacteria lacking the R-factor occurred counts were also made using selective media which was designed to prevent completely growth of recipient bacteria lacking the R-factor but allow complete growth of the R-factor carrying bacteria. The antibiotics used were Sm, Tc or Su at a concentration of 5 μg./ml. or Ap 2 μg./ml. in DM agar where appropriate with respect to the innate sensitivity of the host strain. At least two antibiotics were used to check each strain. With all the strains studied such segregation was insignificant (< 1 %) with the exception of *Serratia marcescens*/1818, which segregated organisms lacking the R-factor at a frequency of about 8 %. Such segregants were excluded from calculations of penicillinase activity.

Bacterial cultures were centrifuged and the viable count of the supernatant fluid estimated. This was subtracted from the culture count and the resulting number used in calculations for penicillinase activity. These procedures were found to be necessary since, depending on the strain studied, different fractions of the bacteria were left in the supernatant fluid and resuspension of the pellet in phosphate buffer killed a fraction of the bacteria it contained.

Penicillinase assay. The pellet was resuspended in 5 ml. 25 mM-sodium phosphate buffer pH 7·4 and cooled to 4°. To remove crypticity (Cohen & Monod, 1957) the suspension was treated in an MSE ultrasonic disintegrator at 1·5 A. and 4° for 4 min. Suitable dilutions of the disrupted bacteria were made in 25 mM-sodium phosphate buffer pH 7·4 at 4° and substrate added to a concentration of 5 mM. The reaction mixture was warmed at 30° in a water bath and the amount of penicillin remaining determined by the hydroxylamine assay as described by Smith (1963). These precautions were necessary as it was found that, irrespective of the host strain, the enzyme produced by the R-factor 1818 was thermolabile at temperatures above 33° especially when dilute. This thermolability was decreased when the enzyme was protected by substrate. Hence to avoid thermal inactivation completely substrate was added before the reaction mixture was incubated and an assay temperature of 30° was used.

The destruction of penicillins was followed by using substrate concentrations sufficient to saturate the enzyme. Control tests showed that, with all strains studied, the penicillinase activity was largely intracellular since > 95 % of the total penicillinase activity of a culture could be recovered in the disrupted pellet. Results were expressed in μmole of substrate destroyed per min. which are units recommended by the International Commission on Enzymes (Thompson, 1962).

RESULTS

Transfer of R-factor 1818

With Escherichia coli J6-2 recipient. The results (Table 3, Expt. 1) show that the transfer frequency is similar irrespective of the antibiotic used and probably reflects the simultaneous transmission of all four resistances. Furthermore, the penicillinase activity of strain J6-2 (1818) isolated independently on media containing each antibiotic was found to be similar to that of strain K12 (1818). Indeed when J6-2 (1818) was used as donor and K12 as recipient similar findings as regards transfer frequency and penicillinase activity were made.

Table 3. *Transfer of R-factor 1818 to different species of bacteria*

Expt. no.	Recipient	Selective agent	Frequency of transfer per bacterium: Donor	Frequency of transfer per bacterium: Recipient
1	*Escherichia coli* J6–2	Tc	$1{\cdot}3\times10^{-2}$	$8{\cdot}9\times10^{-3}$
		Ap	$1{\cdot}4\times10^{-2}$	$9{\cdot}7\times10^{-3}$
		Sm	$1{\cdot}5\times10^{-2}$	$1{\cdot}0\times10^{-2}$
		Su	$1{\cdot}6\times10^{-2}$	$1{\cdot}1\times10^{-2}$
2	*Serratia marcescens*	Sm	$2{\cdot}0\times10^{-3}$*	$1{\cdot}4\times10^{-5}$
3	*Proteus mirabilis* 130	Sm	$1{\cdot}4\times10^{-1}$	$3{\cdot}3\times10^{-2}$
		Su	$1{\cdot}7\times10^{-1}$	$4{\cdot}0\times10^{-2}$
		Ap	$1{\cdot}6\times10^{-1}$	$3{\cdot}8\times10^{-2}$
	P. mirabilis F67	Su	$3{\cdot}3\times10^{-1}$	$9{\cdot}2\times10^{-2}$
		Ap	$2{\cdot}9\times10^{-1}$	$8{\cdot}1\times10^{-2}$
4	*Alkalescens* sp. V2	Tc	$3{\cdot}0\times10^{-9}$	$1{\cdot}3\times10^{-8}$
5	*Aerobacter aerogenes* 418	Tc & Sm	$1{\cdot}9\times10^{-6}$	$3{\cdot}0\times10^{-7}$

* Per input donor.

The donor strain K12 (1818) was mixed at a ratio of 1 : 10 with the recipient strain in fresh medium as indicated in the text. After 6 hr incubation the mixtures were plated to isolate donor bacteria, recipient bacteria and recipient bacteria carrying R-factor 1818.

With Serratia marcescens recipient. When the transfer of R-factor to *Serratia marcescens* was studied it was found that the K12 donor was rapidly killed, presumably due to a phage or colicin carried by the *S. marcescens*, but nevertheless R-factor transfer was observed (Table 3, Expt. 2). A similar phenomenon has been reported by Watanabe (1963).

With Proteus mirabilis recipients. The transfer of R-factor 1818 from K12 (1818) to *Proteus mirabilis* strains 130 and F67 occurred at high frequency which was similar irrespective of the drug used to select *P. mirabilis* (1818) (Table 3, Expt. 3).

With Alkalescens sp. strain V2 recipient. Numerous unsuccessful attempts were made to transfer from K12 (1818) to V2 by using nutrient broth cultures. The recipient is very mucoid which is possibly the reason why its dry weight is highest of all strains used here (Table 1). It was thought that large amounts of capsular material could interfere with conjugation. Since growth of this organism on DM agar was much less mucoid it seemed possible that R-factor transfer might occur using DM liquid medium. When DM-grown donor and recipient bacteria were mixed and culture continued in DM medium R-factor transfer was observed, albeit at very low rates (see Table 3, Expt. 4), using Tc to select for V2 (1818). Streptomycin (Sm) was not suitable since the

natural mutation rate of V2 was higher than the transfer rate (see Table 1) and neither Ap nor Su were suitable due to reasons stated in the methods.

With Aerobacter aerogenes 418 recipient. This recipient behaved like *Alkalescens* sp. strain V2 in that, despite repeated attempts, transfer was not observed in nutrient broth. Since this is also a mucoid strain attempts were made to obtain transfer using DM medium and Sm as selective agent, again without success. Ap could not be used as the recipient strain produces penicillinase (Smith & Hamilton-Miller, 1963) and Su was also unsuitable (see methods). Tc suffered from the defect that the natural mutation rate of 418 to Tc resistance was significant (see Table 1) and precluded its use as a direct selective agent under conditions of low frequency transfer. However, when DM grown bacteria were cultured together in DM medium and plated on Tc it was found that colonies of 418 (1818) could be differentiated from those of 418. Tc^r by replication on Sm 10 μg./ml., which selected for R-factor containing colonies. Thus transfer was achieved and the frequencies are shown in Table 3, Expt. 5. It is unknown why Sm failed to isolate this organism directly after mixed culture; subsequent investigation showed that resistance to Sm as well as resistance to the other three drugs was present in 418 (1818). Generally Sm^r is more rapidly expressed when the allele is on a R-factor in comparison to a chromosomal location but wide variations in R-factor Sm^r expression do occur (Watanabe, 1963) and it is possible that there is an abnormally long segregation lag in the Aerobacter recipient.

Penicillinase activities of bacteria

The substrate profile of the penicillinase activity of R-factor 1818 has been published by Datta & Kontomichalou (1965). In addition to the substrates studied by these authors it was found that oxacillin was very rapidly hydrolysed by this enzyme (J. M. T. Hamilton-Miller, personal communication). Therefore when the penicillinase activity of the strains studied here was investigated both oxacillin and benzylpenicillin were used as substrates. In addition, oxacillin was particularly useful as a sensitive index of R-factor 1818 penicillinase activity since most if not all other penicillinases studied do not significantly hydrolyse this substrate (Smith & Hamilton-Miller, 1963). As the number of organisms studied here was too large to investigate simultaneously with accuracy each separate experiment was conducted using J6-2 (1818) and K12 (1818) as internal controls. All species were investigated with and without the R-factor. However, it was found that, with the exception of *Aerobacter aerogenes* 418, all strains lacking the R-factor were penicillinase-negative or produced insignificantly low amounts of penicillinase. It is interesting that the penicillinase produced by 418 does not hydrolyse oxacillin, whereas that of 418 (1818) does (Table 4). Both enzymes, however, hydrolyse benzylpenicillin. The activity of 418 (1818) on benzylpenicillin was 49·6 milli units, whereas with 418 it was 12·6 milli units. The difference (37·0) is theoretically the benzylpenicillin activity due to the R-factor enzyme and agrees well with the activity of the *Escherichia coli* controls. Since 418 penicillinase does not attack oxacillin the penicillinase activity of 418 (1818) on oxacillin should reflect its R-factor penicillinase alone and this was similar to the activities of the *E. coli* controls. Furthermore, the oxacillin:benzylpenicillin ratio 236·3:37·0 = 6·4 agrees reasonably with the ratios obtained with all other R-factor containing organisms.

With this consideration in mind it can be seen (Table 4) that all strains which possess R-factor 1818, with the exception of *Proteus mirabilis* strains 130 (1818), and F67 (1818),

Table 4. *Penicillinase activities of R-factor 1818*

Expt. no.	Organism	Antibiotic used to select R-factor isolate used	Penicillinase activity (m-units per 10^9 bacteria using)		Penicillinase activity of R-factor 1818 as % relevant controls using		Ratio of oxacillin: benzylpenicillin activity
			Benzylpenicillin	Oxacillin	Benzylpenicillin	Oxacillin	
1	*Serratia marcescens* (1818)	Sm	35·3	225·1	110·6	112·2	6·4
	Escherichia coli J6-2 (1818)	Tc	33·5	212·4			6·3
	E. coli K12 (1818)	—	30·3	188·9			6·2
2	*Alkalescens* sp. V2 (1818)	Tc	38·8	245·5	114·6	119·0	6·3
	E. coli J6-2 (1818)	Tc	34·9	217·8			6·2
	E. coli K12 (1818)	—	32·8	194·7			5·9
3	*Acrobacter aerogenes* 418	—	12·6	0			—
	A. aerogenes 418 (1818)	Tc & Sm	49·6	236·3	109·0*	115·3	4·8†
	E. coli J6-2 (1818)	Tc	33·0	193·5			5·9
	E. coli K12 (1818)	—	34·9	216·3			6·2
4	*Proteus mirabilis* F67 (1818)	Ap	1·7	10·5	5·4	5·3	6·2
	P. mirabilis 130 (1818)	Ap	2·1	12·8	6·7	6·5	6·1
	E. coli J6-2 (1818)	Tc	33·9	214·4			6·3
	E. coli K12 (1818)	—	28·8	178·4			6·2

* When account is taken of the penicillinase produced by this strain without the R-factor.

† When account is taken of the penicillinase produced by this strain without the R-factor this ratio becomes 6·4 (see Text),

The viable counts of overnight nutrient broth cultures were estimated, the cells deposited by centrifugation and ultrasonically treated to remove crypticity. The rate of destruction of both substrates was followed at concentrations sufficient to saturate the enzyme (initial substrate concentration 5 mM) pH 7·4 and 30° using the hydroxylamine assay. The results are expressed as mμ mole substrate hydrolysed per min./10^9 viable units.

In addition the percentage penicillinase activity of the strains studied in each of the four experiments is calculated in relation to the mean-penicillinase activities found with the two *E. coli* controls on both substrates in that particular experiment.

have penicillinase activities similar to those of the two *Escherichia coli* controls. The *P. mirabilis*, (1818) strains, make much less penicillinase (about $\frac{1}{20}$th) than all the other strains studied here. Rownd *et al.* (1966) presented evidence showing that *P. mirabilis* F67 contained 10 times as much R-factor satellite DNA as *E. coli* K12 and if this is applicable here it would be expected that the *P. mirabilis*, (1818) strains, would make more, rather than less, penicillinase than *E. coli* K12 (1818). Consequently further investigations were made into the penicillinase activity of the two *P. mirabilis* strains studied here. These included a careful examination into the amount of enzyme produced (*a*) extracellularly, (*b*) by log. phase cultures, (*c*) by 130 (1818) and F67 (1818) isolated on Su and 130 (1818) isolated on Sm, and (*d*) by bacteria cultured in DM medium. In no case was any discrepancy noted which could be used to explain the low penicillinase activity of these Proteus strains.

Tests were done to see if there was any synergism or antagonism in mixtures of crude enzyme preparations from *Escherichia coli*, (1818), and *Proteus mirabilis*, 1818, and in no case was any effect other than additive observed. Thus *P. mirabilis* cell sap does not contain any inhibitor of the R-factor penicillinase nor does the cell sap of *E. coli* contain any stimulatory factor for the R-factor penicillinase from *P. mirabilis*. Furthermore, although the dry weights of Proteus strains were about 33 % lower than those of *E. coli*, *Aerobacter aerogenes* and Serratia (Table 1) this was not sufficient to account for the difference in penicillinase levels.

Table 5. *Penicillinase activity of 3 R-factors in Proteus mirabilis*

Organism	Strain no.	R-factor	Penicillinase activity using benzylpenicillin substrate (m-units/10^9 bacteria)	Percentage penicillinase activity compared with same R-factor in *E. coli* K 12
Proteus mirabilis	130	(TEM)	7·8	0·8
P. mirabilis	F 67		7·3	0·9
Escherichia coli	K 12		907·3	100
P. mirabilis	130	(7268)	9·0	7·2
P. mirabilis	F 67		8·9	7·1
E. coli	K 12		124·9	100
P. mirabilis	130	(1818)*	2·1	6·7
P. mirabilis	F 67		1·7	5·4
E. coli	K 12		28·8	100

* Data taken from Table 4 for the sake of comparison.

The viable counts of overnight broth cultures were estimated, the cells deposited by centrifugation and ultrasonically treated to remove crypticity. The rate of destruction of benzylpenicillin was followed at concentrations sufficient to saturate the enzyme (initial concentration 5 mM) pH 7·4 and 30° using the hydroxylamine assay. The results are expressed as mμ mole of benzylpenicillin hydrolysed per min./10^9 viable units.

Since it was possible that these results could be explained by R-factor modification in the Proteus strains it was transferred from them to *Escherichia coli* K12 which was then tested for penicillinase production and a level similar to that produced by the original K12 (1818) donor was detected. These results indicate that, at least as regards the penicillinase gene, no alteration to the R-factor had occurred as a result of its transfer into and out of *Proteus mirabilis*. Kontomichalou (1967) has also reported this finding.

Investigation of R-factors TEM and 7268 in Proteus mirabilis strains 130 and F67

Since the substrate specificity of the penicillinase of R-factor 1818 was found to be unlike that of any penicillinase studied in this laboratory and its thermolability was also unusual it was thought possible that other R-factor penicillinases may behave differently in *Proteus mirabilis*. The penicillinases specified by R-factors TEM and 7268 have been stated (Datta & Kontomichalou, 1965) to be similar to those produced by Aerobacter strains (Smith & Hamilton-Miller, 1963). Accordingly, these were transferred from a K12 donor using Ap 10 μg./ml. as selection for *P. mirabilis* (TEM) and *P. mirabilis* (7268). The penicillinase activities (Table 5) are interesting in that by comparison with the corresponding K12 strain the level due to R-factor 7268 in either strain of Proteus is similar to that produced by R-factor 1818 (about $\frac{1}{20}$th). The amount of enzyme produced by R-factor TEM is even more depressed as 130 (TEM) and F67 (TEM) produced less than 1% of the penicillinase activity of K12 (TEM). Thus two different strains of *P. mirabilis* containing three different R-factors produce much less penicillinase than the corresponding *Escherichia coli* K12 hosts.

DISCUSSION

When the R-factor 1818 was introduced into Escherichia, Serratia, Aerobacter and Alkalescens strains a similar degree of R-factor specific penicillinase was produced. It is tempting to assume that the gene dosage effect (Jacob, Schaeffer & Wollman, 1960) is applicable to these four species. Conclusions drawn from this effect have been criticized (Jacob, Brenner & Cuzin, 1963) on the grounds that Jacob & Monod (1961) found that β-galactosidase produced by a F-*lac* episome was 2·5 to 3 times more than that produced by normal haploid bacteria where the *lac* gene occupied a chromosomal position. Calculations by Yoshikawa & Sueoka (1963) have shown that this difference may be attributed, not to a larger number of episomal genes, but to the likely possibility that episomal replication is completed long before that of the bacterial chromosome. However, the studies here have been with episomal genes only and no comparison has been drawn with levels of enzyme produced by the same gene in a chromosomal location. Another factor is that if a gene is subject to regulation it may be regulated differently in different cytoplasmic environments as, noted by Colby & Hu (1968). However, this criticism does not apply here as preliminary work indicates that all these bacterial species were constitutive as regards their penicillinase. Indeed, the same applies to the penicillinase produced by chromosomal genes in many species of Gram-negative bacteria (Smith & Hamilton-Miller, 1963). However, to prove gene dosage it will be necessary to estimate directly the amount of genetic material attributable to the R-factor in the species studied here and this project is being considered. It should be noted that experiments along these lines have been done with Escherichia and Serratia by Rownd *et al.* (1966), who found similar quantities of R-factor specific satellite DNA in these two species.

The extremely low level of R-factor penicillinase detected in *Proteus mirabilis* is difficult to explain. It would seem that since every organism can grow on antibiotic concentrations sufficient to inhibit completely the growth of bacteria lacking R-factor there must be at least one R-factor in each Proteus organism. Moreover, if the results of Rownd *et al.* (1966) are applicable here it is possible that *P. mirabilis* possesses

about ten R-factor copies per bacterium and it seems improbable that gene dosage is applicable to this species. Kontomichalou (1967) also found that *P. mirabilis* produced less R-factor penicillinase than *Escherichia coli*. However, in relation to the *E. coli* level all her R-factors including TEM produced about 30 times more penicillinase in her strain of *P. mirabilis* than TEM in two different strains of this species studied here. Furthermore, one of the latter strains (F67) is the *P. mirabilis* of Rownd *et al.* (1966). It is not known whether this discrepancy can be attributed solely to strain differences.

Okamoto, Suzuki, Mise & Nakaya (1967) found that the level of chloramphenicol inactivating enzyme was similar in *Escherichia coli* and in *Proteus mirabilis*. The R-factor 7268 studied here carries a Cm^r allele and preliminary work has shown that it too mediates resistance by an inactivation mechanism. Moreover, the level of enzyme produced is similar in the same *E. coli* and *P. mirabilis* cells which show such marked differences in penicillinase levels. This it would seem that the cytoplasm of *P. mirabilis* can control some but not all R-factor gene functions. It is strange that although the work of Rownd *et al.* (1966) suggests that *P. mirabilis* cannot control the replication of its R-factors the work here suggests that they may partially compensate for this by controlling the phenotypic expression of some but not all R-factor genes.

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The Mutagenic Action of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on *Coprinus lagopus*

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SUMMARY

Optimal conditions, defined by the use of a known auxotrophic strain, are described for the mutagenesis of *Coprinus lagopus* oidia by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), which is a very effective mutagen for bacteria and yeast. The oidia were best treated in phosphate buffer (pH 6·8) with a concentration of 15 μg. NTG/ml. for about 70 min. The yield of auxotrophs was considerably less than might be expected from a comparison of other systems in which NTG has been used as a mutagen.

INTRODUCTION

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) has been widely used to induce mutations in bacteria. It has proved highly effective, so much so that it has been suggested to be the most potent chemical mutagen yet discovered (Adelberg, Mandel & Chen, 1965). Adelberg *et al.* (1965) found that mutations to valine resistance and to auxotrophy occurred at high frequency (up to 42·5% auxotrophs) after exposure of *Escherichia coli* to NTG under conditions such that about 5% of the treated bacteria remained viable. NTG has also proved a very effective mutagen for yeast, though the results are less dramatic than with *E. coli*. Megnet (1965) with *Schizosaccharomyces pombe* found that, without selection, NTG-induced auxotrophs increased to a maximum frequency of about 8% at 20% survival. On the other hand, Nordström (1967) obtained up to 50% petite mutants among survivors of *Saccharomyces cerevisiae* after NTG treatment.

To date the vast majority of auxotrophs obtained in *Coprinus lagopus* have been isolated following exposure to ultraviolet (u.v.) radiation. The reported successes in the use of NTG as a mutagen in other organisms prompted the experiments reported below. It can be concluded that, under the conditions tested, NTG was no more advantageous than u.v. radiation in inducing mutants of *C. lagopus*, though it should prove to be a useful alternative.

METHODS

A strain of *Coprinus lagopus*, stock number ZMR1/66, with nutritional requirements for methionine (*met-8* allele) + nicotinic acid (*nic-4*) was used.

Complete and minimal media were made as described previously (Moore, 1968). Normal media contained 100 mM-D-glucose; sorbose media contained 100 mM-L-sorbose + 2·5 mM-D-glucose. Except where otherwise stated, nicotinic acid was added to minimal media to a final concentration of 2 μg./ml.; where required methionine was added to a final concentration of 100 μg./ml.

NTG treatment was done on oidial suspensions prepared from slope cultures growing on normal complete medium (Moore, 1968). Oidia were suspended in a buffered solution (pH 6·8) which was one of the stock medium constituents. In addition to the buffer salts (10 mM-KH_2PO_4 + 10 mM-Na_2HPO_4) this solution contained 3·3 mM-ammonium tartrate, 2 mM-Na_2SO_4 and 1×10^{-4}% (w/v) thiamine hydrochloride. Freshly harvested oidia were centrifuged and the pellet washed twice with buffer solution before being suspended in fresh buffer.

NTG was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. Solutions were made in sterile buffer immediately before use, and used without further sterilization.

All incubations and treatments were made at 37°; NTG solutions and prepared oidial suspensions were equilibrated to this temperature before mixing. The final concentration of viable oidia treated varied from 2 to 8×10^5 in 10 ml.

Treatment was terminated by filtration through a sterile Oxoid membrane filter (5 cm. size, standard grade). Oidia retained by the filter were washed with 50 ml. cold sterile buffer and then the filter was transferred to a sterile 1 oz. universal bottle containing 10 ml. buffer. Oidia were resuspended by vigorous hand shaking and the filter membrane removed. This technique enabled the rapid and complete removal of extracellular NTG. The filtration step did not significantly affect the concentration of the oidial suspension.

The filtered suspension was analysed for both survival and methionine independence. Survival was measured by plating appropriately diluted samples on sorbose complete medium. Colonies were counted after about 48 hr incubation. To determine the production of methionine-independent mutants from the methionine-dependent parent strain 1 ml. quantities of the undiluted post-treatment suspension were added to small tubes containing 2 ml. melted water agar (1 %, w/v) at 45°. After mixing the contents of the tube were poured over the surface of a plate of sorbose minimal agar. Colonies were counted after 48 to 60 hr incubation. The frequency of spontaneous methionine-independent mutants varied from 3·4 to 28×10^{-3}.

RESULTS AND DISCUSSION

Effect of different NTG concentrations. The numbers in Table 1 were derived from experiments in which oidia were exposed to NTG for a standard period of 30 min. NTG clearly exerted both toxic and mutagenic effects on *Coprinus lagopus*. This organism is evidently much more sensitive to the toxic effects of NTG than either *Schizosaccharomyces pombe* or *Escherichia coli*. It is not possible to make a precise comparison from the published data. However, with a 30 min. treatment time only 39 % of *E. coli* organisms were killed by 300 μg. NTG/ml. (Adelberg *et al.* 1965), while 80 % of treated organisms of *S. pombe* were killed by exposure to 2 mg. NTG/ml., again for 30 min. (Megnet, 1965). Very approximately, therefore, *C. lagopus* appears to be 100 times more sensitive than *E. coli*, and 200 times more sensitive than *S. pombe* to the killing effects of NTG under comparable conditions.

Effect of time of exposure to NTG. 15 μg. NTG/ml. (about 0·1 mM) was chosen as a standard concentration for further experiments. The effect of time of exposure on survival and frequency of methionine-independent mutants was determined for this concentration. Results representative of the four experiments performed are presented

in Table 2. It is clear that exposure to 15 μg. NTG/ml. for between 50 and 70 min. gave the best balance between toxicity and mutagenicity of NTG.

Effect of the suspending medium. Adelberg *et al.* (1965) and Nordström (1967) reported that NTG showed a much greater toxicity towards *Escherichia coli* and *Saccharomyces cerevisiae* when the organisms were treated in media which permitted

Table 1. *Survival and mutagenesis in Coprinus lagopus as a function of NTG concentration*

NTG concentration (μg./ml.) (30 min. exposure)	Survival (%)	Methionine-independent mutants/10^3 survivors
0	100	0·003
1	87	0·007
2	92	0·024
3	66	0·026
5	53	0·031
10	36	0·063
15	16	0·107
30	4	Not scored

Table 2. *Coprinus lagopus. Survival and mutagenesis as a function of time*

Duration of exposure to 15 μg. NTG/ml. (min.)	Survival (%)	Methionine-independent mutants/10^3 survivors
5	74	0·11
10	80	0·07
15	33	0·13
25	16	0·29
40	7	0·26
55	3	0·84
70	1·7	0·89
85	0·6	0·68

Table 3. *Coprinus lagopus. Survival as a function of the suspending medium*

Duration of exposure to 15 μg. NTG/ml. (min.)	Survival (%)		Minimal medium + methionine + nicotinic acid
	Buffer	Minimal medium	
5	86	58	40
10	73	28	12
15	38	13	5

growth than when they were exposed to the agent in buffer. A similar effect was observed with *Coprinus lagopus* strain ZMR1/66 (Table 3). Adelberg *et al.* (1965) found that the fraction of mutants among survivors was the same whether the organisms were treated in buffer or in growth-permitting medium. Though no such comparison has been made here, it is likely that a similar condition applies. In any case the use of buffer as the suspending medium is obligatory when the wild-type organism is to be treated.

Effect of NTG on the wild type strain BC9/66 of Coprinus lagopus. Experiments with

the auxotrophic strain ZMR1/66 established conditions for NTG treatment which appeared likely to be optimal for the production of mutations in wild-type oidia. The response of wild-type oidia to exposure for various times to 15 μg. NTG/ml. in buffer is shown in Table 4. The survival of BC9/66 oidia is very similar to the survival pattern of ZMR1/66 (Table 2), suggesting that mutagenic conditions shown to be optimal for ZMR1/66 most likely also applied to BC9/66. Accordingly survivors from an experiment in which BC9/66 oidia were exposed in buffer to 15 μg. NTG/ml. for 70 min. (1·85% survival) were examined and mutants isolated; 575 survivors were isolated individually for examination. Of this number three proved to be auxotrophic and seven were isolated as reliable morphological variants. One of these latter showed the 'purple' phenotype described by Morgan (1966). This result can be compared with an experiment in which BC9/66 oidia were u.v.-irradiated. Survival was 4·6% and 1050 survivors were isolated for examination; five auxotrophs were recovered. The yields of auxotrophs from these two experiments were thus approximately the same.

Table 4. *Coprinus lagopus. Survival of wild type (BC9/66) oidia as a function of time*

Duration of exposure to 15 μg. NTG/ml. (min.)	Survival (%)
10	68
20	28
40	7
70	2

Table 5. *Coprinus lagopus. Survival of wild-type oidia following incubation in minimal medium*

Duration of pre-incubation in minimal medium (hr)	Survival (%)	Auxotrophs among 250 survivors tested
0	2·6	2
1	1·9	1
3	3·8	2
5	13·1	Not tested
7	27·7	Not tested
9	60·5	Not tested
11	84·1	Not tested

Oidia were separated by filtration from the minimal medium, washed rapidly with buffer and then suspended in buffer+NTG (15 μg./ml.) for 70 min.

Incubation of BC9/66 oidia in liquid minimal medium before exposure to NTG in buffer did not lead to an increased yield of auxotrophs (Table 5). It appears that the over-all effects of the two mutagens u.v.-radiation and NTG were similar and that, at least under the conditions tested, NTG was no more effective in inducing mutations of *Coprinus lagopus* than was u.v.-radiation.

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The Evaluation of the Metabolic-inhibition Technique for the Study of *Mycoplasma gallisepticum*

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SUMMARY

Antisera to nine strains of *Mycoplasma gallisepticum* were prepared in rabbits, chickens and turkeys, the response of individual rabbits or birds varying widely. Minor antigenic differences between some of the strains were shown in metabolic inhibition (m.i.) tests with rabbit antisera. The strains could be divided into at least three subtypes. In general, antibody diminished so that it was barely detectable 6 months after inoculation.

Antibody titres measured by m.i. were lower in chicken and turkey antisera than in rabbit antisera. The choice of the mycoplasma strain to be used in the m.i. test for the measurement of antibody in the bird sera was of fundamental importance since not all strains were capable of detecting antibody; strain T37 was the most useful.

Antibody measured by m.i. was not destroyed by repeated freezing and thawing of antisera, and the antibody titres were not, in general, increased by the addition of unheated guinea-pig serum to the tests. The titres of antibody which inhibited metabolism of the mycoplasma strains were closely similar to those which inhibited their growth in liquid medium. On solid medium, however, some rabbit antisera with high m.i. antibody titres did not inhibit or inhibited poorly the development of colonies of the homologous strains. Only one of the nine rabbit antisera inhibited the development of colonies of all the strains, a feature of importance in mycoplasma identification.

INTRODUCTION

Agglutination (Adler, 1953) and haemagglutination inhibition (Jungherr, Luginbuhl & Jacobs, 1953) have been the serological techniques used most extensively for the study of mycoplasmas from birds. Growth inhibition by antiserum incorporated into agar medium was used by Edward & Kanarek (1960), by Fabricant (1960), and more recently by Dierks, Newman & Pomeroy (1967) for the classification of mycoplasmas of avian origin. This method is relatively insensitive compared to the technique of metabolic inhibition (Taylor-Robinson, Purcell, Wong & Chanock, 1966) which has been used to detect minor antigenic differences betwen mycoplasmas within one serotype (Purcell *et al.* 1967). This paper reports on the use of the metabolic inhibition technique for the antigenic study of *Mycoplasma gallisepticum* strains. In addition, other factors, such as the use of a particular strain for measuring antibody and the possible requirement for unheated guinea-pig serum, have been evaluated and

results obtained by metabolic inhibition have been compared with those obtained by growth inhibition in liquid and on agar media.

METHODS

Mycoplasma strains. Mycoplasma gallisepticum strains T34, T37, MG2, C54, J1 and A514, isolated from field infections in this country, were obtained from Dr D. H. Roberts, Dr F. T. W. Jordan and Dr A. G. Newnham. Strains S6 and A5969, isolated in the U.S.A., were obtained from Dr D. H. Roberts. The prototype strain X95 (Edward & Kanarek, 1960) was obtained from Dr R. H. Leach.

Media. The media (A) used at Glaxo Laboratories were Brucella broth and agar described by Newnham (1964). The liquid and solid media (B) used in Salisbury were basically those described by Hayflick (1965), and have been reported previously (Manchee & Taylor-Robinson, 1968).

Antigen production. All strains were cloned three times on medium A except strains S6 and C54 which were cloned on medium B. Thereafter, organisms to be used for metabolic inhibition and disc-growth inhibition tests were grown in liquid medium A by incubating at 37° until the medium pH, initially 7·8, decreased by at least one unit. The cultures were then divided into lots, stored at −70° and a sample titrated by the 'colour-change' technique (Taylor-Robinson & Purcell, 1966) for its content of viable organisms. One colour-changing unit (c.c.u.) of activity was defined as the highest dilution which produced a colour change. Antigens for rabbit and bird inoculation were prepared from organisms propagated in a similar manner; organisms were concentrated 100-fold by centrifugation at 10,000 rev./min. for 20 min. and resuspension in phosphate-buffered saline. These antigens were then inactivated by incubation with 1/1000 β-propiolactone for 2 hr at 37°.

Antiserum production. Antisera were prepared in groups of rabbits and 6-week-old *Mycoplasma gallisepticum*-free chickens and turkeys. Each of these was inoculated intramuscularly with 0·5 ml. of inactivated antigen and this procedure was repeated 14 days later. In addition, each chicken and turkey was inoculated intrathoracically with 10^8 c.c.u. of the homologous viable mycoplasma 28 days after the first inoculation. Sera were obtained before inoculation, 28 days after the first inoculation and at weekly intervals thereafter. They were stored at −20° and were inactivated at 56° for 30 min. before use.

Guinea-pig serum (g.p.s.). This was obtained from animals killed without anaesthesia. The serum was separated immediately and was stored without preservatives at −70°.

Metabolic inhibition (m.i.) tests. These were done in Linbro Disposo-trays (IS-MRC-96; Linbro Chemical Co. Inc., New Haven, Connecticut, U.S.A.) as described previously by Taylor-Robinson *et al.* (1966) with liquid media A and B. Unheated g.p.s. was not used unless otherwise stated. The trays were incubated at 37° for 15 min. before they were sealed at this temperature with sellotape. This prevented detachment of the sellotape from the trays that otherwise sometimes occurred on incubation. The results were recorded when a colour change of about half a pH unit had occurred in the controls containing mycoplasma organisms but no antiserum.

Disc growth inhibition (g.i.) tests. These were done as described previously by Clyde (1964) using solid medium B. Stock cultures, previously titrated, were diluted in liquid medium so that 0·1 ml. contained about 10^5 c.c.u. This was spread on the agar

medium. Then filter-paper discs of 7 mm. diameter, soaked with 0·025 ml. of undiluted antiserum, were placed on the agar. Zones of inhibition were measured from the edge of the disc to the edge of colony development after incubation of the cultures at 37° in a humid atmosphere of 95 % (v/v) N_2 + 5 % (v/v) CO_2.

RESULTS

Effect of age of culture on the m.i. test. It was considered that the age of the culture used as inoculum in the m.i. test might affect the result. Therefore, the A5969 strain of *Mycoplasma gallisepticum* was grown in liquid medium B, and at 4 times over a period of 118 hr a sample was harvested and stored at −70°. The samples were titrated for viable organisms; a variation between 10^4 and 10^9 c.c.u./ml. occurred. Then each sample was diluted so that when used as inoculum in the m.i. test it contained 10^3–10^4 c.c.u. The m.i. titre of a rabbit antiserum did not vary more than four-fold (1/2560–1/10,240) when tested with the four different inocula. Thus, variations greater than this in subsequent experiments were unlikely to be due to the use of cultures of different age. Nevertheless, as described in Methods, some standardization was achieved by harvesting organisms of each *M. gallisepticum* strain when the pH of the medium had fallen at least one unit, at which time the culture contained at least 10^8 c.c.u./ml.

Table 1. *M.i. tests with rabbit antisera to Mycoplasma gallisepticum strains*

	M.i. titre with mycoplasma strain								
	Subtype 1					Sub-type 2	Subtype 3		
Antiserum* to strain	T34	T37	A5969	S6	X95	A514	JI	MG2	C54
T34	**5,120**	2,560	1,280	1,280	1,280	320	80	320	160
T37	10,240	**10,240**	5,120	2,560	2,560	640	80	640	320
A5969	10,240	5,120	**5,120**	2,560	5,120	640	80	160	160
S6	1,280	1,280	640	**640**	1,280	160	40	80	320
X95	2,560	10,240	1,280	2,560	**20,480**	640	160	640	320
A514	160	320	160	160	320	**2,560**	80	160	320
JI	320	320	80	160	640	320	**5,120**	320	320
MG2	160	160	40	80	320	160	320	**320**	160

* Prepared by inoculating on day 0 and 14 and bleeding 21 days later.

M.i. tests with rabbit antisera

The antisera prepared against each mycoplasma strain were tested against all the strains in one test (Table 1). Strains T34, T37, A5969, S6 and X95 were closely related to one another (subtype 1). Strain A514 (subtype 2) was different from subtype 1 strains, because antisera to subtype 1 strains inhibited strain A514 4- to 16-fold less than they inhibited their homologous strains within subtype 1. In addition, antiserum to strain A514 inhibited the other strains 8- to 16-fold less than it inhibited its homologous organism. Strain JI (subtype 3) was different from all the other mycoplasma strains. Antisera to the other strains were 64- to 128-fold less effective in inhibiting strain JI than in inhibiting their homologous strains, and JI antiserum was 16- to 64-fold more active against its homologous strain than against the other strains. Strain MG2 was similar to strain A514 in its reaction to the other antisera but it was probably different

from strain A514; A514 antiserum inhibited its homologous organism 16-fold more than it inhibited strain MG2. Although antiserum to strain C54 was not available, this strain behaved in a similar way to strain MG2 in its reaction to the other antisera. These results were confirmed in m.i. tests in which three other antisera prepared against each strain were tested. It was thus apparent that on this basis *Mycoplasma gallisepticum* comprised at least three serological subtypes.

Duration of antibody persistence. Antisera obtained 1, 2 and 6 months after inoculation of rabbits with the S6 strain were tested against this strain and against heterologous strains. The m.i. antibody titres at 2 months were between 4- and 50-fold lower than at 1 month, the decline being least in tests with the homologous organism. By 6 months the antibody titres had fallen to barely detectable levels.

Table 2. *M.i. tests with chicken antisera to Mycoplasma gallisepticum strains*

Antiserum* to strain	M.i. titre† with mycoplasma strain								
	T34	T37	A5969	S6	X95	A514	J1	MG2	C54
T34	16	64	4	64	< 2	< 2	< 2	< 2	2
T37	16	64	4	64	< 2	< 2	< 2	< 2	2
A5969	8	32	8	16	2	< 2	< 2	< 2	2
S6	8	64	8	32	< 2	< 2	< 2	2	2
X95	8	64	4	32	< 2	< 2	< 2	< 2	< 2
A514	16	128	8	64	2	4	16	2	4
J1	8	32	4	8	2	< 2	16	4	2
MG2	8	16	8	16	4	< 2	16	4	2
C54	< 2	8	< 2	< 2	< 2	< 2	< 2	< 2	< 2

* Prepared by inoculating on day 0 and 14, challenging with viable organisms on day 28 and bleeding 14 days later.

† M.i. titre of all pre-inoculation sera < 2.

M.i. tests with chicken and turkey antisera

Effect of different strains on detection of antibody. Antisera obtained 2 weeks after intrathoracic inoculation of chickens and turkeys were studied. The results of cross-testing all the strains against one of several chicken antisera prepared against each strain are shown in Table 2. Antibody was measured successfully in tests in which strains T34, T37, A5969 and S6 were used; strain T37 was most useful for this purpose. Antibody was detected infrequently or not at all when the antisera were tested with the other mycoplasma strains, even though the strain used in the m.i. test was the same as that which had been used to prepare the chicken antiserum. In addition, when antibody was detectable, subjection of the antisera to nine cycles of freezing and thawing did not diminish their antibody titres in tests with strain T37.

It may be noted that the titre of antibody in chicken antisera was at least 16-fold lower than observed with the corresponding rabbit antisera. In addition, it was not possible to subtype the various mycoplasma strains by using chicken antisera.

The results of testing all the strains against one of several turkey antisera prepared against each strain were similar to the results obtained with the chicken antisera, except that the antibody titres were generally 2- to 4-fold greater with turkey antisera.

Variation in antibody response. Several chicken antisera prepared against the S6 strain were tested against both homologous and heterologous mycoplasma strains. The wide variation in antibody response of the different chickens is shown in Fig. 1.

The results of similar tests with several turkey antisera prepared against the S6 strain are shown in Fig. 2. They are similar to those obtained with chicken antisera since wide variations occurred in the titre of antibody in the sera of different birds given the same course of inoculations of a particular strain, and antibody in both chickens and turkeys was best detected by using strains T34, T37, A5969, S6 and X95,

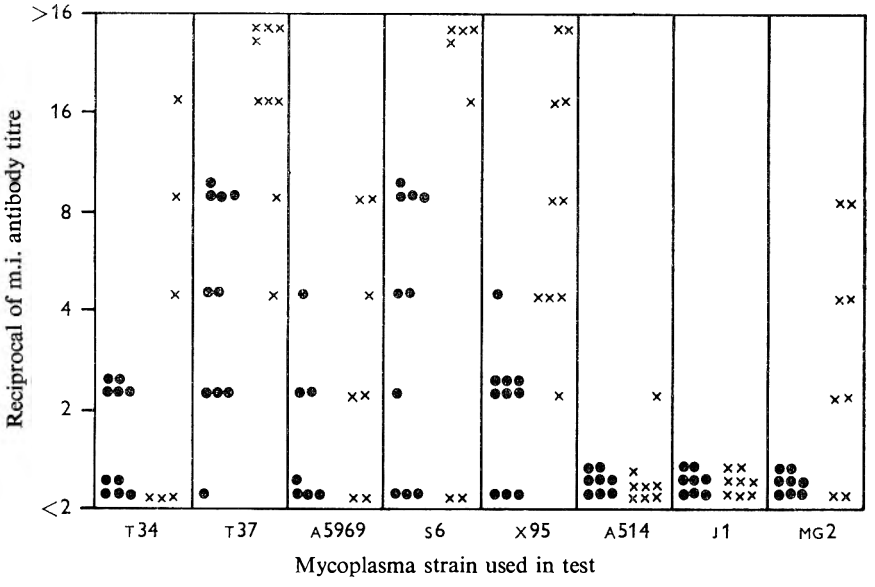

Fig. 1. M.i. titres of antisera from chickens inoculated with *Mycoplasma gallisepticum* strain S6. Sera taken after inoculation with inactivated antigen (●) and inactivated antigen and viable organisms (×).

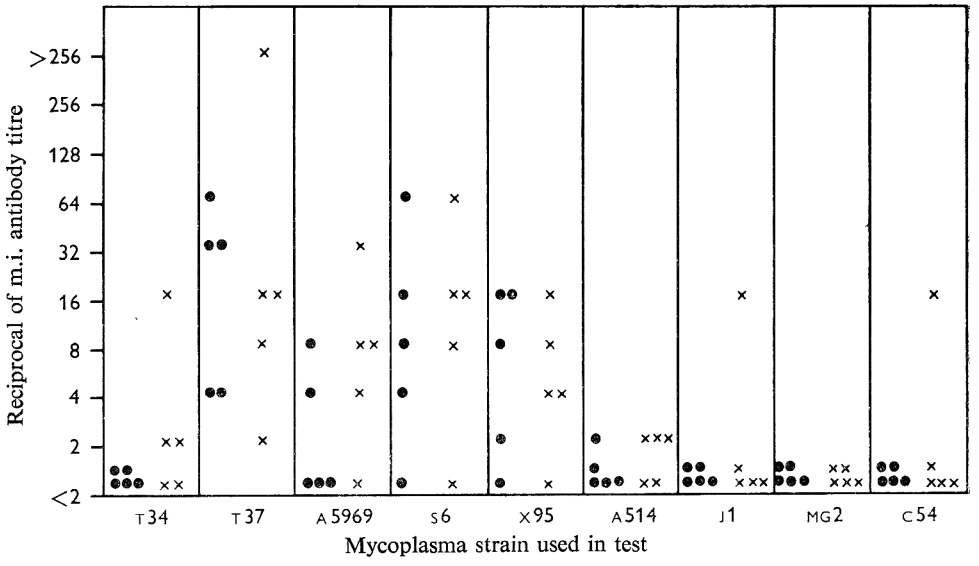

Fig. 2. M.i. titres of antisera from turkeys inoculated with *Mycoplasma gallisepticum* strain S6. Sera taken after inoculation with inactivated antigen (●) and inactivated antigen and viable organisms (×).

The use of unheated g.p.s. in the m.i. test

Previous results (Taylor-Robinson *et al.* 1966; Purcell *et al.* 1967) indicated that an accessory factor, such as unheated g.p.s., might be essential to demonstrate antibody or be required to enhance the antibody titre in the m.i. test.

Effect of unheated g.p.s. on mycoplasma growth. Since unheated g.p.s., in the absence of antiserum containing antibody, was known to inhibit the growth of some mycoplasmas, all the strains under study were titrated in the absence and in the presence of 10% unheated g.p.s. in the medium. Two strains were titrated also in medium containing 10% g.p.s. which had been heated at 56° for 30 min. The results are shown in Table 3. The growth of some strains was inhibited by unheated g.p.s., whereas the growth of others was unaffected. Those strains most inhibited by unheated g.p.s. were those which were found previously to be most suitable for the detection of m.i. antibody in chicken and turkey sera. Heated g.p.s. did not inhibit the growth of the two strains tested.

Table 3. *Inhibition of growth of Mycoplasma gallisepticum strains by 10% guinea pig serum (g.p.s.)*

Mycoplasma strain	Initial titre (log_{10}) of viable organisms	Reduction in titre (log_{10}) by	
		Unheated g.p.s.	Heated g.p.s.
T34	9	3	n.t.
T37	9	3	n.t.
A5969	8	4	Nil
S6	8	4	Nil
X95	9	Nil	n.t.
A514	8	Nil	n.t.
J1	9	2	n.t.
MG2	9	Nil	n.t.

n.t. = not tested.

Table 4. *Effect of guinea pig serum (g.p.s.) on the m.i. titre of rabbit and chicken antisera*

Antiserum to strain		M.i. titre with (+) and without (−) additional 6% g.p.s. in tests with mycoplasma strain					
		T34		A514		MG2	
		(+)	(−)	(+)	(−)	(+)	(−)
Rabbit	T34	256	4096	32	128	16	32
	A514	1280	80	2560	640	320	80
	MG2	320	160	80	80	320	320
Chicken	T34	2	16	n.t.	16	n.t.	8
	A514	< 2	< 2	2	4	< 2	< 2
	MG2	< 2	< 2	< 2	2	< 2	4

n.t. = not tested.

Effect of unheated g.p.s. on the m.i. antibody titre. The results which have been presented were obtained in tests done without unheated g.p.s. However, all the rabbit antisera and most of the chicken antisera were tested also with additional unheated

g.p.s. and some of the results are shown in Table 4. With few exceptions, notably strain A514 tested against rabbit antisera, the addition of unheated g.p.s. did not enhance the m.i. antibody titre and sometimes it depressed the titre. However, it was found that unheated g.p.s. stabilized the m.i. titre in some instances (not shown in Table); in other words, in the absence of unheated g.p.s., colour changes that occurred

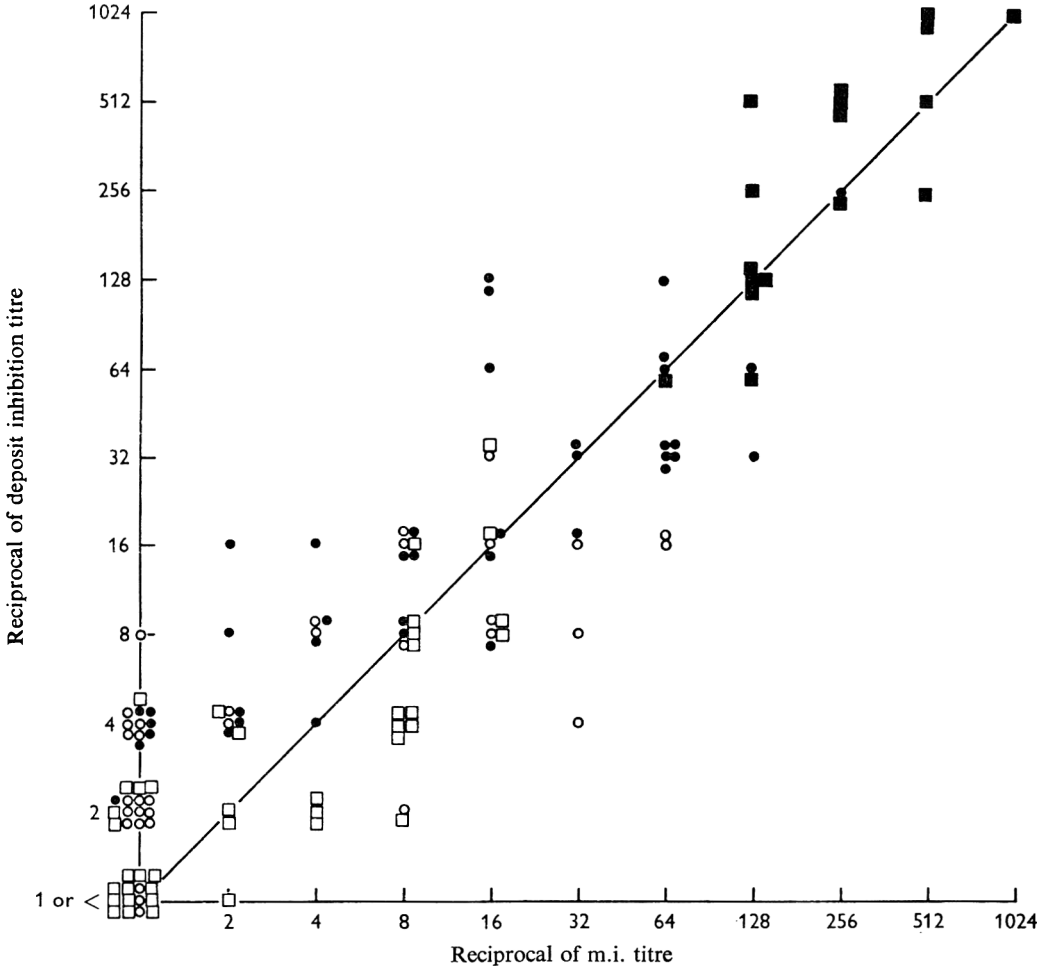

Fig. 3. Relationship between inhibition of growth deposit and inhibition of metabolism of four strains of *Mycoplasma gallisepticum*. ○, S 6; ●, T 37; □, T 34, ■, A 514.

in cups which contained high dilutions of antiserum, sometimes occurred in cups with lower dilutions of antiserum upon continued incubation of the test. This did not happen in the presence of unheated g.p.s. Therefore, in tests performed in the absence of g.p.s., it was advisable to record the m.i. antibody titres as soon as a colour change was clearly visible, that is at a time when the pH of the medium containing organisms but no antiserum had changed by about half a pH unit.

Correlation between antibody titres measured by m.i. and g.i. in liquid medium

Purcell *et al.* (1967) demonstrated a close correlation between the dilution of antiserum which inhibited a colour change in the m.i. test and the dilution which inhibited the growth in liquid medium of several mycoplasmas. An opportunity to test this correlation with strains of *Mycoplasma gallisepticum* arose when it was observed that, on continued incubation of some m.i. tests, buttons of deposit formed in cups which contained organisms without antiserum or organisms with high dilutions of antiserum. The deposits formed after the colour change had commenced and were produced by strains T34, T37, S6, A5969 and A514. Microscopic examination of some of the deposits revealed mycoplasma cells. However, it cannot be excluded that, in part, these deposits consisted of by-products of metabolism. In a number of tests with rabbit and chicken antisera (Fig. 3), m.i. antibody titres were recorded, and the occurrence of deposits also when those in cups without antiserum were first discernible; the highest dilution of antiserum which prevented the formation of a deposit was regarded as the deposit inhibition titre. A close correlation was found between m.i. titres and deposit inhibition titres which indicated that antibody measured by the m.i. test was identical or closely related to deposit-inhibiting antibody, and thus presumably closely related to growth-inhibiting antibody.

Table 5. *Disc growth inhibition tests with rabbit antisera to Mycoplasma gallisepticum strains*

Antiserum to strain	Zone of inhibition in mm. with mycoplasma strain								
	T34	T37	A5969	S6	X95	A514	J1	MG2	C54
T34	0·2	1·7	1·6	1	1	Nil	0·5	0·7	1
T37	2·6	3	1·6	1·6	3	2	1	Nil	1·5
A5969	1·4	3	2	2	3·3	0·7	Nil	1·5	0·2
S6	Nil	2	0·2	1·2	Nil	Nil	Nil	Nil	0·2
X95	1	4·2	0·5	2·5	3	1·5	1·7	3	2·7
A514	Nil	1·5	Nil	0·8	1	3·2	Nil	1·2	Nil
J1	0·5	0·5	0·2	Nil	3·2	Nil	8	2·5	2·2
MG2	Nil	Nil	Nil	Nil	0·7	0·2	1	1·7	Nil

Disc g.i. tests with rabbit antisera

Most of the rabbit antisera inhibited the development of colonies of most of the strains (Table 5). Sometimes, however, colony development although inhibited by one antiserum was not necessarily inhibited by an antiserum against another strain. Indeed, only strain X95 antiserum inhibited the development of colonies of all the strains. It was possible to differentiate strain J1 from the other strains; J1 antiserum inhibited the development of colonies of its homologous strain to a much greater extent than it inhibited colony-formation by the heterologous strains. This apart, it was not possible to distinguish between the other mycoplasma strains on the basis of the disc g.i. test. Although the results of m.i. and g.i. tests in liquid media were comparable, there was poor correlation between the results of m.i. and disc g.i. tests since some antisera with high m.i. titres did not produce zones of inhibition.

DISCUSSION

The results of m.i. tests with rabbit antisera showed minor antigenic differences between *Mycoplasma gallisepticum* strains and they may be divided into at least three subtypes within this serotype. This conclusion is valid even though the organisms of each strain used as inoculum in the tests were not harvested at the same stage of growth. The development of antibody, as measured by the m.i. test, was poorer in turkeys, and even more so in chickens, than in rabbits. However, to some extent the results observed with chicken and turkey antisera were in keeping with those observed with rabbit antisera. Thus, when strains A514, J1, MG2 and C54 were used to measure antibody in rabbit antisera the titres were low, and the same strains failed, in the majority of instances, to detect antibody in chicken or turkey antisera. This correlation was not complete, however, because high titres of antibody were demonstrated in rabbit antisera by using strain X95, but it sometimes failed to detect antibody in chicken or turkey antisera. It would be reasonable to assume that the most suitable mycoplasma strain to use for detecting antibody in chicken and turkey sera was that used to inoculate the birds. However, antibody was often only detectable by using a heterologous strain and it is clear that the choice of the mycoplasma strain for inoculum in the m.i. test is of fundamental importance for successful detection of antibody in chicken and turkey sera. The fact that birds vary widely in their antibody response to a single strain indicates that the sera of a large number of birds from a flock should be examined in order to obtain a realistic picture of the antibody status of the flock as a whole.

It is known that the effect of additional unheated g.p.s. in the m.i. test may be threefold. First, it may itself inhibit mycoplasma growth as reported previously for different mycoplasma serotypes (Taylor-Robinson *et al.* 1966), but its variable effect upon different strains within a serotype has not been reported. The *Mycoplasma gallisepticum* strains most inhibited by unheated g.p.s. were those most useful for detection of m.i. antibody, which suggests that the inhibitory effect of unheated g.p.s. might be due to it containing antibody. Contrary to this idea is the fact that g.p.s. heated at 56° was not inhibitory. It is possible, however, that antibody in the g.p.s. is only active in the presence of heat-labile complement factors. Secondly, unheated g.p.s. may stabilize the m.i. titre so that it does not decrease on continued incubation of the test (Taylor-Robinson *et al.* 1966; Purcell *et al.* 1967); this was observed in some of the present experiments. Thirdly, unheated g.p.s. may enhance the specific inhibitory activity of an antiserum (Taylor-Robinson *et al.* 1966; Purcell *et al.* 1967; Fallon & Jackson, 1967). Indeed, Barker & Patt (1967) found that the addition of unheated g.p.s. was necessary to inhibit the growth of a strain of *M. gallisepticum* by antiserum. In most of our tests additional unheated g.p.s. did not increase but sometimes decreased the m.i. titre. It is possible that the unheated horse serum in the medium contained sufficient of the required accessory factor.

Dinter, Danielsson & Bakos (1965), and Hayflick & Stanbridge (1967) experienced difficulties in studying *Mycoplasma hyorhinis* with the disc g.i. technique. Likewise we encountered problems, since a rabbit antiserum which inhibited a strain of *M. gallisepticum* to high titre in the m.i. test did not necessarily produce a zone of inhibition on agar. However, we observed that antibody titres measured in the m.i. test correlated closely with the titres of antibody which inhibited the formation of buttons of deposit in liquid medium. It seems reasonable to assume that the deposits consisted

mainly of mycoplasma growth. It is probable, therefore, that the difference between the results of m.i. tests and those of disc g.i. tests arises because of the difference in the tests systems and not because antisera affect growth and metabolism differently.

The disc g.i. technique is generally considered to be specific (Clyde, 1964; Taylor-Robinson, Canchola, Fox & Chanock, 1964). However, Smith, Dunlop & Strout (1966) indicated that the type of culture medium used to propagate *Mycoplasma gallisepticum* for antiserum production might influence the specificity of the test since they found that antiserum to medium alone was inhibitory. Although we did not examine this possibility in detail we noted that the results of m.i. tests carried out in each laboratory corresponded closely, even though the medium used to produce antigens for rabbit immunization was the same as that used for subsequent m.i. tests in one laboratory and quite different from that used for m.i. tests in the other laboratory. In addition, we have found incidentally that antisera prepared in rabbits with organisms of other mycoplasma species grown in medium containing horse serum have not produced zones of inhibition with *M. gallisepticum*. On the contrary, in the present experiments some *M. gallisepticum* antisera did not inhibit all strains even though care was taken to avoid an excess of colonies on the agar medium. Only X95 antiserum was capable of inhibiting all the strains tested. It is clear that antiserum prepared against a single strain may be inadequate in a diagnostic scheme.

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Some Factors Affecting Production and Assay of *Escherichia coli* Haemolysins

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SUMMARY

The amount of haemolysin produced by *Escherichia coli* grown under various gas phases was determined by the amount of growth obtained under these conditions. Calcium or strontium was required for activation of haemolysin. The haemolytic reaction was stopped by sodium citrate. The loss of haemolytic activity after incubation with trypsin and chymotrypsin indicated that at least the active group of the haemolytic molecule is protein or a peptide.

INTRODUCTION

Several studies have shown discordant results about the production and assay of *Escherichia coli* haemolysins. This paper attempts to clarify the effects of various gas phases on the production of haemolysin and to demonstrate a cation requirement for activation of the haemolysin.

METHODS

Organism. A haemolysin-producing strain of *Escherichia coli* type 06 (Iowa Stock Culture, ISC, no. 447), originally obtained from a patient with pyelonephritis, was used. A standard inoculum was obtained as previously described (Snyder & Koch, 1966).

Measurement of haemolytic activity. Total haemolysin content of the cultures was obtained by two-fold dilution of whole cultures. Filterable haemolysin was determined in the same manner with culture filtrates passed through a Millipore filter (0·45 mμ porosity). The amount of haemolysin/ml. culture or filtrate was determined as previously described (Snyder & Koch, 1966).

Measurement of growth and pH value. Turbidometric measurements of growth were obtained with a Spectronic 20 spectrophotometer at a wavelength of 625 mμ. The pH value of the culture was determined with a Beckman Zeromatic II pH meter.

Preparation of media. Beef heart for infusion medium (BHI) was prepared from Difco Beef Heart for infusion as described by the manufacturer. When glucose was added to the media, 1 ml. of a Millipore-filtered solution 20% (w/v) glucose was added to 100 ml. autoclaved media. In media buffered with phosphate, 0·23 g. K_2HPO_4 and 0·078 g. KH_2PO_4 were added to each 100 ml. of medium before sterilization. Alkaline meat-extract broth was prepared as described by Smith (1963).

Incubation of cultures. Anaerobic growth was obtained by using Brewer jars. The jars were flushed with nitrogen three times and filled with hydrogen, a palladium catalyst being used for removal of residual oxygen. Incubation in a CO_2 atmosphere

was done in jars evacuated and filled with CO_2 to a concentration of 20 % CO_2 and 80 % air (v/v). Shake cultures were incubated on a rotary shaker at 174 cycles/min over a one-inch circle. All cultures were incubated at 37°.

RESULTS

Role of cations in haemolysis

Filtrates of *Escherichia coli* cultures grown in BHI medium did not cause haemolysis of sheep erythrocytes unless Ca was added to the filtrate or to the diluent used for titration of haemolytic activity. This indicated that Ca was required for activation but not for production of haemolysin. Addition of various amounts of the Ca showed that a final concentration of 0·005 M-$CaCl_2$ caused maximal activation of the haemolysin (Table 1).

To determine whether other cations could substitute for calcium, various concentrations of other cations were added to the 0·15 M-NaCl used as for assay. Neither barium nor magnesium salts were effective; however, strontium replaced calcium (Table 1).

Table 1. *Effect of various salts on haemolytic activation*

	Concentration of salts		
Salt	0·01 M	0·005 M	0·0025 M
	Haemolytic titre (reciprocal)		
$CaCl_2$	32	32	16
$MgSO_4$	0	0	0
$MgCl_2$	0	0	0
$BaCl_2$	0	0	0
$SrCl_2$	32	16	16
$Sr(NO_3)_2$	8	16	16

Table 2. *Effect of medium preparation on the calcium requirement for haemolytic activity*

	Reciprocal of titre	
Type of infusion	Without calcium	With calcium
Fresh veal (121°)*	64	128
Fresh beef heart (121°)	32	128
BHI (121°)	0	64
Fresh veal (56°)	0	128
Fresh beef heart (56°)	0	64
BHI (56°)	0	64

* Temperature of infusion.

Addition of $CaCl_2$ to culture filtrates caused a precipitate. To determine whether the role of Ca was in removing an inhibitor, uninoculated medium was precipitated with $CaCl_2$, the precipitate removed by filtration and the medium was dialyzed to remove excess Ca. This medium was then sterilized and inoculated with *Escherichia coli*. Haemolytic activity was not shown by the filtrates from cultures grown in the dialyzed medium unless calcium was added.

Smith (1963) reported production of haemolysin by *Escherichia coli* grown in

alkaline broth prepared by autoclaving 1 part fresh veal in 2 parts water, 1 % proteose-peptone and 0·5 % NaCl at a pH 7·6. A calcium requirement for activation of haemolysin was not reported. To determine whether the method of media preparation or type of medium affected the requirement for calcium, the experiment shown in Table 2 was done. Filtrates of cultures grown in fresh meat infusions prepared by the method of Smith did not require added Ca for haemolytic activity; however, dehydrated beef heart for infusion (Difco, Detroit, Michigan) prepared in the same manner required Ca. Haemolytic activity could not be obtained with filtrates from alkaline fresh meat infusions prepared at 56° unless calcium was added. Addition of Ca to the filtrates from cultures grown in autoclaved infusions increased the haemolytic activity. Autoclaved fresh veal infusion dialyzed before inoculation with *E. coli* did not permit production of activated haemolysin. However, addition of calcium to the filtrate resulted in haemolytic activity.

Attempts to measure quantitatively Ca in the autoclaved infusions by several methods (Ferro & Ham, 1957; Clark & Collip, 1925; Diehl & Ellingbee, 1956) were unsuccessful. However, small amounts of calcium were detected in the infusions prepared by autoclaving. Calcium was not found in the other media.

Inhibition of haemolytic activity. Since the haemolytic reaction required calcium, the effects of sodium citrate and EDTA on inhibition of the reaction were investigated. Addition of 0·1 ml. of 1·0 M-sodium citrate to 2 ml. of an incubating haemolysin-erythrocyte mixture stopped the haemolytic reaction. EDTA partially inhibited the haemolysin.

Effect of enzymes on the haemolysin. Samples of haemolysin were incubated with trypsin, chymotrypsin, deoxyribonuclease and ribonuclease. Inactivation of the haemolysin was obtained only with trypsin and chymotrypsin, suggesting that the haemolysin or active portion of the molecule is a protein or peptide.

Effect of gaseous environment on haemolysin production

Figures 1, 2 show the relationships between growth in BHI media, haemolysin production and pH value in cultures of *Escherichia coli* type 06, ISC 447, grown aerobically in a shake culture (Fig. 1) and a stationary culture (Fig. 2). Calcium was added to the culture and filtrates for the haemolysin assay. The same measurements were made with cultures grown in 20 % CO_2 (v/v) and air (Fig. 3) and anaerobically (Fig. 4). Filterable and non-filterable haemolysin was produced in all media, however, more haemolysin was produced by organisms grown aerobically than by those grown under the other environmental conditions tested. The production of filterable haemolysin during the logarithmic phase of growth confirms a previous report (Snyder & Koch, 1966). Both the stationary aerobic culture (Fig. 2) and the cultures grown in CO_2 (Fig. 3) showed a diauxic type of growth with an increase in both total and filterable haemolysin during each accelerated growth phase. In all cultures an increase in haemolysin production was associated with a decrease in culture pH value.

To determine whether better growth of the organisms would result in increased production of haemolysin, glucose and a phosphate buffer was added to BHI medium and the previous experiments were repeated. Increased growth, as indicated by O.D. about 0·7, was obtained in all cultures except the aerobic shaken culture. Diauxic growth was not obtained in the stationary aerobic culture or the cultures grown in CO_2 and haemolysin was produced only during the single logarithmic growth phase

When compared with cultures grown without glucose there was an increase in total haemolysin produced by cultures grown in stationary aerobic culture as well as in CO_2 and anaerobically. However, there was no significant increase in the yield of filterable haemolysin.

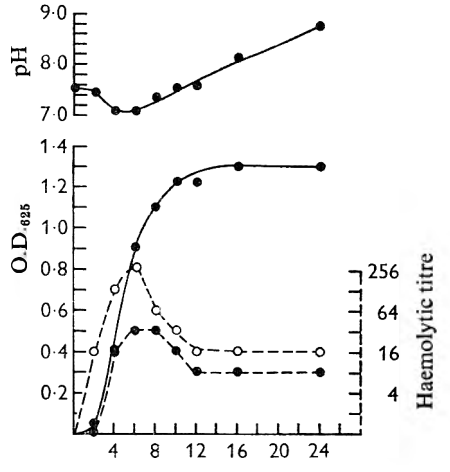
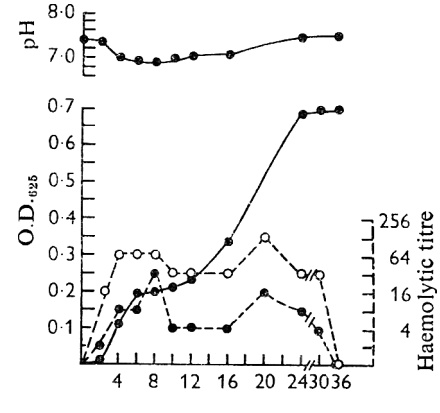


Fig. 1



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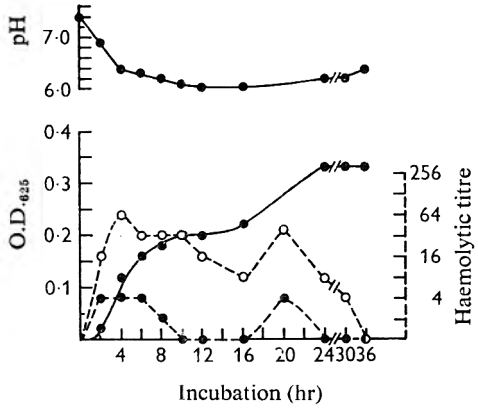

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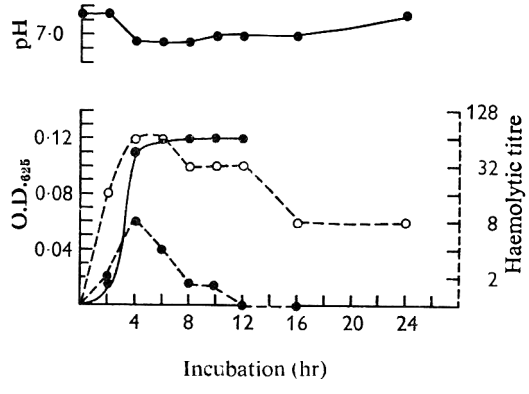

Fig. 4

Fig. 1. Effect of agitation on haemolysin production in medium without added carbohydrate. ●—● Growth, ○ - - ○ total haemolysin in culture, ● - - ● filterable haemolysin.

Fig. 2. Effect of stationary growth on haemolysin production in medium without added carbohydrate. ●—● Growth, ○ - - ○ total haemolysin in culture, ● - - ● filterable haemolysin.

Fig. 3. Effect of CO_2 on haemolysin production in medium without added carbohydrate. ●—● Growth, ○ - - ○ total haemolysin in culture, ● - - ● filterable haemolysin.

Fig. 4. Effect of anaerobiosis on haemolysin production in medium without added carbohydrate. ●—● Growth, ○ - - ○ total haemolysin in culture, ● - - ● filterable haemolysin.

DISCUSSION

The data presented show that production of *Escherichia coli* haemolysin was obtained during growth in various gas phases. More haemolysin was produced aerobically either in shaken or stationary cultures than in cultures grown in CO_2 or anaerobically. However, since better bacterial growth was obtained in cultures growing aerobically, glucose and phosphate were added to the cultures to improve growth. These additions increased bacterial growth especially in the cultures grown in CO_2 and anaerobically and increased the production of haemolysin. No significant increase in filterable haemolysin was obtained. Tomic-Karovic (1955) found more haemolysin was produced when *E. coli* was grown on blood agar in an atmosphere of CO_2 than in air and that the least haemolysin was produced in an anaerobic atmosphere. Ishii (1960) found greater production of haemolysin under CO_2 than in air. Lovell & Rees (1960) reported less haemolytic activity in cultures grown in CO_2 or hydrogen than those grown aerobically. Widholm (1953) found that various mixtures of oxygen and nitrogen did not affect haemolysin production. The differences in the reports most likely can be attributed to differences in time of sampling the culture filtrates. Cultures growing under more ideal environmental conditions will produce haemolysin at a faster rate than other cultures. In all cases, production of haemolysin is associated with a decrease in pH value and with growth of the organisms.

Our experiments on the role of cations in haemolysis show that cations are responsible for activation of the haemolysin and are not responsible for production. Bamforth & Dudgeon (1952) reported that calcium was required for haemolytic activity and that barium and strontium could be substituted for calcium; we were not able to show haemolytic activity with barium. Other workers (Lovell & Rees, 1960; Smith, 1963) did not report a requirement for calcium. However, these workers used an alkaline meat extract broth. Our studies with the alkaline meat extract show (1) that some calcium is present in the alkaline meat extract, and (2) that haemolysin cannot be demonstrated in cultures of *Escherichia coli* grown in dialyzed broth unless calcium is added.

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Induction and Repression of Steroid Hydroxylases and Dehydrogenases in Mixed Culture Fermentations

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SUMMARY

Some factors affecting induction and repression of steroid-transforming enzymes were studied in single and mixed cultures of *Arthrobacter simplex*, *Nocardia restrictus* and *Streptomyces roseochromogenes*. In single or mixed cultures induction and repression of 1-dehydrogenase, 16α-hydroxylase, and 20-ketoreductase were found to be dependent on the type of steroid substrate and the composition of the medium. In mixed cultures patterns of growth as well as enzyme induction and repression were altered by culture interaction. An observation having practical significance was that 16α-hydroxylation and 1-dehydrogenation of 9α-fluorohydrocortisone to triamcinolone could be done, in a single fermentation operation, by a mixed culture of *A. simplex* and *S. roseochromogenes*. The practicality of this transformation derives from the fact that 20-ketoreductase, an enzyme responsible for the production of undesirable by-products in cultures of *A. simplex*, was completely repressed in the mixed culture system. Although growth of *A. simplex* was suppressed by metabolites of *S. roseochromogenes*, conversion of steroids with this mixed culture was reproducible and controllable within a wide range of ratios of organisms in the starting mixtures.

INTRODUCTION

Multiple steroid transformations by mixed cultures have been reported by McAleer, Dulaney & Dulaney (1959) and Spalla, Modelli & Amici (1962). However, they added different micro-organisms sequentially and did multiple conversions in stepwise operations without isolation of the intermediates. The first practical mixed-culture fermentation for steroid conversion was reported by Shull (1959). He mixed a diluted culture of *Curvularia lunata* (11β-hydroxylator) with a fresh inoculum of *Mycobacterium phlei* (1-dehydrogenator) and did the conversion of cortexolone to prednisolone in a single operation. Kimura (1962) reported the transformation of cortexolone to prednisolone by mixed cultures of *C. lunata* and *Bacillus sphaericus*.

We have found that *Arthrobacter simplex* (1-dehydrogenator) and *Streptomyces roseochromogenes* (16α-hydroxylator) can transform 9α-fluorohydrocortisone (F2) to 1-dehydro-16α-hydroxy-9α-fluorohydrocortisone (triamcinolone, F4) in a one-step fermentation. In our mixed culture fermentation with *A. simplex* and *S. roseochromogenes*, F2 is first converted to the intermediate, 1-dehydro-9α-fluorohydrocortisone (Δ^1-F2), which is subsequently transformed to F4 (Fig. 1). 16α-Hydroxy-9α-fluorohydrocortisone (F3) is not detected. Unless the mixed culture fermentation is carried out under conditions in which 20-ketoreductase is repressed, however, all of the substrate (F2) and the intermediate (Δ^1-F2) formed in the early stage of the fermenta-

tion process are reduced at the 20-keto position. Thus the success of carrying out the multiple conversion of F2 to F4 depends entirely upon providing conditions in which 1-dehydrogenase and 16α-hydroxylase are selectively induced and 20-ketoreductase is repressed. We have found that the undesirable enzyme, 20-ketoreductase, which is induced when *A. simplex* is grown alone in soybean meal medium, is repressed when cultures of *A. simplex* are mixed with *S. roseochromogenes*.

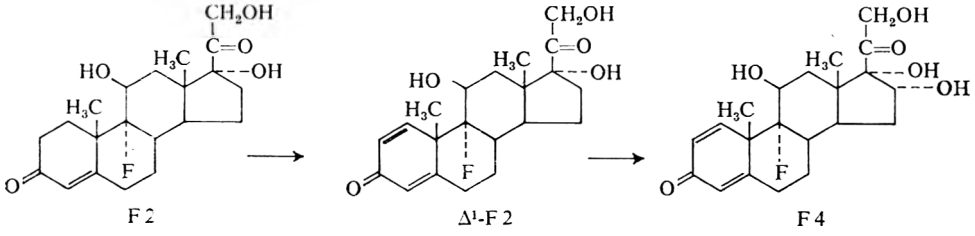

Fig. 1. Conversion steps of 9α-fluorohydrocortisone (F2) to 1-dehydro-16α-hydroxy-9α-fluorohydrocortisone (F4) by mixed culture of *Arthrobacter simplex* and *Streptomyces roseochromogenes*.

METHODS

Organisms. The organisms used were: *Arthrobacter simplex*-X^{s} (original Squibb Culture No. 6062-V3, which is sensitive to an antibiotic substance or substances (X) produced by *S. roseochromogenes*); *Arthrobacter simplex*-X^{r} (Squibb Culture No. 6216-V1, which is resistant to X); *Streptomyces roseochromogenes* (Squibb Culture No. 6186); *Nocardia restrictus* (Squibb Culture No. 2914). The *A. simplex* strains were maintained on yeast beef agar slopes, and the *S. roseochromogenes* and *N. restrictus* cultures on glucose yeast extract agar slants. Stock cultures were stored as suspensions in skimmed milk, frozen and held over liquid nitrogen at about −150°.

Culture growth. All cultures were grown in 100 ml. medium in 500 ml. Erlenmeyer flasks and shaken at 25° on a Gyrotory G-52 shaking machine (New Brunswick Scientific Co.) at 280 cycles/min. in a 2-inch diameter circle. Inoculum was developed in two 48-hr stages. For the fermentation stage 5 ml. portions of each vegetative culture were used to inoculate 100 ml. of the broth. Samples (5 ml.) were removed at intervals for colony counts and steroid measurement. Steroid substrate and organisms were added at zero time.

Media. Germination medium E33 contained (g.) 15, extracted soybean meal (Archer-Daniels-Midland, Minneapolis); 11, glucose; 2, $CaCO_3$; 2·2, soybean oil; in 1 l. distilled water. Fermentation medium E34 was made of (g.) 20, extracted soybean meal; 33, glucose; 7·5, $CaCO_3$, 2·2, soybean oil; 1, KH_2PO_4; 1, K_2HPO_4; in 1 l. distilled water.

Growth curves. Growth of each organism in pure culture and mixed culture systems was followed by colony counts of samples drawn, at intervals, from the fermentation broth.

Inoculum ratios. The ratios were determined on the basis of colony counts of samples drawn, at zero time, from the mixed culture fermentation broth.

Preparation of samples for steroid measurement. Five-ml. samples were drawn at intervals from the fermentation broth, and each sample extracted with 2 ml. 4-methyl-2-pentanone (MIBK).

Paper chromatography. Steroid samples (0·1 ml. each of the MIBK extracts) were spotted and developed by descending chromatography on Whatman No. 1 paper in benzene ethanol water (50 + 25 + 50, by vol.) solvent.

Steroid identification and measurement. Following development of the chromatograms, steroid spots were detected by ultraviolet (u.v.) scanning. For identification heavy reliance was placed on comparative mobility (R_F) values, established with authentic materials prepared in our laboratories in fermentations conducted with these organisms. One or more standards were placed on each papergram. For quantitative estimation the detected spots were cut out, eluted with 95 % (v/v) ethanol in water, and read at wavelength 240 mμ on a Beckman DU spectrophotometer. The concentration of steroid in each sample was then estimated by comparison with a standard solution equilibrated with MIBK and chromatographed as were the samples.

Enzyme identification. Qualitative identification of enzyme activities was made by noting the presence or absence of characteristic steroid transformation products. Positive identification of steroids was made by isolating and characterizing products in a few mixed culture experiments. The transformation products to be expected with *Streptomyces roseochromogenes* in single culture with these steroid substrates have been described by Smith, Foell & Goodman (1962). The transformation products to be expected with pure cultures of *Arthrobacter simplex* and *Nocardia restrictus* have been described by Goodman, May & Smith (1960).

RESULTS

Enzyme induction and repression in pure cultures of Arthrobacter simplex-X^s

In the transformation of F2 to Δ^1-F2, 1-dehydrogenase and 20-ketoreductase play critical roles. Both 1-dehydrogenase and 20-ketoreductase were induced, when *Arthrobacter simplex*-X^s was germinated in soybean meal medium E33 and then fermented in soybean meal medium E34 containing F2 as substrate. As a result, the organism transformed some of the substrate F2 to Δ^1-F2, and then simultaneously converted compounds F2 and Δ^1-F2 to 20-dihydro-F2 and Δ^1-20-dihydro-F2. Consequently, only 20-dihydro products were seen in fermentations lasting longer than 23 hr. In contrast, selective induction of 1-dehydrogenase and repression of 20-ketoreductase were observed when *A. simplex*-X^s was germinated in two stages, using peptone yeast extract (Difco, Detroit) medium and Yeastamin (Vico Products, Chicago) medium and then fermented in Yeastamin medium containing steroid substrate, F2.

The enzymic response of *Arthrobacter simplex*-X^s to a different steroid, 16α-hydroxycortexolone 16,17-acetonide, was studied in a different series of media. Both 1-dehydrogenase and 20-ketoreductase were repressed, when *A. simplex*-X^s was germinated in two stages of soybean meal medium E33 and fermented in cornsteep liquor medium E24. However, selective induction of 1-dehydrogenase and repression of 20-ketoreductase were seen when *A. simplex*-X^s, germinated in two stages of cornsteep liquor medium E24, was fermented in the same cornsteep liquor medium.

Enzyme induction and repression in pure cultures of Streptomyces roseochromogenes

Induction of 16α-hydroxylase and repression of 20-ketoreductase were observed when *Streptomyces roseochromogenes* was germinated in soybean meal medium E33

and fermented in soybean meal medium E34 containing F2 steroid substrate. Under these conditions the organism converted all of the F2 substrate to F3 in 97 hr, without producing any detectable 20-dihydro-product. When *S. roseochromogenes* was exposed to a different steroid, cortexolone, in the same sequence of soybean meal media (E33 → E34), however, both 16α-hydroxylase and 20-ketoreductase were induced. On the other hand, the two enzymes were both repressed when the organism was germinated in two stages of peptone-yeast extract medium E6 and Yeastamin medium E21, and fermented in E21 with F2 steroid substrate.

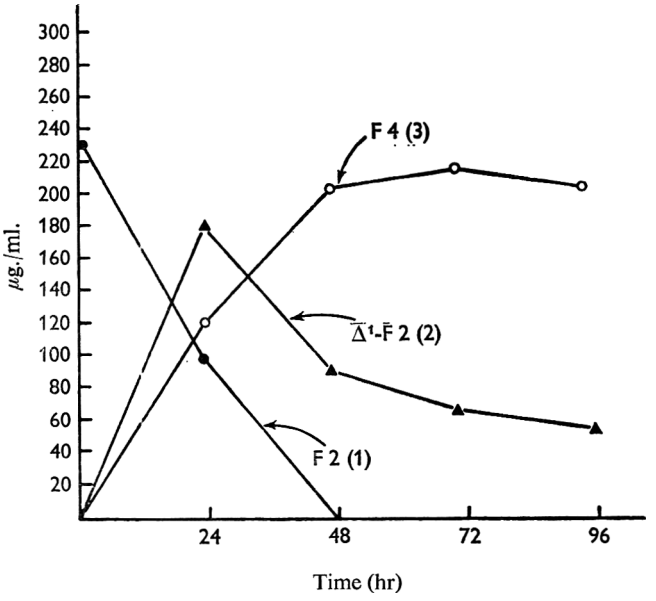

Fig. 2. Steroid conversion curves by mixed cultures of *Arthrobacter simplex* and *Streptomyces roseochromogenes*. The two organisms were grown separately in two stages of medium E33, and inoculated together into E34 containing F2 230 μg./ml. (1) F2: 9α-fluorohydrocortisone (substrate); (2) Δ¹-F2: 1-dehydro-9α-fluorohydrocortisone (intermediate); (3) F4: 1-dehydro-16α-hydroxy-9α-fluorohydrocortisone (product).

Patterns of enzyme induction and repression in the mixed culture system of Arthrobacter simplex-X^s and Streptomyces roseochromogenes

When the two organisms were grown separately in two stages of soybean meal medium E33, mixed and fermented in soybean meal medium E34 containing F2 steroid substrate, induction of 1-dehydrogenase by *Arthrobacter simplex*-X^s and 16α-hydroxylase by *Streptomyces roseochromogenes* was seen. However, 20-keto-reductase which was induced in the pure culture system of *A. simplex*-X^s was repressed in the mixed culture system of this organism and *S. roseochromogenes*. Thus the mixed system allowed development of conditions that influenced a change from induction to repression of the 20-ketoreductase of *A. simplex*-X^s, the induction of which is not desirable in the transformation of F2 to F4. A time course experiment was done with this F2 to F4 conversion system. Substrate F2 (230 μg./ml.) was almost completely converted to Δ¹-F2 intermediate within 48 hr. Conversion of Δ¹-F2 to F4

was initiated early and continued after F2 had disappeared. The maximal titre of final product was achieved in 72 hr in this experiment (Fig. 2).

Enzyme induction and repression in pure cultures of Nocardia restrictus and in the mixed culture system of this organism and Streptomyces roseochromogenes

Nocardia restrictus (SC 2914) is known to possess both 1-dehydrogenase and 20-ketoreductase. For comparison with *Arthrobacter simplex*-X^s, this organism was therefore tested in a single culture system and in a mixed culture system with *Streptomyces roseochromogenes*. Induction of 1-dehydrogenase and repression of 20-ketoreductase was seen in the single culture system of *N. restrictus* when tested in soybean

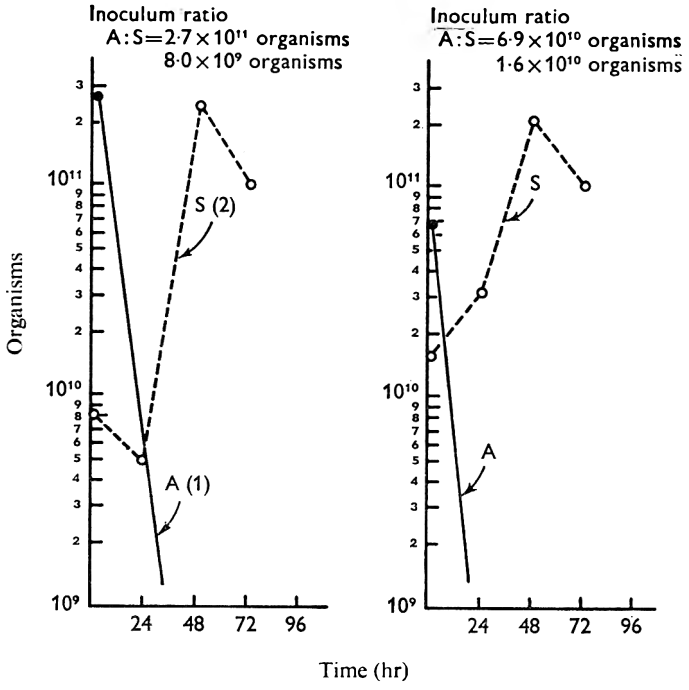

Fig. 3. Effects of changing initial ratios of *Arthrobacter simplex*-X^s inoculum and *Streptomyces roseochromogenes* inoculum on growth in E34 medium. A: *A. simplex*-X^s; S: *S. roseochromogenes*.

meal media (E33 → E33 → E34). In the mixed culture system of the organism with *S. roseochromogenes*, on the other hand, selective induction of 16α-hydroxylase and repression of both 1-dehydrogenase and 20-ketoreductase were seen. Thus the results illustrate a different type of altered enzyme induction and repression with a different mixture of organisms.

The effects of changing initial ratios of inoculum of Arthrobacter simplex-X^s and Streptomyces roseochromogenes on growth curves and steroid transformation

It was suspected that one of the factors controlling the success of the multiple steroid transformation by the mixed cultures might be the ratio in which the two organisms were mixed. By changing the volumes of inoculum, five different ratios of

viable organisms (colony count) were tested: (1) $5{\cdot}0 \times 10^{10}$ *Arthrobacter simplex*-X^s: $6{\cdot}0 \times 10^9$ *Streptomyces roseochromogenes* (8·3:1) at 0 time; (2) $3{\cdot}0 \times 10^{10}$: $7{\cdot}0 \times 10^9$ (4·3:1) at 0 time; and $5{\cdot}5 \times 10^{10}$: 0 at 23 hr; (3) $1{\cdot}0 \times 10^{11}$: $8{\cdot}5 \times 10^9$ (11·8:1) at 0 time; (4) $2{\cdot}7 \times 10^{11}$: $8{\cdot}0 \times 10^9$ (33·8:1) at 0 time; (5) $6{\cdot}9 \times 10^{10}$: $1{\cdot}6 \times 10^{10}$ (4·3:1) at 0 time. For each condition, growth curves of *A. simplex*-X^s and *S. roseochromogenes* were followed up to 71 hr and F4 product was measured after 71 hr of fermentation. In the range of mixture ratios covered, similar growth patterns were observed (Fig. 3). The patterns of steroid transformation were also alike in the different sets of conditions, and the overall yields ranged from 70 to 89 % (mole/mole) (Table 1). Thus the ratio of inocula in the range covered did not have any significant effect on the multiple steroid transformation.

Table 1. *Effect on steroid conversion of changing ratios of inocula of Arthrobacter simplex-X^s and Streptomyces roseochromogenes*

Sample*	Fermentation time (hr)	F2 input μg./ml. broth	F4 found μg./ml. broth	Conversion mole (%)
L6–1	0	234	—	—
	71	—	199	83
L6–2	0	226	—	—
	71	—	187	81
L6–3	0	264	—	—
	71	—	244	89
L6–4	0	286	—	—
	71	—	236	81
L6–5	0	224	—	—
	71	—	162	70

Inoculum size was determined by viable organism count.

* Samples. Ratios of *A. simplex* X^s organisms to *S. roseochromogenes* organisms at 0 time,

L 6–1 8·3:1
L 6–2 4·3:1 and $5{\cdot}5 \times 10^{10}$; 0 at 23 hr
L 6–3 11·8:1
L 6–4 33·8:1
L 6–5 4·3:1

Growth of Arthrobacter simplex strains and Streptomyces roseochromogenes under mixed culture conditions

The characteristics of growth of each organism were studied during the mixed culture fermentation with *Arthrobacter simplex*-X^s or *A. simplex*-X^r and *Streptomyces roseochromogenes*. When *A. simplex*-X^s was cultured alone in soybean meal medium E34 growth reached a maximum within 24 hr and the colony count remained more or less unchanged up to 120 hr. On the other hand, the maximum growth of *S. roseochromogenes* in pure culture was attained at 96 hr and then decreased at 120 hr, probably because of exhaustion of nutrients. When the two organisms were mixed and grown together in soybean meal medium E34, *A. simplex*-X^s decreased in number and nearly disappeared in less than 48 hr. *A. simplex* resistant to *S. roseochromogenes* metabolites appeared after 72 hr.

The antibiotic-producing capability of *Streptomyces roseochromogenes* has been reported by several laboratories, since the earliest report by Ishida (1950). In these

studies, we have made no effort to identify the inhibitory substances produced by the strain of *S. roseochromogenes* used. Earlier we had recognized that an antagonism between these cultures existed and could be shown by conventional techniques, namely, cross-streaking on agar, or tube-dilution assaying of *S. roseochromogenes* culture fluid with *Arthrobacter simplex* as the test organism.

A resistant mutant (*Arthrobacter simplex*-X^r) was isolated from 240 hr fermentation broth of a mixed culture of the parent *A. simplex*-X^s and *Streptomyces roseochromogenes*.

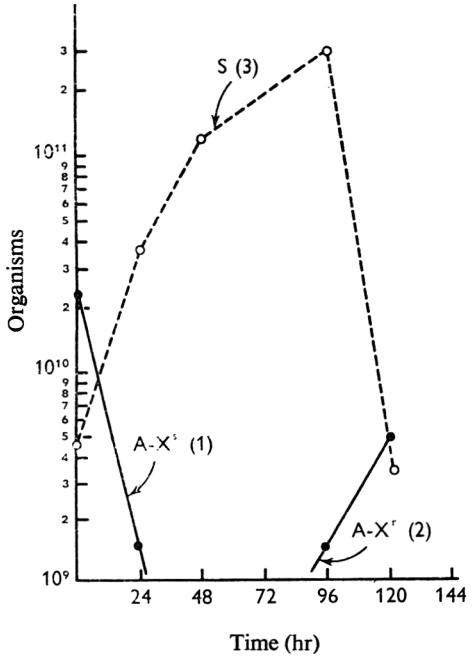

Fig. 4

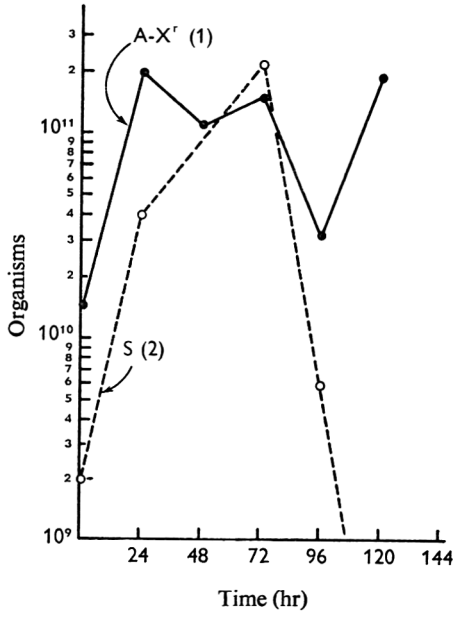

Fig. 5

Fig. 4. Growth curves of *A. simplex*-X^s and *S. roseochromogenes* in mixed culture fermentation during which *A. simplex*-X^r mutants are developed. (1) A-X^s: *A. simplex*-X^s, (2) A-X^r: *A. simplex*-X^r, (3) S: *S. roseochromogenes*.

Fig. 5. Growth curves of *A. simplex*-X^r and *S. roseochromogenes* in mixed culture fermentation in E34 medium. (1) A-X^r: *A. simplex*-X^r, (2) S: *S. roseochromogenes*.

The growth curve of the resistant *A. simplex* mutant was then compared with that of the original sensitive strain under mixed conditions with *S. roseochromogenes*. The mutant was found to be distinctly different from the parent strain in its response to *S. roseochromogenes* metabolites (Fig. 4, 5). The growth curve of the resistant mutant fluctuated with a period of about 48 hr, suggesting that the transmission of the resistance property to daughter organisms was not uniform. Under mixed conditions with the parent sensitive *A. simplex*, *S. roseochromogenes* grew without any interference and its pattern of growth was similar to that seen in the single culture of the organism (Fig. 4). When *S. roseochromogenes* was cultured with the resistant *A. simplex* mutant, a smaller number of viable *S. roseochromogenes* organisms (2×10^9 ml. vs. 3×10^9 ml.) was counted (Fig. 5). The *S. roseochromogenes* organisms may have been in competition for growth supporting substances with the resistant *A. simplex* mutant under the

mixed condition. In spite of the fact that the growth in the mixed culture system of resistant *A. simplex* (X^r strain) and *S. roseochromogenes* differed from the growth pattern seen in the mixed culture system of the *A. simplex* parent (X^s strain) and *S. roseochromogenes*, the amounts of steroid turnover in the two systems were alike, being 79 and 78 % (mole/mole), respectively.

DISCUSSION

The use of a mixed culture fermentation for steroid conversion has economic importance if, by its use, isolation of an intermediate can be eliminated or better control of the bioconversion steps can be achieved. One mixed-culture fermentation we have described has both advantages. In the conventional process for producing triamcinolone from 9α-fluorohydrocortisone, the substrate is first 16-hydroxylated in a *Streptomyces roseochromogenes* fermentation and the intermediate, 16α-hydroxy-9α-fluorohydrocortisone, isolated. Substantial losses of the intermediate occur during the isolation step. The isolated intermediate is then converted by fermentation with *Arthrobacter simplex*. to triamcinolone, isolated and purified. The second advantage, better control of the bioconversion steps, is achieved by the complete repression in the mixed culture of the 20-ketoreductase enzyme. This enzyme accounts for considerable by-product formation in the single culture conversion of 16α-hydroxy-9α-fluorohydrocortisone to triamcinolone with *A. simplex*, and consequently close monitoring of the fermentation is needed to ensure that the fermentation is terminated when the conversion to desired product is at a maximum and yet the formation of undesired product, 20-dihydro-triamcinolone, is still at a minimum.

The phenomena of enzyme induction and repression have been recognized for many years (Monod & Cohn, 1952; Monod & Cohen-Bazire, 1953). We have found that enzyme induction and repression patterns relating to the transformation of steroids can be influenced markedly by medium composition, by steroid substrate and, unexpectedly, by the interaction of organisms in mixed culture systems. Two enzymes, 1-dehydrogenase synthesized by *Arthrobacter simplex*, and 16α-hydroxylase produced by *Streptomyces roseochromogenes*, play critical roles in the multiple conversion of 9α-fluorohydrocortisone to triamcinolone by mixed cultures of the two organisms. The multiple transformation of 9α-fluorohydrocortisone to triamcinolone in the mixed culture system depends entirely upon repressing selectively a third enzyme, 20-ketoreductase, which can be synthesized by both organisms under conditions where induction is favoured. We found that the 20-ketoreductase enzyme, which was induced in the pure culture system of *A. simplex* grown in soybean meal medium with 9α-fluorohydrocortisone present, was repressed in the mixed culture system of the organism with *S. roseochromogenes*. Changes in enzyme induction and repression patterns were also seen in the mixed culture system of *Nocardia restrictus* and *S. roseochromogenes*. Both 1-dehydrogenase and 20-ketoreductase, which were inducible in the pure culture system of *N. restrictus* grown in the same soybean meal medium, were repressed in the mixed culture system of the organism with *S. roseochromogenes*.

We postulate, therefore, that certain inducing substances present in the medium in our mixed culture system are rapidly metabolized to certain substances, through the action of the micro-organisms, in the very early stage of fermentation, and these metabolites then no longer inactivate specific repressors and therefore changes from induction to repression of certain enzymes are effected.

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Bacitracin Action on Membranes of Mycobacteria

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SUMMARY

Bacitracin at concentrations of 6·5–13·0 μg./ml. inhibited the growth of *Mycobacterium smegmatis* ATCC 607. Concentrations 10 times higher were required to produce a similar effect on *M. tuberculosis* BCG. Assays of the bacitracin effect in Sauton medium showed no significant drug inhibition due to the cancelling effect of citrate ions present in this medium. Low concentrations of bacitracin produced smooth-surface colonies consisting of bacteria which showed a modified cell-wall appearance, in contrast to the rough colonies formed by control bacteria. Ultrastructure studies indicated that the main target of bacitracin action on mycobacteria may be a direct effect on the membrane system.

INTRODUCTION

Studies on the action of bacitracin on Gram-positive bacteria have shown the antibiotic to possess a variety of biological activities, such as induction of cell lysis (Smith & Weinberg, 1962), accumulation of cell-wall intermediates (Abraham & Newton, 1958), and inhibition of the incorporation of labelled amino acids into cell-wall mucopeptides (Hancock & Fitz-James, 1964). All these findings suggest that bacitracin acts by interfering with the synthesis of the cell walls of susceptible organisms. Although Sutter & Vaughan (1955) reported that bacitracin is an effective inhibitor of the growth of *Streptomyces bovis*, which is taxonomically related to the genus *Mycobacterium*, no report is available about the action of bacitracin on mycobacteria. It has now been found that when added during the early stages of growth, bacitracin is bactericidal to mycobacteria *in vitro*. In agreement with its postulated role as a cell-wall antibiotic, the present work shows that bacitracin seems to affect synthesis of the mycobacterial cell wall. The present report also shows that bacitracin acts on mycobacteria by causing marked alterations in their membranous structures.

METHODS

Stock cultures and organisms. The cultures used in the present experiments were maintained by adding 1 ml. of a 4-day stock culture of *Mycobacterium smegmatis* ATCC 607 to 150 ml. of liquid medium. Similar procedures were followed for *M. tuberculosis* BCG which was subcultured by adding inocula from 10-day stock cultures into the corresponding media.

Conditions for growth. The cultures were incubated at 37° on a reciprocal shaker and growth was followed in triplicate by measuring extinction at 450 mμ (E 450) in colorimetric tubes (2·54 cm.) with a Bausch & Lomb Spectronic-20 Colorimeter.

Mycobacterium smegmatis ATCC 607 was studied in (*a*) Sauton medium containing 2 % glucose instead of glycerol; (*b*) Penassay medium (Difco Lab., Detroit, Mich.); (*c*) Middlebrook 7H9 medium (Difco). *M. tuberculosis* BCG was studied only in Sauton and Penassay media. Tween 80 (polyoxyethylene sorbitan monooleate, Sigma Chemical Co., St Louis, Mo., U.S.A.) was added to all media to a final concentration of 0·05 % (v/v).

The viability of organisms was estimated by plating samples of experimental cultures on brain heart infusion agar (Difco) and incubating at 37° for 4 to 5 days to allow for the slower growth of bacitracin-treated cultures.

Bacitracin (Sigma) 73·9 units/mg. previously sterilized by filtration was added to the cultures.

Electron microscopy. Bacitracin-treated and control organisms were fixed by a slight modification of the Stoeckenius & Rowen's method (1967). The salt solution consisted of 5 % (w/v) NaCl plus 1 % (w/v) $CaCl_2$; 1 % (w/v) osmium tetroxide was used for post-fixation instead of potassium permanganate. After fixation followed by uranyl nitrate treatment, organisms were dehydrated in graded acetone plus water mixtures and embedded in Araldite. Ultrathin sections were doubly stained with uranyl acetate and lead citrate. An aqueous solution of sodium silicotungstate (2 %, w/v), at pH 7·6, was used for negative staining of unfixed organisms. The material was examined with Hitachi HU-11B and Jeol JEM-7A electron microscopes.

RESULTS

Effect of bacitracin on the growth of mycobacteria

The inhibition caused by bacitracin on the growth of *Mycobacterium smegmatis* ATCC 607 is shown in Fig. 1. When bacitracin was added during the early stages of growth, there was a complete inhibition, at concentrations of 13·0 μg./ml. Examination of the viability of the bacteria by plating out concentrated samples showed that bacitracin was highly bactericidal even at 6·5 μg./ml. Addition of bacitracin after the bacteria had reached their exponential stage of growth, did produce some inhibition although not as marked as previously quoted (Fig. 1). Bacteria which survived after bacitracin treatment, formed on agar media smooth surface colonies differing from the rough appearance of the control colonies. A higher proportion of smooth colonies was also observed by using the gradient plate technique (Freimer, Krause & McCarthy, 1959) with 100 μg. bacitracin/ml. in the centre well.

Similar results with regard to growth inhibition were obtained with *Mycobacterium tuberculosis* BCG, although considerably higher concentrations of bacitracin were required to cause a strong inhibition (Fig. 2).

Influence of culture media on the effects of bacitracin

Bacitracin showed an inhibitory action against the mycobacterial species examined when tested in Penassay or Middlebrook media (Difco). However, this effect was considerably weakened when *Mycobacterium smegmatis* ATCC 607 and *Mycobacterium tuberculosis* BCG grown in Sauton medium were tested for their susceptibility to bacitracin. Further examination pointed towards a possible inhibitor being present in this medium which presumably interfered with the action of bacitracin.

In agreement with reports on the requirements for divalent cations for the micro-

biological activity of bacitracin (Adler & Snoke, 1962), it was found that high concentrations of citrate (2 g./l.) were responsible for the annullment of the action of bacitracin in Sauton medium. Attempts to prepare this medium with much lower concentrations of citrate were unsuccessful because of abundant precipitation. It was also observed that when citrate (2 g./l.) or EDTA (10^{-5} M) were added to the other media tested, in which bacitracin was highly effective, a comparable result of decreasing the drug effect was obtained.

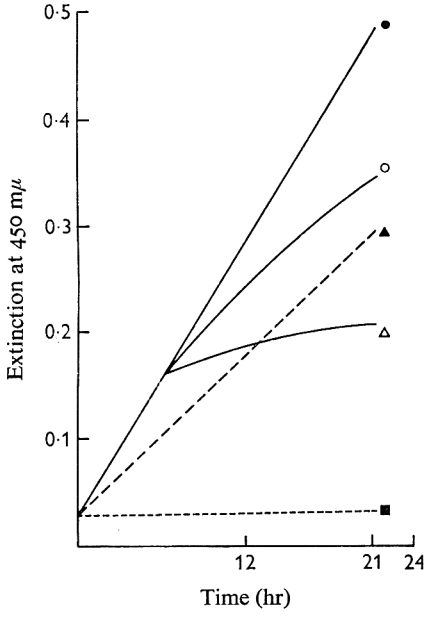

Fig 1

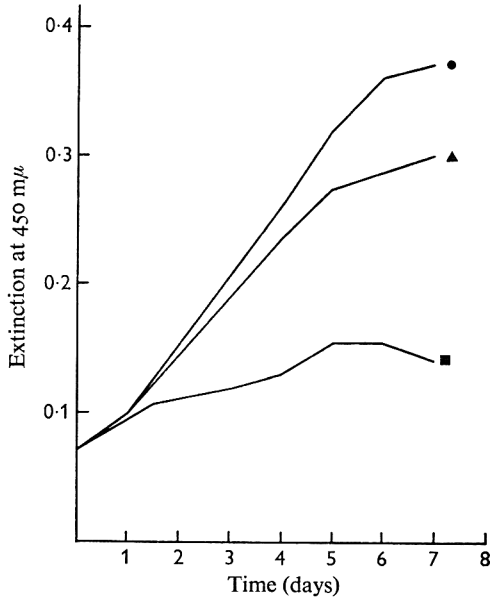

Fig 2

Fig. 1. Effect of bacitracin on the growth of *Mycobacterium smegmatis* ATCC 607. ● Control; ▲ 6·5 μg./ml.; ■ and ○ 13·0 μg./ml.; △ 130 μg./ml. The drug was added at 6 hr.

Fig. 2. Effect of bacitracin on the growth of *Mycobacterium tuberculosis* BCG. ● Control; ▲ 13·0 μg./ml.; ■ 130 μg./ml.

It has been reported (Snoke & Cornell, 1965) that the antibiotic effect of bacitracin is promoted by cadmium ions at 10^{-4} to 10^{-5} M. Although concentrations of cadmium ions at 10^{-5} M or higher were apparently effective in enhancing the effect of bacitracin when tested in Sauton medium, it was found that such concentrations of cadmium were by themselves inhibitory to the mycobacteria tested.

Ultrastructure studies

In exponentially growing mycobacteria treated with bacitracin, multilamellaf structures occurred nearby the plasma membrane (Pl. 1, fig. 1, 2). The thickness or each lamella, measuring approximately 25 Å, was identical to that of the plasma membrane. Frequently, these lamellar structures surround low-density spaces which contain granular substances (Pl. 1, fig. 2). The fact that the outer two layers of the lamellae continued to the plasma membrane (Pl. 1, fig. 2) indicated the lamellar struc-

tures were derived from the plasma membrane. Noteworthy was the finding that multitubular structures of mesosomes observed in the control bacteria at the same growth (Pl. 1, fig. 3) were not seen after bacitracin treatment.

Some of the bacteria contained no ribosomes nor well-defined nuclear filaments, leading us to suggest that they had undergone degeneration (Pl. 1, fig. 4). In these bacteria, lamellar structures usually composed of three layers containing a denser and slightly thicker central layer were distributed throughout the cytoplasm. Specimens at various sectioning angles did not evidence any cross-sectioned features of tubules, and furthermore, no tubular substance was encountered in negatively stained materials after disruption of the bacteria. These findings implied that the lamellar structures in ultrathin sections were three-dimensionally constituted of membranous layers.

Negative staining with moderate positive stain effect showed cell walls of surviving bacteria after bacitracin treatment at low concentration which differed significantly from those of control bacteria in exponential growth. Fibrillar components in mycobacterial cell walls of untreated bacteria (Pl. 2, fig. 5) (Imaeda, Kanetsuna & Galindo, 1968) were partially evident on the bacitracin-treated bacterial surface which was mainly covered with irregularly shaped substances (Pl. 2, fig. 6). This cell wall alteration cannot be visualized in ultrathin sections, because of non-osmiophilic characteristics of these fibrils (Imaeda, 1965).

Because of the effect of bacitracin on plasma membranes and mesosomes, a possible action of bacitracin on isolated mesosomes was examined. Mesosomes were prepared from exponentially growing *Mycobacterium smegmatis* ATCC 607 by differential centrifugation in sucrose 0·25M plus phosphate buffer (pH 7·0; Cesari, Imaeda & Rieber, unpublished data). Ultrastructural examination of the fractions exposed to bacitracin up to 1 mg./ml. showed no appreciable difference from the untreated controls.

DISCUSSION

The observations described here show that mycobacterial growth is inhibited by bacitracin. The fact that higher concentrations of bacitracin were required to inhibit the growth of *Mycobacterium tuberculosis* BCG may be due to permeability factors resulting from the higher lipid content in the wall of BCG as compared with that of the rapidly growing species (Imaeda, Kanetsuna & Galindo, 1968). The significant decrease of the effect of bacitracin in Sauton medium emphasizes the importance of selecting an adequate culture medium, without a high concentration of chelators, when assaying a cation-requiring antibiotic.

Although bacitracin is highly bactericidal to mycobacteria during the middle or late exponential growth phase, its addition did not produce an immediate effect. This action was only manifested after a lapse of time after the addition of bacitracin, as shown in Fig. 1. Furthermore, the initial sign of ultrastructural changes was represented by derangement of plasma membranes and mesosomes. Low concentrations of bacitracin modified cell-wall components resulting in the formation of smooth surface colonies on solid media, showing minimal structural damage of membranes without influencing cellular viability (Pl. 1, fig. 1, 2). However, higher concentrations of bacitracin produced severe changes in the membrane causing the disappearance of mesosomes (Pl. 1, fig. 4) and finally death of the organism. From this evidence, it is presumed that bacitracin interferes with membrane components, which not only

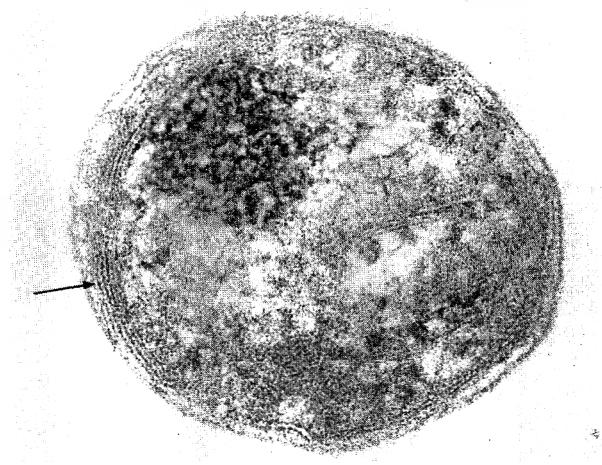

Fig. 1

Fig. 2

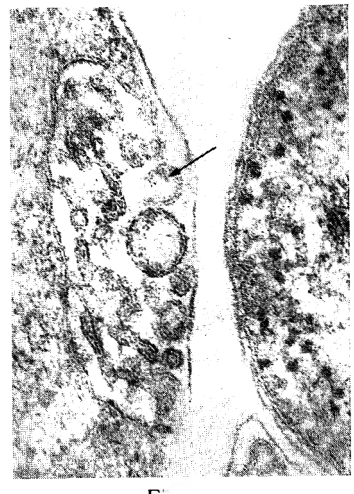

Fig. 3

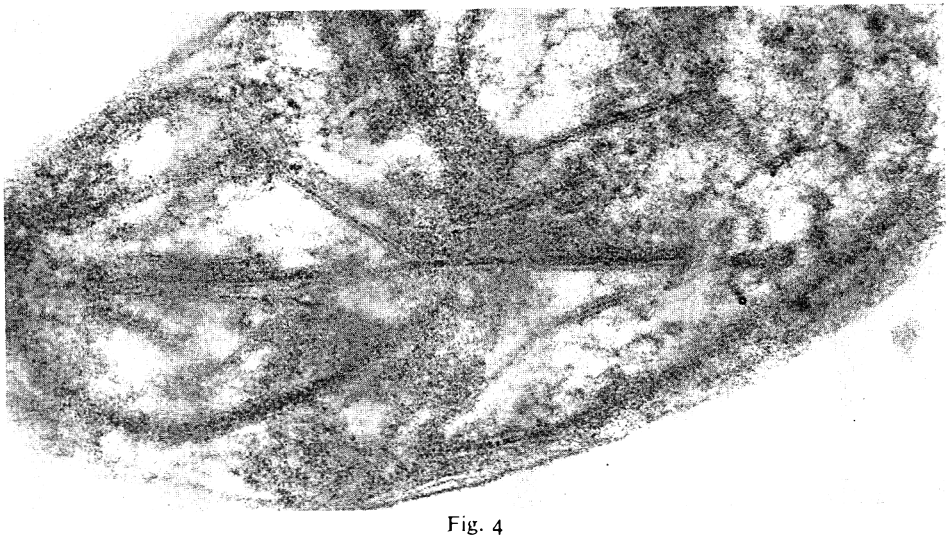

Fig. 4

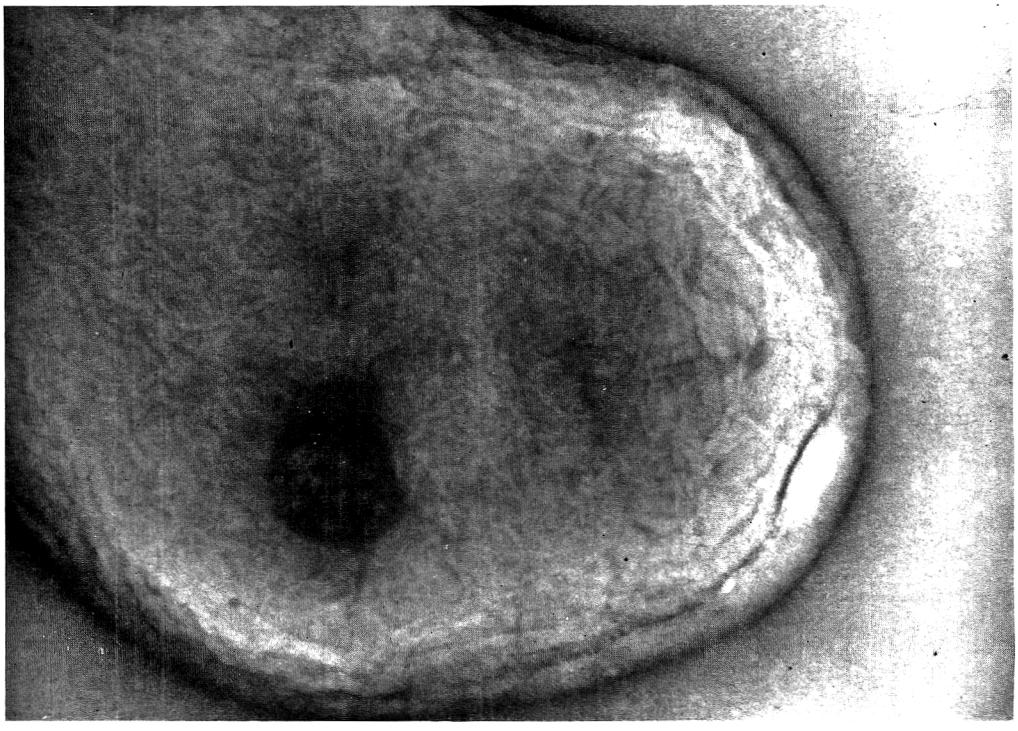

Fig. 5

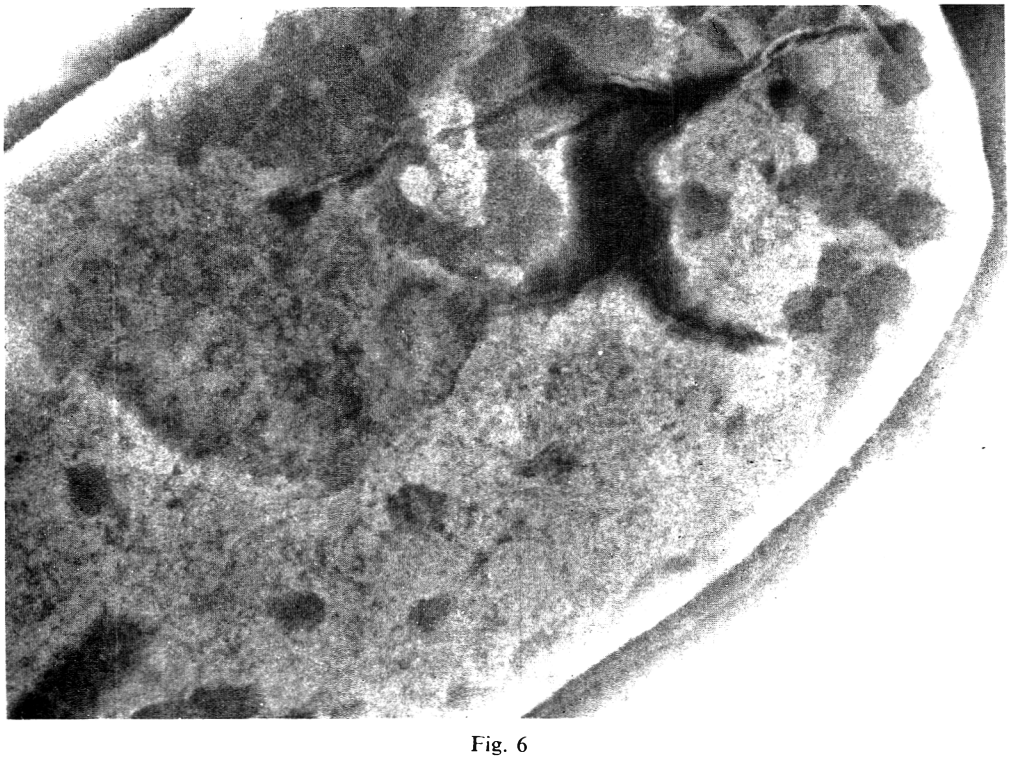

Fig. 6

maintain cellular integrity but may also contain enzymatic systems to synthesize cell-wall constituents (Hancock & Fitz-James, 1964; Imaeda & Ogura, 1963). It is proposed that bacitracin interferes with cellular division and leads to bacterial death because of membrane disorganization.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Bacitracin (6·5 μg./ml.) treated *Mycobacterium smegmatis*. Note a multilamellar structure (arrow). Magnification: × 200,000.

Fig. 2. Bacitracin (6·5 μg./ml.) treated *Mycobacterium smegmatis*. Multilamellae continue to the plasma membrane (arrow). Granular substances are surrounded by these lamellae. Magnification: × 150,000.

Fig. 3. Control cell at exponential growth phase. Tubular infoldings form a mesosome (arrow). Magnification: × 150,000.

Fig. 4. *Mycobacterium smegmatis* treated with bacitracin (13·0 μg./ml.). Lamellar structures are scattered throughout the cytoplasm. Magnification: × 225,000.

PLATE 2

Fig. 5. Negatively stained control of *Mycobacterium smegmatis* with moderate positive staining effect at exponential growth phase. Note fibrillar substances covering the cell surface. Magnification: × 120,000.

Fig. 6. Negatively stained *Mycobacterium smegmatis* with moderately positive staining effect after the treatment of bacitracin (6·5 μg./ml.). Fibrillar substances are scarcely distributed in the cell wall. Magnification: × 120,000.