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# THE BASIS OF PATHOGENICITY IN *AGROBACTERIUM*

by

WILLIAM PHILIP ROBERTS

B.Sc. (Hons.)
Australian National University

Department of Plant Pathology,

Waite Agricultural Research Institute,

University of Adelaide,

South Australia.

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## SUMMARY

A number of non-pathogenic strains of *Agrobacterium* were converted to pathogens using the method of Kerr (1971). The converted pathogenic strains were compared with the original non-pathogenic strains by serology, isoenzyme patterns after electrophoresis, reaction to mitomycin C, nucleic acid analysis and sensitivity to bacteriocin 84 produced by *Agrobacterium* strain 84 (Roberts and Kerr 1974). There was a close correlation between bacteriocin 84 sensitivity and pathogenicity. No other consistent difference between non-pathogens and converted pathogens was found. In contrast to the work of Van Larebeke, Zaenen, Teuchy and Schell (1973) no evidence was found for the presence of a plasmid in the pathogenic strains used in this study.

Purification of bacteriocin 84 was attempted using gel filtration, chromatography and electrophoresis. Although good purification was not achieved some characteristics of the bacteriocin were determined. In contrast to bacteriocins produced by other bacteria, bacteriocin 84 is of relatively low molecular weight and not a protein. It is rapidly degraded at temperatures above approximately 80°C and by alkaline pH. It is strongly polar and has a residual negative charge which is due to the presence of a phosphate group.

The mode of action of bacteriocin 84 did not follow the same kinetics that have been reported for other bacteriocins. When bacteriocin 84 was added to a culture of sensitive bacteria there was a

rapid decrease in the number of bacteria able to form colonies. Although there was little immediate effect on the rate of oxygen consumption of a culture of sensitive bacteria after bacteriocin treatment, the normal increase in optical density with time stopped and it is suggested that bacteriocin 84 acts by stopping cell division without grossly affecting the metabolism of the cell. Radioisotope tracer studies showed that DNA synthesis stops very soon after adding bacteriocin and it is possible that this is the primary mechanism of bacteriocin action.

Although bacteriocin 84 affected all the converted pathogenic strains used in this study, no effect on the original non-pathogenic strains was observed. The possible mechanism of bacteriocin sensitivity and its relationship to pathogenicity is discussed.

## STATEMENT

This thesis contains no material which has been accepted for the award of any degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

W.P. Roberts

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# *PART A*

## GENERAL INTRODUCTION

Crown gall is a plant disease that is characterized by the production of a gall generally on the crown of the plant but often on the general root system or even sometimes on the aerial parts of the plant. These generally grow at a fast rate often reaching a very large size and represent a large diversion of the growth resources of the plant as well as interfering with the movement of substances through the plant.

The disease has been shown to affect at least 142 genera of plants from both the gymnosperms and the dicotyledonous angiosperms (Elliott 1951). In South Australia the disease is only really of serious economic importance on stone fruit trees although it is commonly found on roses and sometimes on apples (New 1972).

Smith and Townsend (1907) showed that the disease was caused by a bacterium commonly present in the rhizosphere and although there have been many studies since this report the precise mechanism of gall induction has still not been determined.

Conn (1942) created the genus *Agrobacterium* to include *Agrobacterium tumefaciens*, the pathogenic organism, and *Agrobacterium radiobacter*, a closely related non-pathogenic organism often isolated from the soil. This non-pathogenic bacterium is often used in

comparative studies on pathogenic and non-pathogenic agrobacteria and a number of workers have questioned the validity of distinguishing species solely on the basis of pathogenicity (DeLey, Bernearts, Rassel and Guilmot 1966, Keane, Kerr and New 1970). Conn (1942) recognised that the agrobacteria were closely related to rhizobia and a number of reports have suggested that the two genera be merged (Graham 1964, Herberlein, DeLey and Tijtgat 1967, DeLey 1968, Moffett and Colwell 1968, White 1972).

Keane *et al.* (1970) recognised two distinct biotypes of *Agrobacterium* which could be distinguished by several biochemical and physiological tests. Non-pathogenic and pathogenic bacteria of both biotypes were reported and it was suggested that the character of pathogenicity should be reduced to a varietal status. Similar biotypes were also found in a number of other studies (White 1972, Kersters, DeLey, Sneath and Sackin 1973, Panagopoulos and Psallidas 1973). The nomenclature of Keane *et al.* (1970) is used throughout this study and further evidence for the similarity of pathogenic and non-pathogenic agrobacteria is presented.

Pathogenic agrobacteria are thought to be good soil colonizers that can survive long periods in soil without a suitable host plant (Schroth, Weinhold, McCain, Hildebrand and Ross 1971). Although New (1972) demonstrated that there was a significant rhizosphere effect, suggesting that the presence of plant roots may assist long term survival of agrobacteria, survival of both non-pathogenic and pathogenic agrobacteria in soil without plants was still high.

It is generally thought that pathogenic agrobacteria are introduced into new areas on the roots of host plants (Scroth *et al.* 1971, New 1972). Although it is illegal to sell galled trees in most areas, New (1972), in a study of an almond nursery in South Australia, was able to isolate pathogenic *Agrobacterium* from the roots of 10% to 40% of trees in bundles prepared for distribution. Every bundle contained at least one tree with pathogenic *Agrobacterium* on the roots. He could not isolate any pathogenic bacteria from an area in this nursery that had not been used for growing stone fruit suggesting that the disease was originally introduced on planting material.

Control of the disease once it is established is a difficult problem. Schroth *et al.* (1971) reviewed the possible chemical methods and concluded that with the exception of Bacticin treatment, chemical control was impracticable or uneconomic. Even Bacticin treatment is unlikely to be satisfactory for large orchards as galls have to be exposed and treated individually.

New and Kerr (1972) and Kerr (1972) reported that biological control could be achieved by dipping seeds and plant roots in a suspension of non-pathogenic *Agrobacterium* strain 84 before planting. This treatment did not prevent galls already induced from growing but it did stop healthy plants from becoming galled. This method is being used by a number of growers in South Australia and has proved very effective. Kerr and Htay (1974) reported that the

biological control was due to strain 84 producing a bacteriocin which was active against pathogenic strains. Part of this thesis describes some properties and the mode of action of this bacteriocin.

Perhaps the main factor that has led to so much interest in the crown gall problem is the demonstration that once galls are induced the bacteria are no longer involved in the growth of the gall. Crown gall then, is a true plant cancer and it is the ease of production and maintenance of these galls that has stimulated much research in the hope that an understanding of such a system will contribute basic concepts to the whole cancer problem.

Although a number of early workers suggested that once a gall was induced the bacteria were no longer necessary, it was not until the demonstration that galls could be cultured bacteria-free on a medium containing only basic mineral salts and a carbon source that this was generally accepted (White and Braun 1941, White and Braun 1942). Braun and Mandle (1948) proposed that the bacteria produce a "tumour inducing principle" (T.I.P.) that somehow alters the plant cell to produce a gall. Most of the recent work on crown gall has been involved with determining the nature of the T.I.P. and the mechanism of crown gall induction.

Although wounding was shown early to be an essential process in crown gall induction (Riker and Berge 1935) the precise role of the wound in this process has yet to be determined. There are a number of different theories which can be broadly divided into two groups:

1. that wounding allows entry of the T.I.P. or close contact of the bacteria and the plant cell, and 2. that processes involved in the wound healing process make the plant cells susceptible to gall induction. On the available evidence it is not really possible to decide between these two possibilities.

Braun (1947, 1952) and Braun and Mandle (1948) carried out an extensive study on the effect of the wound healing process on gall induction. They found that a conditioning period of the wound was necessary before gall induction could take place. This conditioning process could occur at 25°C or 32°C without the bacteria being present although gall induction only occurred below approximately 28°C. Using different temperature conditions they were able to establish that there was a distinct optimum length of conditioning period after wounding for maximum transformation. Conditioning periods on either side of the optimum produced smaller, slower-growing galls. Braun (1952, 1954) reported that plant cells were most susceptible to transformation just before the onset of the first cell division after wounding and suggested that the conditioning process may involve alterations in the permeability of the plant cell and so allow entry of the T.I.P. Lipetz (1965, 1966) extended these findings and reported that the time taken to the onset of the first cell division decreased with temperature as did the time to reach maximum sensitivity to gall induction. At all temperatures greatest

sensitivity to induction was found just before the first cell division after wounding. These results suggest that the process of gall induction is closely related to cell division. However, cell division alone is not a satisfactory condition for gall induction, for bacteria added to the surface of actively dividing callus tissue do not produce galls unless wounding occurs (Riker 1923, Riker and Banfield 1932). There are also reports of gall induction on pinto bean leaves (Lippincott and Herberlein 1965a) and decapitated pea seedlings (Kurkdjian, Manigault and Beardsley 1969), where gall induction occurs without wounding inducing cell division.

Klein (1954) suggested that wounds were essential to release substances that were necessary for gall induction. He reported that washing the wound with sterile water prevented gall induction. In another report (Klein 1955) he showed that carrot discs of a variety which normally respond only weakly to pathogenic bacteria gave a much stronger response when treated with an extract from carrots which normally respond strongly. Hildebrand (1942) obtained a similar increase in tumorogenesis when he added wound sap to small wounds containing bacteria. The possible importance of wound sap composition is emphasised by the studies of Lippincott and Lippincott (1966, 1969b) who showed that bacterial nutrition was a significant factor in the expression of virulence, particularly when only small wounds were involved. There is one report, however (Therman and Kupila-Ahvenniemi 1971) which indicates that wound washing enhances tumorogenesis.

These workers attributed this to a promotion of the first cell division after wounding. A similar promotion of the first cell division after wounding by washing has been reported by Lipetz (1967).

Klein (1952, 1953) and Klein, Rasch and Swift (1953) reported that a peak of DNA synthesis occurred one day after wounding in wounds inoculated with virulent bacteria but not in wounds inoculated with avirulent bacteria. They showed that this was not due to plant nuclear DNA synthesis and suggested that the virulent bacteria were synthesizing specific tumour inducing DNA under the influence of the wound sap. These results were later shown to be erroneous by Kupila and Stern (1961), who demonstrated that a peak in DNA synthesis occurred in wounded tissue irrespective of whether it was inoculated and they suggested that this peak of DNA synthesis was necessary for gall induction. A similar peak in DNA synthesis was reported by Lipetz (1966, 1967) and Kuplia-Ahvenniemi and Therman (1968, 1971). The latter authors reported that the peak of DNA synthesis in wounded tissue occurred just before the first cell division which corresponded to the period of maximum sensitivity to gall induction.

There is some evidence that suggests that wounding may expose specific sites on the plant cells that are involved in gall induction. Lippincott and Lippincott (1969a) showed that avirulent bacteria could prevent gall induction if added to the wound site before or at the same time as virulent bacteria. They showed that there was a 1 : 1 relationship between virulent and avirulent bacteria and suggested that

both virulent and avirulent bacteria were competing for specific bacterial binding sites on the plant cells that were involved in gall induction. Similar results were obtained by Schilperoort (1969) who was also able to find further evidence for bacterial binding sites using electron microscopy. Yajko and Hegeman (1971) reported that specific DNA transfer between virulent agrobacteria and carrot tissue was decreased in a competitive manner by avirulent agrobacteria, again suggesting the presence of specific bacterial binding sites.

Although crown gall induction is often considered as an "all or none" response this is far from the true situation. Both the characteristics of the bacteria and the host plant are important in determining the final characteristics of the induced galls.

The genus *Agrobacterium* consists of a wide range of bacteria from highly virulent to completely avirulent (see, for example, Keane *et al.* 1970). Even within the group that is considered *A. tumefaciens* there are wide differences in virulence. For example, Lippincott and Herberlein (1965b) developed a quantitative assay for virulence and they found that compared to strain B6 the other strains they tested showed virulence ranging from 10% to 100%. Lippincott and Lippincott (1966, 1969b) carried out studies on the virulence of naturally occurring and induced auxotrophic mutants and reported that virulence could often be increased by adding the required nutrient to the wound. They interpreted this to mean that the bacteria needed metabolic activity when present in the wound either to stay viable until the plant cell

was conditioned or to produce the T.I.P. Langley and Kado (1972) carried out a similar study with induced amino acid requiring mutants but adding the required amino acid did not restore or increase virulence of the auxotrophs, suggesting that nutritional conditions were not the limiting factor in this case.

Locke, Riker and Duggar (1938) reported that attenuated strains of *Agrobacterium* only produced fast growing galls when inoculated below a gall produced by a normal strain. This suggests that growth substances from the normal gall are influencing the gall produced by the attenuated strain. Braun and Laskaris (1942) and Thomas and Riker (1948) showed that certain strains of *Agrobacterium* only produced rapidly growing galls when auxin was added. As well as influencing the growth rate of a gall the inducing bacterial strain may also determine the final morphology of the gall. For example, Braun (1953) reported that some strains of agrobacteria induced teratoma type galls which showed organisation into shoots, roots and vascular elements in contrast to the completely undifferentiated gall tissue produced by many strains.

Although there appears to have been very few studies on the host specificity of *Agrobacterium* strains Wormald (1945) reported that a number of strains of *Agrobacterium* isolated in England showed distinct strain specificity and Panagopoulos and Psallidas (1973) reported that a number of Greek *Agrobacterium* isolates were pathogenic on grape vines but not on a number of other plants.

The host species is also important in determining the characteristics of the gall produced. For example, tobacco plants often form a teratoma tumour when inoculated with virulent bacteria but sunflower plants normally form a disorganised gall (Braun 1953, 1959). These are stable characteristics which are maintained when bacteria-free tumours are grown in tissue culture. Galls of the teratoma type can be forced to grow rapidly if grafted onto normal host plants and sometimes regenerate normal plants (Braun 1959). Sacrista and Melchers (1969) reported that even the unorganised type of tumour will regenerate buds, leaves and roots if placed on a suitable medium. Although these structures were highly abnormal, it was reported that they needed all the usual growth substances to grow in culture, indicating that they were no longer tumorous and it was suggested that the abnormalities were due to chromosomal changes unrelated to tumorogenesis.

Any model of crown gall induction has to account for the wide range of virulence observed in *Agrobacterium* and the diversity of characteristics and growth rates of galls. Braun (1970) proposed that tumours of any type can be due to either (1) changes in the genetic information in the cell (genetic changes), or (2) changes in the pattern of expression of the normal genetic information present in the cell (epigenetic changes). Genetic changes would be expected to lead to a permanent tumorous state that could not be reversed and

are unlikely to produce a range of tumour types from highly organised to completely disorganised. This is not the situation with crown gall and it seems likely that gall induction is an epigenetic process. This implies that all cells of sensitive host plants carry the complete genetic information for the production of a gall, but it is only under certain conditions that this information is expressed. Support for this hypothesis can be seen in the reports of Limasset and Gautheret (1950) and Kandler (1952) who showed that sunflower tissue can be habituated with auxin to form tumours with similar physiological and morphological features to tumours produced by *Agrobacterium*.

Changes in the pattern of expression of genetic information are the basis for the normal development in most biological systems. Although these processes are generally under close control, little is known about the basic mechanisms which trigger changes in genetic expression. Much of the work on crown gall has concentrated on determining the nature of the T.I.P. that is produced by the bacteria and induces the gall in the plant. An understanding of the nature and mode of action of the T.I.P. will contribute, not only to our understanding of crown gall, but also to our understanding of the control mechanisms present in plant cells.

Many of the early studies were concerned with demonstrating that galls were induced by hormones produced by the bacteria. For example, Brown and Gardner (1936) were able to reproduce the morphological characteristics of typical galls using auxin and extracts

from bacterial cultures. However, no correlation between auxin production and pathogenicity could be established (Locke, Riker and Duggar 1938, 1939). The demonstration by Braun and White (1943) that secondary tumours did not contain bacteria and the cultivation of bacterial free gall tissue on medium containing no plant growth substances (White and Braun 1942) conclusively demonstrated that growth substances produced by the bacteria were not responsible for gall production. In a more recent study Galsky and Lippincott (1969) could find no correlation between pathogenicity and the production of a gibberelin in culture by a number of *Agrobacterium* strains.

Since the discovery of widespread lysogenicity in the genus *Agrobacterium* (Beardsley 1955) there have been many reports attempting to show that bacteriophage is involved in gall induction. Kurkdjian (1968, 1970), Kurkdjian, Beardsley and Manigault (1968) and Hoursiangou-Neubrun and Puiseux-Dao (1969) all reported that bacteriophages could be detected in tissues of plants inoculated with *Agrobacterium*. However phage was observed when avirulent as well as virulent bacteria were added to wounds. Parsons and Beardsley (1968) and Tourneur and Morel (1970) were able to detect phage in bacteria-free crown gall tissue that had been in culture for over 10 years and they suggested that the phage genome must be present in the gall genome. These results have been criticised by Schilperoort, Sittert and Schell (1973) who claim that the phage sensitive indicator strain used in these studies still carries a defective phage and suggest that the

phage apparently detected from gall tissue may come from spontaneous lysis of a small proportion of the indicator strain. They were unable to detect any phage from gall tissue in their experiments.

The enhancement of infectivity of agrobacteria by ultraviolet light treatment (Duggar and Riker 1940, Herberlein and Lippincott 1965, 1967a) and mitomycin C treatment (Herberlein and Lippincott 1967b) could be explained on the basis of the T.I.P. being a phage as these are both efficient bacteriophage inducing agents.

There have been a number of attempts to induce galls using purified bacteriophages isolated from pathogenic agrobacteria, most of which were unsuccessful (Klein and Beardsley 1957, Stonier, McSharry and Speitel 1967, Roussaux, Kurkdjian and Beardsley 1968). Leff and Beardsley (1970) suggested that the failure to induce galls with phage preparations may be due to the absence of a suitable enzyme in the plant cell to remove the phage coat and expose the DNA. They reported that phage DNA preparations sometimes induced limited overgrowths on a number of plants but the result of a number of experiments were not consistent and the growth rates of these "galls" were generally much slower than normal galls. Beiderbeck, Herberlein and Lippincott (1973), in an attempt to confirm this report, were unable to induce any overgrowths on a wide variety of test plants using purified phage DNA.

Schilperoort *et al.* (1973) reported that they could detect phage DNA sequences in sterile gall tissue induced by strain A6 even though they could not detect phage DNA sequences in this bacterium and

they suggested that the phage may be coded for by a plasmid which is lost in the normal bacterial DNA isolation procedure. This would have to be a cryptic bacteriophage as no phage could be isolated from strain A6 (Zaenen, van Larebeke, Teuchy, van Montagu and Schell 1974). Zaenen *et al.* (1974) report that they have evidence that a number of other *Agrobacterium* strains may carry cryptic prophage. This is further supported by Bourgin-Rosenberg, Garnier-Expert and Tourneur (1974) who reported that when two *Agrobacterium* strains that were considered to be phage free were treated with a mutagen they produced normal phage and suggested that these strains must carry a cryptic prophage. Despite all of these studies the precise role (if any) of bacteriophage in crown gall induction has still to be determined.

The other possibility for the T.I.P. that has been most studied is bacterial nucleic acid and there is some evidence that part of the genome of the bacteria is expressed in the plant gall. Schilperoort (1969) showed that antisera against crown gall tissue (but not normal tissue) reacted with *Agrobacterium* antigens, although he was not able to show the reverse reaction. Chadha and Srivastava (1971) were able to show a similar reaction using antisera against *Agrobacterium* and crown gall antigens. They showed that crown gall tissue had three proteins not present in callus tissue, two of which were bacteria specific.

Until recently the production of either octopine or nopaline by crown gall tissue was thought to be due to the expression of the genome of the gall-inducing bacteria (Goldman, Tempe and Morel 1968) but the demonstration that small amounts of octopine can be detected in normal tissue (Wendt-Gallitelli and Dobrigkeit 1973, Johnson, Guderian, Eden, Chilton, Gordon and Nester 1974) shows that this is invalid. However, the bacteria do specifically determine which of these compounds is produced by a gall.

A number of workers have attempted to show more directly the presence of *Agrobacterium* DNA in crown gall tissue. Stroun, Anker and Ledoux (1967) and Stroun and Anker (1971) reported that when agrobacteria were added to plants a DNA with a buoyant density intermediate between that of the plant DNA and the bacterial DNA could be isolated. When these DNA samples were sheared two bands were obtained, one corresponding to the buoyant density of plant DNA and the other to the buoyant density of bacterial DNA. They suggested that the bacterial DNA was joined to the plant DNA to give a DNA of intermediate density. Srivastava and Chadha (1970) carried out similar experiments and although they were unable to detect a DNA of intermediate density they did detect DNA at the buoyant density of bacterial DNA when the DNA samples were sheared. Schilperoort (1969), using similar techniques, could not find any evidence for DNA with intermediate density or DNA with the density of bacterial DNA, in crown gall tissue cultures. Using radioactive DNA, Yajko and Hegeman (1971)

were able to show that pathogenic agrobacteria transformed DNA to carrot tissue at a higher rate than non-pathogenic agrobacteria. Non-pathogenic agrobacteria competitively decreased the transfer of DNA by pathogenic agrobacteria.

There has been a number of reports of nucleic acid hybridization experiments which show the presence of *Agrobacterium* DNA base sequences in crown gall tissue. Schilperoort, Veldstra, Warnaar, Mulder and Cowen (1967) and Schilperoort (1969) reported that synthetic RNA complementary to *Agrobacterium* DNA showed a much greater homology with crown gall DNA than normal plant DNA. Similar DNA base sequence homology between *Agrobacterium* and crown gall tissue has been reported by Quetier, Huguet and Guille (1969), Srivastava (1970) and Schilperoort *et al.* (1973). Milo and Srivastava (1969) reported that DNA from *Agrobacterium* hybridized at a higher level with RNA extracted from crown gall tissue compared with RNA from normal plant tissue. However the validity of all these reports has been seriously questioned by a number of recent papers (Chilton, Currier, Farrand, Bendich, Gordon and Nester 1974, Drlica and Kado 1974, Eden, Farrand, Powell, Bendich, Chilton, Nester and Gordon 1974) which report that there is no detectable hybridization between *Agrobacterium* nucleic acid and crown gall nucleic acid at levels well below the degree of homology previously reported. They attribute the homology observed in the other studies to the presence of polysaccharide in the nucleic acid

preparations and less stringent hybridization conditions and claim that there must be less than approximately 0.2 bacterial genomes per tumour cell. These results suggest that the whole bacterial genome does not act as the T.I.P. but do not eliminate the possibility that a small part of the bacterial genome may be important in gall induction.

Although there has been a number of claims that galls can be induced by sterile DNA extracts of pathogenic bacteria, these results must be treated with caution. For example, Klein and Braun (1960) showed that a number of claims of sterile gall induction (Klein 1954, Manigault, Commandor and Slizewicz 1956, Klein and Knupp 1957, Bender and Brucker 1959, Thomas and Klein 1959) were erroneous as the precautions taken to ensure sterility of the extracts were inadequate. Manigault and Stoll (1958a, 1958b, 1960) were able to induce overgrowths on *Datura* plants with bacterial DNA but these collapsed after a few weeks. Similar results were obtained by Kado, Heskett and Langley (1972). Koovor (1967) and Beltra and deLecea (1971) claim to have induced galls with nucleic acid fractions from *Agrobacterium* but these reports have not yet been confirmed by other workers.

Swain and Rier (1972) and Beljanski, Aaron-Dar Cunha, Beljanski, Manigault and Bourgarel (1974) claim to have induced galls with sterile *Agrobacterium* RNA fractions. Beljanski *et al.* isolated two RNA fractions, one associated with an RNA directed DNA

polymerase and the other associated with DNA, from both virulent and avirulent bacteria which they claim were able to induce galls. They suggest that the difference between virulent and avirulent bacteria must be in the ability to transfer this RNA to the plant cell. These studies on RNA were initiated by the report of Braun and Wood (1966) who found that ribonuclease enzyme inhibited tumour induction. They could not find any effect of ribonuclease on the bacteria and they suggested that the T.I.P. may be ribonuclease sensitive. However Nevins, Grant and Baker (1970) demonstrated that ribonuclease had a marked effect on *Agrobacterium in vitro*, so the inhibition of tumour induction by ribonuclease observed by Braun and Wood (1966) may be due to non-specific effects on the bacteria rather than degradation of the T.I.P.

There have also been numerous negative reports on sterile induction of galls with nucleic acids (Braun and Stonier 1958, Klein and Braun 1960, Bieber and Sarfert 1968, Gribnau and Veldstra 1969, Stroun and Anker 1971) and it must be concluded from the available evidence that viable bacteria are necessary for gall induction.

Recently there has been a number of reports that virulent *Agrobacterium* contain a large plasmid which is not present in avirulent strains (Van Larebeke, Zaenen, Teuchy and Schell 1973, Zaenen *et al.* 1974, Van Larebeke, Engler, Holsters, Van den Elsacker, Zaenen, Schilperoort and Schell 1974) and it is suggested that this may be

the T.I.P. A similar study by Kado *et al.* (1972) using a slightly different technique did not find any evidence of a plasmid and it remains to be seen whether the work of Van Larebeke *et al.* can be confirmed in other laboratories.

There have been a few studies attempting to transfer the ability to induce tumours from one bacteria to another. Klein and Klein (1953, 1956) reported that they could transfer pathogenicity from virulent agrobacteria to avirulent agrobacteria and to rhizobia using DNA extracted from virulent bacteria. However these results seem doubtful as the sterility precautions taken in their method of DNA preparation have been shown to be inadequate (Klein and Braun 1960). Kern (1965a, b, c) claims to have repeated and extended this work but his results have not been confirmed by other workers.

Kerr (1971) described the transfer of virulence between pathogenic and non-pathogenic agrobacteria when non-pathogenic bacteria were placed on developing galls. Antibiotic markers and selective media ensured that this was a true genetic transfer and preliminary results confirmed that the transformed pathogenic bacteria were very similar to the original non-pathogenic bacteria.

This thesis describes experiments designed to detect differences between non-pathogenic bacteria and pathogenic bacteria which have acquired virulence using the method of Kerr (1971), in an attempt to understand the basis of crown gall induction. Some properties and the mode of action of the bacteriocin important in biological control of crown gall are also described.

# *PART B*

## GENERAL MATERIALS AND METHODS

### Growth and maintenance of bacterial cultures

Bacteria were routinely maintained on yeast-mannitol agar medium (Appendix 1) at 25°C. Cultures could be stored for periods up to 2 months on yeast-mannitol slopes at 4°C. For longer storage cultures were freeze-dried and sealed in glass ampoules under vacuum. These were kept at 4°C and quick recovery of the bacteria was still possible after over 2 years of storage. Some cultures were also stored by soaking sterile ceramic beads in overnight cultures of bacteria in glucose-nutrient broth (Appendix 1). After soaking the beads were removed from the cultures and stored at 4°C in sterile tubes containing silica gel.

For most studies bacteria were grown in mannitol-glutamic acid medium (Appendix 1) in conical flasks with cotton wool plugs. Generally inoculum was prepared by suspending a well grown yeast-mannitol slope of the required strain in sterile distilled water, then transferring part of this to the conical flask. Flasks were incubated at 25°C on a rotary shaker.

The optical density of growing cultures was conveniently followed in 500 ml conical flasks with side-arms of 1.5 cm diameter.

Part of the contents of the flask was tipped into the side-arm which was then placed in an EEL colorimeter. To extend the range of the colorimeter past 100, a neutral density filter was placed in the light beam then the instrument zeroed with a blank. The side-arm was then inserted, the filter removed and a reading taken. The true optical density equals the instrument reading plus the density of the filter. This method could be used to extend the optical density reading to approximately 160, beyond this samples were removed and diluted to obtain a reading.

Pathogenicity testing

Pathogenicity tests of bacteria were carried out using tomato plants (*Lycopersicon esculentum* cv. South Australian early dwarf red) which were grown from seed for approximately 4-6 weeks. A sterilised multipronged inoculating tool was pushed into the stem of the plant (Kerr 1969), a drop of water placed on the wound site and then a loopful of bacteria rubbed over the site. The plants were placed back in a glasshouse and watered with sub-irrigation to prevent cross contamination. Plants were scored approximately 3 weeks after inoculating. When greater control of the number of bacteria added to a wound was required, a standard puncture wound 3 mm deep by 2 mm diameter was made in the stem of the plant and a small volume of water containing a known number of bacteria was added to the wound.

Bacterial strains

The nomenclature of Keane *et al.* (1970) is used. Pathogenic bacterial strains involved in this study were derived from non-pathogenic strains by acquisition of virulence using the method of Kerr (1971).

Four week old tomato plants were inoculated with *Agrobacterium radiobacter* var. *tumefaciens* biotype 2 strain 27 and galls allowed to develop for 3 weeks. The galls were then surface sterilized with 0.5% sodium hypochlorite. After the chlorine had dispersed *Agrobacterium radiobacter* var. *radiobacter* biotype 1 strains were then swabbed onto the surface of the galls and the plants placed back in the glasshouse.

A number of the strains swabbed onto the gall carried an antibiotic marker. These were produced by plating suspensions of the required strain onto yeast-mannitol agar containing 25 µg, 50 µg or 100 µg chloramphenicol/ml. After incubation, resistant colonies were observed on plates containing 25 µg or 50 µg chloramphenicol/ml but no growth occurred with 100 µg/ml. Selected resistant colonies were streaked on agar plates containing the corresponding chloramphenicol concentration and then isolated colonies transferred to agar slopes containing chloramphenicol before swabbing onto galls.

After three weeks the galls were cut off and macerated in a blender with 100 ml sterile distilled water, diluted 1 : 10 with sterile water and 0.1 ml spread on plates of Schroth's medium (Schroth, Thompson and Hildebrand 1965) which is selective for biotype 1

agrobacteria. These plates were incubated at 25$^{\circ}$C and after colonies had developed those strains that carried antibiotic markers were replica plated onto yeast-mannitol agar plates containing chloramphenicol. There was good correspondence between the original plate and the chloramphenicol-containing plate indicating that the isolated bacteria still carried the antibiotic marker.

Fifteen isolates from each gall were tested for pathogenicity and a number of the pathogenic strains were retained for further study.

The source and characteristics of all bacterial strains used in this study are summarized in Table 1.

*Table 1.* Source and characteristics of bacterial strains.

| Strain | Pathogenicity | Biotype | Source |
|---|---|---|---|
| 57 | - | 1 | John Innes potting compost |
| 57A | + | 1 | Converted from strain 57 |
| 200 | - | 1 | John Innes potting compost |
| 200A | + | 1 | Converted from strain 200 |
| 146 | - | 1 | John Innes potting compost |
| 146A | + | 1 | Converted from strain 146 |
| 147 | - | 1 | John Innes potting compost |
| 147A | + | 1 | Converted from strain 147 |
| 27 | + | 2 | Peach gall South Australia |
| 24 | + | 1 | Peach gall South Australia |
| 84 | - | 2 | Soil South Australia |

All converted pathogenic strains received pathogenicity from strain 27.

Waite Institute culture collection numbers and experiment numbers are given in Appendix 2.

## *PART C*

## COMPARISON OF NON-PATHOGENIC AND PATHOGENIC STRAINS OF *AGROBACTERIUM*

### INTRODUCTION

There have been a large number of studies using a variety of techniques to compare non-pathogenic and pathogenic agrobacteria in an endeavour to determine the basis of crown-gall induction.

Hochester and Cole (1967), using serology, compared virulent *Agrobacterium* strain (B6) with the same strain converted to avirulence by exposure to thioproline. Although they detected an antigen in the virulent strain which was absent in the avirulent strain they concluded that this antigen was not related to pathogenicity as it was not present in any other virulent strains that they tested. Similar results were obtained by Schroth *et al.* (1971) who were able to distinguish some pathogens from non-pathogens using serology but there were a number of pathogenic strains which could not be distinguished. Keane *et al.* (1970) were able to distinguish what they called biotypes by serology but they were not able to distinguish between pathogens and non-pathogens within biotypes. Graham (1971), studying the taxonomy of a number of *Agrobacterium* and *Rhizobium* strains, again observed that pathogenic and non-pathogenic agrobacteria could not be

distinguished by serology.

Clark (1972) compared the esterase and catalase isoenzymes from a number of non-pathogenic and pathogenic *Agrobacterium* strains and *Rhizobium* strains after electrophoretic separation. Although he was studying the taxonomy of the group it is obvious from his results that there were no consistent differences between the pathogenic and the non-pathogenic agrobacteria studied. New (1972) and Keane *et al.* (1970) studied the protein patterns after electrophoresis of extracts from a large number of *Agrobacterium* strains but although they could distinguish biotypes, no consistent differences between pathogens and non-pathogens were observed.

Although the sensitivity of *Agrobacterium* to bacteriophage was reported by Coons and Kotila in 1925, it was not until the discovery of lysogeny in *Agrobacterium* by Beardsley (1955) that phage was seriously considered to be involved in crown gall induction. Roslycky, Allen and McCoy (1962) studied the sensitivity of a number of *Agrobacterium* strains to different phages from *Agrobacterium*, but although they demonstrated that these phages were highly specific for the genus *Agrobacterium* they could find no correlation between susceptibility to specific phages and pathogenicity. A similar study was carried out by Boyd, Hildebrandt and Allen (1970) but again no correlation between sensitivity to phage and pathogenicity was found. Beardsley (1955) suggested that the phage itself may be the tumour inducing principle

and this idea gained further support with the report by Parsons and Beardsley (1968) of bacteriophage activity in homogenates of crown-gall tissue that had been in culture for 10 years. A number of studies have been carried out to test this hypothesis. Zimmerer, Hamilton and Pootjes (1966) showed that lysogeny was widespread in the agrobacteria but they could not find any correlation between lysogeny and pathogenicity. Similar studies were carried out by Manasse, Staples, Granados and Barnes (1972) and by Beiderbeck (1972) with the same result. DeLey, Gillis, Pootjes, Kersters, Tyjtgat and van Braekel (1972) studied a number of bacteriophages from different *Agrobacterium* and concluded that if a phage was the tumour-inducing principle it could not be of the omega group, which includes the phages found in most agrobacteria. They did, however, report the presence of another phage type in a pathogenic strain which appeared to be absent in a closely related non-pathogenic strain.

There have been a few studies on the nucleic acids of *Agrobacterium* but most of these have either been concerned with taxonomy (Herberlein, DeLey and Tijtgat 1967, DeLey, Tijtgat, deSmedt and Michiels 1973, Gibbins and Gregory 1972) or with demonstrating that bacterial DNA is incorporated into crown gall DNA (Schilperoort *et al.* 1967, Schilperoort 1969, Quetier *et al.* 1969, Milo and Srivastava 1969, Srivastava 1970, Yajko and Hegeman 1971, Chilton *et al.* 1974, Drlica and Kado 1974, Eden *et al.* 1974). Larsen and Zaitlin (1971) compared the total nucleic acids (DNA and RNA) extracted from pathogenic and

non-pathogenic agrobacteria and concluded that there were no detectable differences. Kado *et al.* (1972) analysed the nucleic acids of pathogenic and non-pathogenic agrobacteria using a dye-buoyant density method and concluded that there was no small closed circular DNA in either the pathogen or the non-pathogen. However, similar studies by Van Larebeke *et al.* (1973, 1974) and Zaenen *et al.* (1974) indicated that there was a closed circular DNA molecule in all of the pathogenic but none of the non-pathogenic strains of *Agrobacterium* they studied.

After an extensive study of the chemical composition of cell envelopes from pathogenic and non-pathogenic agrobacteria, Manasse and Corpe (1967) concluded that there were no differences which could be correlated with pathogenicity.

There has been a number of studies on the nutritional requirements of *Agrobacterium* strains in an attempt to understand the taxonomy of this group (Sagen, Riker and Baldwin 1934, Hendrickson, Baldwin and Riker 1934, Riker, Lyneis and Locke 1941, Starr 1946, Keane *et al.* 1970) but consistent differences between pathogens and non-pathogens were never found. Lippincott and Lippincott (1969b) undertook a study specifically to compare the nutritional requirements of non-pathogens and pathogens and concluded that there was no obvious correlation between nutritional requirements and infectivity. Petit, Delhaye, Tempé and Morel (1970) reported that most galls produced either octopine or nopaline and that this correlated with the ability of the gall-inducing strain to utilize one of these compounds. The loss of pathogenicity in *Agrobacterium* strain B6, which induces galls

that produce octopine, was found to correlate with the loss of ability to utilize this compound (Petit and Tourneur 1972). Lippincott, Beiderbeck and Lippincott (1973) carried out a study of 60 strains of *Agrobacterium* and confirmed that the ability to utilize octopine or nopaline was highly correlated with pathogenicity. However, the significance of this correlation has yet to be determined.

The problem with all of these comparative studies is that the non-pathogenic and pathogenic bacteria being compared are often isolated from quite different sources and so are likely to have many different characteristics which are completely unrelated to pathogenicity. Attenuated or mutagen treated bacteria are sometimes used in an attempt to overcome this problem but these treatments often produce changes in characteristics other than virulence. Because of these problems most studies have used a large number of strains from different sources to account for any variation between bacteria which is unrelated to pathogenicity. Apart from restricting most comparative studies to simple, convenient techniques, the necessity to use a large number of strains with large variations in characteristics makes it difficult to draw valid conclusions from the results obtained.

Kerr (1971) reported that non-pathogenic strains of *Agrobacterium* could acquire virulence if swabbed onto a gall. When re-isolated on selective medium some of the bacteria had become pathogenic although they still retained the other characteristics of the non-pathogenic strain. These converted pathogenic strains and

the corresponding original non-pathogen strains provide a very good system for looking for differences between pathogens and non-pathogens as the genetic variation between them should be minimal.

This section describes experiments comparing converted pathogenic strains with their corresponding non-pathogenic "parents" using serology, isoenzyme studies, nucleic acid analysis and reaction to mitomycin C. Some results of a study by Kerr (Roberts and Kerr 1974) on the bacteriocin sensitivity of a number of non-pathogen : converted pathogen pairs has also been included as it is highly relevant to this work.

## Serological comparison of non-pathogenic and pathogenic agrobacteria

The failure to distinguish between non-pathogenic and pathogenic agrobacteria using serology in previous studies may have been due to the large variation in the characteristics of the different bacterial strains used. It was possible that when very closely related non-pathogenic and pathogenic agrobacteria were studied, consistent serological differences may be observed. This section describes a comparison of a number of non-pathogenic agrobacteria with the corresponding converted pathogenic strains using serological techniques.

For antisera production pathogenic strains 57A, 146A, 147A, 200A and the original donor (strain 27) were grown overnight in mannitol-glutamic acid medium, harvested by centrifugation, washed by

resuspension in sterile saline (0.85% NaCl), recentrifuged and finally resuspended in 0.02M phosphate buffered sterile saline, pH 7.2, to give an optical density of 60 corresponding to approximately $10^9$ cells/ml.

Six week old rabbits were given a series of 4 injections at 10 day intervals. The first 2 were given subcutaneously and the other 2 intravenously. The first injection consisted of 0.5 ml bacterial suspension emulsified with 0.5 ml Freund's complete adjuvant. The second was similar except the bacteria were disrupted for 5 min in an MSE ultrasonic disintegrator before emulsifying with adjuvant. The third injection consisted of 1 ml bacterial suspension and the fourth of 2 ml bacterial suspension disrupted in an MSE ultrasonic disintegrator. Ten days after the last injection the rabbits were bled from the marginal ear vein. The blood was allowed to clot at room temperature for 2 h then placed at $4^oC$ overnight. The clear sera was then removed from the clot and centrifuged to remove a few remaining cells. The sera was stored at $-20^oC$ in small aliquots which were unfrozen only a few times, then discarded.

Bacteria for antigens were grown and washed as for antisera production and suspended at a concentration of 0.5 gm wet weight/ml in buffered saline. This was then disrupted in an MSE ultrasonic disintegrator for 5 min and stored at $-20^oC$.

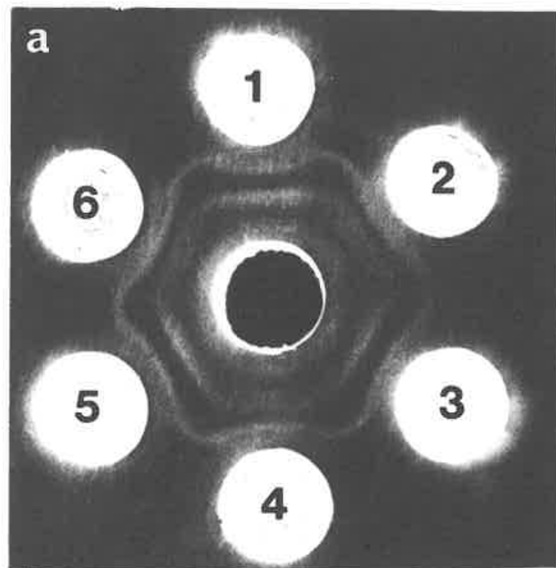
Double diffusion plate analysis of the antigens was carried out using the method of Ouchterlony (1961). Agar containing 8 g NaCl,

1.4 g $Na_2HPO_4$, 7.5 g Bacto-agar and 0.2 g sodium azide per litre water was adjusted to pH 7 with HCl, melted and poured into Petri dishes. After the agar had set, a series of wells was cut in the agar. Antisera was placed in the central well and various antigens in the outer wells. The plates were then incubated at 25$^{o}$C for 3-4 days and the resultant precipitin bands recorded by photography.

Plate 1a, b, c and d show the precipitin bands which were produced when the various antisera were diffused against the homologous converted pathogens and the corresponding heterologous non-pathogens. In most cases a complex pattern is shown but there is no detectable difference between the antigens. Plate 1e and 1f show all the antigens from non-pathogenic and pathogenic strains reacted against antisera produced by strain 27, the strain from which pathogenicity was originally transferred. If some part of the genome of strain 27 was transferred to the other strains in transfer of virulence it was possible that these converted strains would show some serological relationship to strain 27. However, it can be seen that both the non-pathogens and the corresponding converted pathogens do not show any detectable serological relationship to strain 27.

Because the gel diffusion precipitin patterns were complex and it was possible that differences between pathogen and non-pathogen antigens could have been obscured, all of the antigens were subjected to microimmunoelectrophoresis. This was carried out using essentially the method of Ouchterlony (1967). A chamber was constructed containing 2 perspex tanks, with platinum electrodes, spaced to support a standard

*Plate 1.* Gel diffusion test. a. strain 57 and 57A, b. strains 200 and 200A, c. strains 147 and 147A, d. strains 146 and 146A. Peripheral wells 1, 3 and 5 contain antigen to non-pathogens; peripheral wells 2, 4 and 6 contain antigen to pathogen. Centre well contains antisera to pathogen. e. strain 27 antiserum (centre well) against antigens (peripheral wells) 57 (1), 57A (2), 27 (3 and 6), 200 (4), and 200A (5). f. strain 27 antiserum (centre well) against antigens (peripheral wells) 147 (1), 147A (2), 27 (3 and 6), 146 (4) and 146A (5).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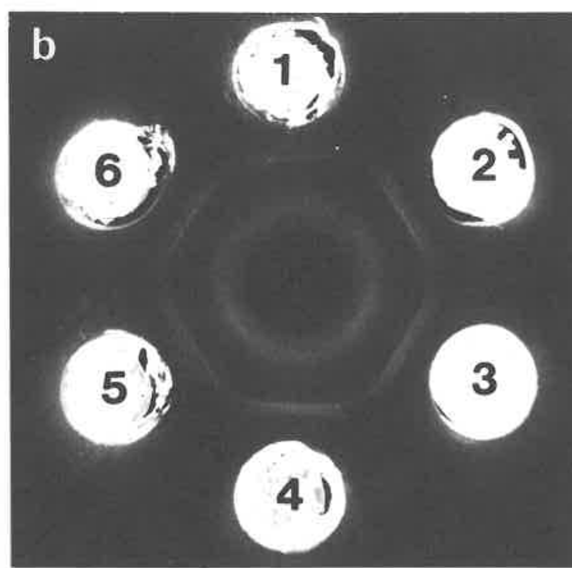
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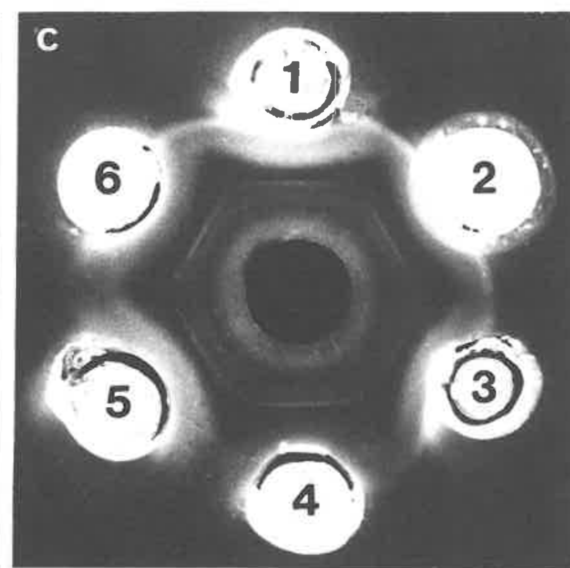
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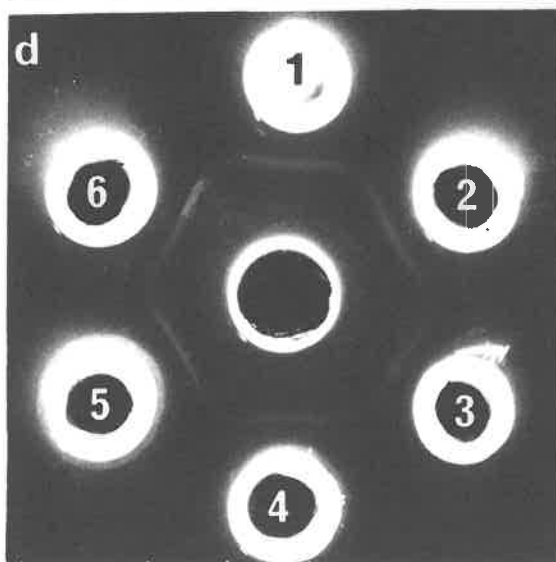
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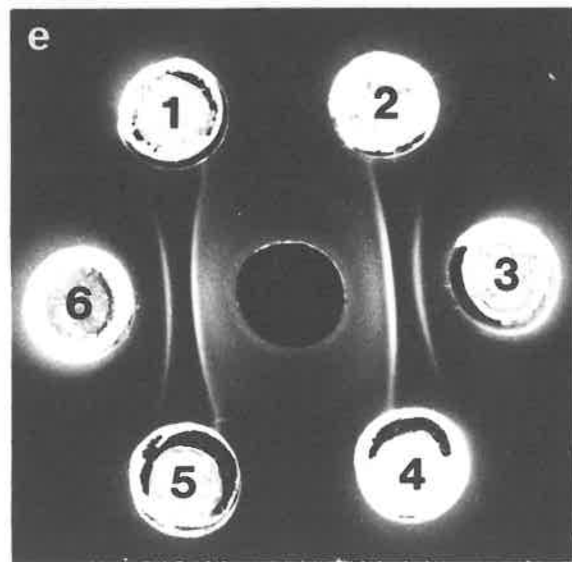
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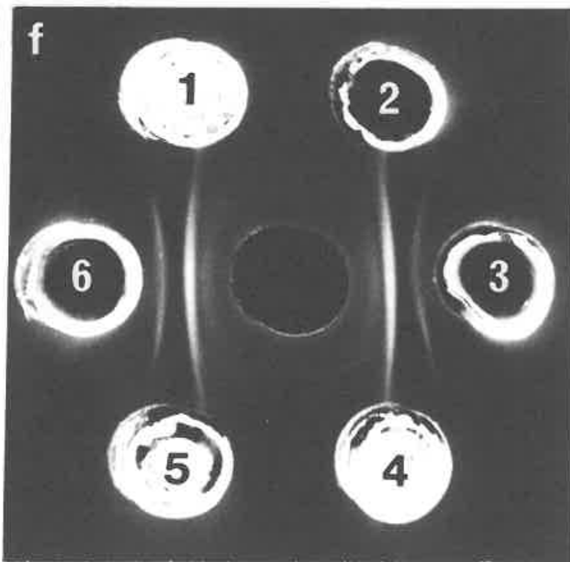
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microscope slide between them (Plate 2). Molten 2% Bacto-agar in 0.025M Borate buffer (pH 8.0) containing 0.25M KCl and 10 ppm merthiolate was pipetted on to microscope slides and filter paper wicks placed in the agar at both ends of the slide. After the agar had set, 2 small wells spaced 1 cm apart were cut in the centre of the slide into which the antigens were placed. The slides were then placed between the 2 tanks with the wicks dipping in Borate buffer (pH 8.0) in the tanks. Water was placed in the bottom of the chamber to keep the humidity high, the electrodes connected to the power source and the whole chamber with a lid placed at 4°C. The electrophoresis was carried out for 1 h 15 min at 140 volts. After the run the wicks were cut off and a 2 mm wide slit cut longitudinally down the centre of each slide. This was filled with antisera and the slides incubated at 25°C in Petri dishes with moistened filter papers. The precipitin bands were recorded after 4-5 days.

Figure 1 shows the results obtained with microimmunoelectrophoresis. Although good resolution of the precipitin bands was observed with this method no consistent differences between pathogens and non-pathogens were noted. Some differences between pathogens and non-pathogens were observed; for example, strains 57 and 57A differ, but similar differences were not observed in other strains. Figure 1e shows that, as in the double diffusion tests, there was no detectable serological relationship between strain 27 and the other pathogen and non-pathogen strains.

*Plate 2.* Microimmunoelectrophoresis apparatus.

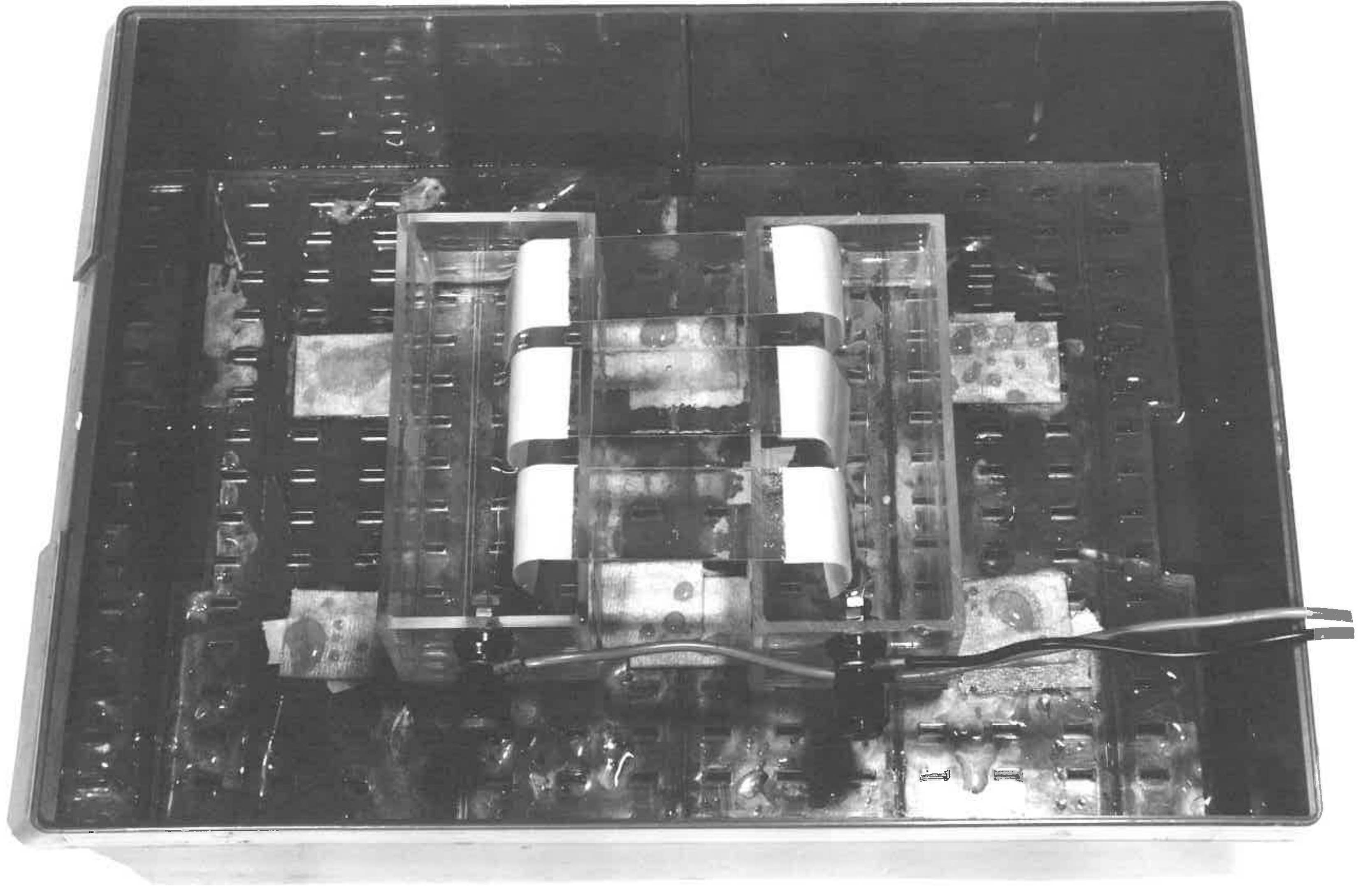
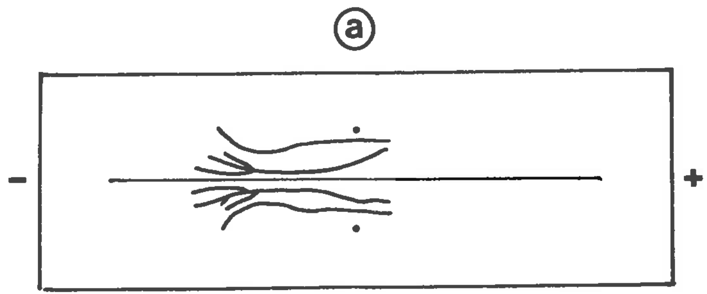


*Figure 1.* Microimmunoelectrophoresis of antigens from: a. strains 200 and 200A, b. strains 57 and 57A, c. strains 147 and 147A, d. strains 146 and 146A. Antigen to pathogen was loaded in top well, antigen to non-pathogen in bottom well. Antiserum to pathogen was loaded in slot cut down centre of slide after the run. e. Typical reaction against antiserum 27. Antigen 27 in bottom well, antigen 200A in top well with antiserum 27 loaded in centre well after run.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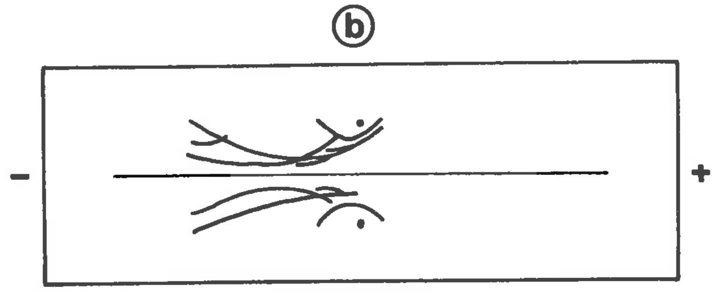
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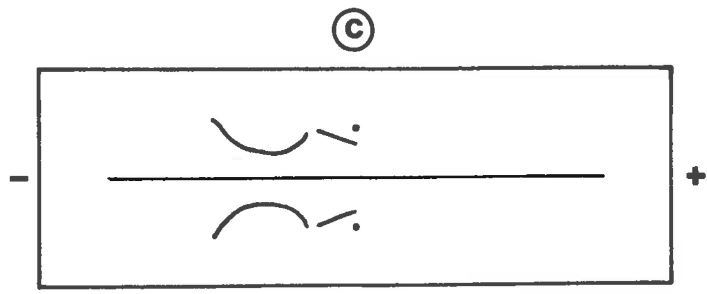
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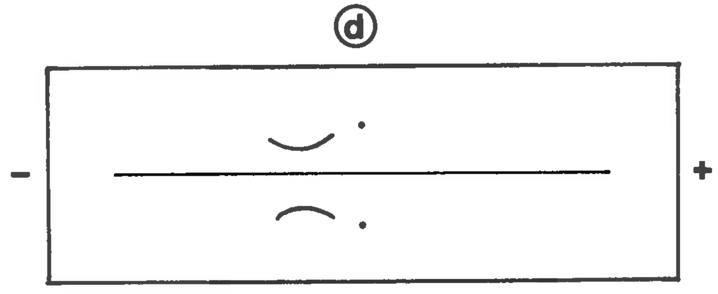
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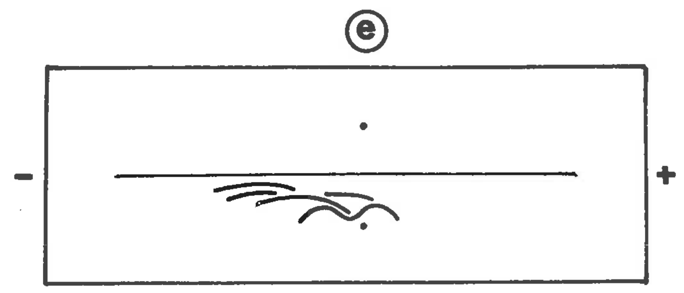
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To further confirm the antigenic similarity of the pathogen : non-pathogen pairs absorption of antisera with the heterologous (non-pathogen) antigen and reaction against the homologous (pathogen) antigen was carried out. This was done in agar using the method of van Regenmortel (1967). Three wells were cut in line in serology agar in Petri dishes. The non-pathogen antigen was placed in the centre well and allowed to diffuse out overnight. Antisera was then added to the central well and the homologous and heterologous antigens in the outer well. Absorption of the antisera occurs as it diffuses through the prediffused antigen. This method was checked using a variety of different antisera and antigens and proved very sensitive in detecting differences between antigens. However, when the non-pathogen and pathogen antigens were tested in this way no precipitin bands were observed; providing further evidence that the non-pathogen and the corresponding converted pathogen in all cases are both very closely related serologically.

Comparison of protein and isoenzyme patterns of non-pathogenic and pathogenic agrobacteria

The genome of any organism is ultimately expressed in the production of structural and enzymatic proteins. Many of these proteins can be separated by electrophoresis and detected using specific staining methods. This technique provides a convenient

method of comparing different organisms and has been used extensively in taxonomic, physiological and biochemical studies (see, for example, Brewer 1970). Polyacrylamide gel electrophoresis of protein extracts from non-pathogen : pathogen *Agrobacterium* pairs followed by staining for specific enzymes was used in an attempt to correlate protein patterns with pathogenicity.

Bacteria were grown for approximately 48 h in mannitol-glutamic acid medium, then harvested by centrifugation. In some of the initial experiments harvested bacteria were stored frozen before extraction but this practice was discontinued when it was discovered that some enzymes were not stable under these conditions. Subsequently all extracts were prepared and run on the same day that the bacteria were harvested.

After harvesting, the bacteria were suspended in the extraction buffer of Staples and Stahmann (1963) (Appendix 3). The suspension was then placed in a water-jacketted glass container with water at $0^{\circ}C$ circulating and disrupted for 5 min in an MSE ultrasonic disintegrator. The extract was then centrifuged 17,000 g for 15 min to remove any non-disrupted cells and the protein content determined using the modified Lowry method of Jennings (1961). Protein standard solution (Armour Pharmaceutical Company) was used to construct a calibration curve.

Tubes (0.6 cm diameter, 9 cm long) of 7.5% polyacrylamide gel were prepared and run using the method of Davis (1964) except that the sample gel was omitted. The samples (50 µg - 200 µg protein N) were layered on the top of the gels after the tubes had been attached to

the buffer tanks. A drop of bromophenol blue was added to the top buffer chamber as a running marker. Because of some variability in the runs, extracts of pathogens and non-pathogens were generally run separately and mixed together. The gels were run at 4 m.a. per tube until the bromophenol blue marker had run to about 0.5 cm from the end of the tube. The gels were then removed and stained for the following proteins:

a. Total proteins (Chrambach, Reisfeld, Wyckoff and Zaccari, 1967).

b. Acid phosphatases (Choudhury and Lundy 1970).

c. Alkaline phosphatases (Weber, Clare and Stahmann 1967).

d. Esterases (Weber *et al.* 1967).

e. Catalases (Woodbury, Spencer and Stahmann 1971).

f. Malate dehydrogenases (Gilbert and Goldberg 1966).

g. Peroxidases (Weber *et al.* 1967).

Full details of the staining method are in Appendix 4. Extracts from each non-pathogen : pathogen pair and for each protein were run on at least 2 separate occasions and there was very little variation between any 2 such runs.

When gels were stained for total proteins there were too many bands to resolve properly and so a reliable comparison between non-pathogens and pathogens could not be made using this system.

The enzyme systems (Fig. 2) showed a much simpler pattern with only a few well-resolved bands in each gel. Although there are some differences detected between single non-pathogen : pathogen pairs, these differences are not consistent in all the strains. For example,

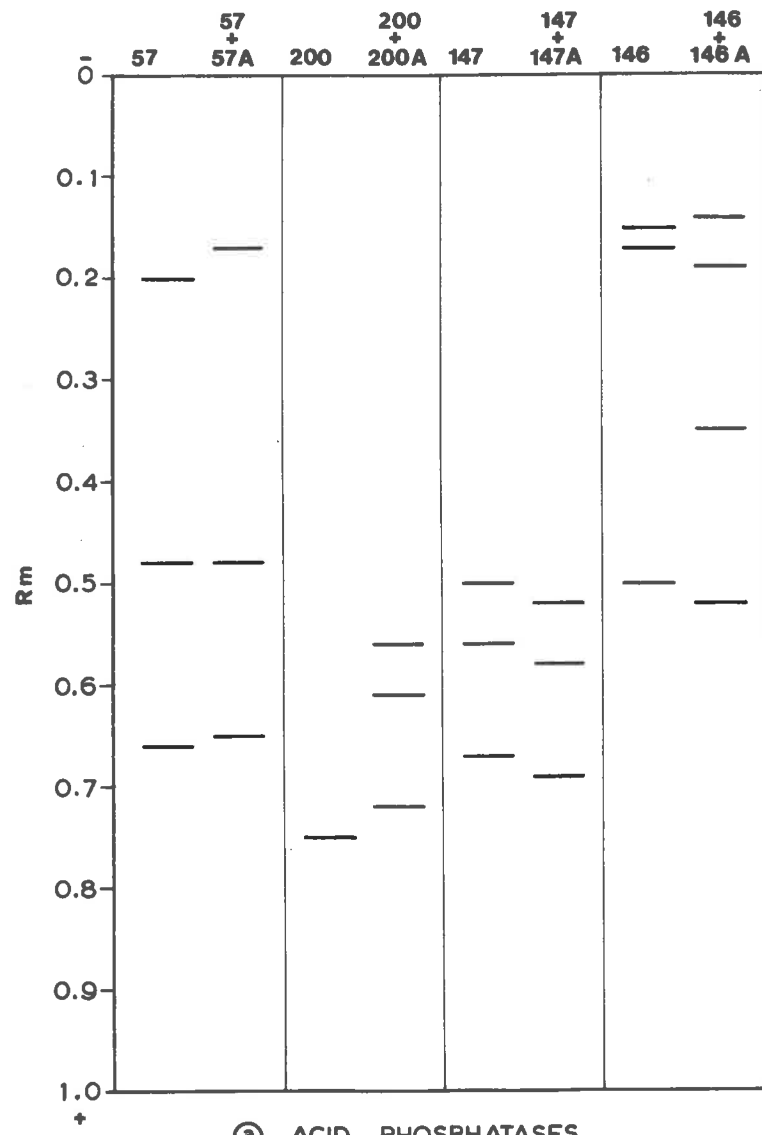
strains 200 and 200A and 146 and 146A both show differences in their acid phosphatase patterns (Fig. 2a) but no differences were detected in strains 57 and 57A. Differences between non-pathogen : pathogen pairs can also be observed in the alkaline phosphatase (Fig. 2b) and malate dehydrogenase (Fig. 2c) but again these differences are not observed in all pairs tested. These results show that isoenzyme patterns are unlikely to be useful in differentiating between closely related pathogens and non-pathogens.

Reaction of non-pathogenic and pathogenic agrobacteria to mitomycin C

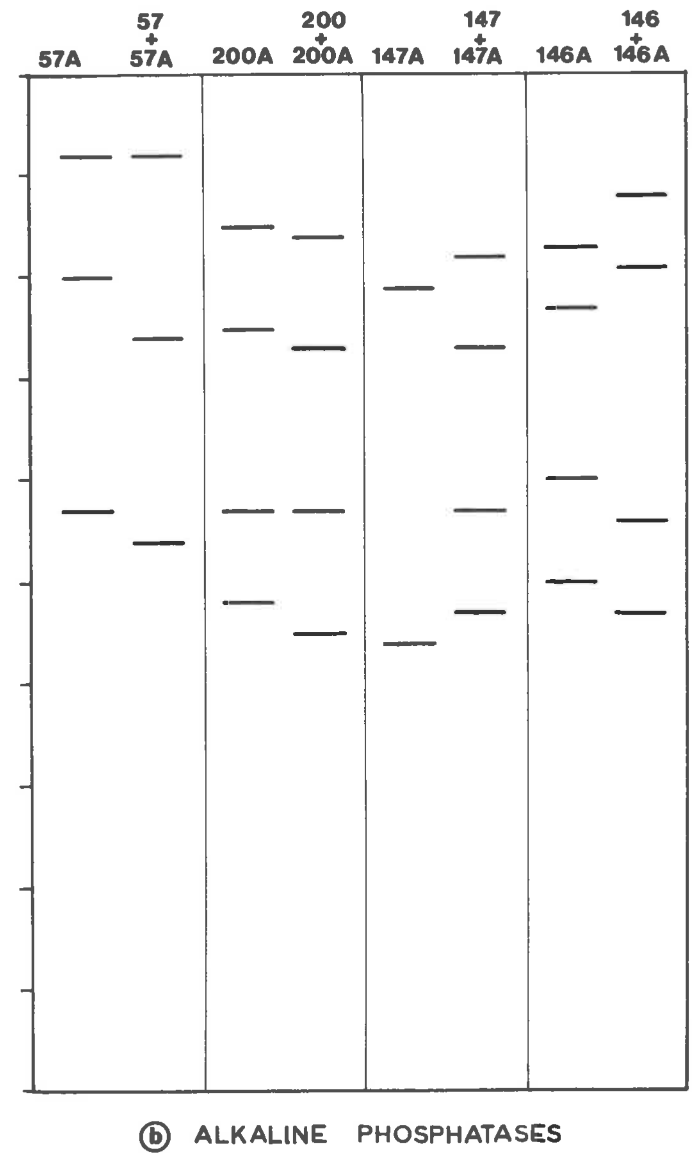
Mitomycin C is an antibiotic substance which has been shown to be an efficient inducing agent for bacteriophages (Levine 1961). It has been used extensively on *Agrobacterium* to induce the production of lysogenic bacteriophages (Brunner and Pootjes 1969, Adler and Pootjes 1972, DeLey *et al.* 1972, Manasse *et al.* 1972). If pathogenicity is dependent on the presence of a bacteriophage then mitomycin C treatment of the non-pathogen : pathogen pairs should only reveal the presence of a bacteriophage in the pathogens. The reaction of the non-pathogen : pathogen pairs to mitomycin C is described in this section.

Inoculated flasks of nutrient broth (DeLey *et al.* 1972, Appendix 1) were placed on a rotary shaker at 25°C. The next day each culture was adjusted to an optical density of 40 (approximately $7 \times 10^8$ cells/ml) with fresh medium and 0.1 ml added to fresh flasks of nutrient broth. These were again placed on the shaker overnight.

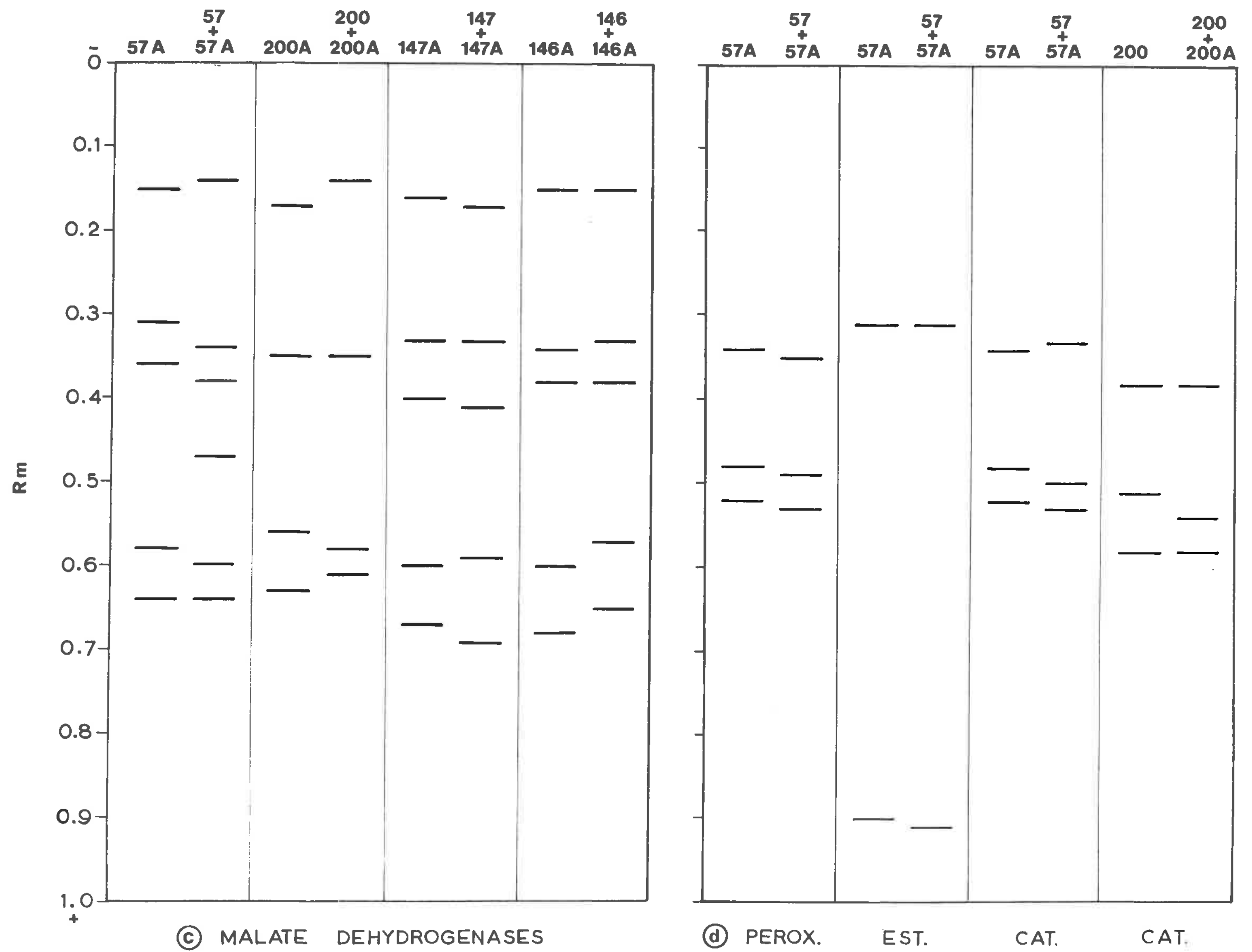
*Figure 2.* Electrophoresis of protein extracts of pathogen : non-pathogen pairs. a. acid phosphatases, b. alkaline phosphatases, c. malate dehydrogenases, d. peroxidases, esterases and catalases. Left column: one isolate run alone; right column: both non-pathogen and pathogen run mixed.




ⓐ ACID PHOSPHATASES




ⓑ ALKALINE PHOSPHATASES


57A
57 + 57A
200A
200 + 200A
147A
147 + 147A
146A
146 + 146A
57A
57 + 57A
57A
57 + 57A
57A
57 + 57A
200
200 + 200A
Rm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
(c) MALATE DEHYDROGENASES
(d) PEROX.
EST.
CAT.
CAT.

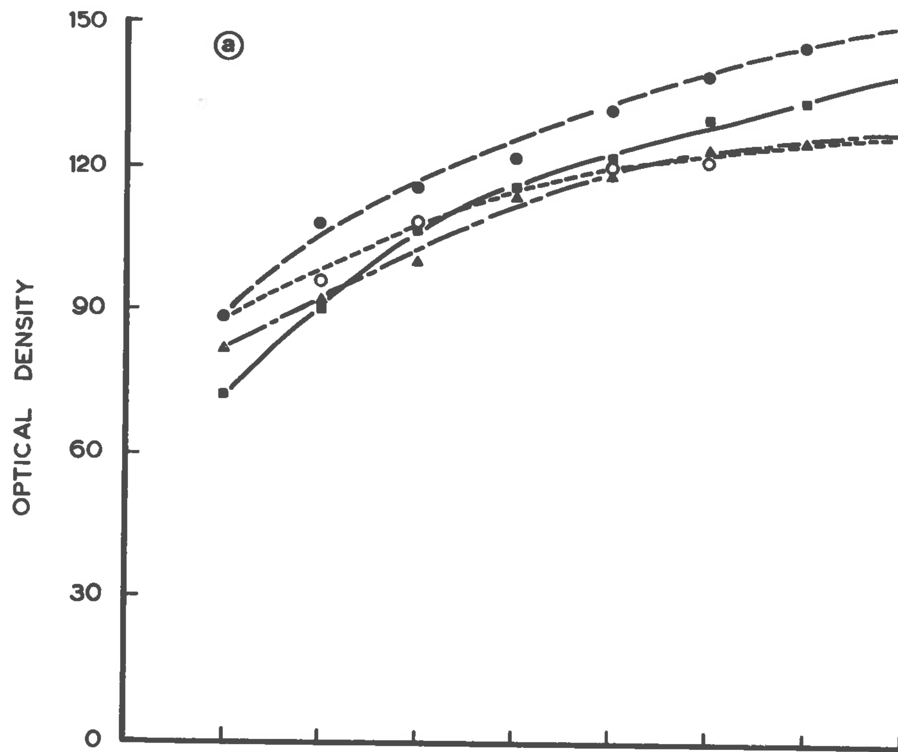
The next morning mitomycin C (Sigma) was then added to the cultures to give a final concentration of 1 μg/ml and the flasks returned to the shaker for 1 h. To remove the mitomycin C the cultures were then centrifuged (15,000 g for 15 min), and the bacterial pellet resuspended in fresh broth in a side-arm flask. This was returned to the shaker and the optical density of the culture determined at intervals for a period of at least 9 h.

The reaction of all the non-pathogen : pathogen pairs to mitomycin C is shown in Figure 3. Mitomycin C had little effect on strains 200 and 200A, and 57 and 57A, but markedly reduced the growth of strains 147 and 147A. Strains 146 and 146A both showed a reduction in optical density starting approximately 4 h after adding mitomycin C. This sort of reaction is typical of cell lysis which occurs when bacteriophages are released (Adams 1959) suggesting that both strain 146 and 146A carry an inducible bacteriophage. The reaction of strain 27 (the original donor strain) to mitomycin C was also tested (Fig. 4) and although there is some reduction in the growth rate there is no suggestion of cell lysis.

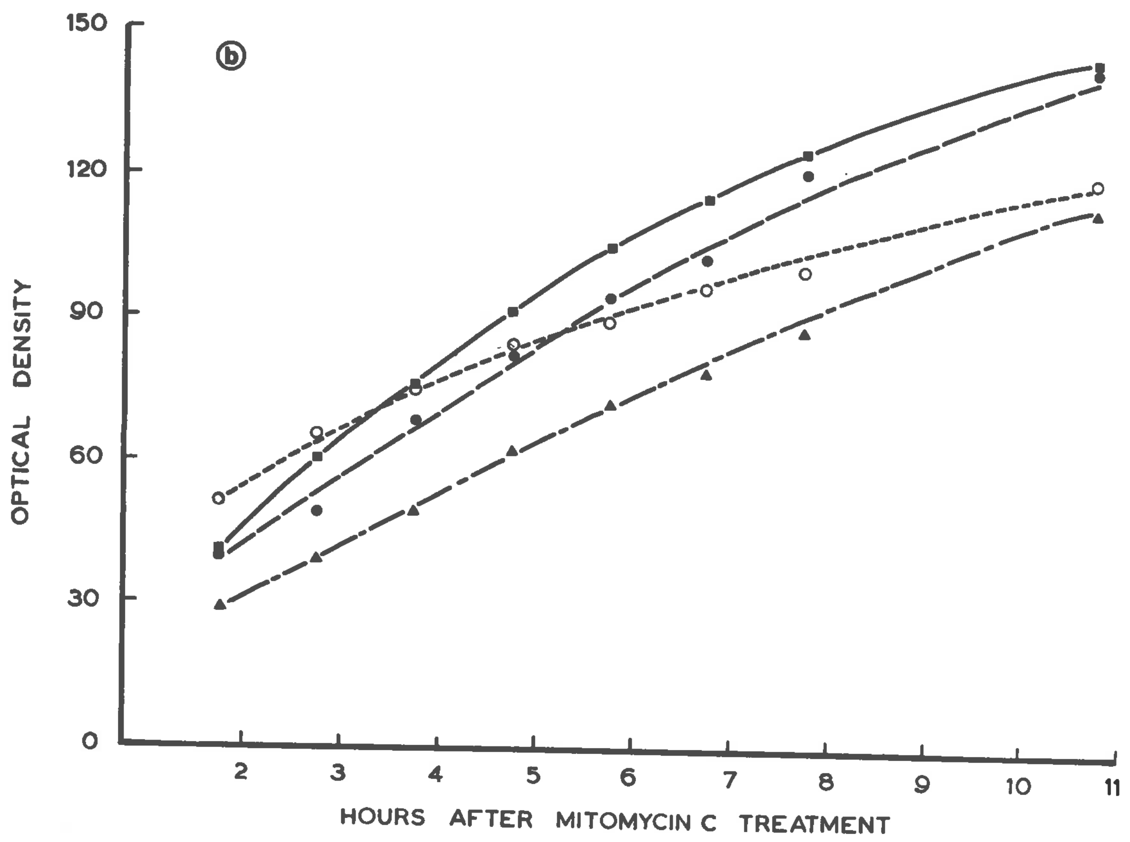
The important point about these results is that in all cases the original non-pathogen and the corresponding converted pathogen both react in the same manner to mitomycin C treatment suggesting that a mitomycin C inducible bacteriophage is not involved in the transfer of pathogenicity or crown gall induction.

Some attempt was made to characterize the reduction in optical density observed in strains 146 and 146A. Figure 5 shows the effect

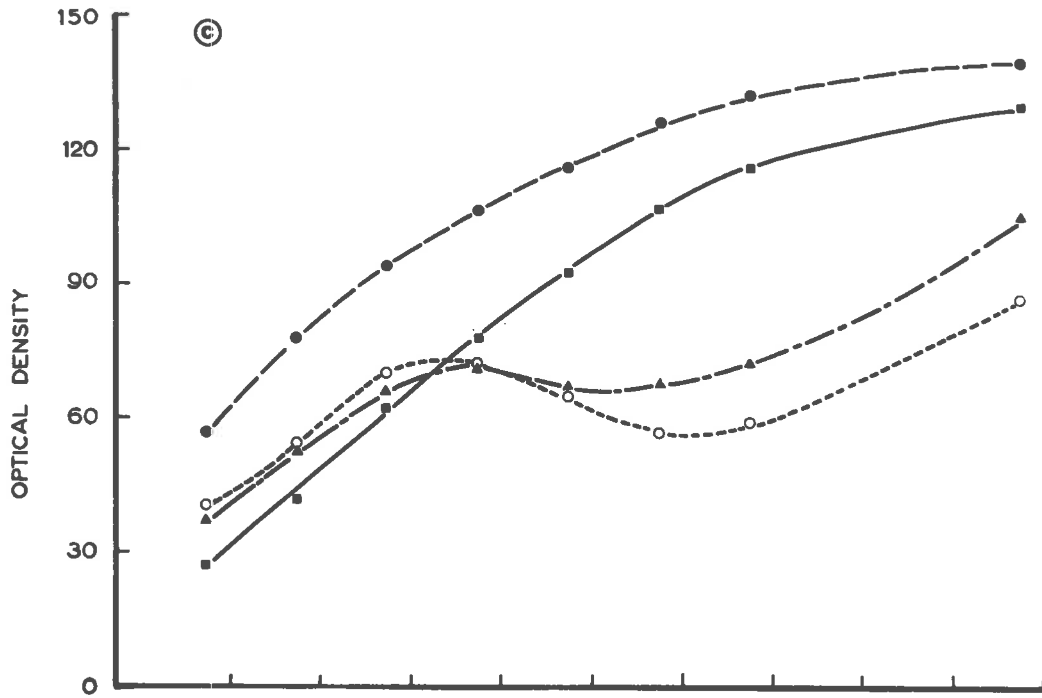
*Figure 3.* The effect of mitomycin C on: a. strains 200 and 200A, b. strains 57 and 57A, c. strains 146 and 146A, d. strains 147 and 147A.
●---● non-pathogen control, ■——■ pathogen control, o---o non-pathogen mitomycin C treatment, ▲— – —▲ pathogen mitomycin C treatment.



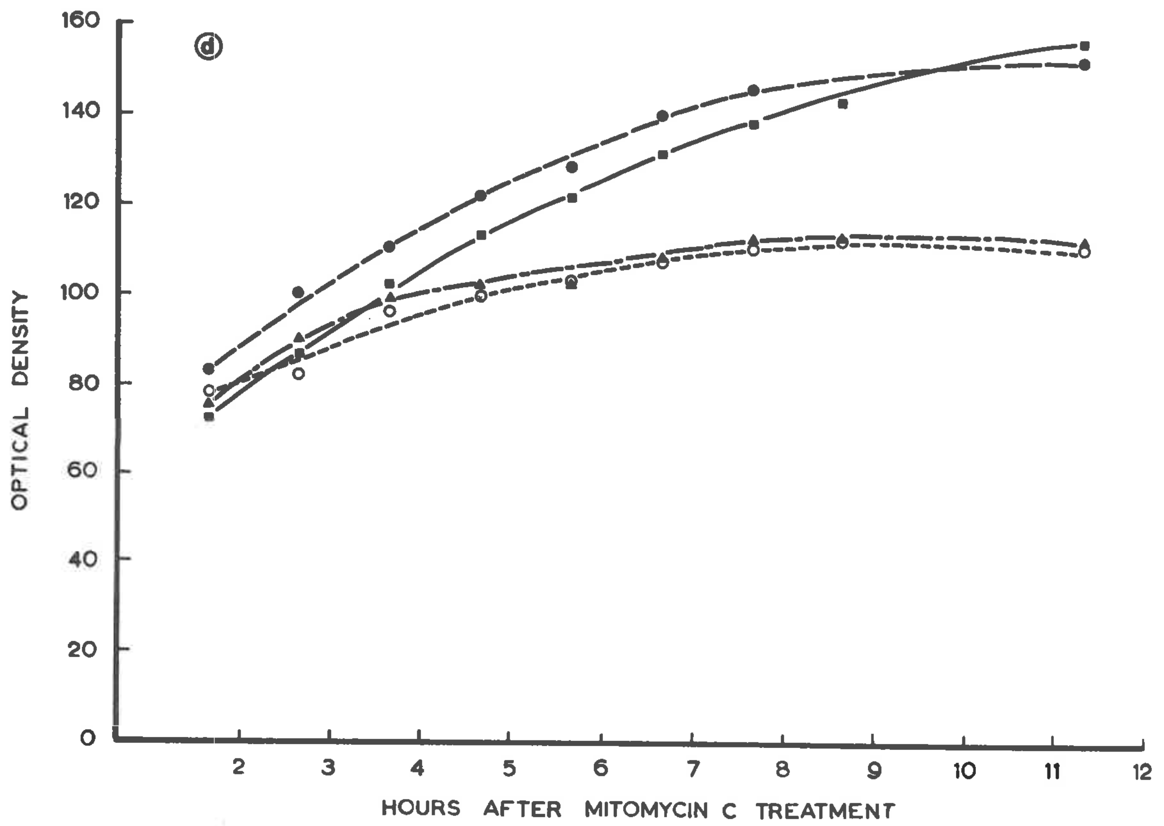
150
120
90
60
30
0
OPTICAL DENSITY
a

150
120
90
60
30
0
OPTICAL DENSITY
b
2
3
4
5
6
7
8
9
10
11
HOURS AFTER MITOMYCIN C TREATMENT



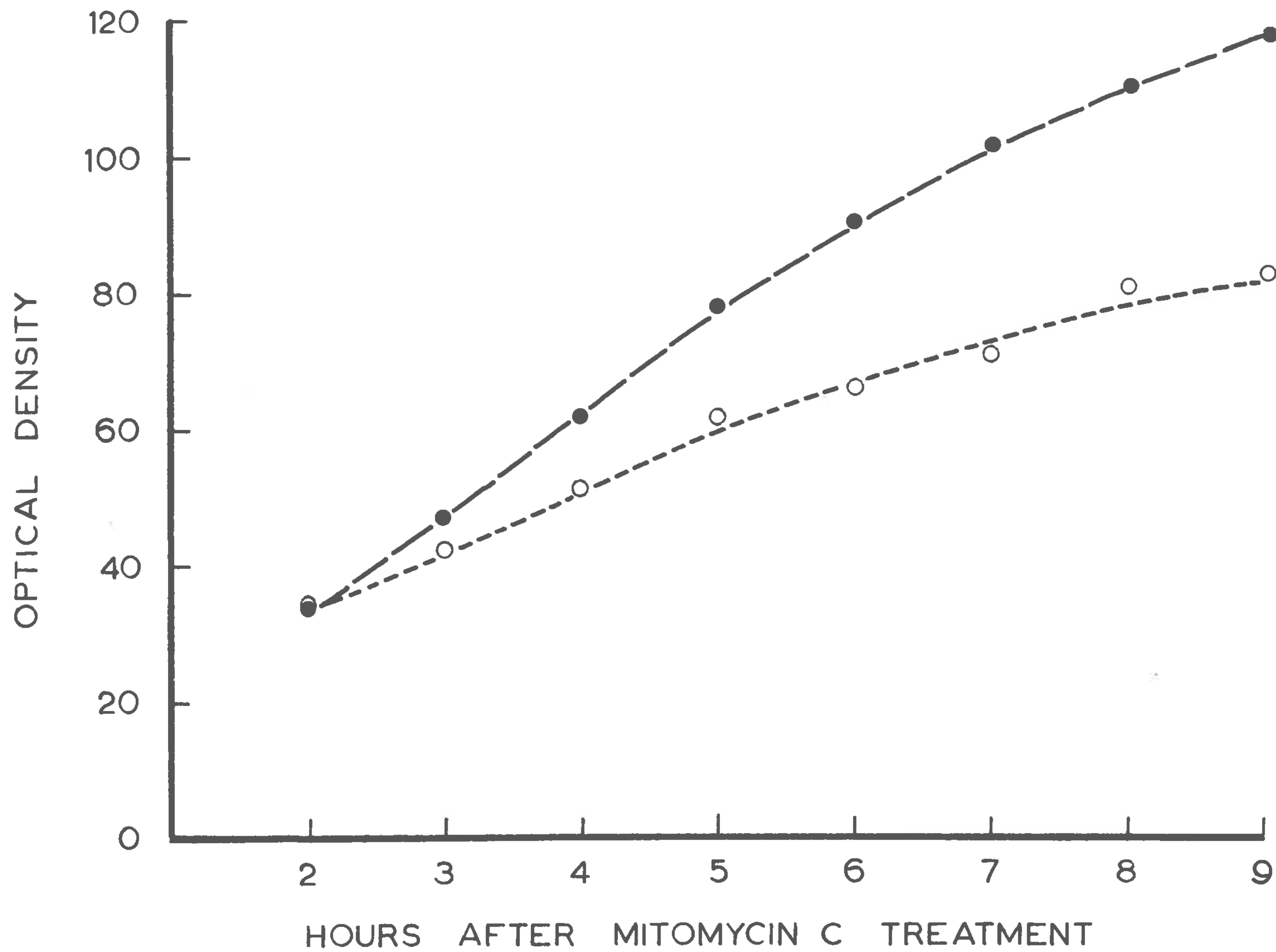
c
150
120
90
60
30
0
OPTICAL DENSITY

d
160
140
120
100
80
60
40
20
0
OPTICAL DENSITY
2
3
4
5
6
7
8
9
10
11
12
HOURS AFTER MITOMYCIN C TREATMENT

*Figure 4.* The effect of mitomycin C on strain 27.

●— — —● control, o----o mitomycin C treatment.


120
100
80
60
40
20
0
OPTICAL DENSITY
2
3
4
5
6
7
8
9
HOURS AFTER MITOMYCIN C TREATMENT

of not removing the mitomycin C from the medium and the effect of 2 different media on the reduction in optical density. No lysis was induced when the bacteria were grown in mannitol-glutamic acid medium. In nutrient broth, although lysis was induced both when the mitomycin C was left in the cultures or removed, the reduction in optical density was greater when it was removed from the culture. The stage of growth of the culture also appeared to influence lysis induction (Fig. 6). Cultures in very early log phase did not show any evidence of lysis while the time taken from addition of mitomycin C to the onset of lysis in older cultures increased with increasing optical density. These results are consistent with the presence of an inducible bacteriophage in strains 146 and 146A.

Attempts were also made to purify the presumptive bacteriophage carried by strains 146 and 146A using basically the method of Yamanto, Alberts, Benzinger, Lawhorne and Treiber (1970). Lysed cultures were centrifuged (15,000 g for 15 min) and the supernatant made up to 8% polyethylene glycol 6000 and 0.5M NaCl. This was placed at 4°C for 1 h and the resultant precipitate collected by centrifugation (5,000 g for 20 min). The pellet was then washed by suspension in 0.01M Tris-HCl buffer, pH 7.3, followed by centrifugation at 15,000 g for 15 min to remove cell debris. The supernatant was then centrifuged at 78,500 g (30,000 r.p.m. type 30 rotor) for 60 min to sediment phage. The resultant pellet, dissolved in a small amount of buffer, was examined in the electron microscope after negative staining with

*Figure 5.* The effect of the medium and removal of mitomycin C on the induction of lysis in strain 146. ●——● control DeLey's medium, ○— —○ mitomycin C left in DeLey's medium, ▲——▲ mitomycin C removed from DeLey's medium, △---△ control mannitol-glutamic acid medium, ■——■ mitomycin left in mannitol-glutamic acid medium, □— — —□ mitomycin C removed from mannitol-glutamic acid medium.

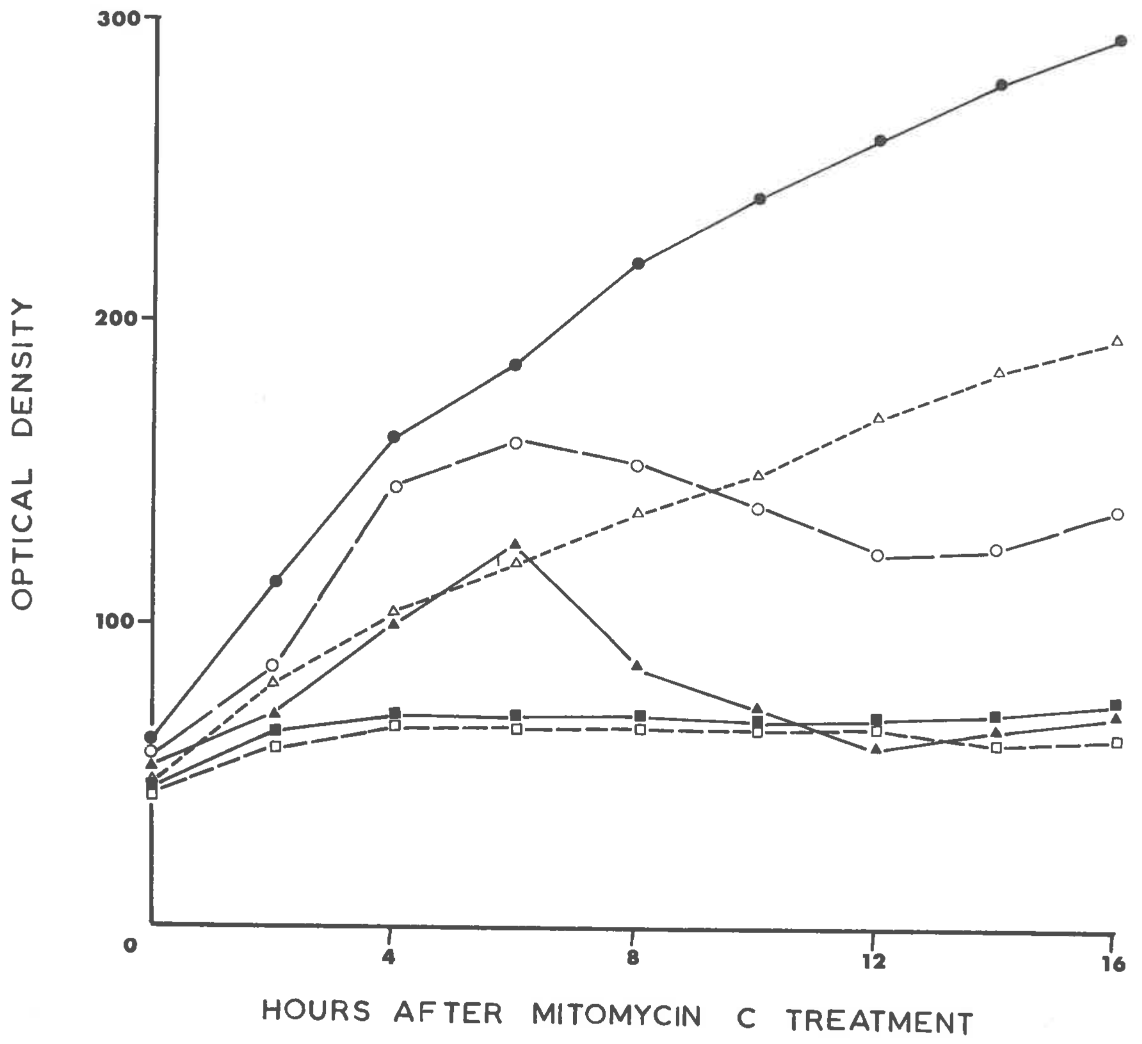
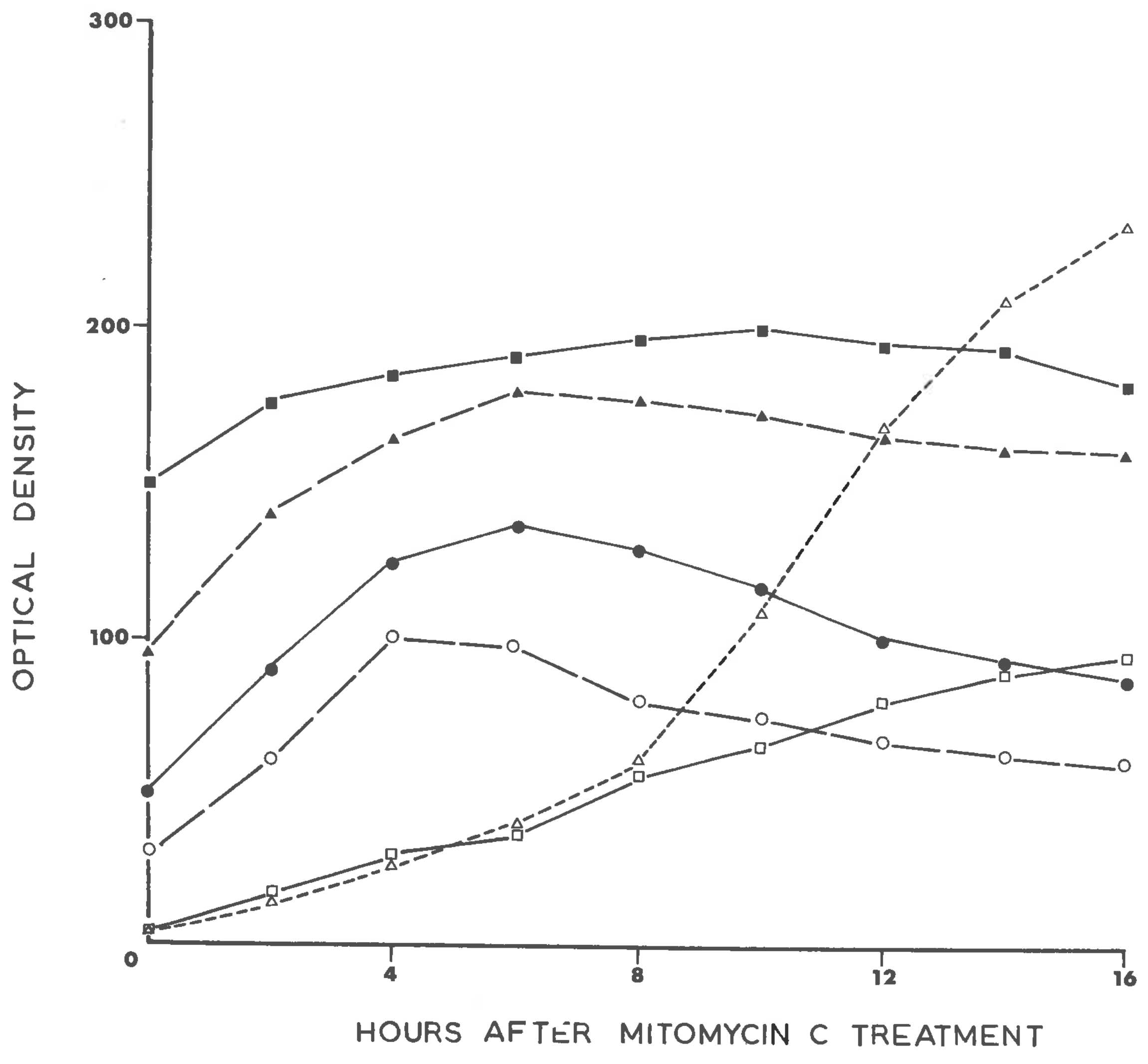

300
200
100
0
OPTICAL DENSITY
4
8
12
16
HOURS AFTER MITOMYCIN C TREATMENT

*Figure 6.* The effect of the stage of growth on lysis of strain 146 induced by mitomycin C. Mitomycin C (1 μg/ml) was added at time 0 to cultures at the indicated optical density (O.D.).

Δ---Δ control, □——□ O.D.6, ○— —○ O.D.30, ●——● O.D.50, ▲— — —▲ O.D.95, ■——■ O.D.150.



300
200
100
0
OPTICAL DENSITY
4
8
12
16
HOURS AFTER MITOMYCIN C TREATMENT

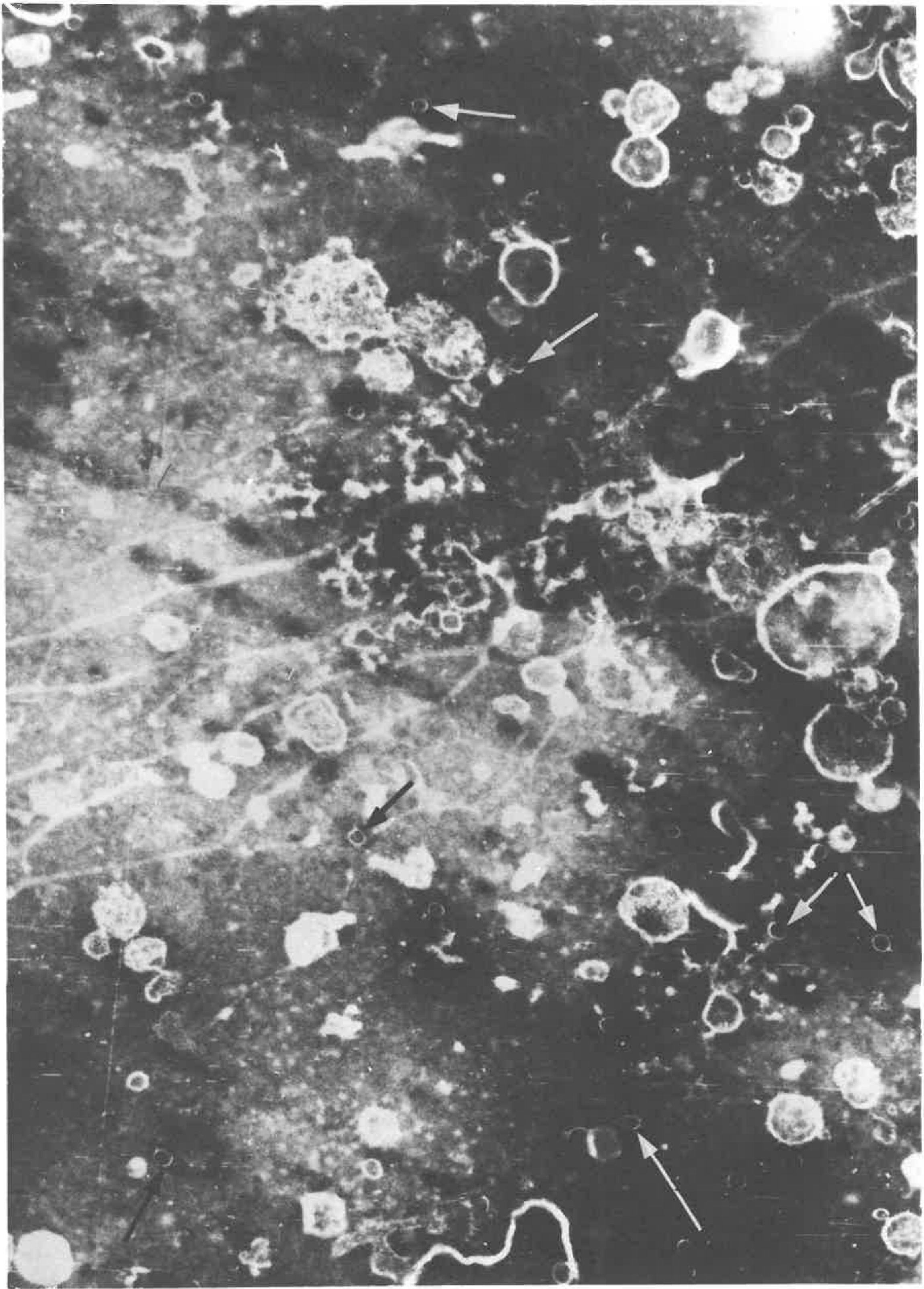
phosphotungstic acid. Part of a typical preparation is shown in Plate 3. This shows a few particles which are similar to *Agrobacterium* bacteriophage P8149 (DeLey *et al.* 1972) and a large amount of membranous material. Some preparations showed a high concentration of what appeared to be bacterial flagella.

## Comparison of nucleic acids from non-pathogenic and pathogenic agrobacteria

As pathogenicity in *Agrobacterium* is a stable inheritable factor it must ultimately be coded for by base sequences on nucleic acids. These base sequences could be carried on: (1) the main chromosome of the bacterial cell, (2) a small independently replicating DNA molecule (a plasmid) or (3) on some other type of inheritable factor such as the double stranded RNA species which is thought to carry the "killer" characteristic in yeasts (Bevan, Herring and Mitchell 1973). This section describes experiments designed to detect differences in nucleic acids of pathogens and non-pathogens.

DNA from a number of non-pathogenic : pathogenic pairs was purified using the method of Schilperoort (1969). After 2 days growth in mannitol-glutamic acid medium the bacteria were harvested by centrifugation and the pellet resuspended in P.E.S.T. buffer, pH 9.0 (Appendix 3) adjusted to 1M NaCl. This suspension was then placed at 75$^{\circ}$C for 15 min to kill the bacteria and destroy nuclease activity. The suspension was then recentrifuged and the pellet weighed and resuspended in 10x volume of buffer (weight/volume). Six-tenths of

*Plate 3.* Electron micrograph of a partially purified bacteriophage preparation. Arrows indicate possible bacteriophage particles.



a volume of Protease (Sigma Type VI 1 mg/ml in 1M NaCl) which had been self-digested at 37°C for 2 h to destroy nuclease activity was added and the suspension made up to 1% sodium dodecyl sulphate (SDS). Incubation of this at 60°C for 3 h resulted in a clear viscous solution which was made up to 6% p-4-aminosalicylate (PAS), 1 volume water saturated phenol, containing 0.1% 8-hydroxyquinoline added and the mixture shaken at 25°C for 30 min. The aqueous phase was then removed, shaken briefly with chloroform and then dialysed overnight against 1SSC-ET buffer (Appendix 3) at 25°C. The next day $\frac{1}{20}$ volume of RNase A+T (2 mg RNase A (Sigma) + 600 units RNase T (Sigma)/ml water) which had been held in a boiling water bath for 10 min to destroy DNase activity was added and dialysis against 1SSC-ET continued for 4 h at 37°C. Sodium chloride to bring the solution to 1M NaCl and 0.6 volume Protease solution was then added to the dialysis bag. Dialysis against 1SSC-ET made up to 1M NaCl was carried out for 16 h at 37°C. The buffer was then replaced with 1SSC-ET without NaCl and dialysis continued for a further 50 h at 37°C. The solution was then adjusted to 6% PAS, 1% SDS and 3% NaCl and shaken with an equal volume of phenol. After 1 h the aqueous phase was removed, shaken again with fresh phenol for 1 h then briefly with chloroform. The DNA was then precipitated with 2 volumes of cold ethanol and the resultant clew wound out on a glass rod and suspended in 0.1 SSC buffer (Appendix 3). This was dialysed overnight against 0.1 SSC to remove phenol and fully dissolve the DNA.

This method yielded highly purified DNA which showed the typical ultraviolet absorption spectrum of nucleic acids. The $E\frac{260}{280}$ and $E\frac{230}{260}$ ratios indicated that the protein contents of the DNA preparations were less than 1%. Yields ranged from 2-4 mg/g wet weight of bacteria. Sucrose density centrifugation of the purified DNA showed that it had an S value of between 25 and 30 indicating that it was highly polymerised with a molecular weight in the range of 12-15 x $10^6$ daltons.

Radioactively labelled DNA was extracted in a similar manner from bacteria which had been grown in mannitol-glutamic acid medium containing 1 μC/ml of $^3$H thymidine (2.0 Ci/mmol, The Radiochemical Centre, Amersham). Generally deoxyadenosine (50 μg/ml final concentration) was added to the cultures as it has been suggested (Boyce and Setlow 1962) that this increases the incorporation of thymidine into DNA by microorganisms. Specific activities of the extracted radioactive DNA ranged from 500 to 1,500 c.p.m./μg DNA.

DNA concentrations were routinely estimated by ultraviolet absorption at 260 nm using the conversion factor of 1 mg/ml equals an optical density of 20.

The purified DNA from both non-pathogens and converted pathogens was characterized by its thermal melting point, sedimentation in sucrose gradients, buoyant density in isopycnic centrifugation and DNA-DNA hybridization.

The melting point of the DNA samples dissolved in 1SSC

(Appendix 3) was determined in a Unicam SP1800 spectrophotometer fitted with a cuvette temperature controller and an X-Y recorder which plotted temperature vs. optical density. Degassed samples in quartz cuvettes with Teflon stoppers were placed in the spectrophotometer set at 260 nm and the temperature controller set to give a 1°C/min temperature rise. The mid-point of the melting curve (the melting point) was determined from the recorder plot.

Figure 7 shows typical melting profiles for DNA extracted from non-pathogenic and pathogenic agrobacteria. The sharpness of the melting curves confirms that the DNA is highly purified. Table 2 shows that there are no significant differences in melting points for the 2 non-pathogen : pathogen pairs tested.

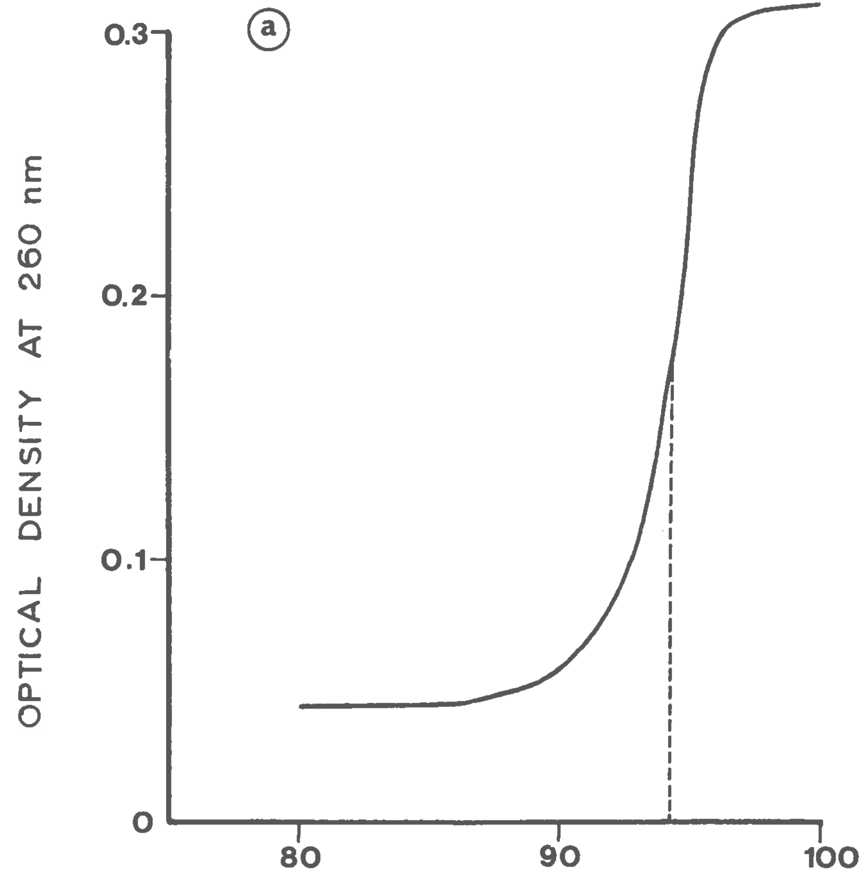
Equilibrium CsCl centrifugation was carried out in a Beckman L2-65 ultracentrifuge. Tubes containing 3 ml CsCl in 0.1 SSC buffer (density 1.718) and approximately 10 μg radioactive DNA were over-layered with oil to fill the tube. They were spun at 42,000 r.p.m. for 24 h at 20°C in an SW50 rotor. After the run the tubes were drop fractionated by bottom puncture onto filter paper discs which were dried, placed in scintillation vials and the radioactivity determined using toluene based scintillation fluid. The density profile of the tubes was determined by weighing known volumes of fractions from a tube without DNA but otherwise treated identically.

The DNA of both non-pathogens and pathogens equilibrated as a sharp peak with no subsidiary peaks (Fig. 8). The buoyant density values of 1.717 for strain 57 and 1.718 for 57A are the same within the

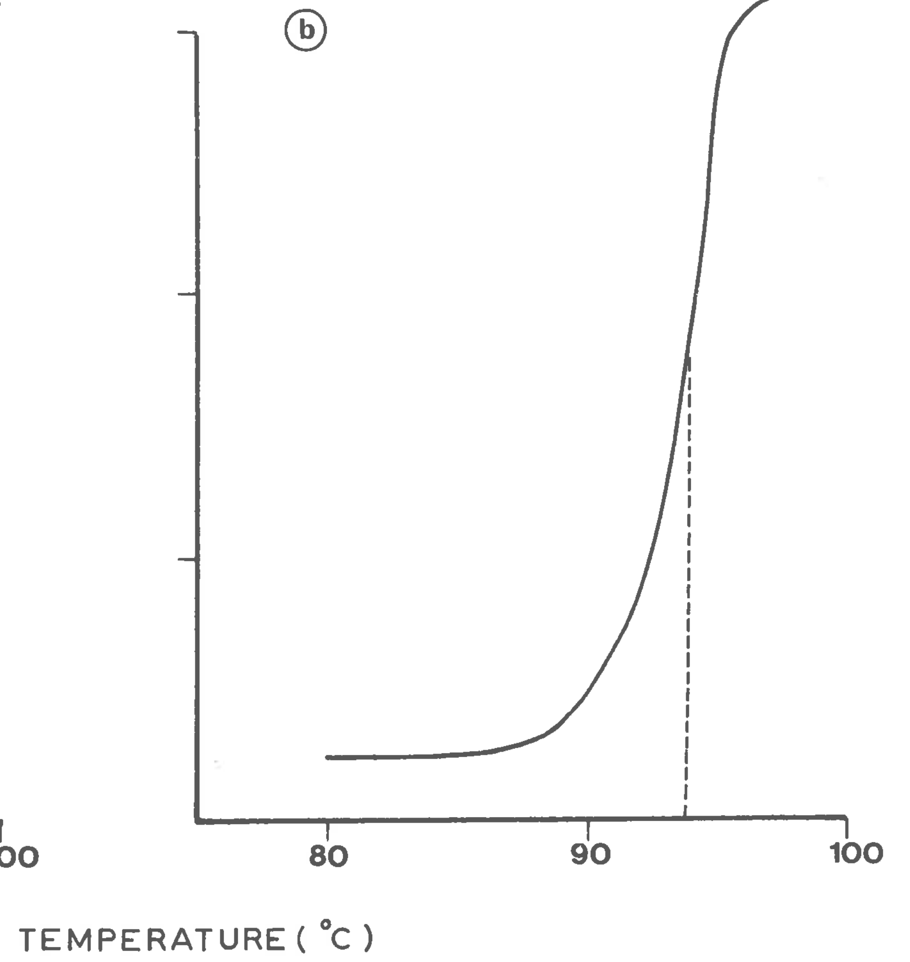
*Figure 7.* Melting profile of DNA extracted from:

a. strain 57, b. strain 57A.

DNA samples were dissolved in 1 SSC buffer.



a
0.3
0.2
0.1
0
OPTICAL DENSITY AT 260 nm
80
90
100

b
80
90
100
TEMPERATURE ( °C )

*Table 2.* Melting points of DNA extracted from 2 non-pathogen : pathogen pairs.

| Strain | Melting point $^{o}C$ |
|---|---|
| 57 | 93.8 |
| 57A | 93.6 |
| 146 | 93.7 |
| 146A | 93.7 |

experimental error of the method of density determination and agree with the results obtained by Schilperoort (1969).

Table 3 shows the %G+C content of the DNA calculated on the basis of the melting points and the buoyant densities. These values all agree very closely with one another and provide strong evidence that the base composition of the DNA from both non-pathogens and converted pathogens is very similar.

One of the powerful methods which has been developed in the last 10 years to study nucleic acids is the technique of nucleic acid hybridization (DeLey 1971). This involves the mixing of "2 species" of nucleic acid which have been made single stranded by heating or alkali treatment. These single stranded molecules are allowed to reanneal for a period, then the remaining single stranded molecules are removed. If one species of nucleic acid is radioactive then the proportion reannealing and so the similarity of the base sequences of the 2 "species" of nucleic acid can be determined. There are many different methods of carrying out nucleic acid hybridization but perhaps the simplest and most direct method is DNA-DNA hybridization carried out with one of the "species" of hybridizing DNA immobilized on a membrane filter. This section describes experiments using this technique on DNA extracted from non-pathogenic and converted pathogenic agrobacteria, using a method similar to that described by Gibbons and Gregory (1972).

DNA solutions (100 μg DNA/ml) in 0.1 SSC buffer were heated for 15 min at 100$^{o}$C followed by rapid dilution to 4 μg DNA/ml with

*Figure 8.* Determination of the buoyant density of DNA from a non-pathogen : pathogen pair by ultra-centrifugation in CsCl solution. ——— strain 57, – – – strain 57A, ●——● density profile of tubes.

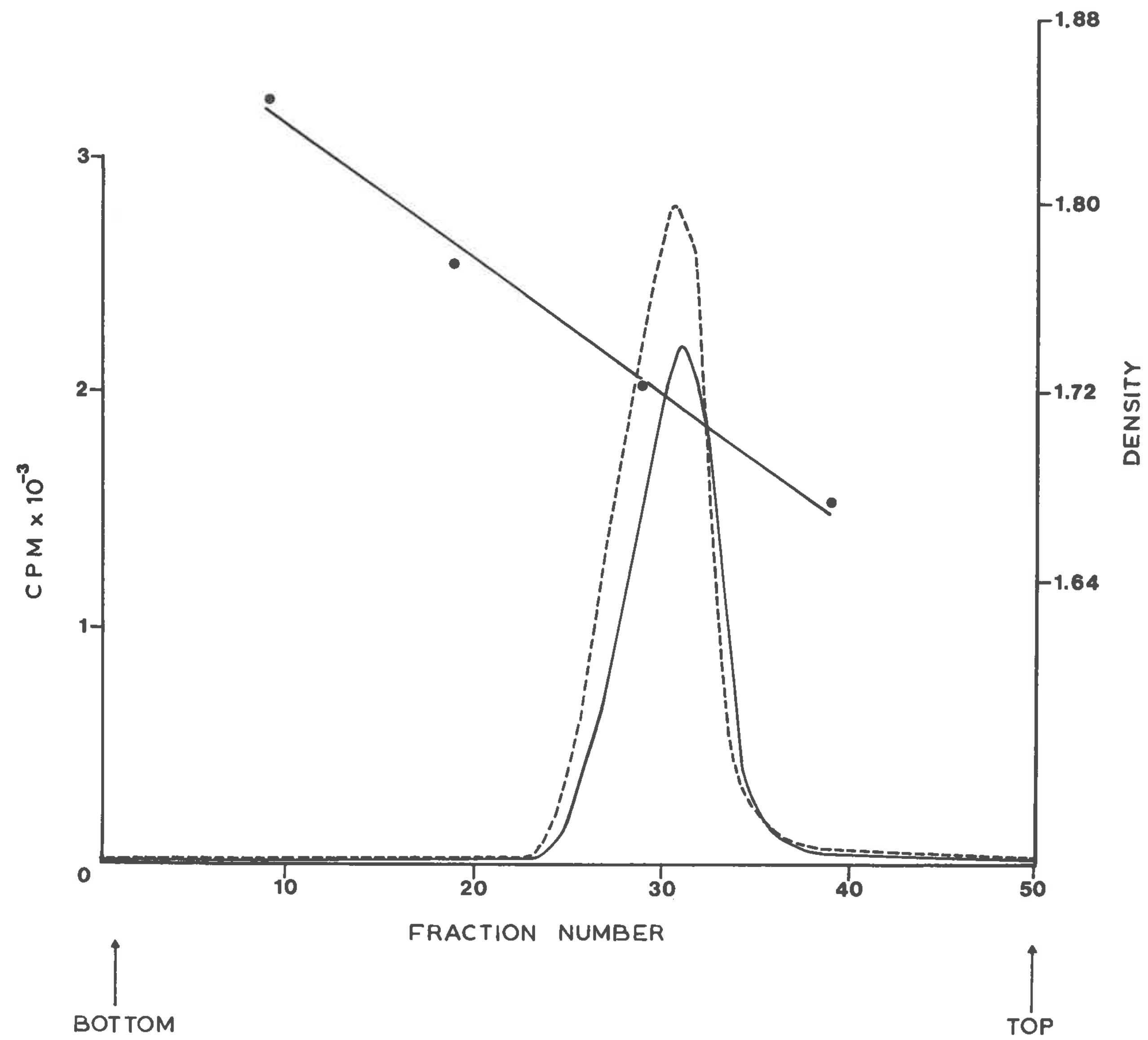

3
2
1
0
CPM x 10^-3
1.88
1.80
1.72
1.64
DENSITY
10
20
30
40
50
FRACTION NUMBER
BOTTOM
TOP

*Table 3.* % G+C content of DNA extracted from pathogen : non-pathogen pairs calculated on the basis of melting points and buoyant densities.

| Strain | % G+C content calculated from: | |
|---|---|---|
| | Buoyant Density+ | Melting Points* |
| 57 | 58.1 | 58.3 |
| 57A | 59.2 | 57.8 |
| 146 | nd | 58.0 |
| 146A | nd | 58.0 |

\+ Calculated using the equation of Schildkraut, Marmur and Doty (1962).

* Calculated using the equation of Marmur and Doty (1962).

nd Not determined.

cold 6 SSC buffer. Five ml aliquots of this solution were slowly passed through 2.5 cm diameter 0.45 μ membrane filters (Sartorius SM11306). These were then washed with cold 6 SSC buffer, air dried overnight, then incubated for 3 h at 80°C in a vacuum oven. The filters were stored at 4°C over silica gel.

Hybridization was carried out in vials containing 50% formamide in 6 SSC buffer, 2 μg radioactive DNA which had been sheared (1 min MSE ultrasonic disintegrator) and made single stranded (15 min at 100°C followed by rapid cooling) and a membrane filter with bound DNA. The vials were incubated at 40°C for 16 h then the filters removed, washed on both sides with cold 6 SSC buffer by filtration, dried and the radioactivity bound to the filter determined.

The amount of DNA on control filters before and after incubation was checked using the method of Burton (1956). Initially a lot of trouble with this technique was experienced, with blanks showing a high optical density and standards being very variable. Both these problems were overcome by recrystallizing the diphenylamine and redistilling the acetic acid before making up the reagent.

Because it was expected that any differences between non-pathogens and pathogens were likely to be small an assessment of the likely variability of counting radioactivity on membrane filters was carried out. This was done by adding known amounts of radioactive thymidine to filters which had been treated in a variety of ways and determining the counting efficiency and channel ratios in the scintillation counter.

Table 4 shows typical results of an experiment hybridizing DNA from a non-pathogen and the corresponding converted pathogen. There are no significant differences in the amount of radioactive DNA bound to the DNA on the membrane filters. Although the standard errors are reasonably small there is some variation in the replicates which could conceivably be obscuring small differences between pathogens and non-pathogens.

One source of this variability could be elution of the bound DNA from the filters during the hybridization period. Determinations of the DNA left on control filters after incubation under the hybridization conditions compared to discs not incubated showed that there was an average loss of DNA from the filters of approximately 18% but this was highly variable, ranging from 3% to 20%. This order of variation is not reflected in the results obtained, however, as the amount of DNA bound to the filter was enough to completely saturate the hybridization reaction so quite large losses in DNA would only give small variations in the amount of radioactive DNA bound.

Another source of variation in the results obtained would be variations in counting efficiency of the radioactive DNA on membrane filters. Table 5 shows the result of a variety of treatments on counting efficiency and channel ratios of radioactivity on membrane filters. It can be seen that the variations are quite significant and apparently unpredictable. For example, 4 filters treated as far as possible identically show counting efficiencies of 22.1, 19.8,

*Table 4.* DNA-DNA hybridization of DNA extracted from strains 57 and 57A.

| DNA on filter | Radioactive DNA added | % of radioactive DNA bound | Standard error |
|---|---|---|---|
| 57 | 57 | 31.4 | 1.0 |
| | 57A | 29.7 | 0.7 |
| 57A | 57A | 28.2 | 1.7 |
| | 57 | 31.3 | 1.7 |

Each value is the average of 5 replicates.

20.2 and 22.6, a variation of 2.8%. When the channel ratios are plotted against the counting efficiency (Fig. 9) there is no obvious relationship which could be used to correct the c.p.m. obtained.

Although the variations in counting efficiency may be eliminated by changing to a more energetic radioactive emitter such as $^{14}C$ or $^{32}P$ to label the DNA, the variation in elution of bound DNA appears to be an uncontrollable source of variation in all DNA hybridization techniques using membrane filters (DeLey and Tijtgat 1970).

One DNA hybridization method which eliminates radioactivity labelled DNA and membrane filters completely is the thermal renaturation rate method of DeLey, Cattior and Reynaerts (1970). In this method different DNA species made single stranded are mixed and the rate of renaturation is followed spectrophotometrically. The relatedness of the 2 DNA "species" can be calculated from this rate. Some preliminary experiments were carried out using this method but although it is simple in principle sufficient replication to obtain reliable results needs many experiments. It also appears to be more variable than filter hybridization (Gibbins and Gregory 1972), and so experiments using this method were not attempted.

<u>Polyacrylamide gel electrophoretic analysis of nucleic acids extracted from non-pathogenic and pathogenic agrobacteria</u>

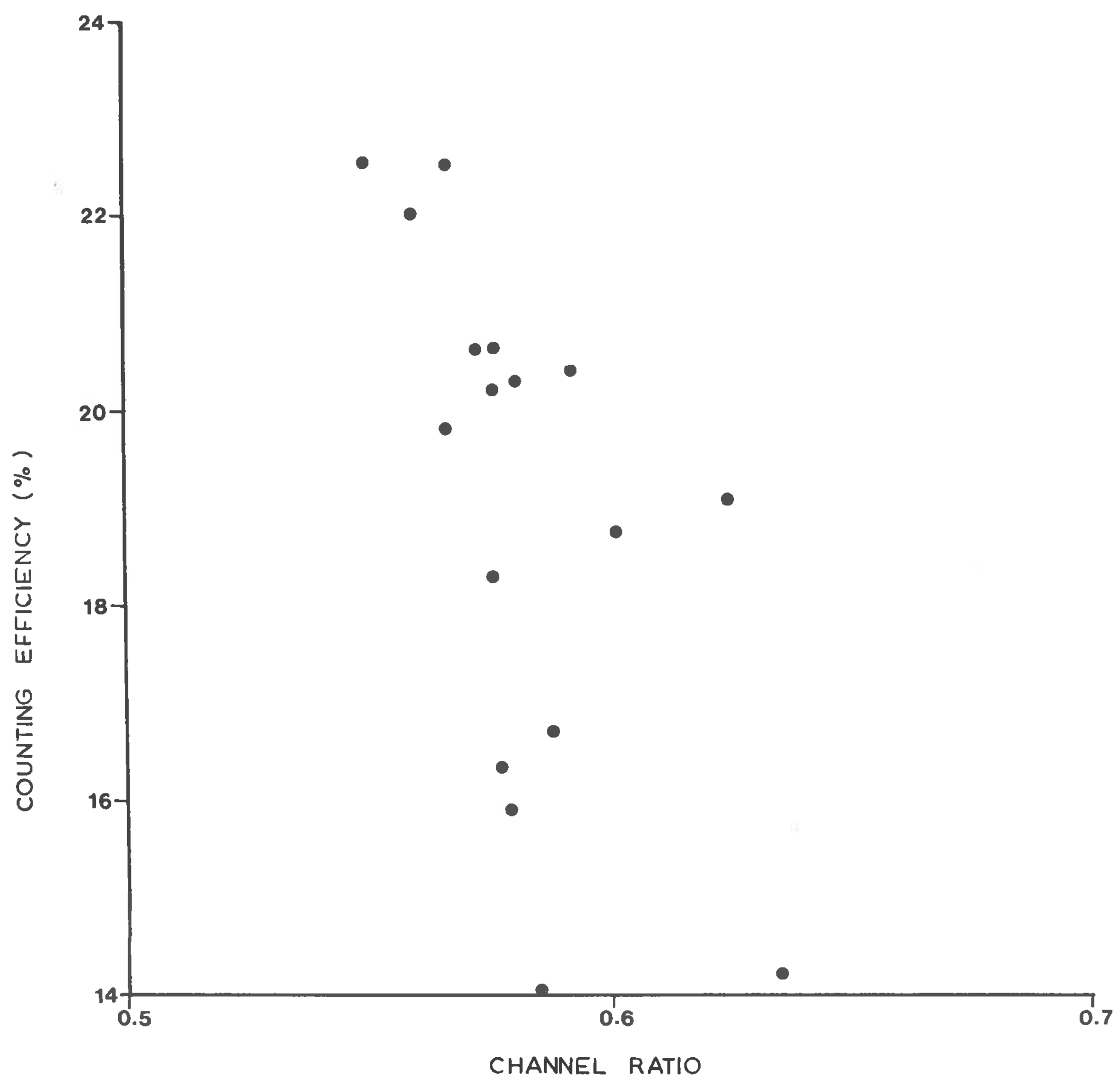
Nucleic acid molecules of different sizes can be separated using polyacrylamide gel electrophoresis. This technique is

*Table 5.* The effect of a variety of treatments on the counting efficiency and channel ratio of $^{3}$H-thymidine on membrane filters. Membrane filters loaded with a known amount of $^{3}$H-thymidine were treated in a variety of ways, then the radioactivity determined.

| Treatment | Counting efficiency | Channel* ratio |
|---|---|---|
| Replicate filters 1 | 22.1 | .559 |
| 2 | 19.8 | .556 |
| 3 | 20.2 | .576 |
| 4 | 22.6 | .566 |
| $^{3}$H-thymidine loaded on circumference of filter | 22.6 | .549 |
| $^{3}$H-thymidine spread evenly on filter | 20.7 | .572 |
| $^{3}$H-thymidine loaded in centre of filter | 20.3 | .580 |
| Filter with 30 μl 24 SSC buffer | 18.7 | .609 |
| Filter with 30 μl 6 SSC buffer | 14.2 | .635 |
| Filter with 30 μl 0.1 SSC buffer | 17.2 | .624 |
| Filter with 1 blank filter on top | 20.7 | .572 |
| Filter with 2 blank filters on top | 20.5 | .592 |
| Filter with 5 μg DNA | 14.0 | .585 |
| Filter with 10 μg DNA | 16.8 | .588 |
| Filter with 20 μg DNA | 16.3 | .577 |
| Filter loaded with DNA for hybridization | 18.3 | .576 |

* Discriminators on 2 channels of a Packard scintillation counter were set 20-1000 and 20-200 with 55% gain. Channel ratio is narrow channel over wide channel.

*Figure 9.* Relationship between channel ratio and counting efficiency of $^{3}H$-thymidine on membrane filters after various treatments. Discriminators on 2 channels of a Packard scintillation counter were set from 20 to 1,000 and 20 to 200 with 55% gain. Channel ratio plotted is narrow channel over wide channel.



24
22
20
18
16
14
COUNTING EFFICIENCY (%)
0.5
0.6
0.7
CHANNEL RATIO

particularly useful for the separation of different RNA molecules and has been used extensively on a wide variety of organisms. DNA molecules are not normally well resolved using this technique as their size and double stranded structure results in only very slow movement through the gel. Polyacrylamide gel electrophoresis of nucleic acids extracted from the non-pathogen : pathogen pairs was carried out to determine whether any distinct nucleic acid "species" was associated with pathogenicity.

Total nucleic acids (DNA and RNA) were extracted from non-pathogen : pathogen pairs using the method of Francki and Jackson (1972). Bacterial strains were grown for approximately 48 h in mannitol-glutamic acid medium and harvested by centrifugation. The pellet was suspended in 3 ml TNE buffer (0.02M Tris, 0.1M NaCl, 1mM EDTA, pH 8.5) and 1 ml 4% S.D.S. and 3 ml water saturated phenol added. This was shaken for 30 min at 25$^{o}$C and then the aqueous phase removed and extracted 3 more times with phenol. Nucleic acids were precipitated by adding 3 volumes absolute ethanol to the final aqueous phase and placing this at 4$^{o}$C overnight. The resultant precipitate was collected by centrifugation, washed with 80% ethanol, then ether, and finally taken up in TMN buffer, pH 7.3 (Tris 0.01M, $MgCl_2$ 0.01M, 0.1M NaCl). Yields ranged from 0.5 mg to 1 mg per wet weight of bacteria.

Flat gels containing 2.4% acrylamide with agarose (Peacock and Dingman 1968) or 7.6% acrylamide were prepared. After the

acrylamide had set the gels were attached to the buffer tanks which were then filled with running buffer, pH 8.3 (Tris 10.8 g, EDTA 0.93 g, Boric acid 5.5 g/litre). The gels were run at 5$^{o}$C for 1 h at 20 m.a. to stabilize the gel and remove impurities. The samples were then layered in the sample slots with bromophenol blue in one sample slot for a running marker. The gels were run at 10 m.a. for 10 min then the current increased to 20 m.a. until the bromophenol blue had run to approximately 1 cm from the end of the gel. The gels were then removed, fixed in 5% trichloroacetic acid for 10 min, washed in distilled water and then stained for 25 min in a freshly prepared solution of 0.02% w/v of toluidine blue. The gels were destained with frequent changes of distilled water.

To prevent possible nuclease activity all solutions were autoclaved or prepared from autoclaved water and glassware was autoclaved or heated at 150$^{o}$C for at least 1 h.

Plate 4(a) shows a typical 7.6% gel which was run with nucleic acids extracted from strains 147 and 147A. Both non-pathogen and pathogen strains show 2 bands sensitive to RNase treatment. When these extracts were run against markers (Tobacco leaf RNA; Rezaian 1974) the front band was shown to correspond to tRNA while the band further back was shown to correspond to 5S RNA. Although the 2 major ribosomal RNA species (16S and 23S RNA) were not present this is unlikely to be due to ribonuclease activity as 4S and 5S RNA are present. It is possible that the $Mg^{++}$ concentration is critical for

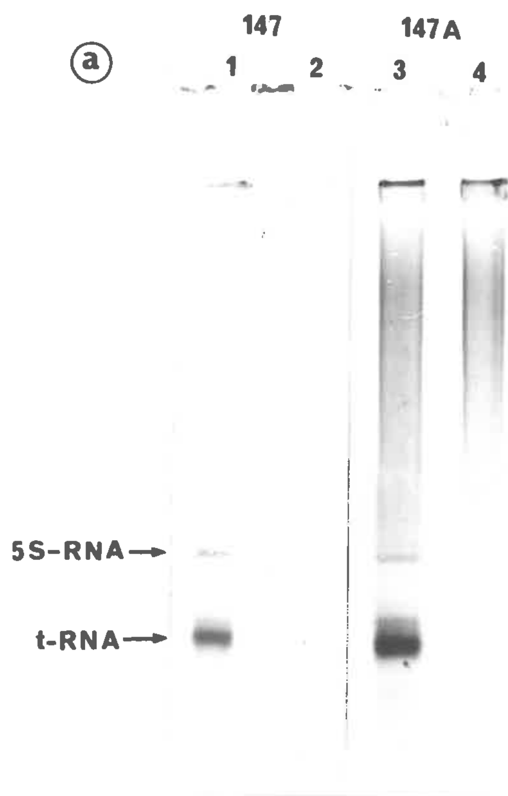
16S and 23S RNA isolation as suggested by Larsen and Zaitlin (1971) but even using their buffer no major ribosomal RNA species were isolated. Another possibility is that the SDS-phenol mixture does not properly disrupt the bacterial cell wall so that the larger ribosomal RNA species are trapped while the smaller RNA species go into the buffer.

Plate 4(b) shows a typical 2.4% acrylamide-agarose gel run with nucleic acids extracted from all 4 non-pathogen : pathogen pairs. The front running, heavy staining band is tRNA while the smaller band just behind it is 5S RNA. The band near the origin is not sensitive to RNase and may well be DNA which is able to penetrate 2.4% gels but not 7.6% gels.

Plate 4(a) shows a diffuse band in the pathogen which is absent in the non-pathogen. A similar band can be observed in strains 147A and 57 in Plate 4(b). It was at first thought that this band was only present in the pathogens but it was subsequently observed in all non-pathogens and pathogens tested. It appears only in extracts from bacteria which have reached the late log phase or stationary phase of growth. This band is insensitive to ribonuclease and deoxyribonuclease enzymes but had a melting profile typical of nucleic acid (Fig. 10). It is possible that it consists of a range of double stranded RNA molecules with different molecular weights. This type of RNA molecule has been found in a number of different organisms (Rezaian 1974).

Beljanski *et al.* (1974) claim that both non-pathogenic and pathogenic bacteria contain RNA associated with DNA and RNA-directed

*Plate 4.* Polyacrylamide gel electrophoresis of nucleic acids extracted from non-pathogen : pathogen pairs. a. Nucleic acid from strains 147 and 147A run on a 7.6% gel. Runs 1 and 3 nucleic acid run without ribonuclease treatment, runs 2 and 4 nucleic acid treated with 5 µg/ml ribonuclease for 30 min at 37°C before run. b. Nucleic acid from strains 147, run (1); 147A, run (2); 57, run (3); 57A run (4); 146, run (5); 146A, run (6); 200, run (7); and 200A, run (8) on a 2.4% gel.


(a)
147
147A
1
2
3
4
5S-RNA
t-RNA

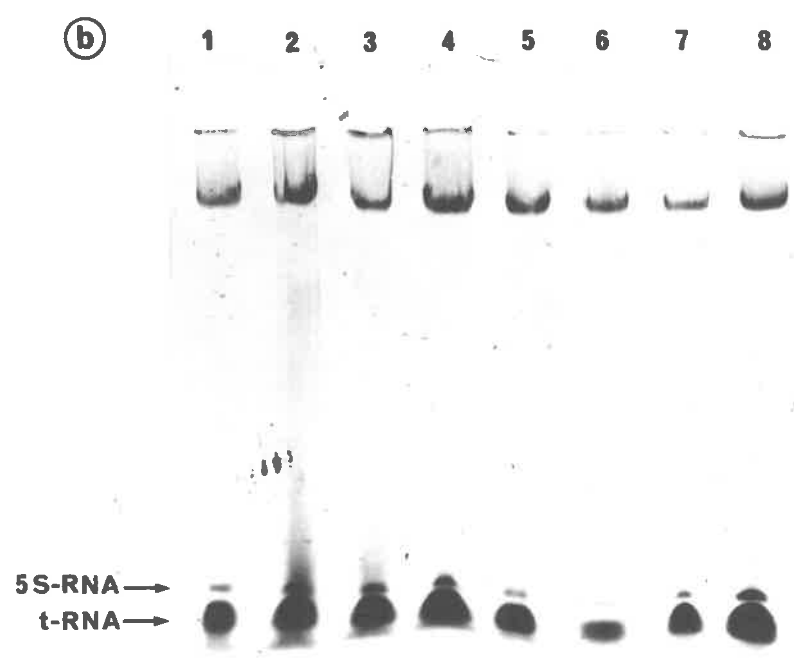
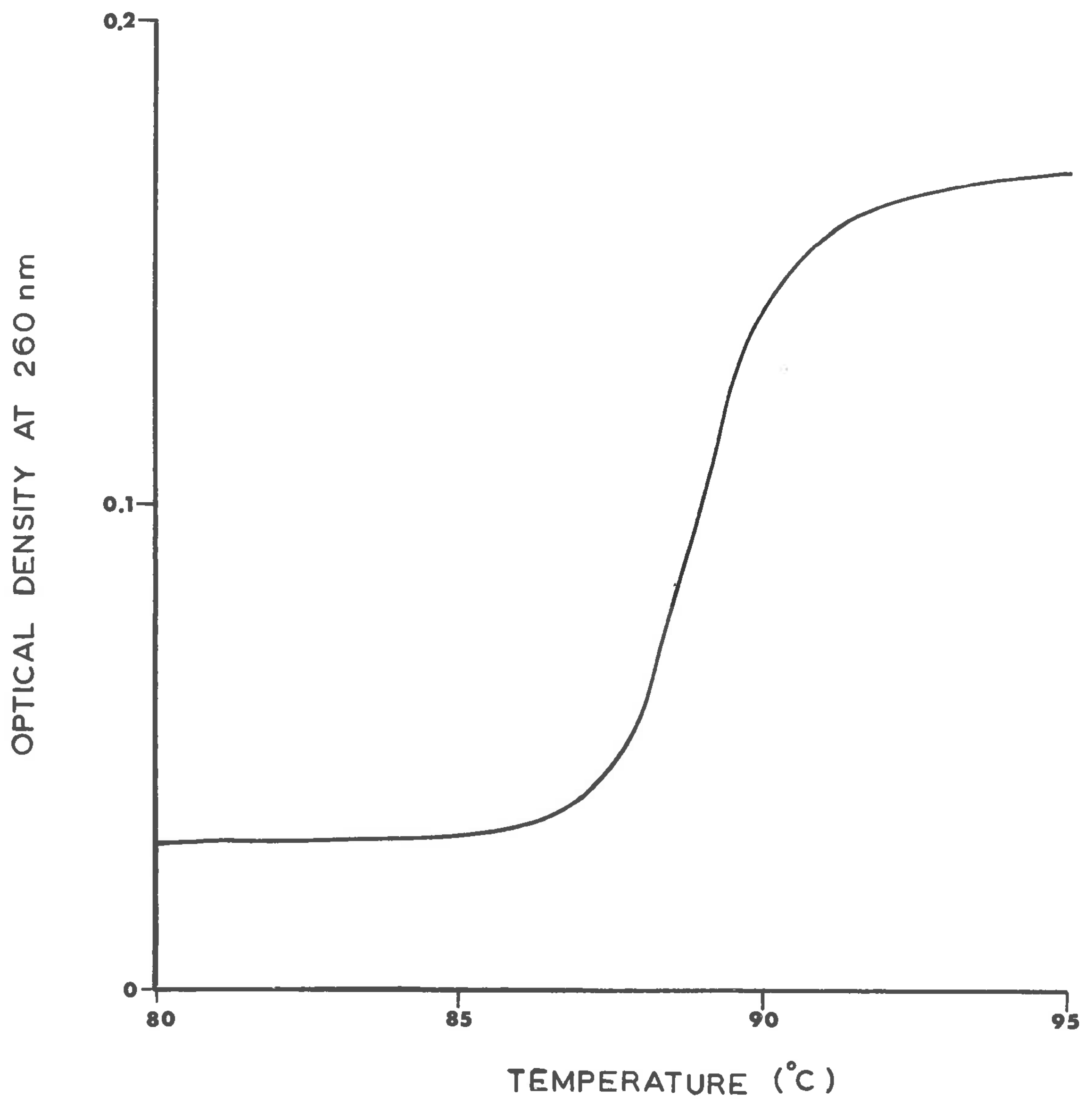

(b)
1
2
3
4
5
6
7
8
5S-RNA
t-RNA

*Figure 10.* Melting profile of the diffuse band after extraction from a polyacrylamide gel. After electrophoresis, the area containing the diffuse band was extracted in 1 SSC buffer and the melting profile determined. A similar size area of gel without the diffuse band was also extracted and used as the blank.



0,2
0,1
0
80
85
90
95
OPTICAL DENSITY AT 260 nm
TEMPERATURE (°C)

DNA polymerase which is capable of gall induction when introduced at wound sites on *Datura*. This tumour inducing RNA is approximately the same size as tRNA and so would not be detected on the poly-acrylamide gels. It is possible that the diffuse band observed in all strains may be this RNA still associated with DNA (the RNA associated with the RNA-dependent DNA polymerase would be released by the phenol treatment). A complex such as this would be expected to run in a diffuse manner on polyacrylamide gels and may show a melting profile typical of a double stranded nucleic acid. Although confirmation of this hypothesis needs further study, these results do indicate that polyacrylamide gel electrophoresis of nucleic acids extracted from agrobacteria does not differentiate between pathogens and non-pathogens.

<u>Analysis of non-pathogenic and pathogenic bacteria for the presence of plasmid DNA</u>

Many studies on a wide range of different bacteria have shown that a large number of characteristics can be determined by small circular DNA molecules (plasmids) which are separate from the main bacterial chromosome. These plasmids are often transferred from bacteria to bacteria during conjugation. Such a DNA molecule is an attractive possibility for T.I.P. with transfer to the plant cell inducing tumours and transfer to non-pathogenic bacteria transferring virulence (Kerr 1971). Experiments to detect plasmid DNA in

agrobacteria using CsCl-Ethidium bromide ultracentrifugation and sucrose gradient ultracentrifugation are described in this section.

Caesium chloride ethidium bromide centrifugation was carried out using basically the method of Hirt (1967). Bacteria were grown for 48 h in mannitol-glutamic acid medium containing 0.5 µCi $^{3}H$ thymidine/ml. They were then harvested by centrifugation, suspended in P.E.S.T. buffer containing 1M NaCl, 0.6 volumes protease added (1 mg/ml) and then made up to 1% SDS. This was incubated at 60°C for 3 h then placed at 4°C overnight. The following morning the precipitate of SDS, protein and high molecular weight nucleic acid was sedimented by centrifugation (17,000 g for 20 min). Aliquots of 50 µl of the supernatant solution were placed in tubes with 3 ml of CsCl solution (density 1.660) containing 100 µg ethidium bromide/ml. This was overlayered with paraffin oil to fill the tubes and they were spun at 40,000 r.p.m. for 24 h at 20°C in an SW50.1 rotor. After the run the tubes were drop-fractionated onto 25 mm diameter filter discs (Whatman 3MM) which were dried, placed in scintillation vials with scintillation fluid and the radioactivity determined.

Sucrose gradient centrifugation was carried out using two quite different lysis methods and a variety of different buffers.

For the method of Guery, LeBlanc and Falkow (1973) bacteria were grown and harvested as for the CsCl-ethidium bromide method, then suspended in 0.05M Tris-HCl buffer, pH 8.0, SDS added to a

concentration of 1%, and incubated at 25°C for 2½ h. The suspension was then adjusted to 1M NaCl and placed at 4°C overnight. The following morning the precipitate was removed by centrifugation and 200 µl aliquots of the supernatant layered on 5% to 20% sucrose gradients prepared in 0.5M NaCl 0.01M potassium phosphate buffer, pH 7.0. The tubes were spun at 40,000 r.p.m. at 15°C for 1 h in an SW41 rotor. Tubes were drop fractionated directly into scintillation vials, Triton X100-toluene scintillation fluid added (Siegel 1971) and radioactivity determined.

The other sucrose gradient centrifugation methods followed Zaenen *et al.* (1974). For alkaline lysis bacteria grown in peptone medium (Appendix 1) containing 1 µCi $^3$H-thymidine/ml were harvested, washed in buffer containing 0.01M $K_2HPO_4$, 0.001M $MgSO_4$, 0.001M $CaCl_2$ and 0.1M NaCl, pH 7.0, and recentrifuged. The pellet was resuspended in lysis buffer (0.05M NaCl, 0.02M EDTA, 0.02M Tris, pH 9.1) and 30 µl of 1% SDS in 0.8M NaOH per 0.2 ml of bacterial suspension was slowly added with constant mixing. The lysed samples were sheared for 30 sec at top speed in a vortex mixer and 0.1 ml aliquots layered on 5% to 20% sucrose gradients prepared in 0.5M NaCl, 0.02M EDTA and 0.3M NaOH.

Neutral lysis was carried out with bacteria grown as for alkaline lysis, then suspended in 0.5M Tris, 0.02M EDTA buffer, pH 8.0, containing 1% SDS and 0.5 mg/ml protease for 2½ h at 37°C. After lysis the samples were sheared for 30 sec at top speed in a

vortex mixer, and 0.1 ml aliquots layered on 5% to 20% sucrose gradients prepared in 0.05M NaCl, 0.005 EDTA and 0.03M Tris, pH 8.0.

Gradients from both alkaline and neutral lysis were spun for 40 min at 40,000 r.p.m. at 15$^{o}$C in an SW41 rotor. After the run the tubes were drop fractionated by bottom puncture directly into scintillation vials. Triton X100-toluene scintillation fluid was added and radioactivity determined.

Figure 11 shows a typical result of CsCl-ethidium bromide centrifugation. The radioactive material from both non-pathogen and pathogen ran as a discrete peak with no detectable subsidiary peaks, suggesting that there was no DNA present as supercoiled circular covalently closed molecules.

The sucrose gradient methods should detect relaxed and super-coiled plasmid molecules (Guerry *et al.* 1973). An examination of the sucrose gradient experiments (Figs. 12 and 13) shows that in all cases the non-pathogen and the pathogen both have the same pattern. The radioactive material sedimented as a large block with a sharp front. Plasmid DNA would be expected to run as a discrete peak ahead of the sharp front. No such peak was detected, irrespective of the bacterial strain or lysis system used.

Bacteriocin sensitivity of non-pathogenic and converted pathogenic agrobacteria

Kerr and Htay (1974) reported that a strain of *Agrobacterium* (Strain 84) used for biological control produced a bacteriocin which

*Figure 11.* Equilibrium centrifugation in CsCl solution containing ethidium bromide of crude lysates from bacteria labelled with $^{3}$H-thymidine

——— lysate from strain 57,

----- lysate from strain 57A.

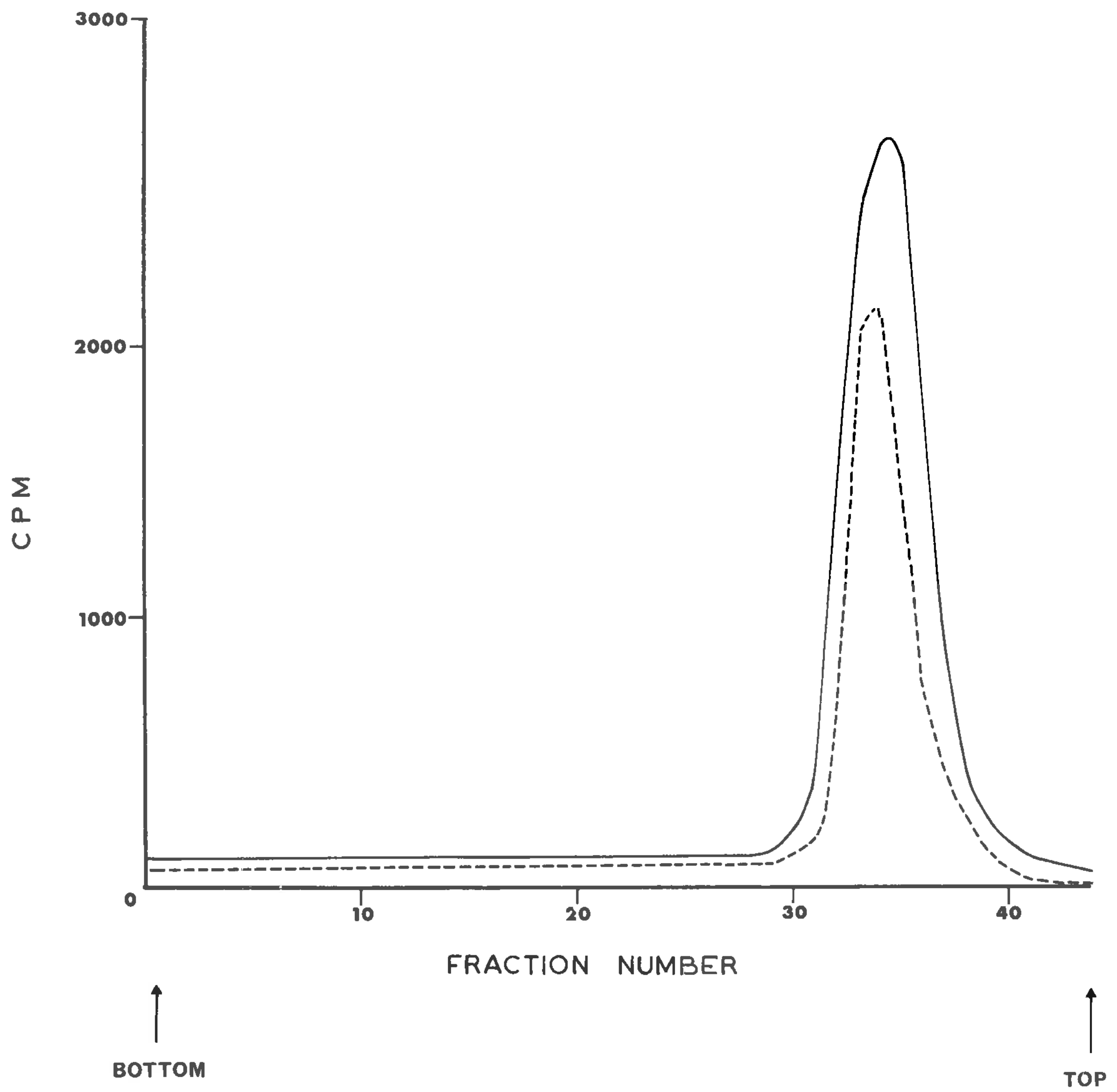

3000
2000
1000
0
C P M
10
20
30
40
FRACTION NUMBER
BOTTOM
TOP

*Figure 12.* Neutral sucrose gradient centrifugation run of crude lysates from bacteria labelled with $^{3}$H-thymidine. a. strain 57, b. strain 57A.

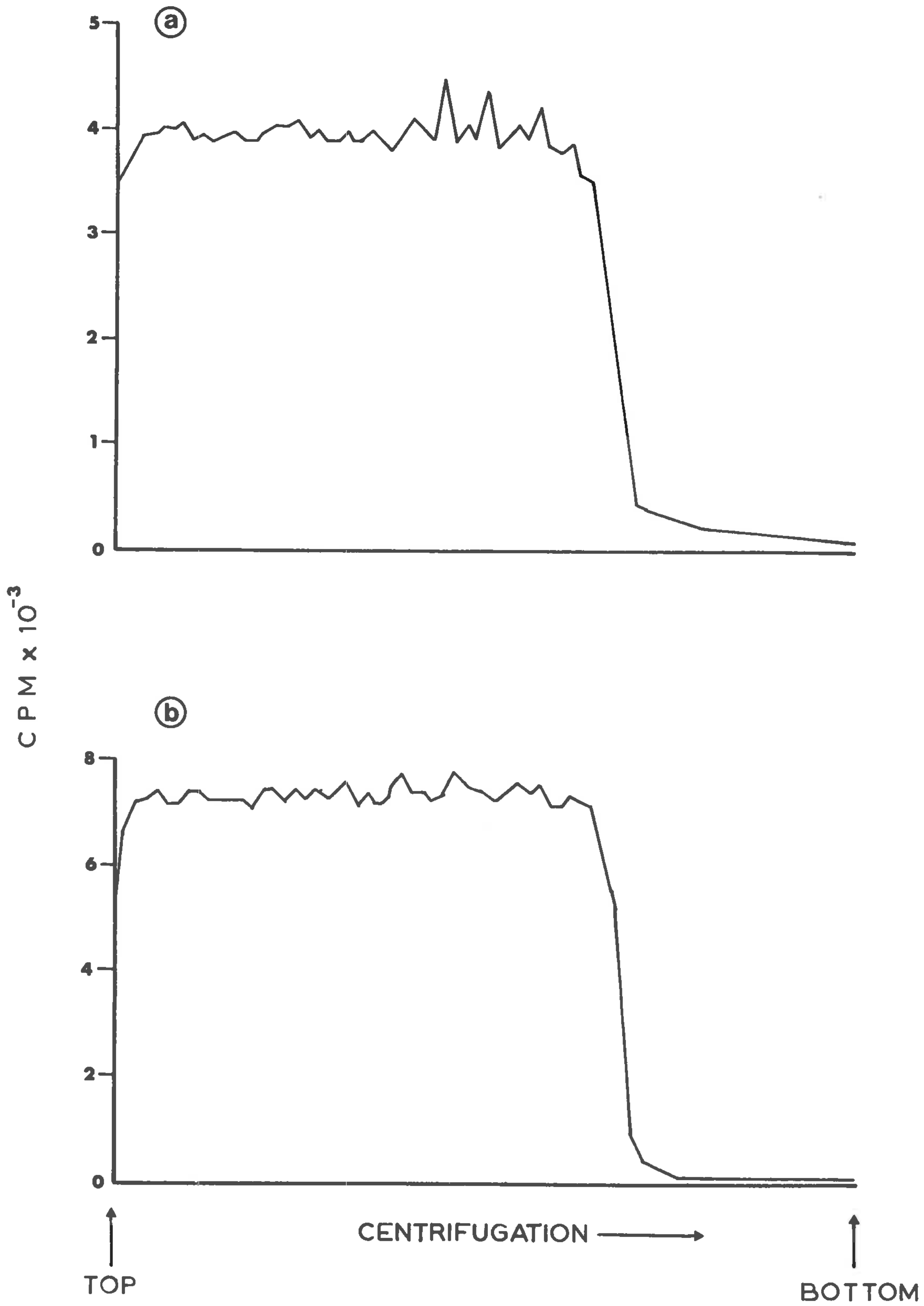
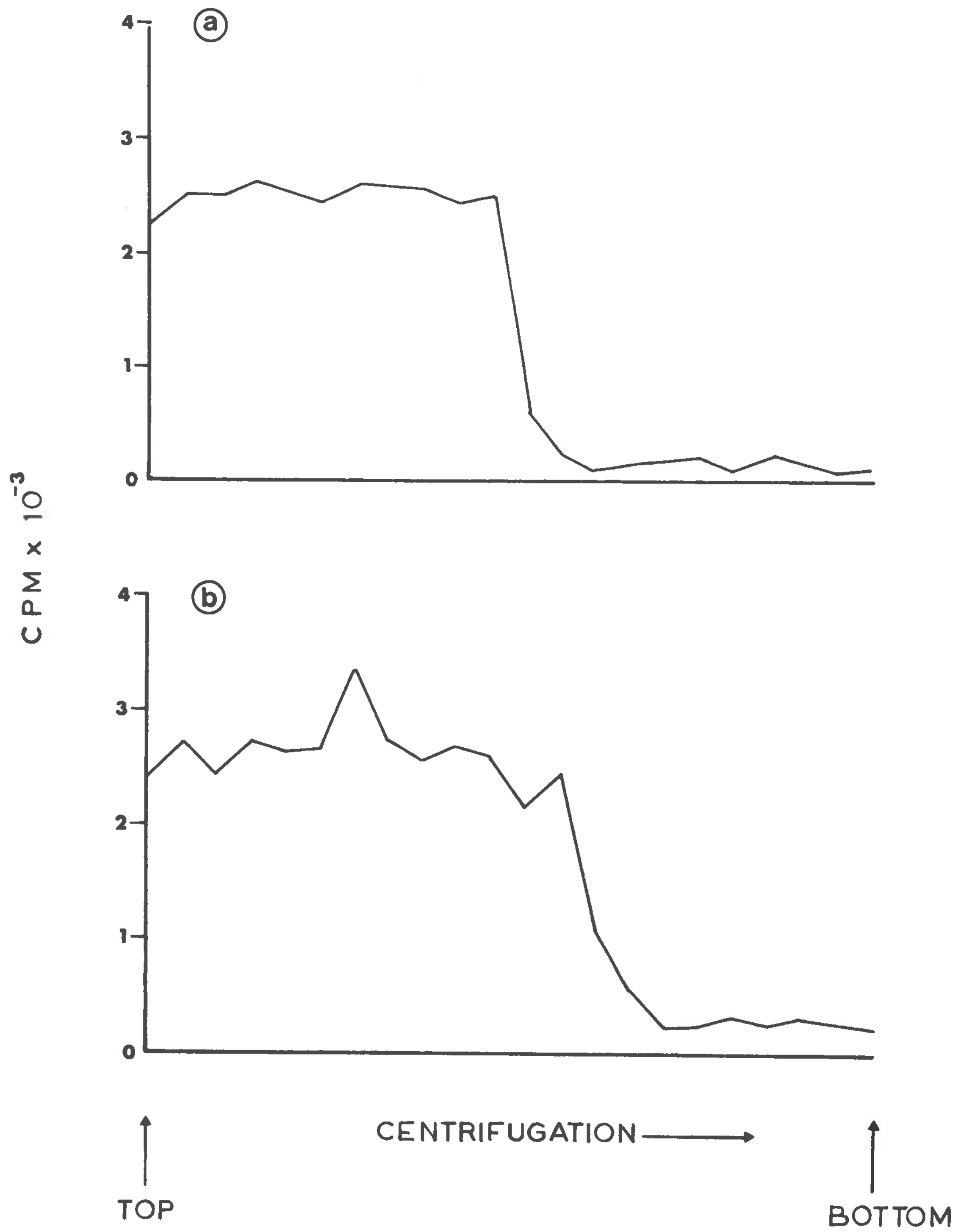

a
b
5
4
3
2
1
0
8
6
4
2
0
CPM x 10⁻³
CENTRIFUGATION
TOP
BOTTOM

*Figure 13.* Alkaline sucrose gradient centrifugation run of crude lysates from bacteria labelled with $^{3}$H-thymidine. a. strain 57, b. strain 57A.



a
b
4
3
2
1
0
CPM x $10^{-3}$
CENTRIFUGATION
TOP
BOTTOM

was active against a wide range of pathogenic agrobacteria. Kerr (Roberts and Kerr 1974) tested a number of non-pathogen : pathogen pairs for bacteriocin sensitivity (Table 6) and showed that with one exception (Strain 23) all of the pathogens were sensitive and all of the non-pathogens were insensitive.

## DISCUSSION

The serology experiments in this section support the studies reviewed in the Introduction in that no constant differences between pathogens and non-pathogens were found. Obviously a wide range of ill-defined antigens have been used to produce the antisera and only a small number have produced a response detectable by the methods used. Possibly any compounds which are unique to pathogenic agrobacteria are either antigenically relatively inactive or are at too low a concentration to give a detectable response.

The isoenzyme studies did not detect any differences which could be correlated with pathogenicity. Possibly if there are any proteins unique to pathogenic bacteria they are at a lower concentration than could be detected or they do not have any of the enzymatic activities that were tested. Although only 6 enzyme systems were studied they represented a broad range of enzymatic activities, suggesting that isoenzyme studies are not a suitable method of differentiating pathogens from non-pathogens.

The experiments on mitomycin C induction of bacteriophage

*Table 6.* Sensitivity of non-pathogens and converted pathogens to bacteriocin 84.

| Strain* | Sensitivity to bacteriocin 84 | Strain* | Sensitivity to bacteriocin 84 | Strain* | Sensitivity to bacteriocin 84 |
|---|---|---|---|---|---|
| 18 | - | 55 | - | 146C | + |
| 18A | + | 55A | + | 146D | + |
| 21 | - | 55B | + | 147 | - |
| 21A | + | 55C | + | 147A | + |
| 23 | + | 57 | - | 147B | + |
| 23A | + | 57A | + | 147C | + |
| 44 | - | 57B | + | 147D | + |
| 44A | + | 57C | + | 200 | - |
| 54 | - | 57D | + | 200A | + |
| 54A | + | 146 | - | 200B | + |
| 54B | + | 146A | + | 200C | + |
| | | 146B | + | 200D | + |

* Non-pathogenic parents are indicated by a number; converted pathogens are indicated by the number of their non-pathogenic parent and by a letter.

\+ = sensitive; - = resistant

From Roberts and Kerr (1974).

suggest that crown-gall induction and transfer of pathogenicity does not involve bacteriophage. This agrees with many other reports which have failed to correlate the production of, or sensitivity to, bacteriophage with pathogenicity. However, it is possible a bacteriophage that is not induced by the normal techniques is involved in pathogenicity. The work of Bourgin-Rosenberg *et al.* (1974) is significant in this regard. If a cryptic prophage such as they found in a number of *Agrobacterium* strains is important in pathogenicity it would not be detected in the experiments reported in this thesis or by the techniques used by many other workers.

The DNA-DNA hybridization studies showed that there was no detectable difference in base sequence between pathogens and non-pathogens. The ability to induce crown-gall is obviously an inheritable factor so its basis must be in DNA base sequences. The experimental results, in agreement with Gibbons and Gregory (1972), suggest that the smallest detectable difference in base sequence would be approximately 5%. Assuming a molecular weight for the bacterial genome of $2 \times 10^9$ (Chilton *et al.* 1974) this could code for approximately 30 proteins of 100,000 molecular weight. Thus, although nucleic acid hybridization techniques are extremely useful for distinguishing between species and genera of bacteria (DeLey 1971) they lack the precision necessary to distinguish between strains of bacteria which possibly differ only in a few base sequences.

The search for plasmid DNA in the non-pathogen : pathogen pairs has been inconclusive. This agrees with Kado *et al.* (1972) but contrasts with the recent reports by Van Larebeke *et al.* (1973, 1974) and Zaenen *et al.* (1974) who claim that all the pathogen strains and none of the non-pathogen strains they tested carried a plasmid. Despite using their methods no evidence for a plasmid in any of the non-pathogen : pathogen pairs was found and it remains to be seen whether their results can be confirmed in other laboratories. The main method they used for screening the strains (neutral sucrose gradients) does not differentiate between supercoiled covalently closed DNA molecules (the form in which most plasmids are thought to exist in the cell (Clowes 1972) and relaxed circular DNA molecules which may have single strand breaks. Relaxed circular DNA molecules have been observed in DNA extracted from *Agrobacterium* by Schilperoort (1969) and it is possible that such molecules are an artifact of the isolation procedure. However, the strong correlation between pathogenicity and the presence of the plasmid appears to eliminate this possibility. Another possibility is that the plasmid is normally integrated into the main bacterial chromosome like Hfr strains of *E. coli* (Hayes 1968) and only released as the free plasmid under certain critical conditions which were not achieved in the experiments reported here. If this were the case then the integrated state may well be important for transfer of the T.I.P. and pathogenicity as Hfr states in many bacteria confer a much higher genetic transfer

than free plasmid.

The bacterial strains used by Van Larebeke *et al.* (1973, 1974) and Zaenen *et al.* (1974) were quite different to those used for the experiments reported in this thesis and it is possible that integration of the plasmid only occurs in some strains. Certainly a plasmid is an attractive hypothesis to explain crown-gall induction and transfer of virulence. If such a plasmid were cryptic (not expressed phenotypically in the bacterial cell) then closely related pathogens and non-pathogens could not be easily distinguished. Due to their small size plasmid molecules would normally be lost in conventional DNA isolation methods and so would not be detected in DNA-DNA hybridization experiments.

The data on bacteriocin sensitivity shows that when non-pathogenic bacteria are converted to pathogens they simultaneously become sensitive to bacteriocin. Kerr and Htay (1974) showed the converse; that when bacteria lose pathogenicity they simultaneously become insensitive to bacteriocin. The possible mechanisms for this strong correlation between bacteriocin sensitivity and pathogenicity are discussed in Part D.

With the exception of the bacteriocin study, rather than differentiate between pathogenic and non-pathogenic bacteria these experiments have emphasised their very close similarity, suggesting that the change from non-pathogen to pathogen is very small indeed.

These studies also highlight the need for more precise techniques in the study of pathogenicity in *Agrobacterium*.

# *PART D*

## A BACTERIOCIN FROM *AGROBACTERIUM*

### INTRODUCTION

In the last section experiments were described (Kerr and Htay 1974, Roberts and Kerr 1974) which indicated that there was a very close correlation between pathogenicity and sensitivity to a bacteriocin produced by *Agrobacterium radiobacter* var. *radiobacter* strain 84. This section describes experiments carried out to determine the nature and mode of action of this bacteriocin in an attempt to understand the close correlation between pathogenicity and bacteriocin sensitivity that had been observed.

Bacteriocins were first reported by Gratia (1925) who showed that one *Escherichia coli* strain produced a substance which had bacteriocidal activity against another strain. Since this initial report bacteriocins have been reported from a wide range of gram-positive and gram-negative bacteria (Reeves 1972).

Bacteriocins are distinguished from other antibiotic substances on the basis of their activity spectra and the large variety of different types that may be produced by the one group of organisms. While most antibiotics are active against organisms that are quite unrelated, bacteriocins are active against bacteria which are generally very closely related to the producing strain. For example, Papavassiliou

(1961) showed that one bacteriocin produced by *E. coli* was active against 100 other *E. coli* strains tested but not active against 77 strains of other bacterial families. That many different bacteriocins can be produced by the same group of organisms is well illustrated by Reeves (1972) who lists 19 distinct categories and many sub-categories of bacteriocin produced by *E. coli* alone. A similar diversity of bacteriocin production is also observed in other bacterial genera.

In most cases where detailed studies have been carried out it has been shown that bacteriocins are proteins with molecular weights ranging from approximately 10,000 to approximately 100,000 (Reeves 1972). The only conclusive report of a low molecular weight bacteriocin is that of Atkinson (1967) who reported that a *Salmonella* strain produced a bacteriocin which readily diffused through dialysis membranes.

When detailed studies of the mode of bacteriocin action have been carried out it has been found that sensitive bacteria have specific binding sites for bacteriocin. For example, Reeves (1965), studying the bacteriocin colicin $E_2$, showed that sensitive bacteria removed colicin activity from solution and that over a suitable range of bacterial and colicin concentrations the amount of colicin adsorbed was directly proportional to the number of bacteria. Resistant mutants did not bind bacteriocin. Maeda and Nomura (1966), using radioactive bacteriocin, showed quite conclusively that bacteriocin adsorbed to sensitive cells but not to resistant cells.

With some bacteria it has been possible to isolate fractions from

the bacteria which show bacteriocin binding *in vitro* (Sabet and Schnaitman 1971, Weltzien and Jesaitis 1971, Braun, Schuller and Wolff 1973, Braun and Wolff 1973). It has been demonstrated in a number of cases that these receptor sites are also important in phage adsorption (for example Fredericq and Gratia 1949, Braun *et al.* 1973).

The kinetics of adsorption of bacteriocin has been worked out in some detail for a few bacteriocins. Shannon and Hedges (1967), using very short sampling periods, showed that the rate of killing was at a maximum immediately the sensitive bacteria and the bacteriocin were mixed, suggesting that only one bacteriocin molecule is needed to kill each bacterium. Although there have only been a limited number of studies on the kinetics of bacteriocin action they all show single-hit kinetics and it has been suggested that this is the normal mode of bacteriocin action (Reeves 1972, Mayr-Harting, Hedges and Berkeley 1972). There is, however, an apparent contradiction as many reports show that a number of bacteriocin molecules have to bind to cause cell death, yet single hit kinetics are still observed and it has been suggested that there may be a number of non-lethal receptors on the cell as well as lethal receptors (Mayr-Harting *et al.* 1972), or that any one of many receptors on the cell has an equal, but low, chance of causing cell death (Reeves 1972).

Bacteriocins have been shown to affect a large number of different cell processes including energy production, protein synthesis, DNA synthesis and RNA synthesis (Reeves 1972). Perhaps one of the most interesting reports is that of Bowman, Sidikaro and Nomura (1971) who

showed that colicin $E_3$ was active in cleavage of 16S ribosomal RNA *in vitro*. Such an effect has already been observed *in vivo* (Bowman, Dahlberg, Ikemura, Konisky and Nomura 1971) but it was thought to be due to the activation of a bacterial ribonuclease.

There have been a number of studies which have shown that at least with some bacteriocins the cells can be rescued by trypsin treatment up to a few hours after adding bacteriocin (for example, Nomura and Nakamura 1962), and it was generally held that bacteriocins mediated their effect from the receptor site located in the cell wall of the bacteria. However, Maeda and Nomura (1966), working with radioactive colicin, showed that a small proportion of bound colicin ultimately became soluble in the cytoplasm of the cell and it is likely that colicin $E_3$ would only be enzymatically active when it is released from the receptor site into the cytoplasm of the cell, suggesting that not all bacteriocins mediate their effect indirectly from receptor sites. It is possible that other bacteriocins may show *in vitro* effects when more detailed studies are carried out.

There has only been one early report of a bacteriocin from *Agrobacterium*. Stonier (1960a), when trying to label agrobacteria with $^{32}P$, noted that virtually all the $^{32}P$ he added was metabolised to organic phosphate compounds and released back into the medium. When he checked to see whether this was due to the release of bacteriophage he discovered that the *Agrobacterium* strain he had been working with produced a bacteriocin-like compound (Stonier 1960b). Beardsley, Bohan and Daly

(1962) reported that glycine attenuation of pathogenicity of *Agrobacterium* strain T37 was correlated with a decrease or loss in bacteriocin production but this preliminary report has not been confirmed.

## CHARACTERIZATION AND PURIFICATION OF THE BACTERIOCIN FROM *AGROBACTERIUM* STRAIN 84

### Bacteriocin assay

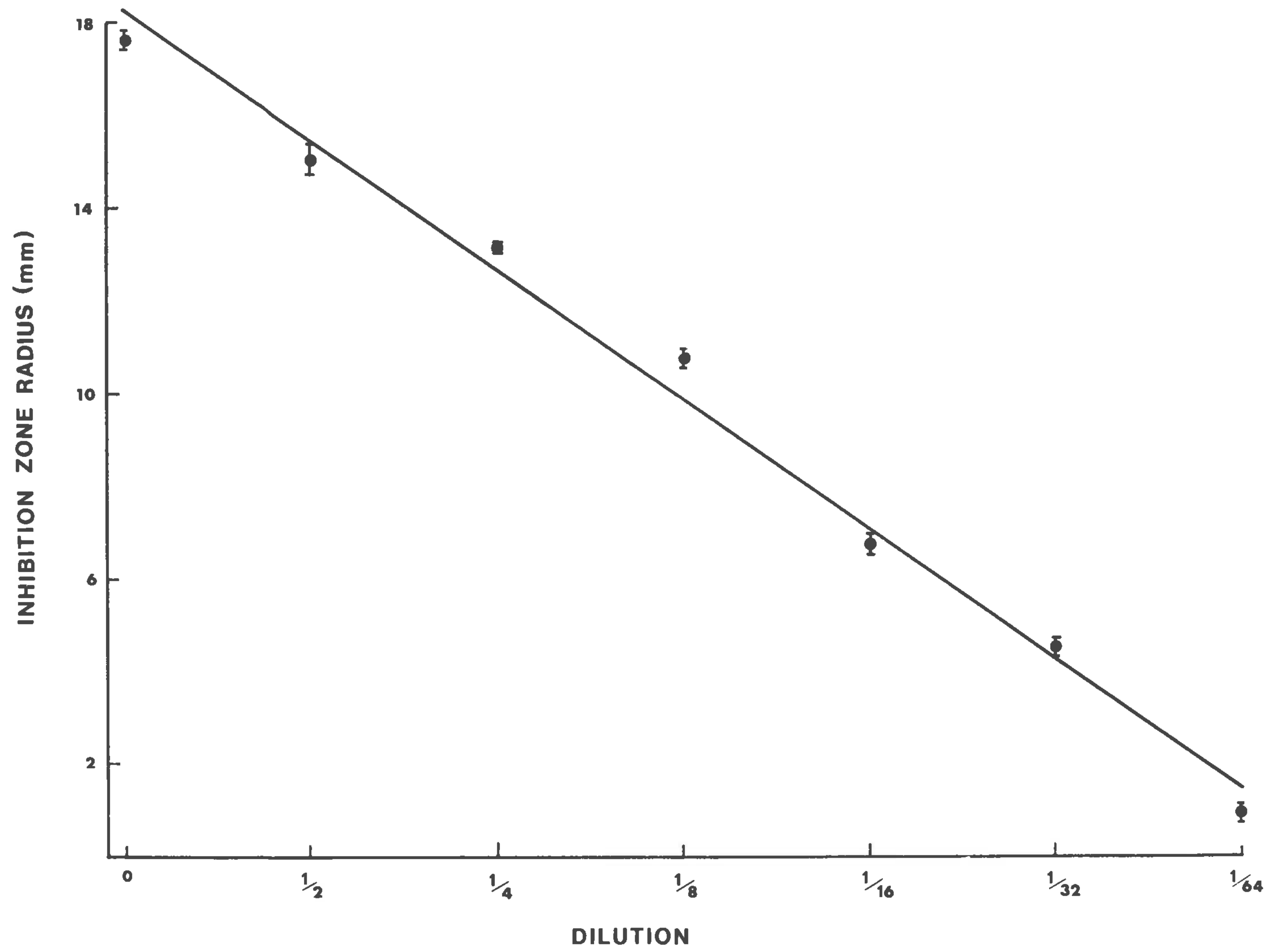
Before any work on the bacteriocin could be carried out it was necessary to develop a bioassay which was sensitive, accurate and convenient. The method used in most studies on bacteriocins (Mayr-Harting *et al.* 1972) is to make a dilution series of a bacteriocin solution, then spot known amounts on to agar plates which have been seeded with a sensitive indicator strain of bacteria. After a period of incubation the end point of growth inhibition can be determined. This method is very simple but rather tedious if a large number of samples are used. There is also some uncertainty in reading the end-point of bacteriocin activity. Preliminary experiments and the work of Stonier (1960b) showed that bacteriocins from *Agrobacterium* had a fast diffusion rate in agar and it seemed likely that the size of the inhibition zone produced would be related to the concentration of bacteriocin. This was tested by cutting 5 mm diameter wells in agar plates of Stonier's medium (Stonier 1960b, Appendix 1) with a cork borer. Into these wells were placed 20 µl aliquots of a two-fold dilution series of an active

bacteriocin solution. To ensure sterility, a lid with an attached filter paper moistened with chloroform was placed over each plate for approximately 20 min. The lid was then replaced with one without filter paper; this lid was left slightly ajar to allow the chloroform to disperse. The plates were then poured with 0.5 ml of a liquid culture of a sensitive indicator strain (strain 24) suspended in 2.0 ml buffered agar (Stonier 1960b, Kerr and Htay 1974). After approximately 40 h incubation at 25$^{o}$C the inhibition zones produced by the bacteriocin solutions were measured.

Figure 14 shows the relationship between the radius of the inhibition zone and the relative concentration of bacteriocin. It can be seen that over quite a wide range of bacteriocin dilution there is a linear relationship between the inhibition zone produced and the log of dilution. The standard errors of each point confirm that there is little variation between replicates of each dilution and show that all points statistically are significantly different. The slope of the line indicates that for a two-fold change in bacteriocin concentration there is a 2.4 mm change in the inhibition zone radius. A value of 2.2 mm change in radius for a two-fold change in bacteriocin concentration was obtained in another experiment using a different preparation of bacteriocin indicating that the relationship between bacteriocin concentration and inhibition zone size was quite consistent.

Further experience with this assay system showed that if very fresh inoculum was used for the sensitive indicator then the assay could be measured after 18 h incubation. The inhibition zones were stable

*Figure 14.* Relationship between the logarithm of dilution and the radius of inhibition zone produced by a bacteriocin solution. Bars represent $\pm$ the standard error.



INHIBITION ZONE RADIUS (mm)
18
14
10
6
2
0
1/2
1/4
1/8
1/16
1/32
1/64
DILUTION

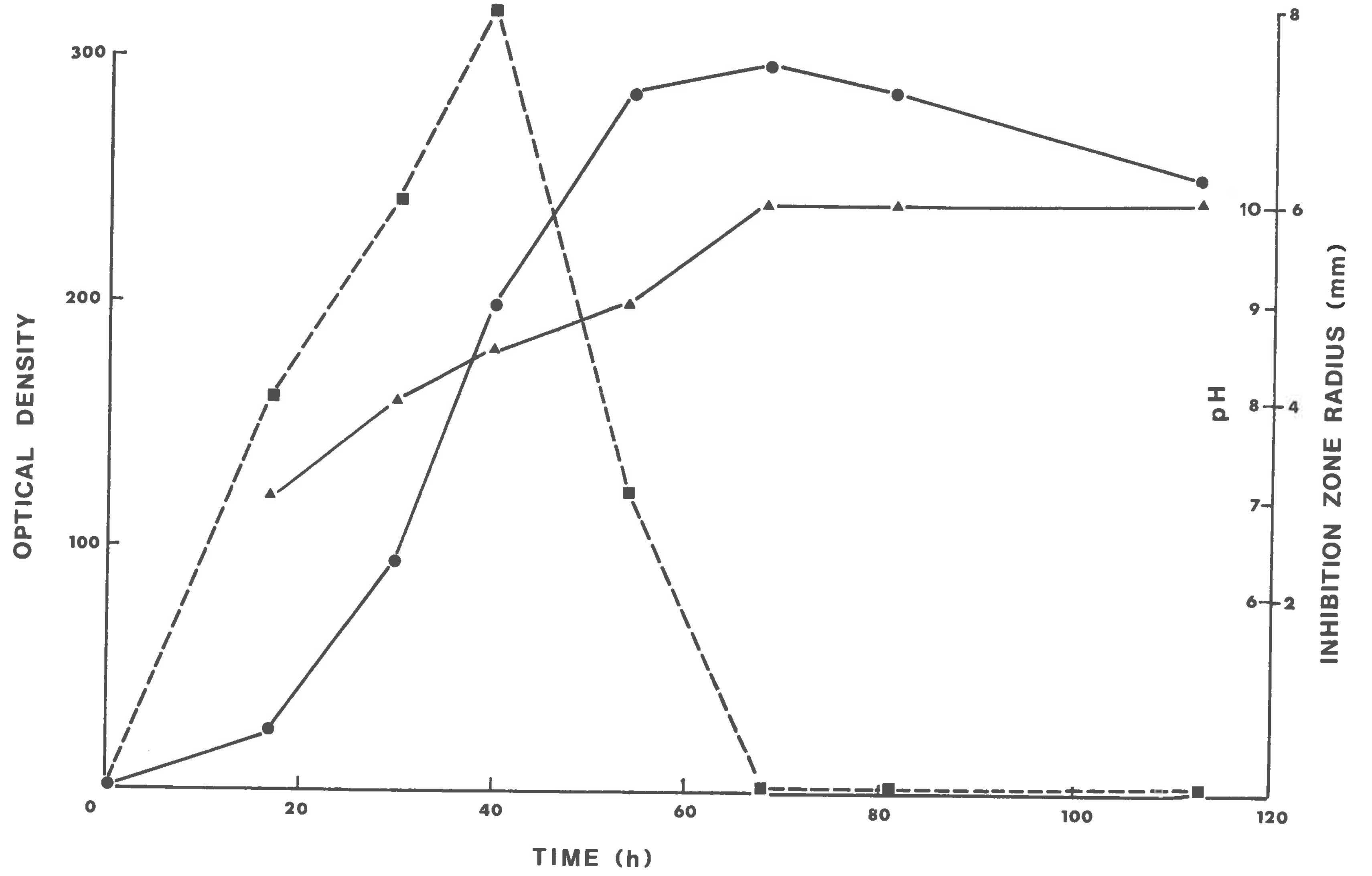
in size up to approximately 48 h but after this resistant mutant growth in the inhibition zone and heavy growth around the zone tended to make the determination of the inhibition zone radius uncertain.

Bacteriocin production

For the preliminary experiments, flasks of Stonier's medium inoculated with strain 84 were incubated for approximately 40 h at 25°C on a rotary shaker. The bacteria were then centrifuged down and the supernatant shaken with a ½ volume of chloroform to sterilize it. Solutions were stored at 4°C with a small volume of chloroform added. This technique, however, gave solutions with highly variable bacteriocin activity. Sometimes very active preparations were obtained while others were completely inactive and there did not appear to be any consistent pattern. At first it was thought that the bacterial cultures were not being incubated long enough, but longer incubation times often produced solutions with no activity. To determine the cause of this variability the bacteriocin activity of cultures of strain 84 were followed with time.

Side-arm flasks containing Stonier's medium were inoculated with strain 84 and placed on a rotary shaker at 25°C. At intervals small samples were removed, the bacteria centrifuged down and the bacteriocin activity of the supernatant determined. At the same time as the removal of samples the optical density and pH of the cultures were measured. The results of this experiment are shown in Figure 15. This shows that the increase in bacteriocin activity parallels the growth of the culture up to 40 h when there is a very sharp drop in activity. The growth of the

*Figure 15.* Bacteriocin production by a liquid culture of strain 84. ●——● optical density of culture, ▲——▲ pH of culture, ■- - -■ bacteriocin activity of culture.



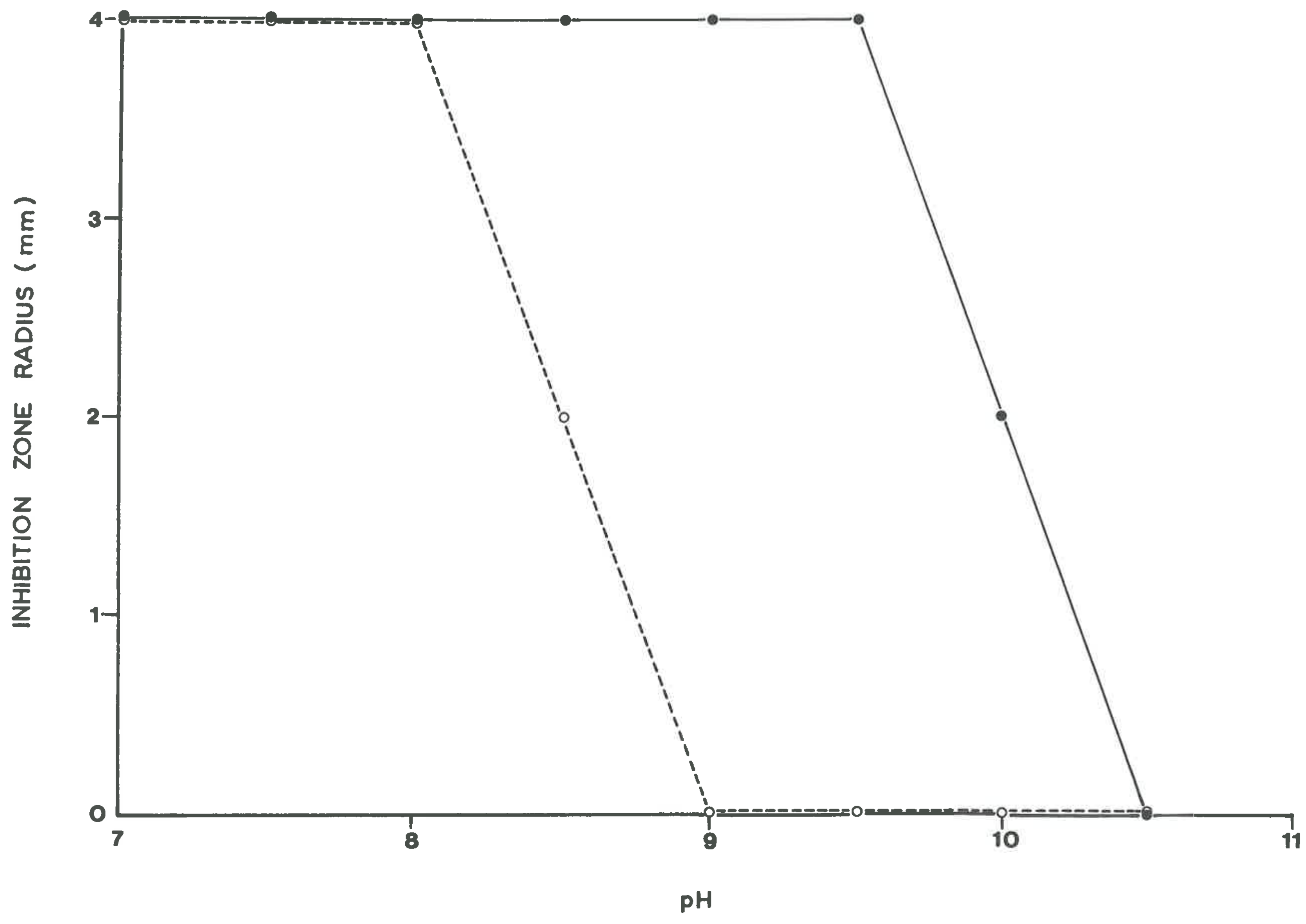
OPTICAL DENSITY
300
200
100
0
20
40
60
80
100
120
TIME (h)
pH
INHIBITION ZONE RADIUS (mm)
8
10
6
9
8
4
7
6
2

bacteria do not reach the stationary phase until 60 h. These results show that after a certain period bacteriocin is degraded, explaining why bacteriocin preparations were often inactive. Figure 15 shows that the pH of the cultures rises steeply during the log phase of growth and it was possible that the degradation observed was due to this.

The effect of pH, $O_2$, $H_2$, ultraviolet light and periodate on on bacteriocin activity

To test whether alkaline pH values had any effect on bacteriocin activity bacteriocin solutions were mixed with an equal volume of 0.1M Tris-HCl buffer at the required pH and incubated at 25°C for 1 h or 3½ h. The pH was then rapidly adjusted back to 7.0 with HCl and bacteriocin activity assayed. Figure 16 shows that a pH of 10.5 for 1 h or 9.0 for 3½ h was sufficient to destroy all bacteriocin activity. It is obvious that there is a time - pH relationship with short incubation at high pH or longer incubation at lower pH both reducing bacteriocin activity. To test whether the reduction of bacteriocin activity was due to oxidation catalysed by high pH, bacteriocin solutions were incubated at high pH under conditions which excluded air. Bacteriocin solutions were degassed under vacuum and then placed under a deep layer of paraffin oil which also had been degassed. An equal volume of 0.2M $Na_2CO_3$ which had been degassed was added (final pH approximately 11). After 15 min incubation at 25°C the bacteriocin solution was rapidly neutralized with HCl, then assayed. Table 7 shows that the reduction in inhibition zone

*Figure 16.* The effect of various alkaline pH values on bacteriocin activity after: ●——● 1 h incubation, o---o 3½ h incubation.



INHIBITION ZONE RADIUS (mm)
4
3
2
1
0
7
8
9
10
11
pH

of samples incubated with and without the layer of oil was the same, suggesting that high pH did not catalyse oxidation.

The effect of oxygen and hydrogen gas on bacteriocin activity was investigated by bubbling the required gas through water (to saturate it and prevent evaporation of the sample) and then through bacteriocin solution at pH 7.0. Platinum catalyst (2 mg/ml) was added to the bacteriocin solution that was treated with hydrogen. At intervals, samples were removed and assayed. Table 8 shows that both oxygen and hydrogen had no effect on the bacteriocin activity. The lack of effect of the hydrogen treatment suggests that the bacteriocin has no double bonds or that if there are double bonds these are not important in determining bacteriocin activity.

Bacteriocin activity was shown to be stable to ultraviolet light, by placing a dish containing a thin layer of bacteriocin solution 4 cm from an ultraviolet light (Oliphant Germicidal Ultraviolet light). No reduction in bacteriocin activity was detected over a period of 6 h.

The effect of heat on bacteriocin activity was investigated by incubating solutions at various temperatures for 10 min, then rapidly cooling and assaying them. Table 9 shows that the bacteriocin activity was completely stable at 60°C for 10 min, slightly degraded at 80°C for 10 min, and completely degraded at 100°C for 10 min.

The effect of sodium periodate, a compound which selectively splits organic molecules between carbon-carbon bonds with both carbon

*Table 7.* The effect of excluding air on the alkaline degradation of bacteriocin.

| Treatment | Inhibition zone radius (mm) |
|---|---|
| Control | 8 |
| 15 min incubation at pH 11 excluding air | 2 |
| 15 min incubation at pH 11 with air present | 2 |

*Table 8.* The effect of oxygen and hydrogen on bacteriocin activity after 3 hours.

| Treatment | Inhibition zone radius (mm) |
|---|---|
| Control | 6 |
| Hydrogen + platinum catalyst | 6 |
| Oxygen | 6 |

*Table 9.* The effect of various temperatures on bacteriocin activity. Samples were incubated at the indicated temperatures for 10 minutes then rapidly cooled and assayed in the standard manner.

| Temperature °C | Inhibition zone radius (mm) |
|---|---|
| Control | 10 |
| 40 | 10 |
| 60 | 10 |
| 80 | 9 |
| 100 | 0 |

atoms carrying hydroxyl groups, was tested by adding sodium periodate to a bacteriocin solution in 0.1M acetate buffer, pH 5.0, to a final concentration of 0.05M and incubating this at 25°C for 23 h. Because sodium periodate proved to be toxic to the bacteria, samples were run on paper electrophoresis (see later) after reaction to separate the bacteriocin from excess periodate. Table 10 shows the result of a typical experiment. It can be seen that periodate destroys all bacteriocin activity suggesting that the bacteriocin may contain a carbon-carbon bond with both carbons having hydroxyl group. The fact that all the bacteriocin activity was destroyed by 1.5 h incubation at 25°C suggests that the hydroxyl groups are in the *cis* configuration. The periodate reaction is generally much slower than this when the hydroxyl groups are in the *trans* configuration.

Although these experiments established some of the important physical and chemical criteria that had to be considered if successful purification of the bacteriocin was to be achieved it is difficult to evaluate the significance of these findings as the effects observed may be the indirect result of the interaction of the treatment, impurities in the preparations and the bacteriocin itself.

Bacteriocin solutions for the following purification experiments were routinely prepared by growing cultures of strain 84 until the optical density reached approximately 80. The bacteria were then removed by centrifugation and the supernatant containing the bacteriocin was

*Table 10.* The effect of sodium periodate treatment on bacteriocin activity. Samples were mixed with an equal volume of 0.1M acetate buffer, pH 5, and sodium periodate added to give a concentration of 0.05M. This was placed at 25°C and at intervals samples were removed, run on high voltage paper electrophoresis in citrate buffer, pH 5, and assayed.

| Treatment | Inhibition at relative mobility of bacteriocin |
|---|---|
| Control | + |
| 1½ h incubation with periodate | - |
| 7 h incubation with periodate | - |
| 23 h incubation with periodate | - |

+ bacteriocin activity

- no bacteriocin activity

adjusted to pH 7.0 with 1M HCl then sterilized by shaking with chloroform. These solutions were stored at 4°C with a small quantity of chloroform and were stable for approximately 1 month.

Paper chromatography of bacteriocin

Although paper chromatography cannot handle the amount of material in large scale purifications it is an extremely useful technique for establishing suitable conditions for such purifications. For this reason crude bacteriocin solutions were subjected to paper chromatography in a variety of solvent systems.

Chromatography was carried out at 25°C on Whatman 3MM paper. Samples of crude bacteriocin solutions were loaded at the origin as thin streaks at a rate of approximately 0.1 ml/cm. The chromatograms were placed in the tank and allowed to equilibrate with the solvent vapour for at least 2 h; then they were run in a descending manner. After the run the chromatograms were allowed to dry, then 1 cm squares cut out along the run, placed on agar plates of Stonier's medium, sterilized with chloroform vapour, then poured with the sensitive indicator strain in phosphate buffered agar. After incubation the inhibition zone radius (measured from the edge of the paper square) was determined.

Table 11 shows the Rf values observed for 4 solvent systems. It can be seen that the Rf increases with increasing water content and increasing polarity of the solvent system suggesting that the bacteriocin may be a polar molecule.

*Table 11.* Rf values of bacteriocin after paper chromatography in a number of solvent systems.

| Solvent system | Rf |
|---|---|
| Butanol : acetic acid : water<br>5 : 2 : 1 | 0.06 |
| Isopropanol : water<br>80 : 20 | 0.15 |
| Isopropanol : water<br>65 : 35 | 0.38 |
| Acetone | 0.45 |

High voltage paper electrophoresis of bacteriocin

Because of the apparent low molecular weight of the bacteriocin it was thought that it may be possible to run it on high-voltage paper electrophoresis. Although this technique is not suitable for large quantities it is very useful in determining whether a molecule is charged or not and can also give information on the nature and magnitude of such charges if observed.

For paper electrophoresis crude bacteriocin solutions were loaded across the centre of a 15 cm by 57 cm strip of Whatman 3MM paper in a similar manner to the paper chromatography. Inorganic phosphate, pyrophosphate, adenosine triphosphate and fructose were loaded as running markers. The paper was then moistened with the required buffer allowing it to creep slowly up to the load streak, then excess buffer blotted off. The paper was placed in a carbon tetrachloride-cooled, high voltage paper electrophoresis apparatus (Tate 1968) and run at 1,500 volts for 1 h. The paper was then removed, dried and assayed in a similar manner to the paper chromatography. The running markers were dipped in phosphate staining reagent (Bartlett 1959), heated at 80°C for 20 min, exposed to ultraviolet light, then the background staining removed by exposure to water vapour from a boiling water bath. The mobility of the bacteriocin relative to pyrophosphate (furthest running marker) and fructose (neutral marker) was determined.

The mobility of the bacteriocin in a variety of buffer systems (Table 12) shows that irrespective of the pH the bacteriocin runs towards the positive electrode. This indicates that the bacteriocin has a

*Table 12.* Relative mobility of bacteriocin activity after high voltage paper electrophoresis in a number of different buffer systems.

| Buffer | pH | Rm |
|---|---|---|
| Tris-citrate | 7.5 | +0.58 |
| Citrate | 5.0 | +0.38 |
| Formic acid - acetic acid | 2.4 | +0.36 |
| Oxalate | 1.5 | +0.29 |

Rm was calculated relative to the position of pyrophosphate and fructose. Composition of buffers is given in Appendix 3.

residual negative charge. The fact that this charge is not suppressed by the low pH of the oxalate buffer indicates that it is unlikely to be a carboxyl group and the two most likely possibilities are either a phosphate or a sulphate group. Although some electrophoretograms were stained with the phosphate reagent in an attempt to demonstrate directly the presence of phosphate at the same mobility as bacteriocin activity this was unsuccessful, suggesting that either the bacteriocin does not contain phosphate or that the stain is not sensitive enough to detect it.

In an attempt to show whether phosphorus was present in the bacteriocin molecule $^{32}P$ was added to a growing culture of strain 84 then the crude bacteriocin solution taken through a number of sequential purification steps. If phosphorus is present in the bacteriocin solution then $^{32}P$ activity should always be associated with bacteriocin activity.

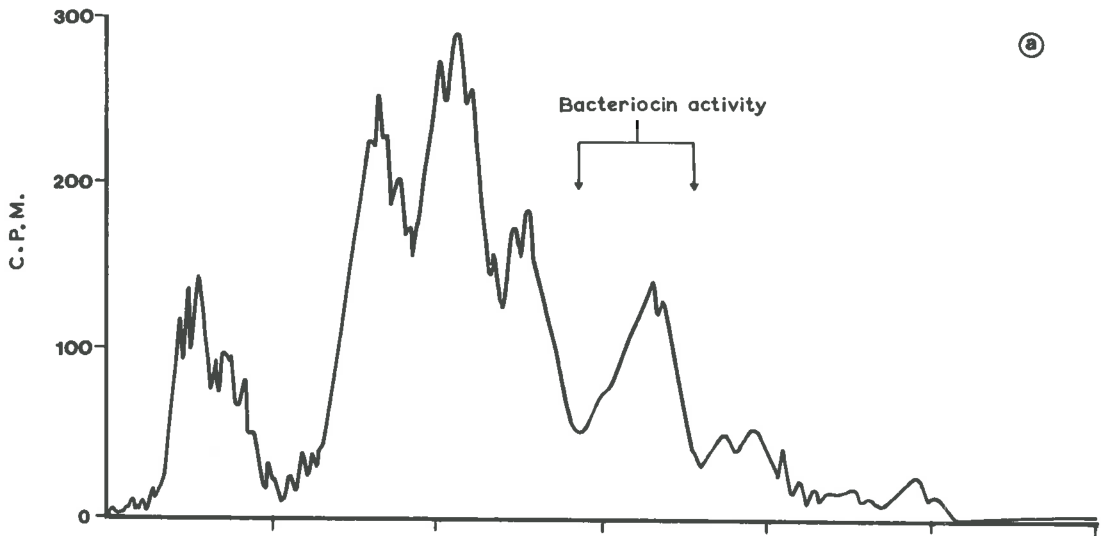
For $^{32}P$ incorporation a culture of strain 84 was grown in low phosphorus (1 µgP/ml) Stonier's medium overnight. This was then used to inoculate another flask of low P Stonier's medium to which was added 1 µC$^{32}$P/ml. This was then grown at 25$^{\circ}$C until the optical density reached approximately 45, the bacteria removed by centrifugation and the supernatant sterilized by shaking with chloroform.

A 0.7 ml aliquot of this solution was loaded as a 7 cm wide streak on Whatman 3MM paper and run at 1,500 V for 1½ h in the high-voltage electrophoresis apparatus in citrate buffer, pH 5.0. After the run

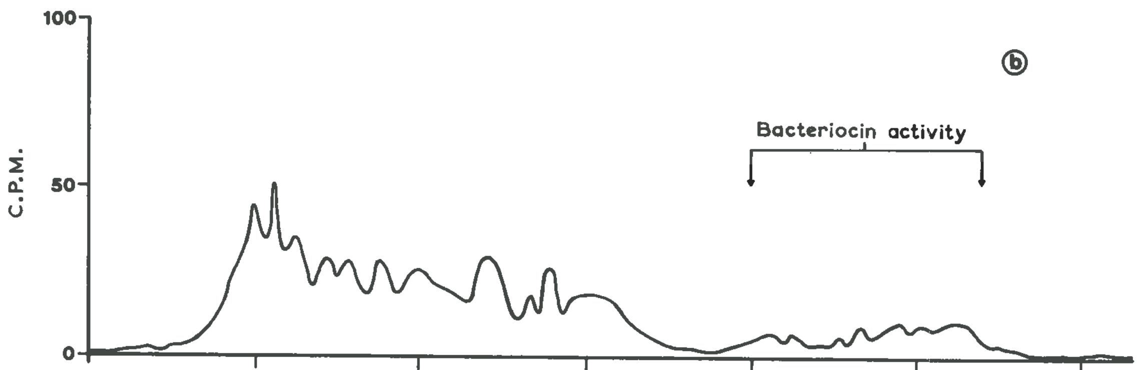
the electrophoretogram was scanned for radioactivity in a Packard radiochromatogram scanner and then assayed for bacteriocin activity. Figure 17(a) shows that most of the $^{32}P$ was incorporated into compounds which did not run very far from the origin but there is a peak of $^{32}P$ activity associated with bacteriocin activity. The electrophoretogram was eluted with distilled water in the area corresponding to bacteriocin activity and this was then loaded on another strip of Whatman 3MM paper. This was run at 1,500 volts for 3 h in formic acid buffer, pH 2.4, then dried, scanned for radioactivity and assayed for bacteriocin activity (Fig. 17(b)). Again there was an area of $^{32}P$ activity that corresponded to bacteriocin activity which was well separated from most of the radioactivity. This electrophoretogram was eluted as before, loaded on a sheet of Whatman 3MM paper and then run overnight in descending chromatography with a solvent system of butanol : acetic acid : water 5 : 1 : 2. This was then dried, scanned for radioactivity and assayed. Again $^{32}P$ activity corresponded to bacteriocin activity (Fig. 17(c)). These experiments provide very strong evidence that the charge associated with the bacteriocin is due to the presence of a phosphate group.

By calculating the amount of radioactive phosphorus in the area of bacteriocin activity and assuming that $^{32}P$ and non-radioactive phosphorus are incorporated into bacteriocin in proportion to their relative concentrations it is possible to estimate approximately the

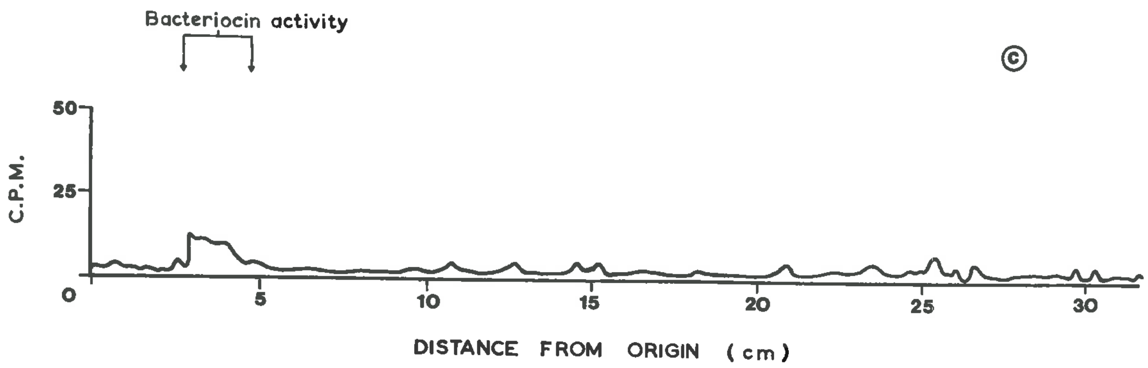
*Figure 17.* Demonstration of the phosphorus content of bacteriocin. a. bacteriocin sample run on high voltage paper electrophoresis with citrate buffer, pH 5.0. b. area of bacteriocin activity extracted from (a) and run on high voltage paper electrophoresis with formic acid-acetic acid buffer, pH 2.4. c. area of bacteriocin extracted from (b) and run on paper chromatography in a solvent system of butanol : acetic acid : water 5 : 1 : 2.



300
200
100
0
C.P.M.
Bacteriocin activity
a

100
50
0
C.P.M.
Bacteriocin activity
b

Bacteriocin activity
c
50
25
0
C.P.M.
5
10
15
20
25
30
DISTANCE FROM ORIGIN (cm)

total amount of phosphorus in this aréa. If it is assumed that there is one phosphorus group per bacteriocin molecule it can be calculated that the bacteriocin is biologically active at a concentration in the order of $10^{-7}$M. This indicates the degree of concentration and purification needed before chemical analysis can successfully be carried out.

Gel filtration of bacteriocin

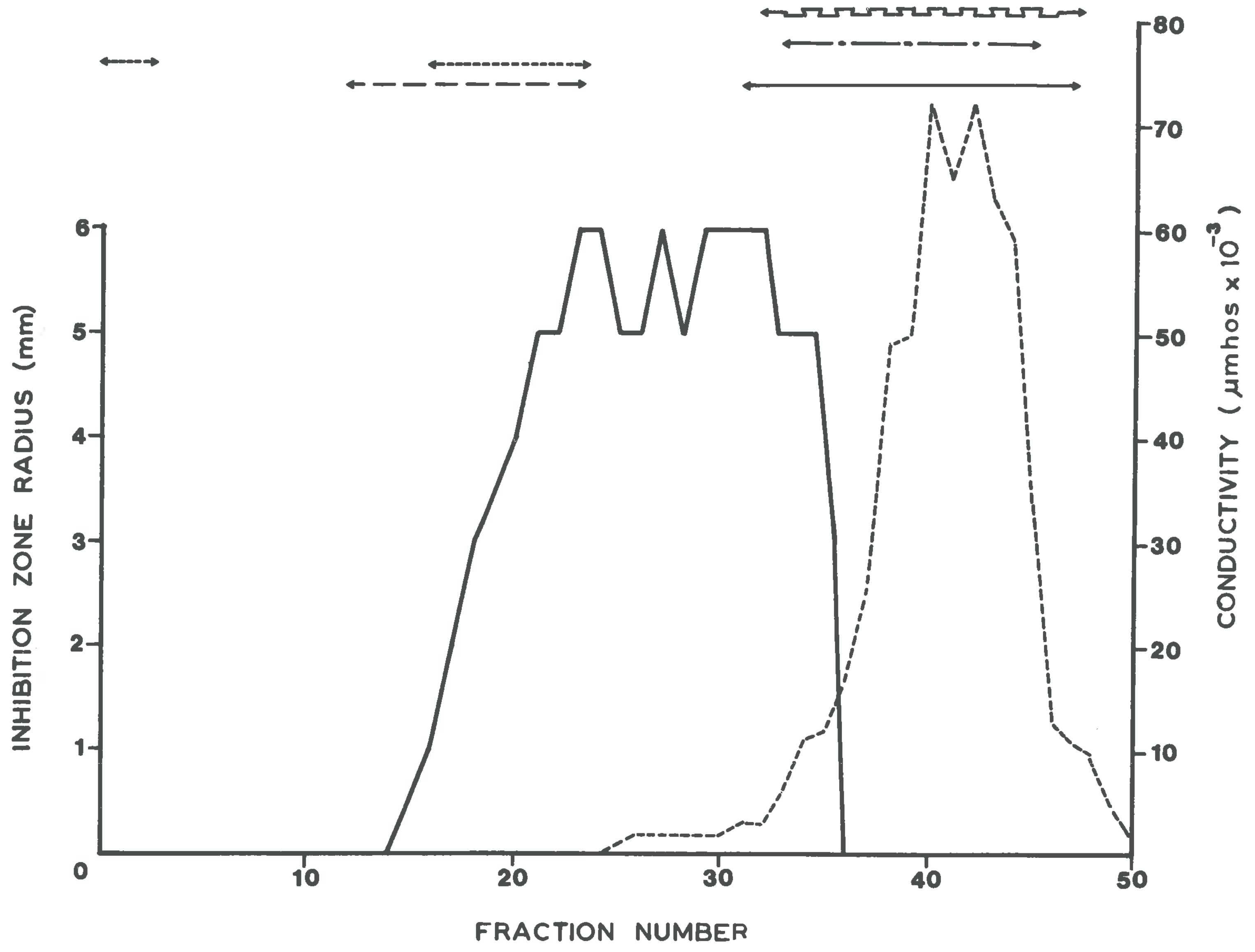
One of the first steps necessary in the purification of compounds from complex mixtures such as culture medium is to remove the bulk of the salts and nutrients. Although the bacteriocin is of relatively low molecular weight it is probably significantly higher than most of the salts and nutrients in the medium, so it seemed likely that the bacteriocin could be separated from the bulk of these impurities on the basis of molecular weight differences. One of the best techniques of separating compounds according to their molecular weight is the technique of gel filtration (Determan 1969). This involves running the mixture through columns packed with a gel that has a carefully controlled pore size. Molecules that can enter the gel are eluted later compared with compounds which do not; so high molecular weight compounds come out of the gel ahead of low molecular weight compounds. The most easily available and convenient packing for such gels is Sephadex, a commercially available cross-linked dextran, available in a range of pore sizes.

Sephadex columns were packed in glass tubes with a glass fibre support and thin outlet tube at one end. The gel, which had been allowed

to swell in water, was poured into these columns which were already partially filled with water. As the gel settled the water was allowed to drip slowly out the bottom and more gel added at the top until the desired column length had been reached. After packing, the columns were routinely washed with a large volume of water to stabilize the gel and remove impurities and free dextran. The packing of the columns was checked using blue dextran 2000 dissolved in NaCl solution. On well packed columns the blue dextran ran as a discrete band with little tailing and the NaCl (determined by conductivity or refractive index measurements) ran as a sharp peak. These compounds were also used to calculate the included and excluded volume of the columns.

Figure 18 shows one run of crude bacteriocin on a Sephadex G25 column using water as the eluant. After the run 20 µl aliquots of each fraction were assayed in the standard manner. It can be seen that the bacteriocin activity ran as a broad peak just in front of the peak of conductivity suggesting that it has a higher molecular weight than the bulk of the salts. Aliquots (0.1 ml) of each fraction were also placed on filter paper strips and then these were stained for a number of different compounds. No correspondence between any of the staining compounds and bacteriocin activity was observed suggesting that either the bacteriocin belongs to none of the groups of compounds stained for, or that the stains are too insensitive. Figure 18 suggests that G25 Sephadex filtration would be a useful technique for partial purification of bacteriocin. However, when the concentration of bacteriocin was

*Figure 18.* Gel filtration of bacteriocin on Sephadex G25 using water as the eluant. —— bacteriocin activity, --- conductivity, ←-→ nigrosine stain (proteins), ← — → coomassie blue stain (proteins), ←——→ ninhydrin stain (amino acids), ←— — —→ silver nitrate stain (reducing compounds), ←ꟷꟷꟷ→ iodine stain (polysaccharides, lipids).

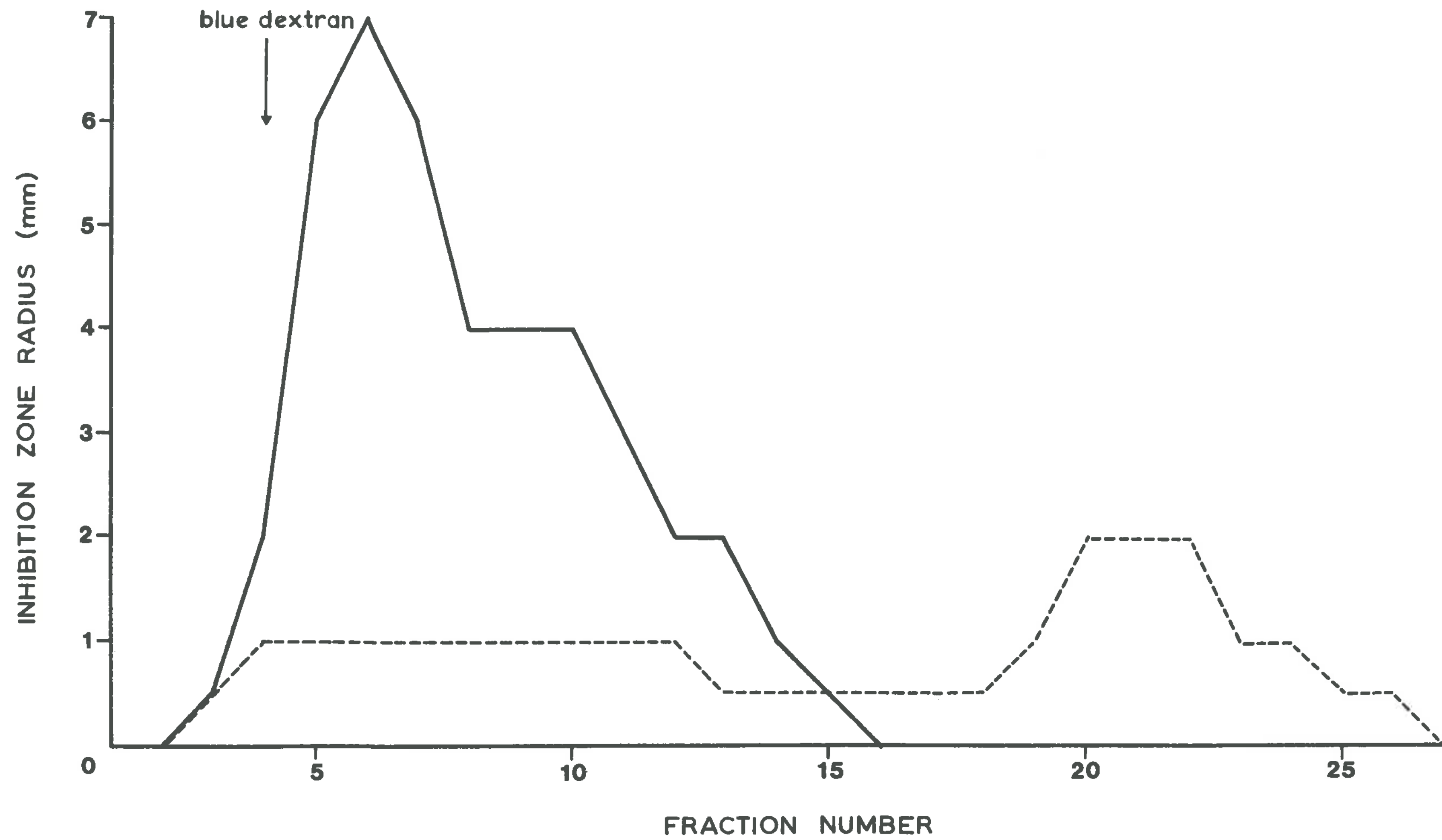


INHIBITION ZONE RADIUS (mm)
CONDUCTIVITY ( μmhos x 10^-3 )
FRACTION NUMBER
0
1
2
3
4
5
6
10
20
30
40
50
60
70
80

increased the bacteriocin showed a very wide spread which often appeared to be resolved into a number of peaks. Obviously the separation of bacteriocin on these columns was very much load dependent. Figure 19 shows the effect of concentration of bacteriocin on separation when all other conditions were kept as constant as possible. When a low concentration was used the bacteriocin eluted as a broad peak just behind the totally excluded blue dextran peak. However, when a more concentrated sample was loaded in the same volume, two peaks of bacteriocin activity were detected, one of which appeared to correspond to the peak in the low concentration run, and another which was apparently of lower molecular weight. When these two peaks were run separately on high voltage paper electrophoresis and paper chromatography they showed identical behaviour suggesting that the resolution observed in Figure 19 is an artifact of the method used.

There are a number of mechanisms which could be involved in producing such artifacts. One possibility is that under certain conditions bacteriocin molecules aggregate and so produce a range of sizes which are partially resolved in these experiments. It is possible that such aggregates may break up under different conditions and so account for the similarity of behaviour of both peaks on other separation methods. However, it would be expected that aggregation would be more likely in highly concentrated samples, yet the high concentration run of Figure 19 shows that apparently low molecular weight bacteriocin is only detected under these conditions. Another possibility is that the bacteriocin interacts in some way with the gel. Gelotte (1960) reported that low

*Figure 19.* The effect of load on the gel filtration of bacteriocin on Sephadex G25 using water as the eluant. ——— 5 ml crude bacteriocin, ---- 50 ml crude bacteriocin concentrated to 5 ml before loading. After the run samples from both the unconcentrated and concentrated load were adjusted to equivalent concentrations before assaying. Fractions were 10 ml. Column 3.5 cm diameter by 43 cm length.



blue dextran
INHIBITION ZONE RADIUS (mm)
0
1
2
3
4
5
6
7
5
10
15
20
25
FRACTION NUMBER

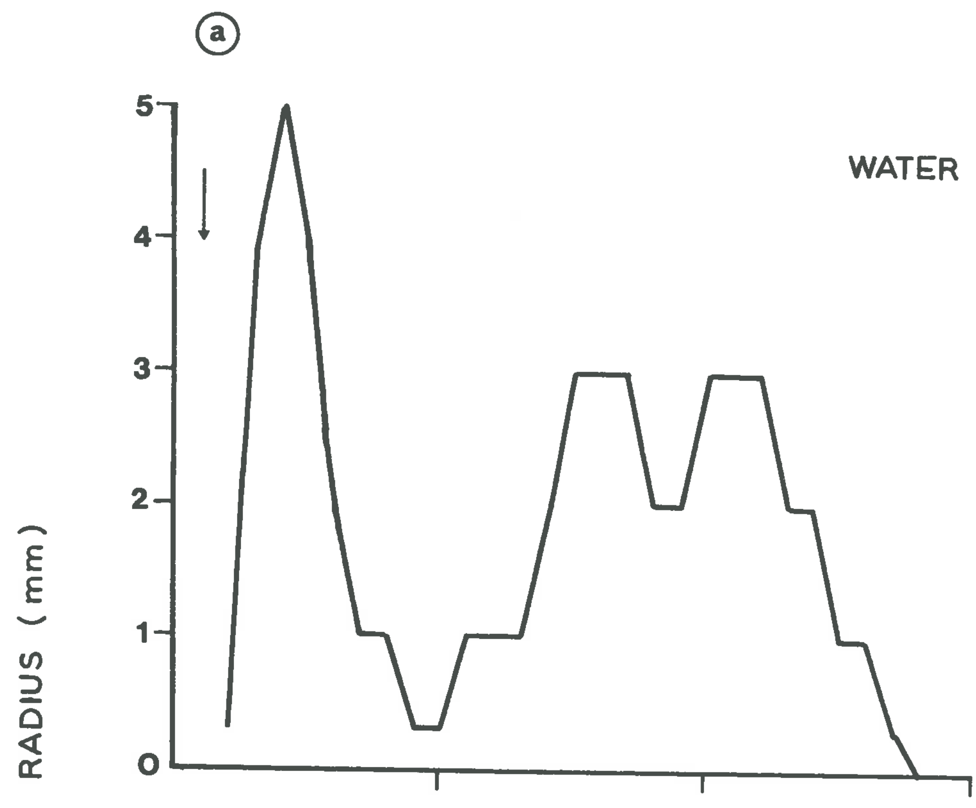
molecular weight anions eluted earlier from Sephadex gels than would be expected on the basis of their molecular weight. Miranda, Rochat and Lissitzky (1962) demonstrated that Sephadex gels have a low proportion of residual negative charges and Neddermyer and Rogers (1968) suggested that low molecular weight anions were partially excluded from these gels by interaction with such charges. They reported that the elution volume of such molecules increased with increasing sample concentration and ionic strength of the buffer and that at high concentrations these molecules eluted strictly according to their molecular size. Similar anion exclusion effects on Sephadex gels were noted by Steward and Tate (1969). As the bacteriocin has a strong residual negative charge it seems likely that such effects are likely to be important in determining the behaviour of bacteriocin on Sephadex gels.

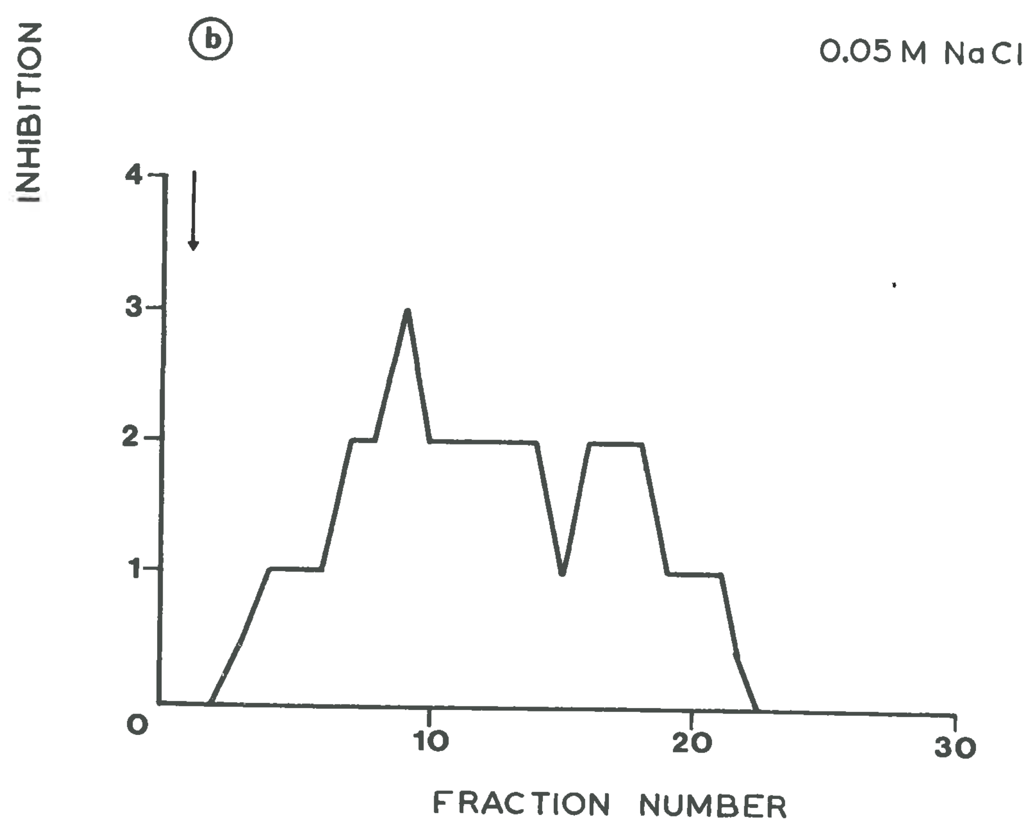
Another possibility is that if the bacteriocin has an aromatic structure there may be some direct interaction with the gel itself. Janson (1967) reviewed these sort of interactions and concluded that they were due to the presence of some sort of adsorption sites on the Sephadex. Adsorption was found to be highest at high salt concentrations and he suggested that this was due either to a reduction in the hydration shell surrounding the aromatic substances allowing stronger interactions with the adsorption sites or an increase in the number of adsorption sites. To determine whether any of these mechanisms may be responsible for the behaviour of the bacteriocin on Sephadex gels a number of samples were run on the same column using different NaCl concentrations in the eluant.

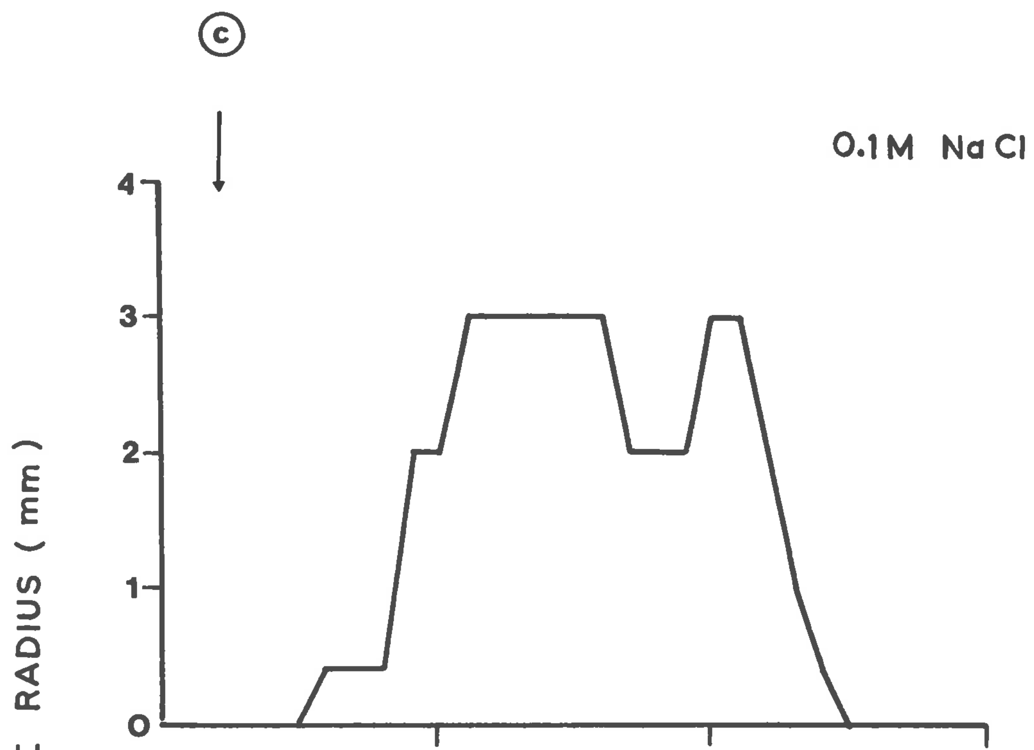
Figure 20(a) shows the result when only distilled water is used. The bacteriocin was resolved into two peaks, one of which ran just behind the blue dextran peak and the other just ahead of the salt peak. When 0.05M NaCl was added to the eluant (Fig. 20(b)) the first peak was no longer observed although there appeared to be some resolution of the activity into two ill-defined peaks. Figure 20(c) shows that 0.1M NaCl had substantially the same effect as 0.05M NaCl. Figure 20(d) shows the effect of adding 0.001M EDTA to the eluant. Two well-resolved peaks were observed which appeared to correspond to the peaks observed with no salt in the eluant.

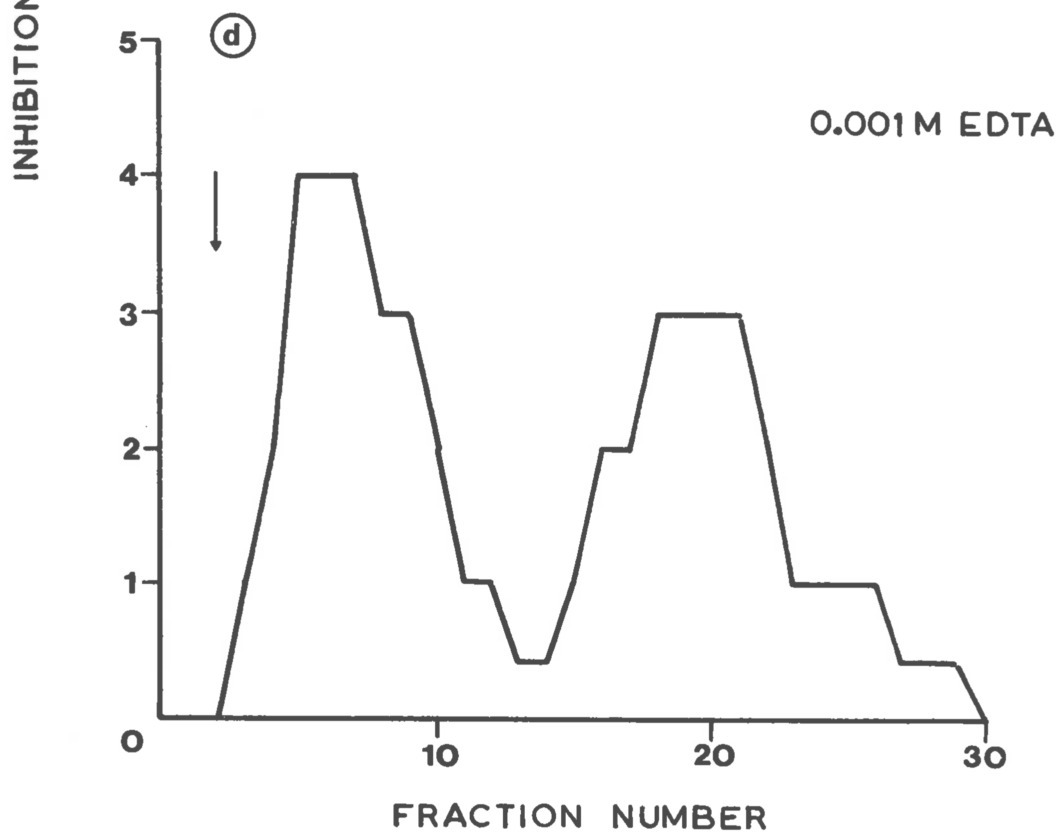
These results and the results with different concentrations of bacteriocin (Fig. 19) suggest that the ionic conditions have a significant effect on the elution pattern of bacteriocin from Sephadex columns. Although it was not possible to determine precisely the mechanism of the ionic concentration effects it appears likely that both anion exclusion and aromatic adsorption effects may be significant. For example, the second peak observed in Figure 20(a) may represent the bacteriocin that was bound to the adsorption sites. However, as the number of sites is limited all the bacteriocin may not be able to bind to such sites and excess free bacteriocin may be influenced by anion exclusion to form the first peak. As the ionic conditions are changed due either to more concentrated samples or adding NaCl to the eluant, so the relative importance of these effects would change. The amount of bacteriocin activity recovered from the Sephadex G25 columns after a run was also dependent on the ionic conditions. For example, samples from both runs

*Figure 20.* The effect of various NaCl concentrations and EDTA on the gel filtration of bacteriocin on Sephadex G25. Two ml of 10x concentrated bacteriocin were loaded on each run. a. distilled water as the eluant. b. 0.05M NaCl as the eluant. c. 0.1M NaCl as the eluant. d. 0.001M EDTA as the eluant. Fractions were 10 ml. Column 3.5 cm diameter by 43 cm length. Arrows indicate blue dextran peaks.


a
WATER
5
4
3
2
1
0
INHIBITION ZONE RADIUS ( mm )


b
0.05 M NaCl
4
3
2
1
0
10
20
30
FRACTION NUMBER


c
0.1M NaCl
4
3
2
1
0


INHIBITION ZONE RADIUS ( mm )
d
0.001M EDTA
5
4
3
2
1
0
10
20
30
FRACTION NUMBER

in Figure 19 were adjusted to give the same final concentration before assay, yet much less bacteriocin activity was detected in the run with the concentrated sample. A similar loss in bacteriocin activity is also evident in the 0.1M NaCl and 0.05M NaCl runs of Figure 20. There are two possible mechanisms by which such a loss could occur under these conditions; either bacteriocin is irreversibly bound to the column or, it elutes as a very broad peak only part of which is active enough to be detected by the bioassay. The experimental data does not discriminate between these two possibilities.

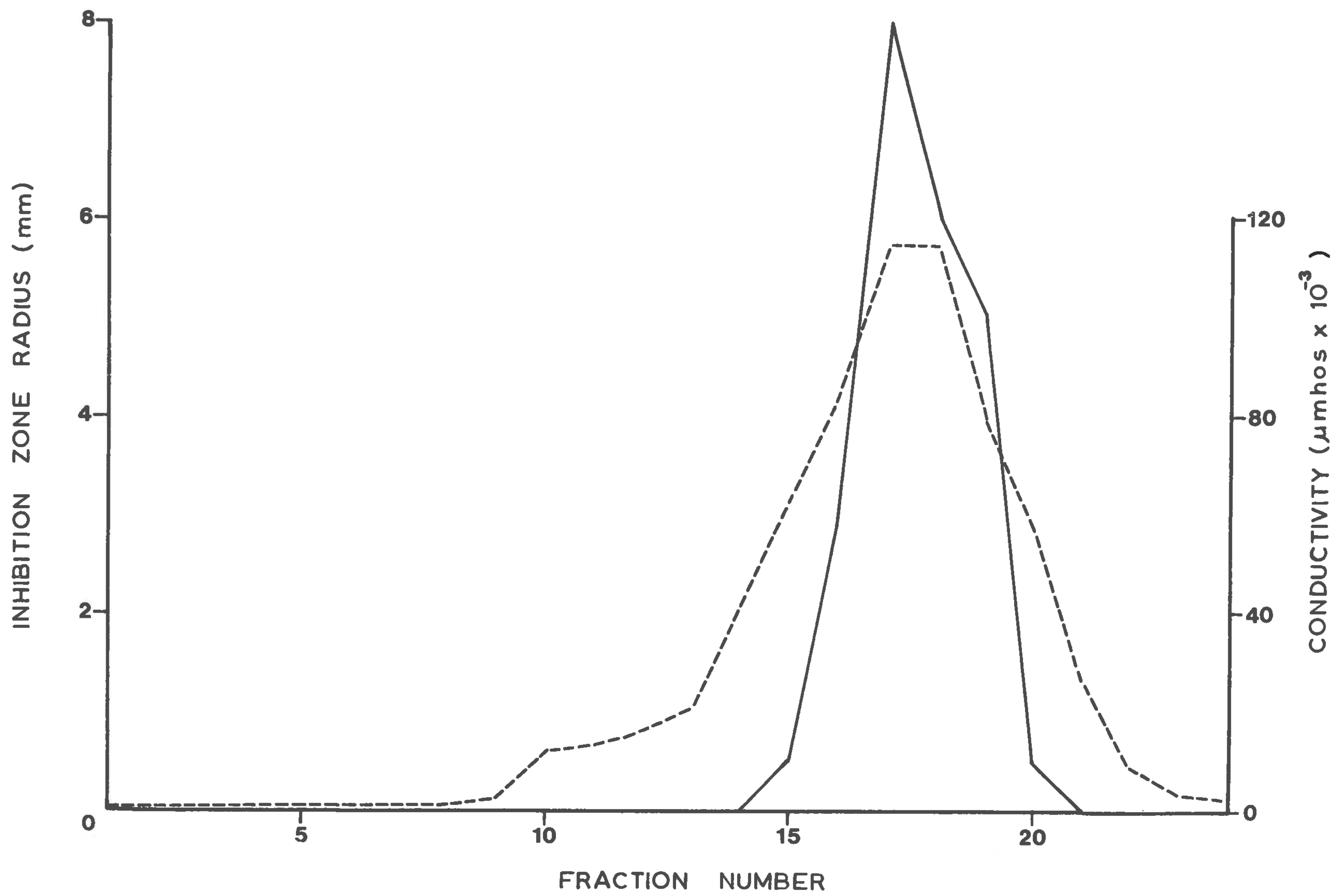
These experiments indicate that Sephadex G25 gel filtration was unlikely to be a useful purification method at least in the first stages as the resolution obtained was strongly influenced by the concentration of the sample and the concentration of ions in the eluant.

The other Sephadex gels (G50, G100 and G200) have much fewer residual negative charges and also would be likely to show less adsorption effects as they have a much lower level of cross-linking. Bacteriocin was run on columns packed with these gels. One run with Sephadex G100 using water as the eluant is shown in Figure 21 where it can be seen that although the bacteriocin ran as a sharp peak no resolution from the salts was achieved. Similar results were obtained with Sephadex G50 and G200 suggesting that these gels with larger pore size would not be useful for purification.

Ion exchange chromatography of bacteriocin

High-voltage paper electrophoresis had demonstrated the presence

*Figure 21.* Gel filtration of bacteriocin on Sephadex G100 using water as the eluant. —— bacteriocin activity, ---- conductivity. Column 2.3 cm diameter by 16 cm length. Fractions were 5 ml.


INHIBITION ZONE RADIUS (mm)
8
6
4
2
0
5
10
15
20
FRACTION NUMBER
120
80
40
0
CONDUCTIVITY (μmhos x 10⁻³)

of a residual negative charge on the bacteriocin molecule which is probably due to the presence of a phosphate group. This suggested that chromatography of the bacteriocin on an anion exchange resin may be a useful technique for purification.

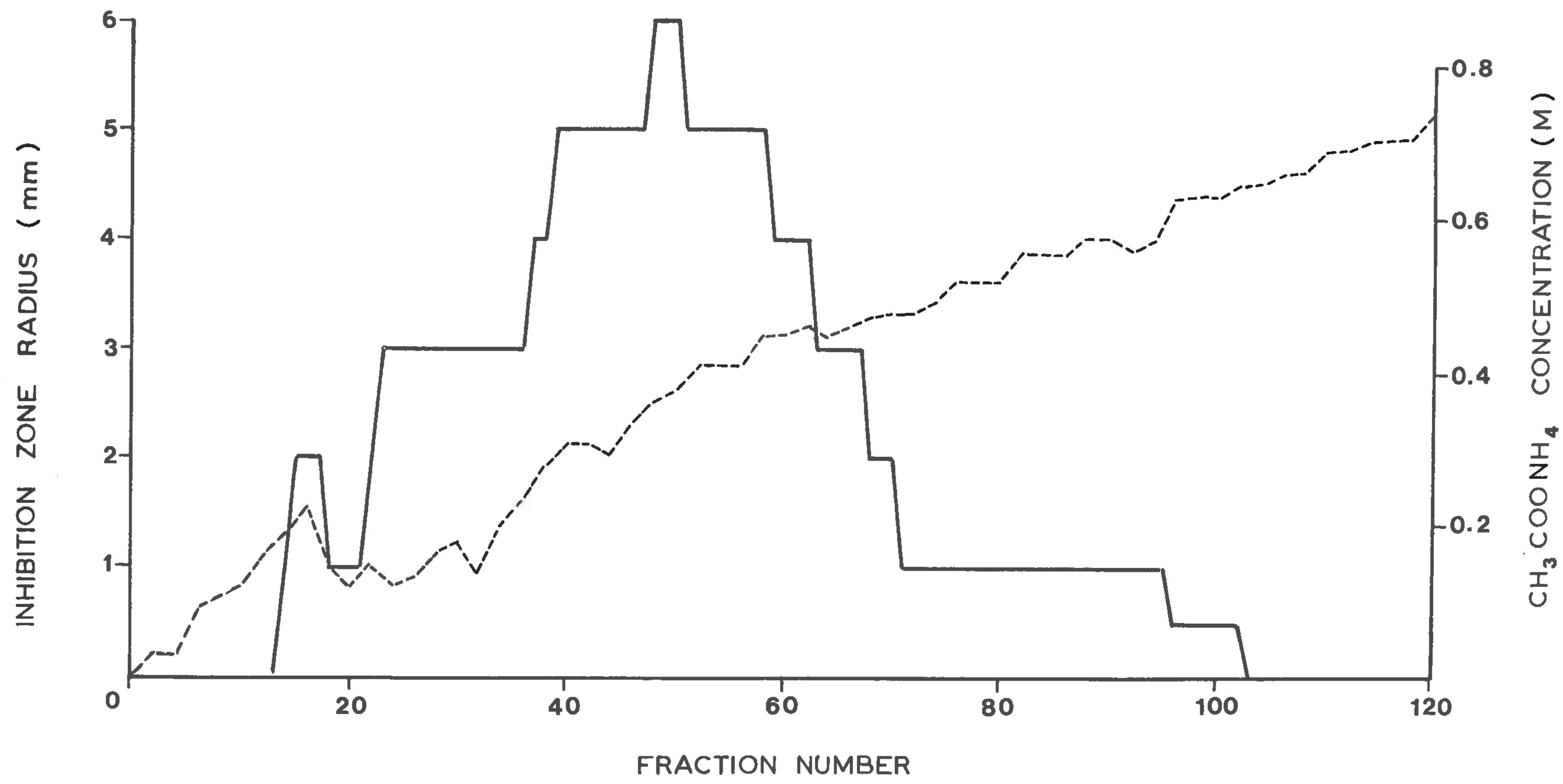
The resin chosen for these experiments was a polystyrene based material containing quaternary ammonium groups as the exchanging sites; Dowex AG1-X8. This is a moderately cross-linked resin with a high exchange capacity. Ammonium acetate was chosen as the displacing salt as preliminary experiments indicated that this could be removed relatively easily by lyophilization in an apparatus using phosphorus pentoxide as a moisture trap. It was considered necessary to choose a volatile salt as the usual method of salt removal after ion exchange chromatography, gel filtration, had already been shown to be unsuitable.

Dowex AG1-X8 was washed with 2M NaOH, then washed extensively with water to swell the gel and remove impurities. The resin was then converted to the acetate form by washing with 2M ammonium acetate followed by water to remove excess free salts. The resin was packed into a column using a similar technique to that for the Sephadex and washed with water to stabilize the column. The bacteriocin sample was run into the column and then water was run through the column to remove any compounds not binding. No bacteriocin was detected in this wash. A linear gradient from 0 to 1M ammonium acetate was then run through the column. The gradient was produced by two vessels of the same size,

one of which contained water and the other 1M ammonium acetate. The vessel with water contained a magnetic stirring bar and was connected to the top of the column and to the other vessel by siphon tubes. As liquid is removed from the stirred vessel, ammonium acetate siphons in from the other vessel producing a linear gradient. After the run 50 µl aliquot samples were placed on 1 cm squares and dried in a stream of air. These squares were then placed on agar plates of Stonier's medium, sterilized with chloroform vapour and then poured with buffered agar containing the sensitive indicator strain. After incubation the inhibition zone radius from the edge of the paper square was measured. This method of assay was used when volatile compounds which may have interfered with the assay were present. The gradient was checked by refractive index measurements.

Figure 22 shows that bacteriocin started to come off the column at an ammonium acetate concentration of 0.35M. However, it was not until the concentration reached 0.46M that the peak was observed and bacteriocin was not completely eluted until the concentration reached 0.65M. This was a very large spread with the bacteriocin activity eluted in a total volume of over 1 litre. The large spread observed suggests that the bacteriocin is being influenced by opposing forces. There is the increasing ionic strength tending to displace the bacteriocin from the exchange sites and some other force tending to make it bind more strongly. If the bacteriocin contained a non-polar group then

*Figure 22.* Dowex ion-exchange chromatography of bacteriocin. ——— bacteriocin activity, ----- increasing gradient of ammonium acetate. Column 3.2 cm diameter by 23.5 cm length. Fractions were 15 ml.


INHIBITION ZONE RADIUS ( mm )
$CH_3COONH_4$ CONCENTRATION (M)
FRACTION NUMBER
0
20
40
60
80
100
120
1
2
3
4
5
6
0.2
0.4
0.6
0.8

hydrophobic forces may be responsible for the spread observed. These forces would increase as the ionic strength of the eluant increased. Another possibility is that the bacteriocin has an aromatic structure and so is adsorbed onto the resin (Reichenberg 1957). Such interactions would probably also increase with increasing salt concentration.

A number of ion-exchange resin columns were run using different displacing salts and sometimes incorporating ethanol into the eluant in an attempt to resolve bacteriocin activity into a sharp peak but none of these methods proved satisfactory. In an attempt to reduce possible aromatic interactions with the ion exchange material bacteriocin was run on columns packed with the anion exchanger, DEAE cellulose. This does not have the strongly cross-linked aromatic structure of Dowex AG1-X8.

DEAE cellulose (Whatman DE11) was placed in 1N NaOH for 30 min then filtered, washed with water and placed in 1N HCl for 30 min. Then the cellulose was again filtered, washed and treated with 1N NaOH for 30 min, then finally washed extensively in water. This treatment removes impurities from the cellulose and exposes the maximum number of exchange sites. The cellulose was then washed with ammonium acetate to convert it to the acetate form and finally washed with water. The cellulose was packed into columns using a similar technique to that for Sephadex. Columns were run and fractions assayed using similar techniques to those for the ion exchange resin.

Figure 23 shows that the resolution obtained on the cellulose column was no better than the resolution on the resin, suggesting that similar forces are operating in both types of material. Although cellulose has not the cross-linked ring structure of the resin it does have an extensive chain structure of cyclic sugars and so interactions similar to the aromatic ring interactions observed in resins may also be important. Hydrophobic forces are also likely to be significant in DEAE cellulose columns.

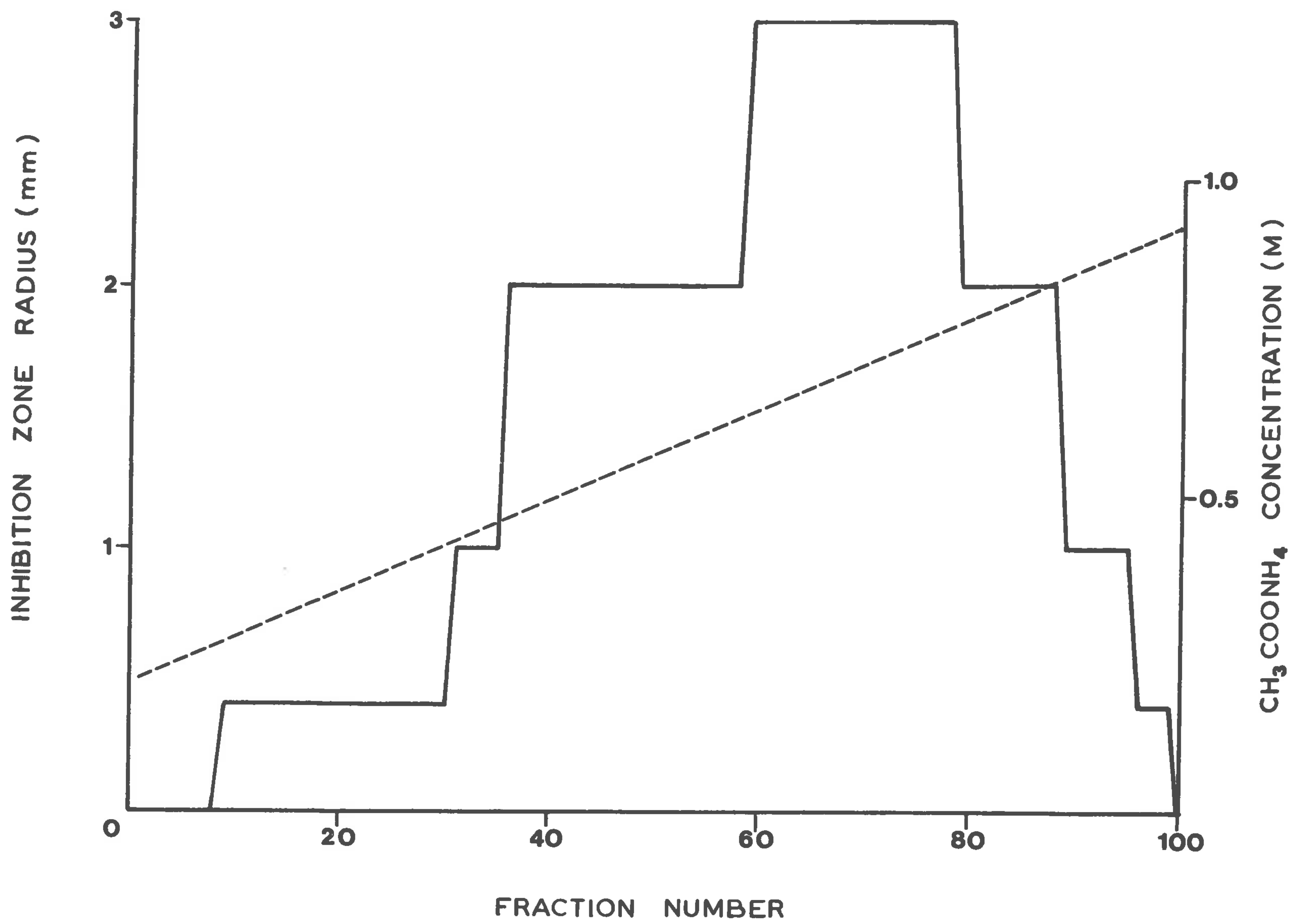
These experiments indicated that hydrophobic forces and adsorption effects may have a significant influence on the behaviour of the bacteriocin on chromatography columns and it was possible that such effects may be useful in purification methods.

Carbon chromatography of bacteriocin

Although the precise mechanism is not clear, charcoal columns can be used to separate molecules on the basis of hydrophobic forces and adsorption phenomena (Morris and Morris 1963) and so purification of the bacteriocin using charcoal was attempted.

Charcoal (BDH decolourising charcoal) was mixed with an equal weight of celite and then digested overnight in concentrated HCl. It was then washed extensively with water until the wash water was close to neutrality and packed into a column with a layer of celite at the bottom. The celite mixed with the charcoal improves the flow while the celite at the bottom of the column prevents leakage of the very fine charcoal. Preliminary experiments carried out by adding charcoal to bacteriocin in

*Figure 23.* DEAE-cellulose chromatography of bacteriocin. ——— bacteriocin activity, ----- increasing gradient of ammonium acetate. Column 2.2 cm diameter by 12 cm length. Fractions were 15 ml.



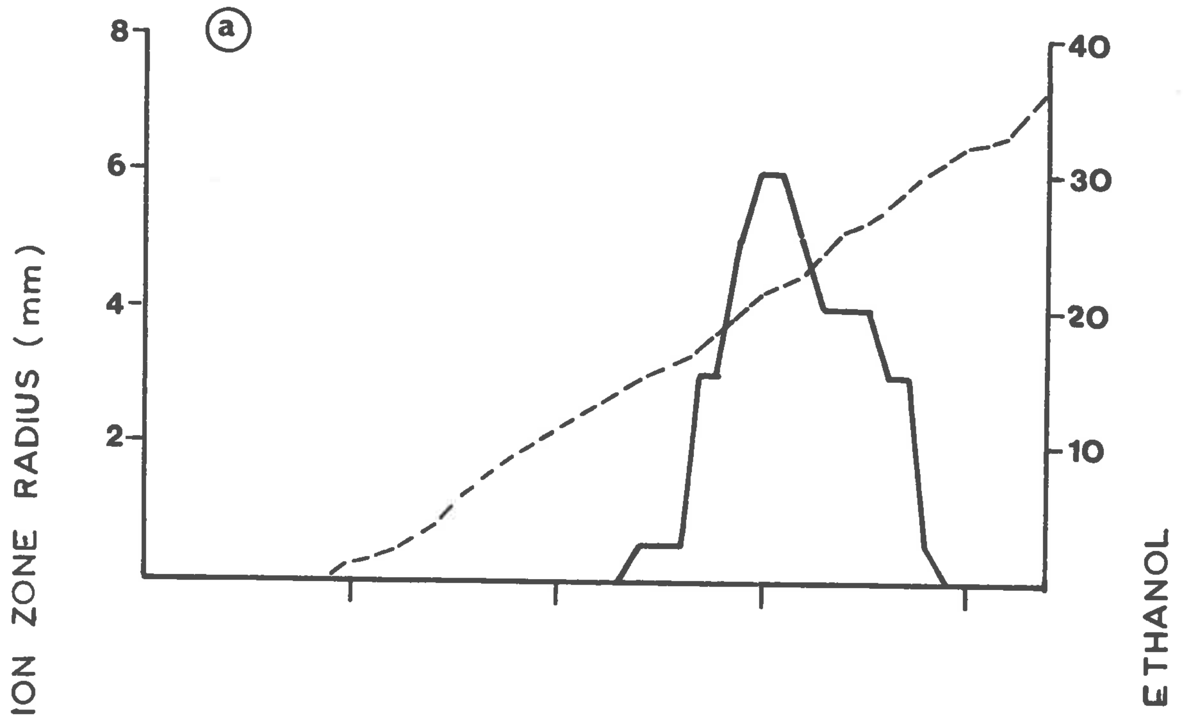
INHIBITION ZONE RADIUS (mm)
3
2
1
0
20
40
60
80
100
FRACTION NUMBER
1.0
0.5
$CH_3COONH_4$ CONCENTRATION (M)

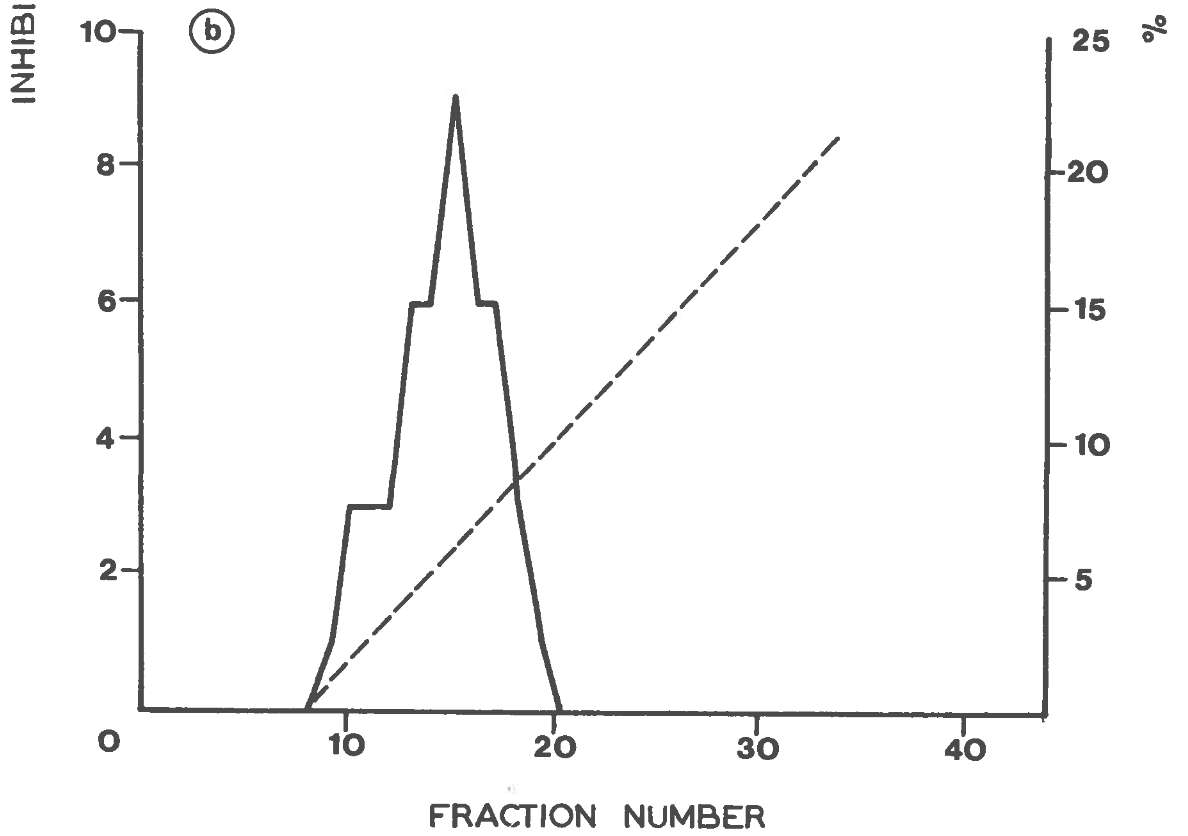
centrifuge tubes, incubating for 10 min, then pelleting the charcoal and assaying the supernatant for bacteriocin activity had indicated that the charcoal had a very high capacity for binding bacteriocin.

Figure 24(b) shows the result of a carbon column run where the bacteriocin was eluted with an increasing gradient of ethanol concentration. Bacteriocin started to be released as soon as the ethanol reached the column but it did not reach a peak until the ethanol concentration reached 3%. Bacteriocin activity was only fully eluted when the ethanol concentration reached 10%. Obviously there is little chance of good resolution of the bacteriocin from other compounds when it is released at such an early stage of the ethanol gradient.

Figure 24(a) shows the result that was obtained when 0.05M ammonium acetate was added to the eluant. This increased the binding forces in the column and so a much higher concentration of ethanol was needed. However, as in Figure 24(b) there is a large spread in the bacteriocin activity suggesting that there are a number of opposing forces involved in bacteriocin binding to the column. It is possible, for example, that although the increasing ethanol concentration decreases the hydrophobic forces it increases the adsorption forces and so produces the result observed. An alternative possibility is that the spread in activity is due to the heterogeneity of the binding sites on the charcoal. Such heterogeneity has been observed in other studies (Synge and Tiselius 1949). Although the considerable spread in the peak of bacteriocin activity observed in Figure 24 suggested that charcoal

*Figure 24.* Carbon column chromatography of bacteriocin using: a. an increasing gradient of ethanol in 0.05 ammonium acetate. b. increasing gradient of ethanol alone. ——— bacteriocin activity, ----- ethanol gradient. Fractions were 10 ml. Column 2.2 cm diameter by 7 cm length.


a
8
6
4
2
40
30
20
10
INHIBITION ZONE RADIUS ( mm )
% ETHANOL


b
10
8
6
4
2
0
25
20
15
10
5
10
20
30
40
FRACTION NUMBER

would not be very suitable for column chromatography of the bacteriocin it was often used in a batch technique to achieve some initial purification and concentration. This was carried out by stirring charcoal (treated as for columns) into crude solutions of bacteriocin. The charcoal was then collected on a filter, washed with water and then the bacteriocin eluted with 10% ethanol. This method was useful in removing much of the salts from the bacteriocin as they did not bind to the carbon. Some concentration could also be achieved by using a small volume of 10% ethanol as the eluting agent.

Cellulose column chromatography of bacteriocin

The behaviour of bacteriocin on paper chromatography has been dealt with earlier. With small amounts of bacteriocin quite good resolution could be achieved in a variety of solvent systems. However, paper chromatography is not really suitable for large scale purification. Cellulose powder can be used to pack columns which show similar separation properties to sheets of paper but have a much greater capacity. The application of cellulose columns to bacteriocin purification is dealt with in this section.

Whatman cellulose powder was mixed at top speed for 2 min in a Sorvall omnimixer with 2 volumes (w/v) of isopropanol : water (4 : 1), packed into a column and then washed with more solvent. Bacteriocin solutions were mixed with enough isopropanol to bring them to the same proportions as the solvent system and then layered directly on the top

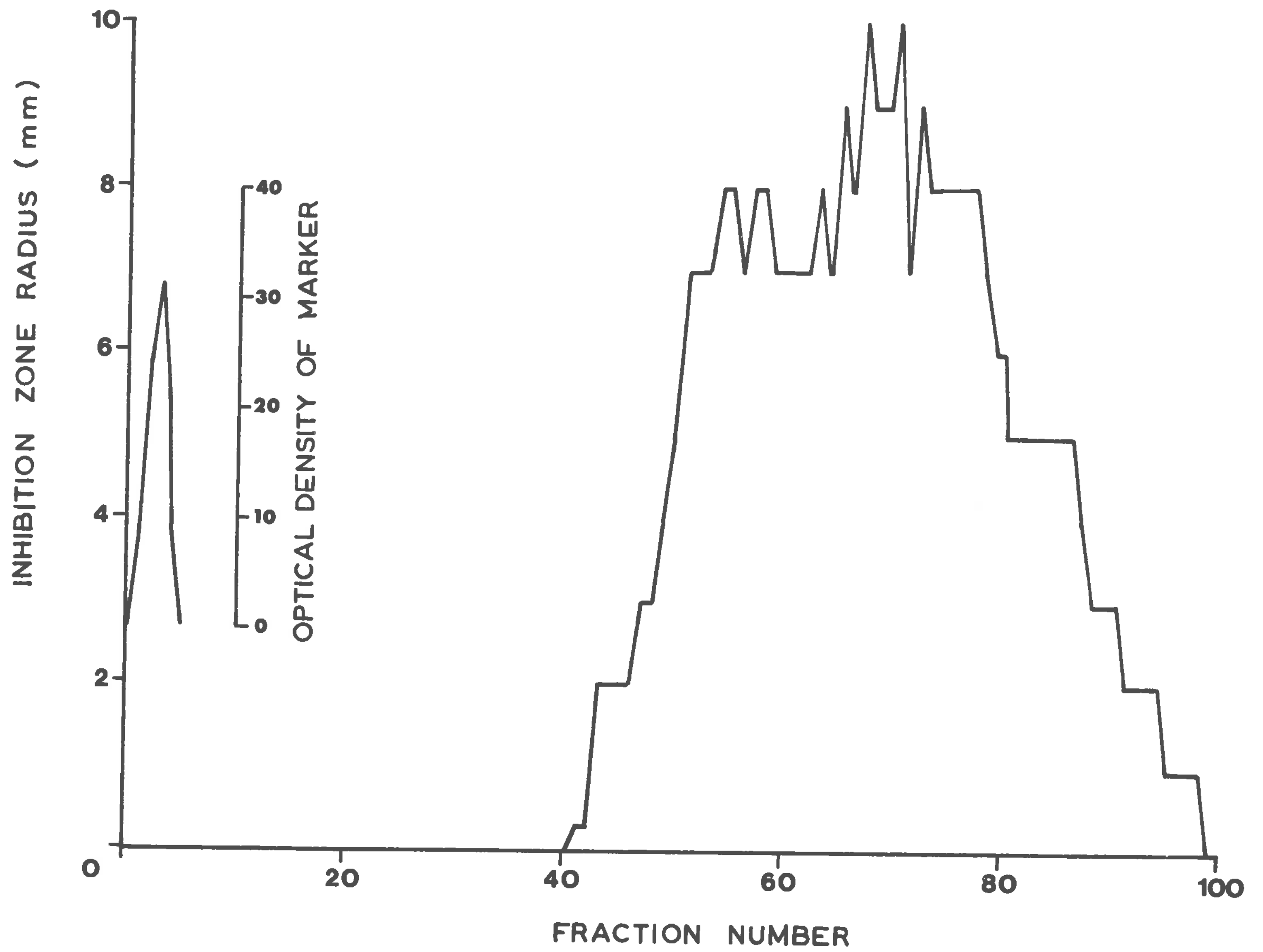
of the column and the run started. Generally a small amount of cotton red was added to the sample as a marker dye. After the run 50 µl aliquots of each sample were placed on 1 cm squares of filter paper, dried with a stream of air and assayed. Bacteriocin ran as a widely spread peak starting approximately 40 fractions behind the dye peak (Fig. 25). It is possible that the wide spread observed is due to gross overloading of the column but the fact that the marker dye ran as a discrete peak as did a yellow coloured impurity that ran well behind the bacteriocin activity suggests that this is not the problem. Presumably the sort of interactions demonstrated with other column packing materials are also important in cellulose columns. Although cellulose columns did not give tight resolution of bacteriocin this technique did not add any non-volatile material to the samples and so it was used in a number of purification attempts.

Bulk purification of bacteriocin

Although most of the purification experiments described already were performed on crude bacteriocin samples attempts were also made to purify the bacteriocin using a series of these techniques.

The usual starting sequence involved the adsorption of bacteriocin from the crude solution using carbon. The bacteriocin was eluted from the carbon with 10% ethanol which was then evaporated to dryness in a Rotavapour apparatus. The bacteriocin was then redissolved in water and 8 volumes of absolute ethanol added to precipitate much of the

*Figure 25.* Cellulose column chromatography of bacteriocin in a solvent system of isopropanol : water 80 : 20. Fractions were 15 ml. Marker dye was cotton red. Column 3.4 cm diameter by 21 cm length.


INHIBITION ZONE RADIUS (mm)
10
8
6
4
2
0
OPTICAL DENSITY OF MARKER
40
30
20
10
0
20
40
60
80
100
FRACTION NUMBER

remaining salts, which were then filtered off. The filtrate, containing the bacteriocin, was then evaporated to dryness, redissolved in a small volume of isopropanol : water (4 : 1) and then run on a cellulose chromatography column. Typically, such a preparation sequence gave a sample of about 50 mg weight with strong bacteriocin activity from 1 litre of culture fluid originally containing about 15 g of nutrients. However, attempts to purify such samples further using paper chromatography and electrophoresis always resulted in a wide spread of bacteriocin activity even if only a few mg were loaded over a full sheet of paper 46 cm wide. This spread was not simply due to overloading as ultra-violet absorbing or fluorescing impurities could be observed as well-resolved sharp bands when the chromatograms and electrophoretograms were examined under ultraviolet light. Presumably the interaction of the bacteriocin and the cellulose supporting medium becomes more important at higher sample loads.

Another problem in purification of the bacteriocin was uncontrollable losses of bacteriocin activity as the purification proceeded. Despite close control of pH, frequent checks for bacteriocin degradation in any of the solvents or buffers used and checks for losses in lyophilization or evaporation, no consistent pattern could be established. It is possible that some impurities in the samples may be important in stabilizing the bacteriocin activity and so, as the sample becomes purer, bacteriocin activity is lost.

Bacteriocin samples were routinely examined at different purification steps by ultraviolet spectroscopy. Although ultraviolet

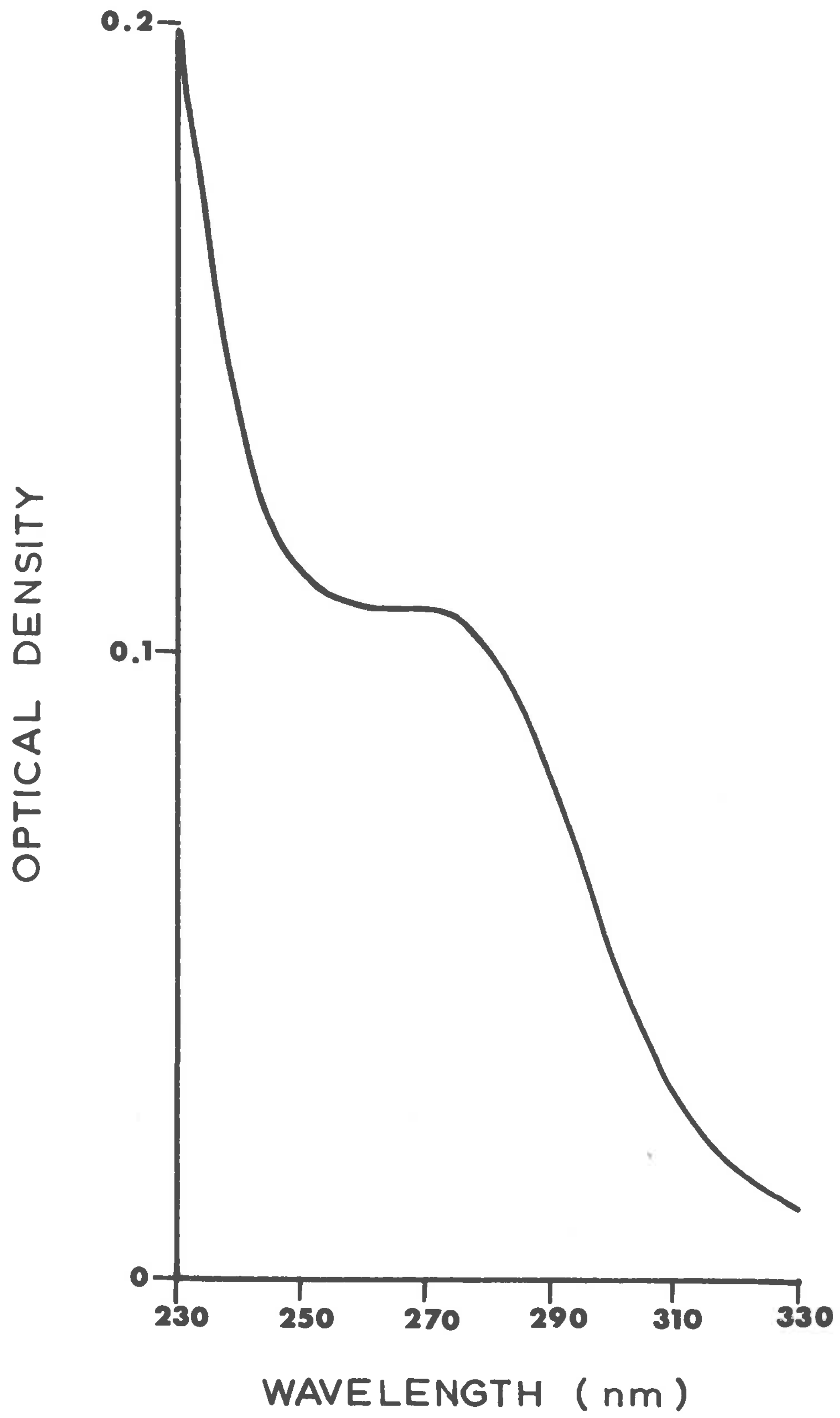
absorption was always associated with bacteriocin activity, due to the uncertainty about the purity of the samples it is difficult to assess the significance of this finding. Figure 26 shows a typical spectrum of a bacteriocin sample. The absorption between 240 nm and 280 nm was consistently present in bacteriocin samples and in some samples a peak could be observed, the position and magnitude of which often depended on the pH of the solution. However, it should be noted that inactive impurities often showed similar ultraviolet spectra and many ultraviolet absorbing or fluorescing bands were observed on chromatograms and electrophoretograms.

## THE EFFECT OF BACTERIOCIN ON SENSITIVE AND INSENSITIVE AGROBACTERIA

The last section described some physical and chemical properties of the bacteriocin and attempts that were made to purify it. Although these attempts gave useful information about the properties of the bacteriocin, purification was not achieved and so experiments on the effect of bacteriocin on agrobacteria were carried out using crude bacteriocin solutions.

These experiments were carried out on the non-pathogenic and converted pathogenic strains of bacteria which were used in the comparison of pathogenic and non-pathogenic bacteria in Part C. The non-pathogenic, non-bacteriocin sensitive strains were used as controls to ensure that any effects observed were due to the bacteriocin itself and not to impurities in the bacteriocin solutions.

*Figure 26.* Typical ultraviolet spectrum of a partially purified bacteriocin sample.


0.2
0.1
0
OPTICAL DENSITY
230
250
270
290
310
330
WAVELENGTH ( nm )

Preparation of bacteriocin solutions

Crude unconcentrated bacteriocin solutions were produced by growing strain 84 in Stonier's medium until the optical density reached 80. The bacteria were then centrifuged down and the supernatant adjusted to pH 7.0 with HCl. Concentrated bacteriocin solutions were prepared by adding 5 volumes of absolute ethanol to unconcentrated solutions, filtering off the precipitated salts, then evaporating the filtrate to dryness in a Rotavapour apparatus with the water bath at 40°C. The residue was then dissolved in a tenth of the original volume of distilled water. Originally the bacteriocin solutions were stored at 4°C with a small volume of chloroform but this practice was discontinued when it was observed that the small amount of chloroform dissolved in the bacteriocin solution could affect the test bacteria. In later experiments samples were stored at -20°C without chloroform. The amounts of bacteriocin added in all the experiments on the effect of bacteriocin on agrobacteria were chosen so that the final concentration never exceeded the concentration of bacteriocin which was present in unconcentrated crude bacteriocin solutions. This was done to ensure that the experiments were carried out with bacteriocin concentrations which might be expected to occur *in vivo*.

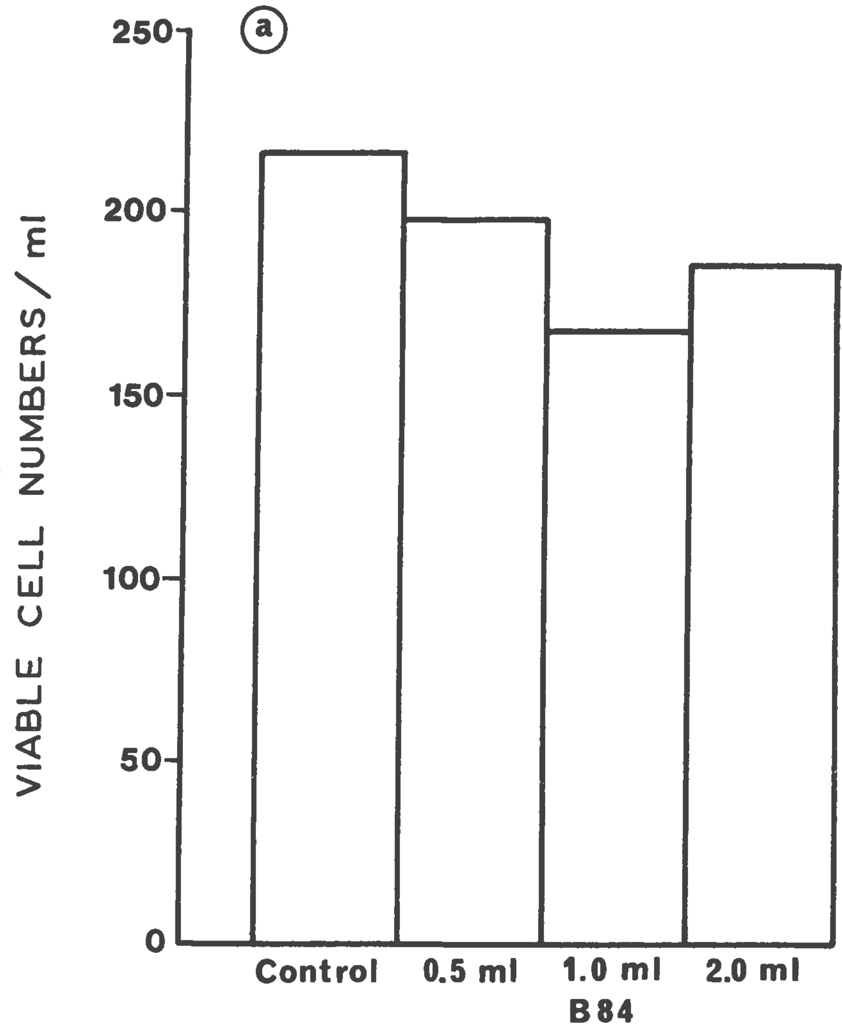
The effect of bacteriocin on viable cell numbers and optical density

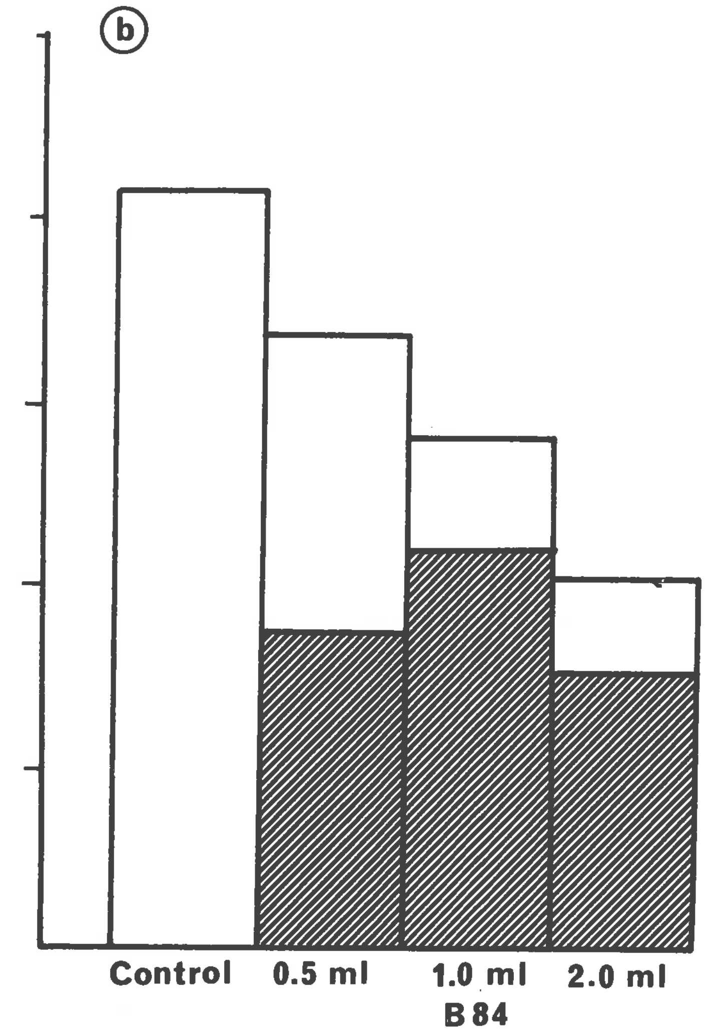
Liquid cultures of strains 57 and 57A were adjusted to an optical density of either 6 (approximately $10^8$ cells/ml) or 20 (approximately $3.3 \times 10^8$ cells/ml), dispensed into flasks and concentrated

or unconcentrated bacteriocin solutions added. At intervals samples were removed, diluted in sterile distilled water and spread on agar plates of Stonier's medium. The optical density of bacteriocin treated cultures in side-arm flasks was followed by tipping the contents of the flask into the side-arm and placing this in an EEL Colorimeter. The effect of cold on sensitivity to bacteriocin was determined by incubating bacteriocin treated cultures in an ice bath and at intervals sampling for viable numbers.

The effect of three concentrations of bacteriocin on the viable number of cells in cultures of strain 57 and 57A after 30 min incubation is shown in Figure 27. There was no significant effect on strain 57 (the non-pathogen). The two highest concentrations of bacteriocin had a marked effect on the viable number of bacteria of strain 57A. Most of the bacteria that did survive, however, took a much longer incubation period to form visible colonies. Plate 5 shows an insensitive and a sensitive strain after bacteriocin treatment. The heterogeneity of colony size on the sensitive plate shows that not all colonies started dividing at the same time. If only the colonies that grew in the normal incubation time are considered then there is an even more marked effect by bacteriocin on sensitive compared with insensitive bacteria. When the colonies that took longer to grow were subcultured they were still pathogenic, still sensitive to bacteriocin and grew at the normal rate. This suggests that exposure to bacteriocin slowed the first few cell divisions of these bacteria necessitating a longer incubation period to produce visible colonies.

*Figure 27.* The effect of 3 concentrations of bacteriocin on: a. strain 57, b. strain 57A, after 30 min incubation.


VIABLE CELL NUMBERS / ml
250
200
150
100
50
0
a
Control
0.5 ml
1.0 ml
2.0 ml
B 84


b
Control
0.5 ml
1.0 ml
2.0 ml
B 84

NORMAL COLONIES
SLOW-TO-GROW COLONIES

*Plate 5.* Agar plates of strain 57A (top) and strain 57 (bottom) after treatment of cultures with bacteriocin solution. Note heterogeneity of colony size of strain 57A indicating that bacteria did not all start dividing at the same time.

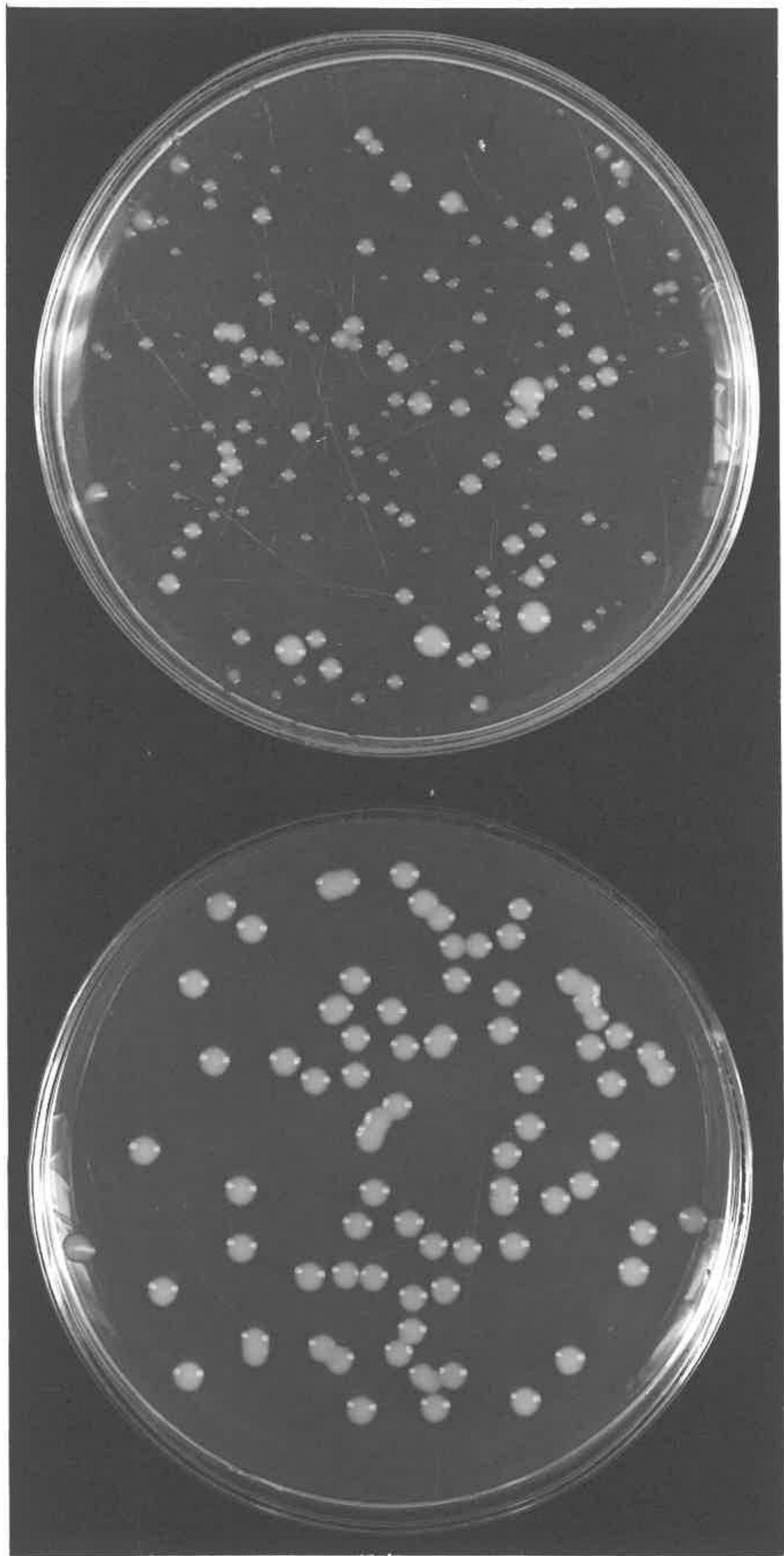
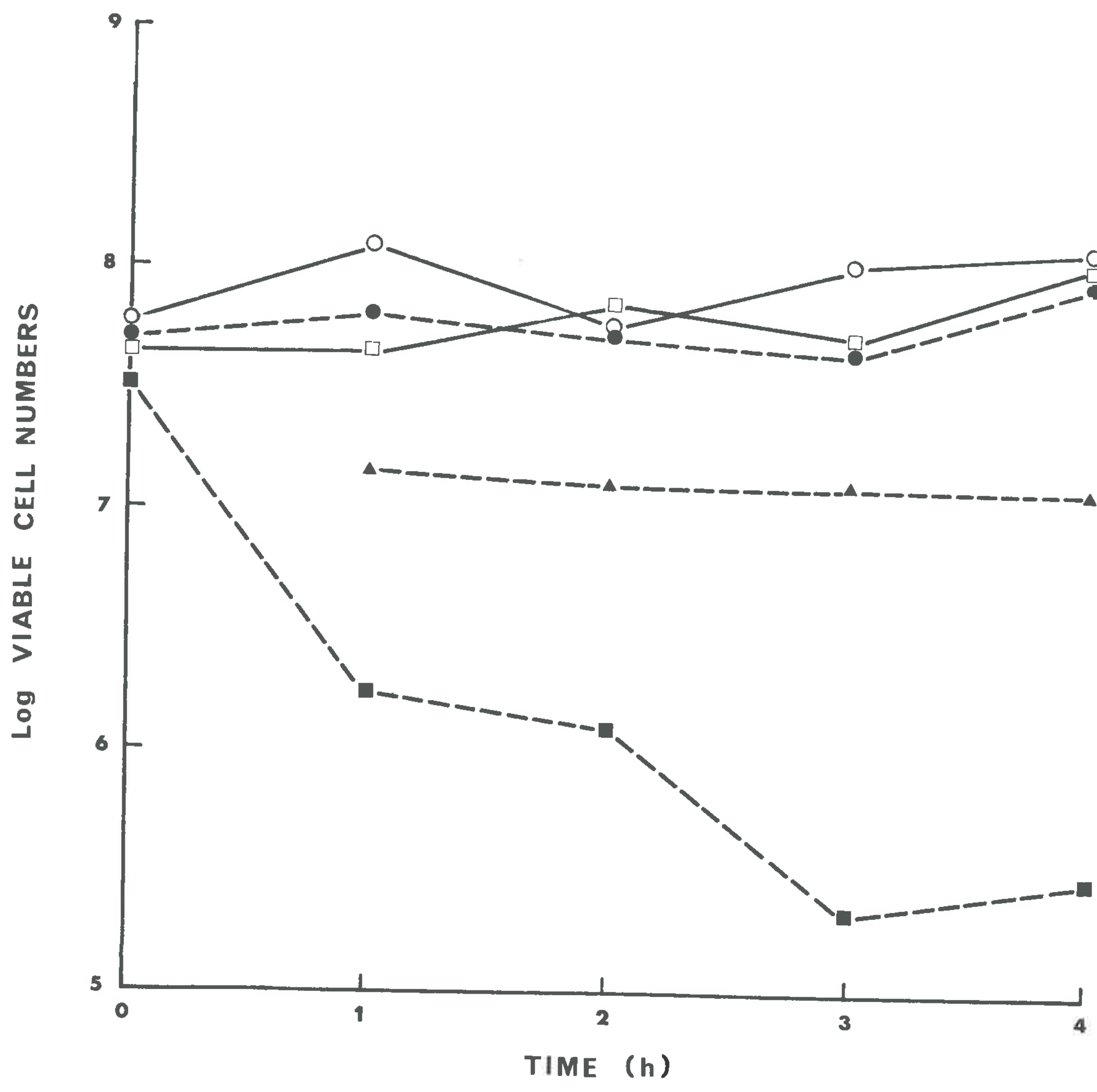


Figure 28 shows the effect of a single concentration of bacteriocin on strains 57 and 57A over a period of 4 h. Again no effect on strain 57 was observed. Strain 57A shows approximately a 99% reduction in viable numbers by 4 h but on longer incubation, many more colonies were formed and so the total final bacterial kill after 4 h was about 77%. The number of bacteria which were slow to grow after bacteriocin treatment remained relatively constant over the whole sampling period, although the number of bacteria killed increased quite sharply with time. Figure 27 indicates that the number of bacteria which were slow to grow was relatively constant irrespective of the bacteriocin concentration. These results suggest that a culture of sensitive cells may consist of a certain proportion of cells which are not killed but produce colonies slowly irrespective of incubation time with, or concentration of, bacteriocin. The rest of the cell population is either unaffected by bacteriocin or killed, depending on bacteriocin concentration and/or length of incubation.

When the effect of bacteriocin on strain 57A was followed at much shorter time intervals (Fig. 29) it was observed that there was a distinct lag period of approximately 15 min before the rate of killing reached a maximum. Although the increases in the rate of killing observed at 25 min and 35 min are not statistically significant, similar changes were observed in another similar experiment. Changes in the rate of killing can also be observed at 1 h and 3 h in Figure 28. It is possible that a sensitive culture of bacteria may contain discrete

*Figure 28.* The effect of bacteriocin on viable cell numbers of strains 57 and 57A over 4 hours. Bacteriocin was added at time 0. o——o 57 control, □——□ 57 with bacteriocin, ●---● 57A control, ■----■ 57A with bacteriocin; colonies appearing after normal incubation period of plates, ▲---▲ 57A with bacteriocin; colonies appearing after extended incubation of plates.



9
8
7
6
5
Log VIABLE CELL NUMBERS
0
1
2
3
4
TIME (h)

populations of different sensitivity to bacteriocin.

Figure 30 shows that when bacteriocin was added to a culture of a sensitive strain the normal increase in optical density with time was markedly reduced, suggesting that the rate of cell division decreased. There was no reduction in optical density indicating that massive cell lysis was not induced. The bacteriocin had no significant effect on the optical density of a closely related non-pathogenic strain.

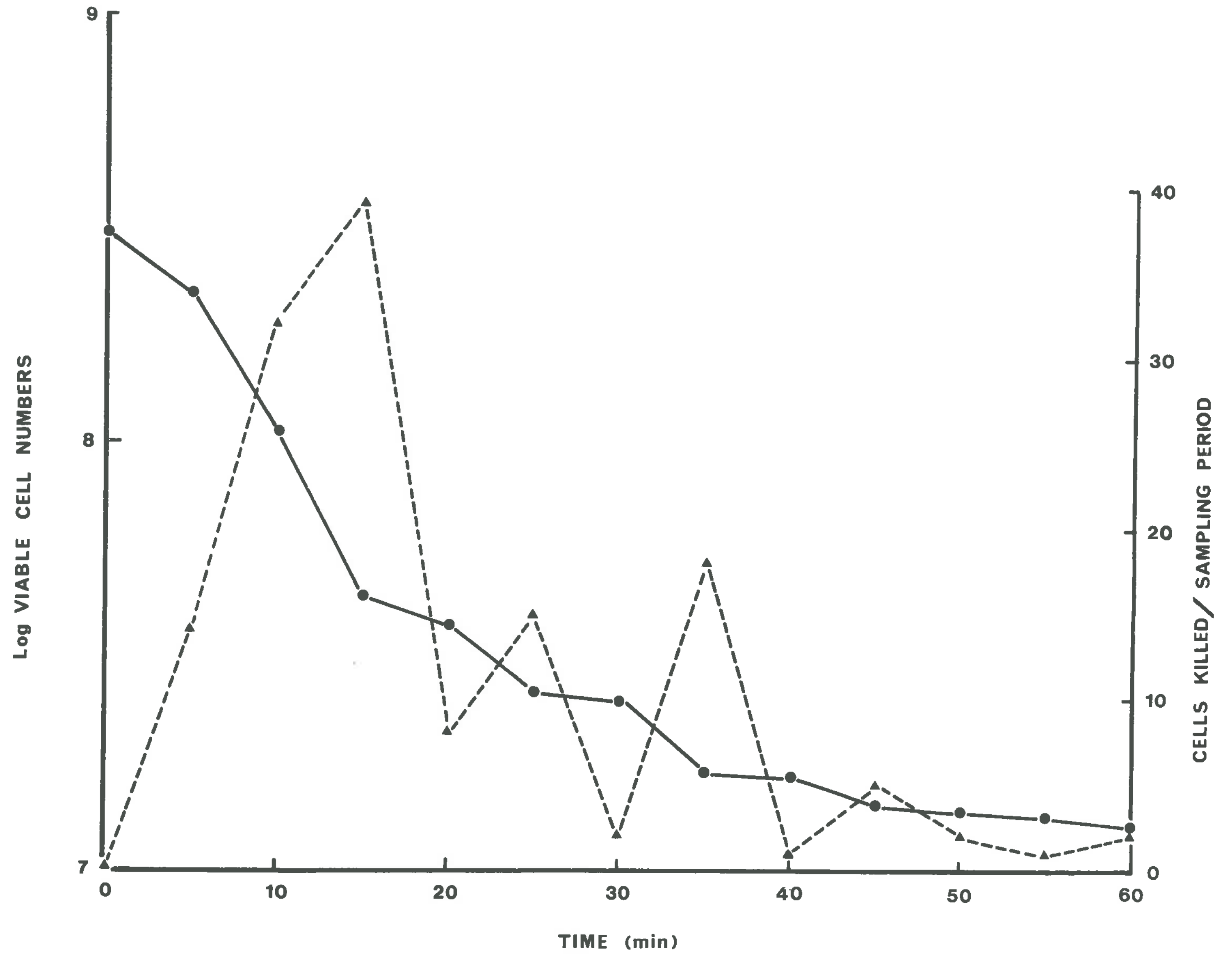
Incubation of a sensitive culture in an ice bath prevented bacteriocin acting, suggesting that active cell metabolism was necessary for bacteriocin to have any effect (Table 13). Presumably metabolic energy is needed for uptake or binding of the bacteriocin to the cell rather than the final lethal event, otherwise cells that bound bacteriocin at $0^{\circ}C$ would subsequently be killed after dilution and incubation at $25^{\circ}C$.

Attempts were made to confirm the necessity for metabolic energy for bacteriocin action, using the metabolic inhibitor 2-4 dinitrophenol (DNP). However, experiments to find the minimum inhibitory concentration of DNP indicated that *Agrobacterium* was only slightly sensitive even at relatively high ($2 \times 10^{-5}$M) concentrations; so further experiments were not attempted.

The effect of bacteriocin on respiration rate

Oxygen consumption of sensitive and insensitive strains of bacteria without and with bacteriocin was followed in a Warburg apparatus. Cultures of strains 57 and 57A were adjusted to an optical density of 20 and 3 ml dispensed into Warburg flasks containing 0.2 ml 10% KOH in the

*Figure 29.* The effect of bacteriocin on viable numbers over short sampling periods. Viable numbers are based on total colonies appearing on plates. ●——● viable numbers with time, ▲---▲ numbers killed/5 minute sampling period. Bacteriocin was added at time 0.



Log VIABLE CELL NUMBERS
9
8
7
CELLS KILLED / SAMPLING PERIOD
40
30
20
10
0
0
10
20
30
40
50
60
TIME (min)

*Figure 30.* The effect of bacteriocin on the optical density of strains 57 and 57A. Bacteriocin was added at time 0. o——o 57 control, ●---● 57 with bacteriocin, ▲——▲ 57A control, ■---■ 57A with bacteriocin.

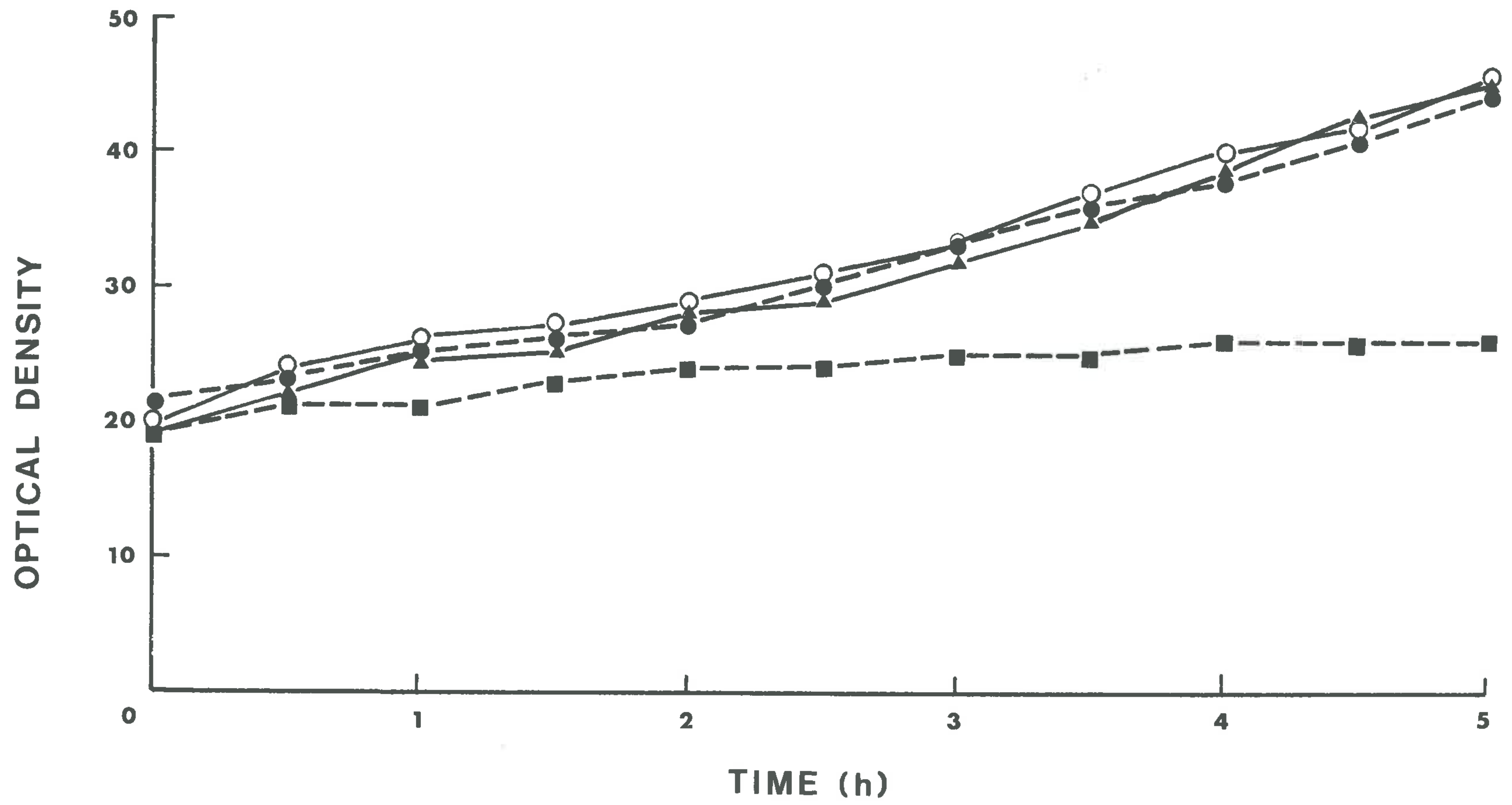

50
40
30
20
10
0
OPTICAL DENSITY
1
2
3
4
5
TIME (h)

*Table 13.* The effect of incubation of cultures at 25°C and in an ice bath on the action of bacteriocin. Bacteriocin was added at time 0. The culture on ice was transferred to 25°C after 2 hours. Values are viable cells/ml x $10^{-8}$.

| Time | 25°C | Ice for 2 hours then 25°C |
|---|---|---|
| 0 | 4.0 | 3.1 |
| 2 hours | 0.3 | 3.0 |
| 3 hours | 0.1 | 0.6 |

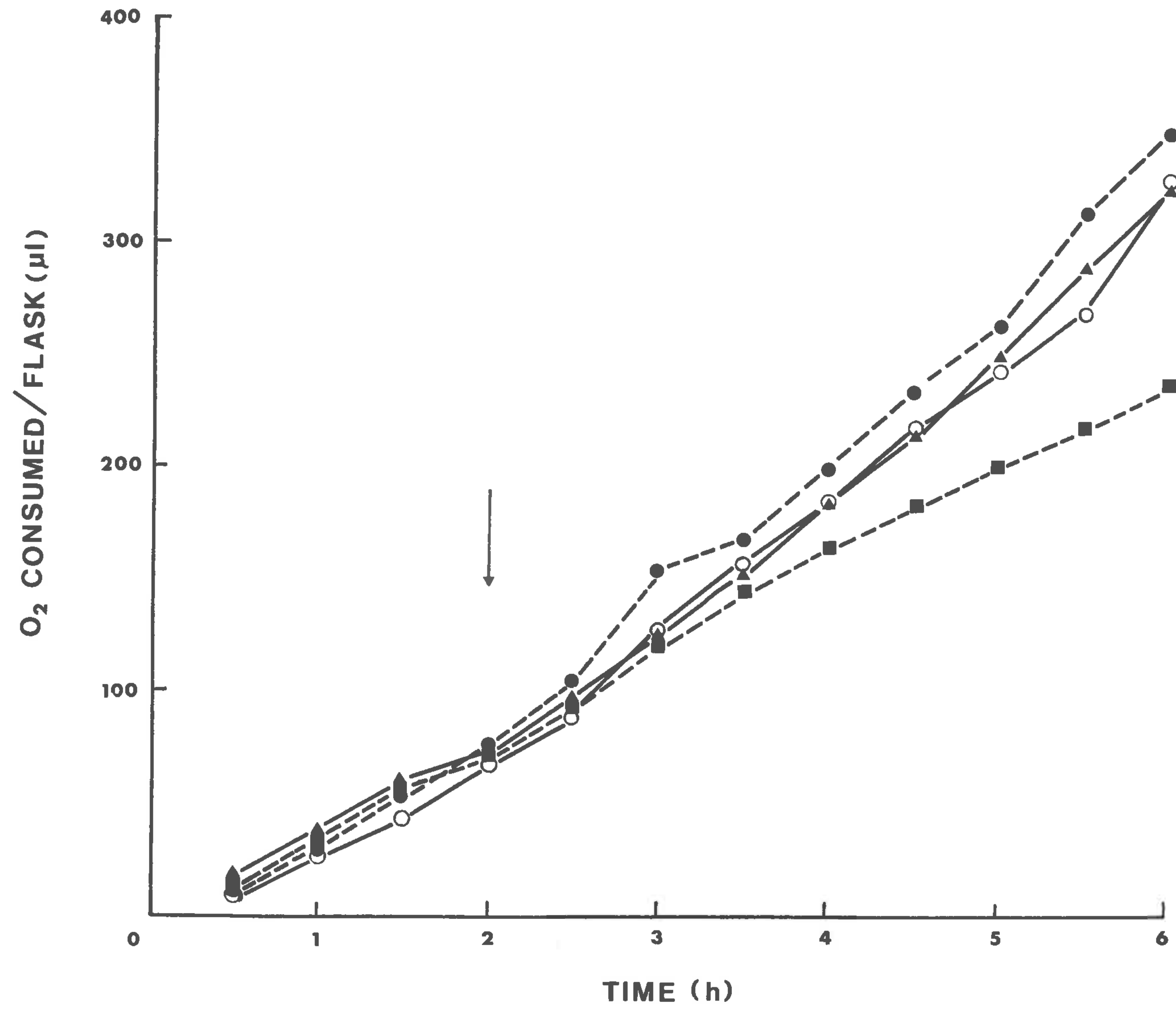
centre well. Bacteriocin solutions were added to the side-arm of each treatment flask and Stonier's medium to the side-arms of the control flasks. The flasks were attached to the manometers and these were placed on the shaker. The waterbath was set at 25°C. After a period of equilibration the manometer taps were closed and the oxygen consumption of all the flasks determined for 2 h. The contents of the side-arms were then tipped and the oxygen consumption of all flasks followed for another 4 h. At the end of this period the number of viable bacteria in each flask was determined.

Figure 31 shows that when bacteriocin was added to a sensitive cell culture the normal increase in respiration rate with increasing cell numbers was stopped. However, there was no sharp change in respiration rate which would be expected if the bacteriocin caused rapid cell death, although Table 14 indicates that only about 6.7% of the sensitive bacteria were able to form colonies after 4 h exposure to bacteriocin. These results suggest that bacteriocin causes a block in cell division without grossly affecting the energy metabolism of the cell. This experiment was repeated a number of times with different amounts of bacteriocin but the results always showed a similar pattern.

The effect of bacteriocin on the uptake and incorporation of radioisotopes

Radioisotopes were obtained from The Radiochemical Centre, Amersham. Cultures of sensitive and insensitive strains of bacteria were adjusted to an optical density of 20 and dispensed into flasks. To follow DNA synthesis $^{3}$H-thymidine (2.0 Ci/mmol) was added while RNA

*Figure 31.* The effect of bacteriocin on the oxygen consumption of strains 57 and 57A. Bacteriocin was added at 2 hours. o——o 57 control, ●-----● 57 with bacteriocin, ▲——▲ 57A control, ■-----■ 57A with bacteriocin.



400
300
200
100
0
1
2
3
4
5
6
$O_2$ CONSUMED/FLASK (µl)
TIME (h)

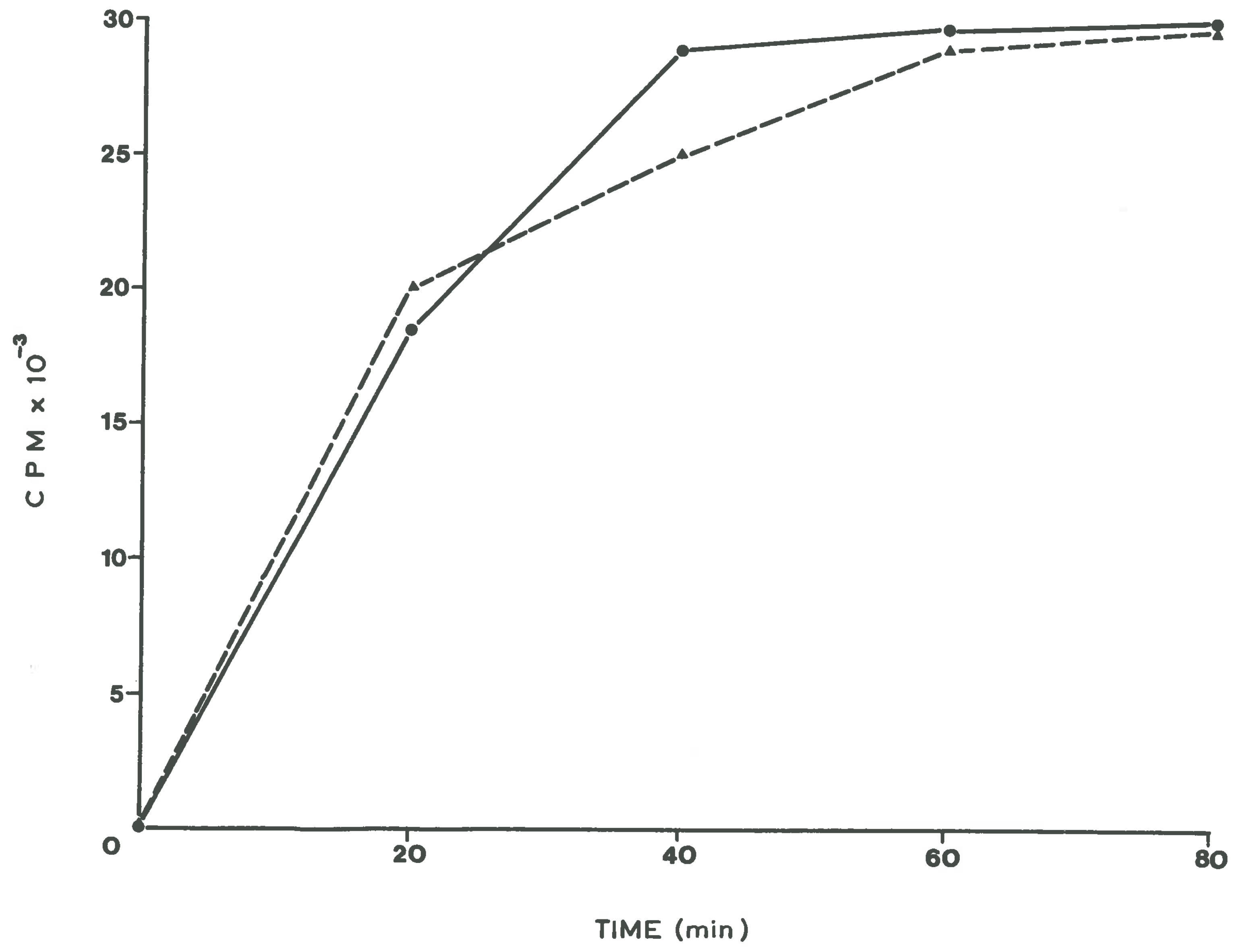
*Table 14.* Viable numbers from Warburg flasks after 6 hours incubation.

| Strain and treatment | Viable numbers/ml x $10^{-8}$ |
|---|---|
| 57 control | 3.5 |
| 57 + bacteriocin | 2.0 |
| 57A control | 1.5 |
| 57A + bacteriocin | 0.1 |

synthesis was followed by adding $^{14}C$-uridine (54 mCi/mmol) and protein synthesis by adding $^{35}S$-methionine (65 Ci/mmol). Bacteriocin was normally added at the same time as the isotope. To measure uptake, duplicate 0.1 ml samples were removed, placed on membrane filters, washed with cold medium, dried and counted in a scintillation counter. At the same time, to measure incorporation, duplicate 0.1 ml samples were added to an equal volume of 10% trichloroacetic acid (TCA) and placed at 4$^{o}$C overnight. The resultant precipitates were then washed onto membrane filters, washed with cold 5% trichloroacetic acid, dried and counted in a scintillation counter.

Initially all of the controls in the uptake and incorporation experiments showed a pattern similar to that shown for thymidine in Figure 32. This suggests that the isotopes were quickly becoming limiting due to fast uptake and incorporation by the bacteria. This possibility was checked by centrifuging down the bacteria from cultures after isotope treatment and then determining the amount of isotope left in the supernatant. Both uridine and methionine rapidly disappeared from the supernatant. The problem with uridine was eliminated by increasing the concentration added to 0.2 µCi/ml of bacterial culture. Because of the very high rate of methionine uptake and its high specific activity it was not found practical to increase the amount of radioactivity. Instead of 10 µl/ml of a 0.1 mM solution of non-radioactive methionine was added with the radioactive isotope. The results with thymidine indicated that most of the radioactivity was still free in the culture fluid. One possibility was that the bacteria rapidly

*Figure 32.* Uptake of a low concentration of $^{3}H$-thymidine by strains 57 and 57A. ●——● strain 57, ▲---▲ strain 57A.

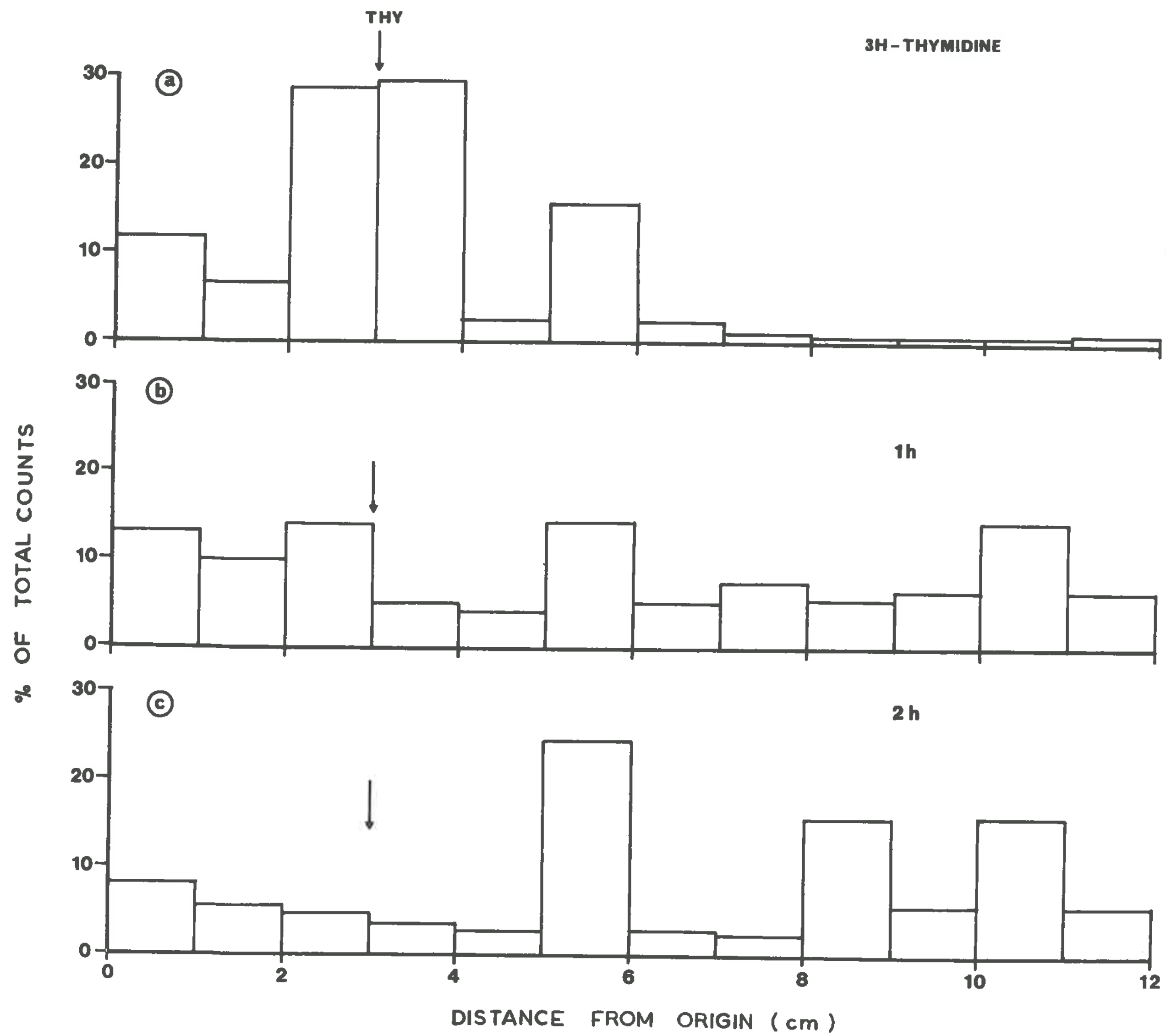


30
25
20
15
10
5
0
20
40
60
80
C P M x $10^{-3}$
TIME (min)

metabolised the thymidine to some compound, which could not be incorporated into DNA, and then released it back into the culture fluid. This possibility was checked by adding $^{3}$H-thymidine to a culture of bacteria and at intervals removing samples, centrifuging the bacteria down, then running aliquots of the supernatant on thin layer chromatography (Eastmann Silica Gel) in the upper phase of a mixture of ethyl acetate : water : formic acid (60 : 35 : 5). Authentic, non-radioactive thymidine and $^{3}$H-thymidine were run as standards. After the run the non-radioactive thymidine was located under ultraviolet light and the radioactivity was located by cutting up the sheet into squares which were placed in scintillation fluid in vials and counted in a scintillation counter. The stock $^{3}$H-thymidine proved to consist of a major peak of thymidine and a minor peak of an unknown impurity (Fig. 33 (a)). Incubation of thymidine with bacteria led rapidly to removal of the thymidine and the appearance of a number of other compounds (Fig. 33 (b) and (c)). By 2 h incubation no thymidine was left but there were substantial amounts of other radioactive compounds. This is strong evidence that the bacteria convert thymidine into other compounds which are released back into the medium. To overcome this problem the amount of $^{3}$H-thymidine added to cultures was increased (to 3 µCi/ml) so that there was sufficient thymidine for both DNA synthesis and the competing biochemical pathway.

Figure 34 (b) shows that there was a marked reduction in the rate of $^{3}$H-thymidine incorporation within 20 min of adding bacteriocin to a sensitive culture. A decrease in uptake was also evident by 60 min.

*Figure 33.* Metabolism of thymidine by *Agrobacterium*. $^3$H-thymidine was added to a culture of strain 57A. At intervals samples were removed, centrifuged and an aliquot of the supernatant run on thin layer chromatography. a. $^3$H-thymidine without bacteria. b. Supernatant after 1 h incubation with bacteria. c. Supernatant after 2 h incubation with bacteria. Arrow indicates position of non-radioactive thymidine.


THY
3H-THYMIDINE
(a)
(b)
1h
(c)
2h
% OF TOTAL COUNTS
30
20
10
0
2
4
6
8
10
12
DISTANCE FROM ORIGIN ( cm )

There was no significant effect on the insensitive culture (Fig. 34(a)).

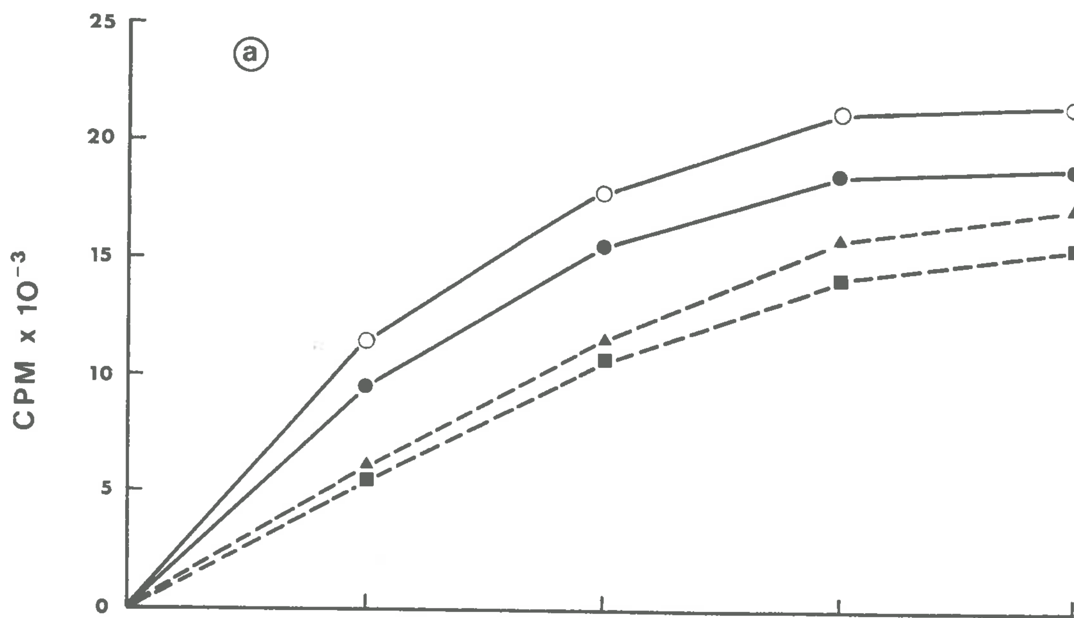
A marked effect on both uptake and incorporation of methionine was observed 40 min after adding bacteriocin to a sensitive culture (Fig. 35(b)) with no effect on insensitive culture (Fig. 35(a)). In some experiments this effect was evident within 20 min.

No significant effect on uridine incorporation was observed until 80 min after adding bacteriocin when a decrease in the rate of uridine incorporation by the sensitive strain was noted (Fig. 36).

Adsorption of bacteriocin by sensitive and insensitive agrobacteria

A number of experiments were carried out to try and determine whether bacteriocin was specifically adsorbed by sensitive but not insensitive bacteria. This was done by incubating strains 57 and 57A with various concentrations of bacteriocin, then centrifuging down the bacteria and assaying the supernatant for bacteriocin activity. A reduction in bacteriocin activity in the supernatant of the sensitive strain compared with the insensitive strain indicates that specific adsorption has occurred. The result of one such experiment is shown in Plate 6 where it can be seen that there was a marked reduction in inhibition zone size by the sensitive strain compared with the insensitive strain and the control. However, this result could not always be repeated and other experiments often showed no adsorption at all. Although no consistent pattern could be established one problem with this method appears to be the relative insensitivity of the diffusion zone assay. Bacteriocin solutions which were not active in the diffusion zone assay were often shown to reduce the number of viable cells quite

*Figure 34.* The effect of bacteriocin on the uptake and incorporation of $^3$H-thymidine by: a. strain 57, b. strain 57A. ○——○ uptake control, ●——● uptake with bacteriocin, ▲---▲ incorporation control, ■---■ incorporation with bacteriocin. Bacteriocin and isotope added at time 0.


a
25
20
15
10
5
0
CPM x 10$^{-3}$

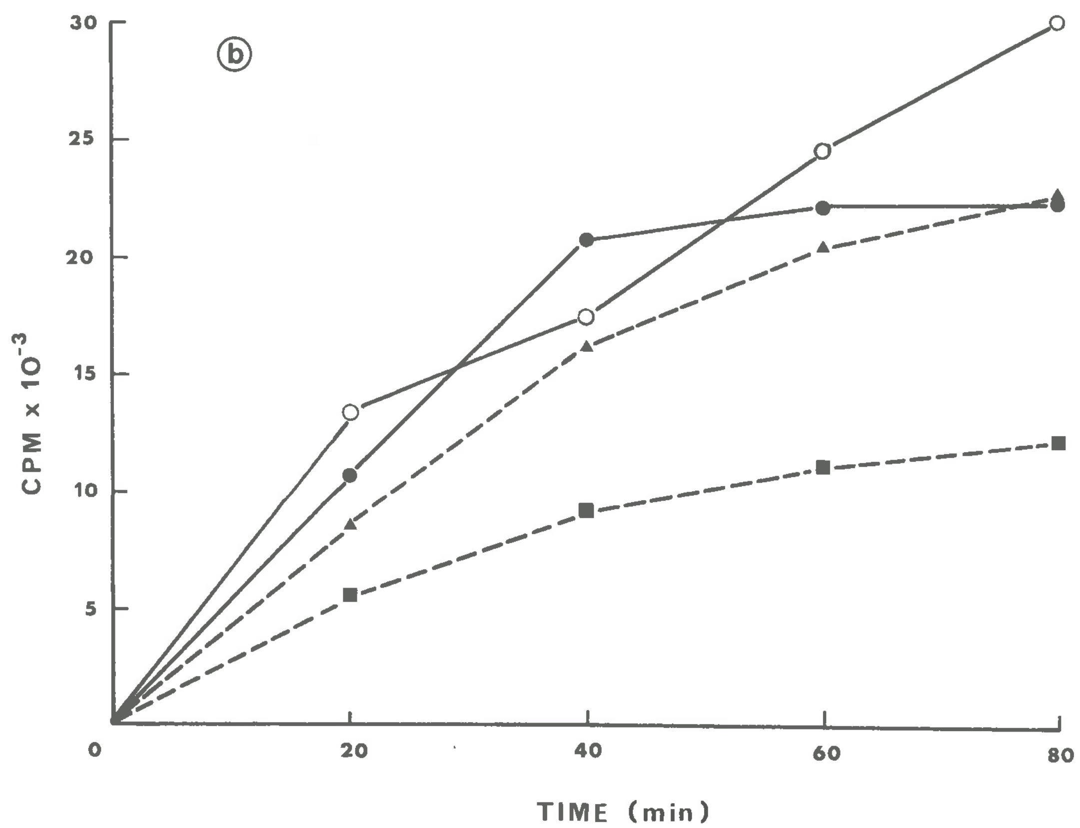
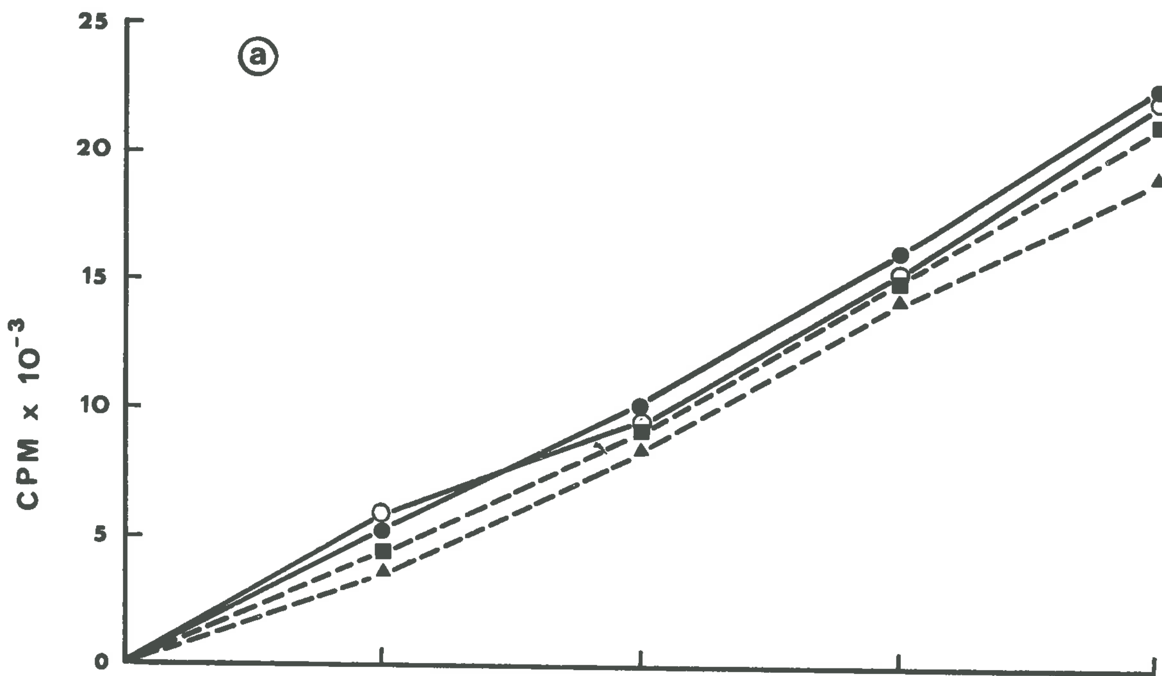
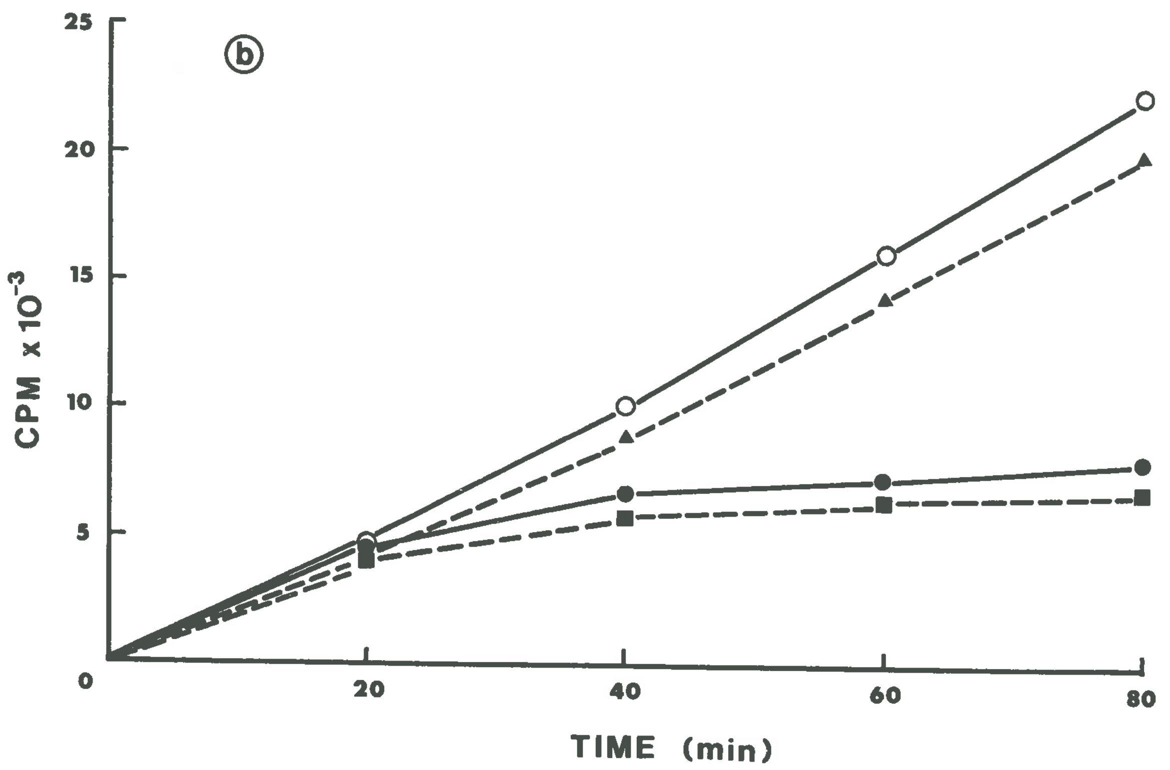

b
30
25
20
15
10
5
0
20
40
60
80
CPM x 10$^{-3}$
TIME (min)

*Figure 35.* The effect of bacteriocin on the uptake and incorporation of $^{35}S$ methionine by: a. strain 57, b. strain 57A. o——o uptake control, ●——● uptake with bacteriocin, ▲---▲ incorporation control, ■---■ incorporation with bacteriocin. Bacteriocin and isotope added at time 0.



a
25
20
15
10
5
0
CPM x 10$^{-3}$

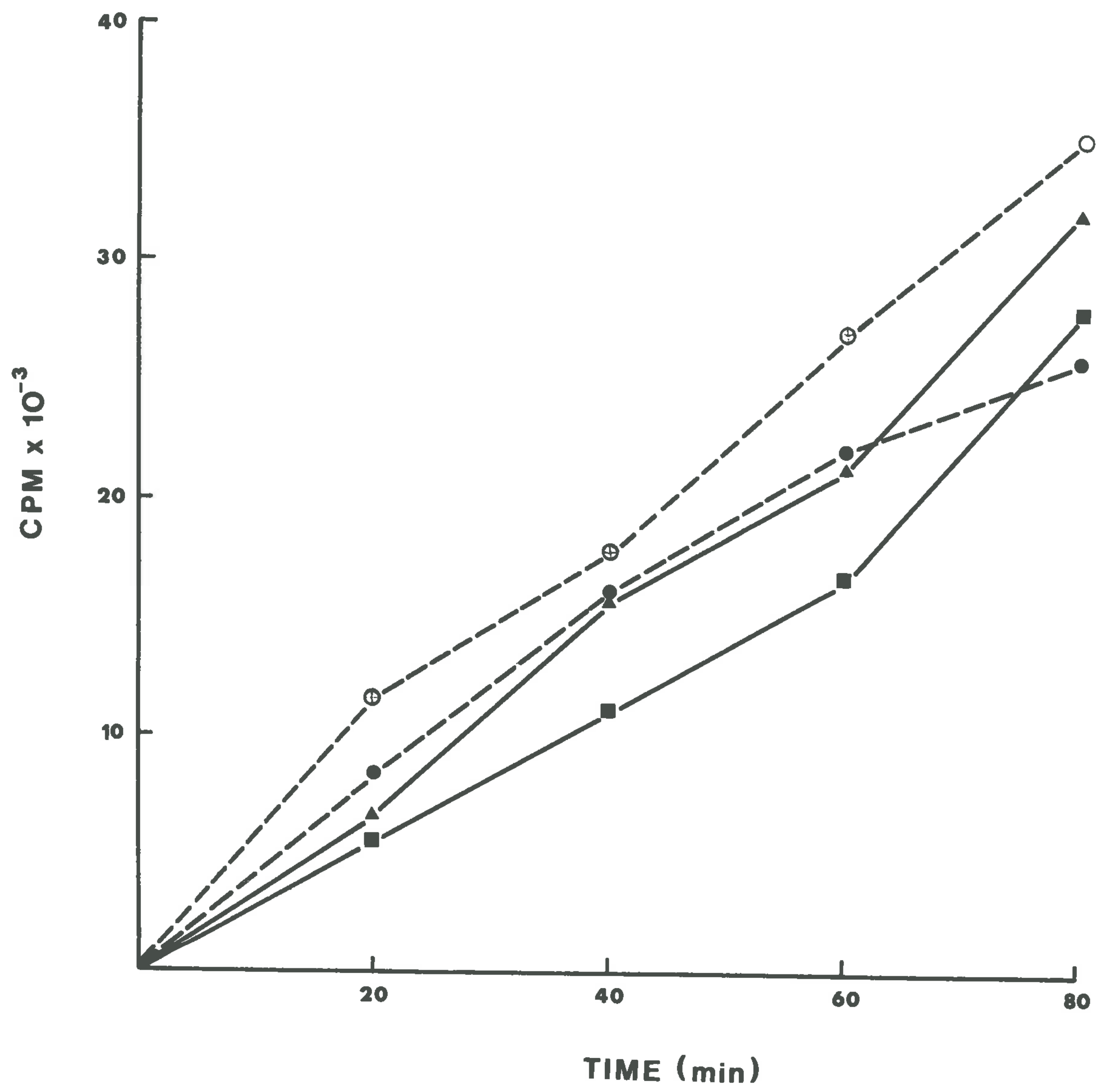
b
25
20
15
10
5
0
CPM x 10$^{-3}$
20
40
60
80
TIME (min)

*Figure 36.* The effect of bacteriocin on the incorporation of $^{14}C$ uridine by strains 57 and 57A.

■——■ 57 control, ▲——▲ 57 with bacteriocin,

o---o 57A control, ●---● 57A with bacteriocin.

Bacteriocin and isotope added at time 0.



40
30
20
10
CPM x $10^{-3}$
20
40
60
80
TIME (min)

significantly when added to a sensitive culture.

In an attempt to overcome this problem experiments were carried out in which bacteriocin was incubated with cultures of sensitive and insensitive bacteria, then the cells centrifuged down and each supernatant added to a pellet of sensitive cells which were then resuspended. This suspension was then incubated for 1 h after which the viable number of bacteria were determined. A typical result of one such experiment is shown in Table 15. If the sensitive culture specifically adsorbed bacteriocin then there should be fewer sensitive bacteria killed by the supernatant from this treatment. An examination of the results, however, reveals that there is no significant difference between the sensitive and insensitive culture, suggesting that specific adsorption may not be occurring.

## DISCUSSION

Although this bacteriocin shows the specificity of a typical bacteriocin (Kerr, personal communication, tested 36 strains of 16 species of 5 genera other than *Agrobacterium* and found them all insensitive) it is unusual in a number of respects.

It diffused rapidly in agar and was readily dialysable, indicating that it had a low molecular weight. This contrasts with most other reported bacteriocins which have a high molecular weight and a correspondingly slow diffusion rate in agar (Mayr-Harting *et al.* 1972, Reeves 1972). There appears to be only one other conclusive report in the literature of a low molecular weight bacteriocin (Atkinson 1967)

*Plate 6.* Adsorption of bacteriocin by sensitive bacteria.

a. bacteriocin solution adsorbed by strain 57A.

b. bacteriocin solution alone at same concentration as a. and c.

c. bacteriocin solution adsorbed by strain 57.

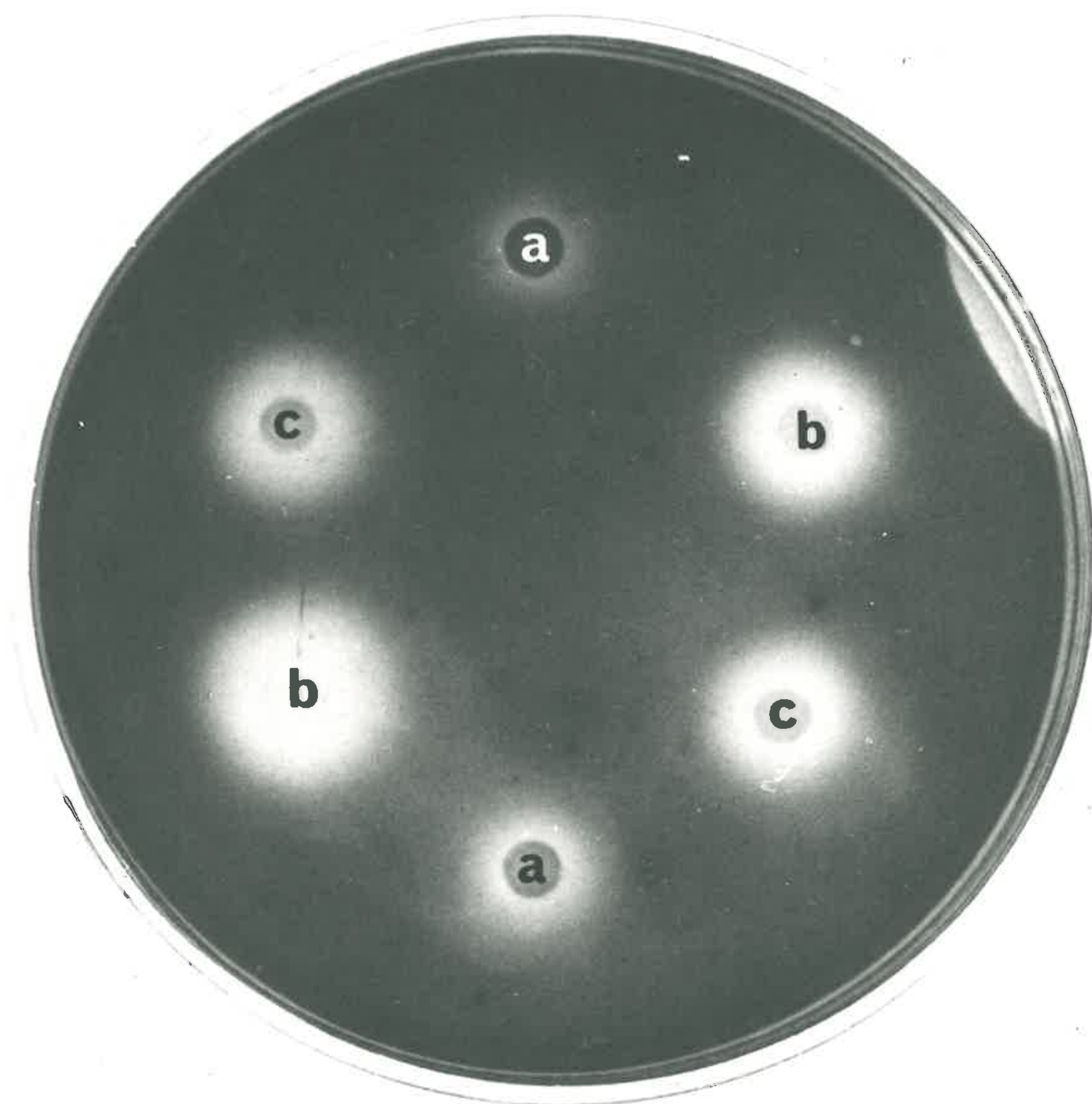

a
c
b
b
c
a

*Table 15.* Viable number assay of bacteriocin after adsorption by sensitive and insensitive bacteria. After bacteriocin solutions were incubated 1 hour with cultures of strain 57 and 57A the bacteria were removed by centrifugation and each supernatant added to a pellet of strain 24 in another tube. This was resuspended, incubated 1 hour and assayed for viable numbers.

| Supernatant | Viable numbers/ml $\times 10^{-7} \pm$ S.E. | % reduction of Bacteriocin treatment compared to control |
|---|---|---|
| Strain 57 control | $13.5 \pm 0.4$ | |
| Strain 57 + bacteriocin | $8.4 \pm 2.0$ | 38% |
| Strain 57A control | $15.2 \pm 0.7$ | |
| Strain 57A + bacteriocin | $10.1 \pm 0.3$ | 34% |

although there is some uncertainty on the molecular weight of colicin V which may exist in two forms (Clowes, personal communication, in Reeves 1972). Due to the fast diffusion rate in agar of the bacteriocin from *Agrobacterium* an estimate of bacteriocin concentration could be made simply by measuring the inhibition zone produced, a method which has only been of limited use in the study of other bacteriocins.

Although none of the methods used in the study of this bacteriocin could give an accurate estimate of its molecular weight, the behaviour of the bacteriocin suggests that the molecular weight could be between 500 and 2,000. Molecules much bigger than this do not generally run at all on paper electrophoresis or chromatography. This molecular weight is well below the 10,000 to 100,000 molecular weights reported for other bacteriocins (Reeves 1972).

Although the experiments with $^{32}P$ indicated quite convincingly that the bacteriocin contains a phosphate group, attempts to further characterize the molecule were largely unsuccessful. This was due mainly to the problems encountered in trying to purify the bacteriocin in amounts useful for chemical analysis. The results suggested that the bacteriocin strongly interacts with most materials used in column chromatographic techniques. These interactions lead to a number of often opposing forces acting simultaneously in any one purification method, and so produce a very wide spread in bacteriocin activity. The alternative explanation, that the bacteriocin is heterogeneous, and so the spread observed is due to the partial resolution of a wide range

of slightly different molecules is not supported, as bacteriocin taken from different areas of the wide spread and subjected to further purification steps always showed similar behaviour. Although strong evidence is not available, it is possible that the bacteriocin consists of a polar charged hydrophilic portion containing the phosphate group and a non-polar hydrophobic portion thus accounting for the behaviour of the bacteriocin during purification attempts.

The experiments with periodate suggest that the bacteriocin molecule contains two adjacent carbon atoms both with hydroxyl groups, probably in the *cis* configuration, that are important for biological activity. Although not conclusive, the results do suggest that the bacteriocin may have an ultraviolet absorption spectra with a peak between 260 and 280 nm. This evidence, together with the phosphate group and the demonstrated action on DNA synthesis, makes it tempting to suggest that the bacteriocin is a DNA base analogue with a phosphate and deoxyribose group; however, this is highly speculative.

All these results, however, demonstrate that this bacteriocin is quite different chemically from any other bacteriocins which have been studied in detail but precise characterization will have to await further purification work.

The rate of killing of this bacteriocin does not follow the single-hit kinetics which has been suggested for other bacteriocins (Mayr-Harting *et al.* 1972, Reeves 1972). When bacteriocin is added to a culture of sensitive cells there is a distinct lag period before the

rate of killing reaches a maximum; this contrasts with other bacteriocins where the rate of killing is at a maximum immediately on mixing bacteriocin and the sensitive culture indicating that apparently only one bacteriocin molecule has to bind to cause cell death. The lag observed in *Agrobacterium* may represent the time needed for the bacteria to accumulate a lethal dose of bacteriocin, suggesting that more than one bacteriocin molecule is needed to kill. Another possibility is that the bacteriocin acts at a site within the cell and the delay represents the time taken for bacteriocin molecules to reach this site. The heterogeneity observed in the rate of killing is distributed in a number of discrete peaks, suggesting that a sensitive culture may contain distinct populations which differ in bacteriocin sensitivity by a constant factor. An alternative explanation is that one stage of the bacterial growth and division cycle is uniquely sensitive to the bacteriocin. The heterogeneity may then be due to bacteria which were in an insensitive stage when the bacteriocin was added but which subsequently became sensitive. However, if this were the case it is unlikely that discrete peaks would be observed unless the cultures were partially synchronised.

The proportion of a sensitive bacterial culture that produces colonies only slowly after exposure to bacteriocin appears to be relatively independent of the length of exposure to or the concentration of bacteriocin, suggesting that these bacteria are not simply the bacteria which have accumulated a sub-lethal dose of bacteria. Possibly a

proportion of the bacteria in a sensitive culture are able to repair the damage (albeit slowly) that the bacteriocin causes. Perhaps a more likely explanation is that at a certain stage of growth the bacteria are less susceptible to the bacteriocin and so it only causes sub-lethal damage. It is possible that this may be when the bacteria are not actively growing; the lag phase and the stationary phase. If this were the case then it would account for some variability between experiments which was observed in the number of bacteria which grew only slowly, as the proportion of a culture in lag phase or stationary phase would depend on the amount of the original inoculum, the age of this inoculum, and the length of incubation of the culture; factors which were not always closely controlled.

The results of the experiments on the specific adsorption of bacteriocin by sensitive cells are inconclusive and contradictory. Most of the experiments appear to indicate that specific adsorption of the bacteriocin does not occur. Such an hypothesis would account for the dependence of the number of bacteria killed on the concentration of bacteriocin and the lack of adsorption observed in a number of experiments. However, this hypothesis does not fit the results of the respiration experiment. This experiment shows that the respiration rate is only slightly below control after 4 h exposure to bacteriocin yet when the viable numbers were determined only a very low proportion were able to form colonies. This suggests that cell death occurs some time after 4 h, although the cells are doomed soon after they are mixed with bacteriocin.

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Unless the bacteriocin is bound in some way to the bacteria it would be diluted out when viable numbers are determined and the number of bacteria killed would only be those that were actually killed when in direct contact with bacteriocin. It is possible, however, that the bacteriocin rapidly causes permanent damage to the bacteria without grossly effecting respiration and so the bacteria are not able to grow even when the bacteriocin is diluted out. If specific adsorption is occurring it may not be reliably detected due to the relative insensitivity of the detection techniques in the presence of an excess of bacteriocin.

In all other bacteriocins that have been studied in detail, specific irreversible adsorption onto sensitive cells has been shown to occur (Reeves 1972). With a number of bacteriocins it has also been demonstrated that specific adsorption of the bacteriocin can be quite distinctly separated in time from cell death (Nomura and Nakamura 1962, Reynolds and Reeves 1969, Elgat and Ben-Gurion 1969).

Incubation of sensitive cells with bacteriocin at 0°C prevented cell death, suggesting that metabolic activity was needed for bacteriocin action. A number of other bacteriocins have been shown to bind to sensitive bacteria at 0°C. Maeda and Nomura (1966), for example, used an incubation period of 2 h at 0°C for survivor count assays.

As the concentration of bacteriocin produced by a culture was correlated directly with cell growth it appears that this bacteriocin is produced continuously. This is not the case with all bacteriocins.

Papavassiliou (1963) and Farkas-Himsley and Seyfried (1963) reported that production of a number of bacteriocins was apparently independent of incubation time but that when the cultures reach a critical stage, a rapid increase in bacteriocin concentration is observed. With the bacteriocin from *Agrobacterium* the results do not differentiate between continuous production of bacteriocin by most of the bacterial population or occasional lethal synthesis by a small proportion of the population. Synthesis of other bacteriocins is generally thought to be lethal to the producing cell (Mayr-Harting *et al.* 1972, Reeves 1972).

This bacteriocin appears to stop cell division without grossly affecting the energy metabolism of the cell. As the incorporation of thymidine was markedly reduced shortly after adding bacteriocin to a sensitive strain it seems likely that the blocking of cell division was caused by a cut-off in DNA synthesis. The bacteriocin could affect DNA synthesis by acting on the enzymatic DNA replication system, binding to DNA directly or as has been suggested for a number of other bacteriocins (Reeves 1972), mediate the effect indirectly from some specific site. If, as suggested before, the bacteriocin is a DNA base analogue then it would probably act by binding to DNA polymerase and inhibiting further enzymatic activity. Such an effect would have to be irreversible otherwise the bacteriocin would only be bacteriostatic.

If the primary effect of the bacteriocin is on DNA synthesis the marked effect on apparent protein synthesis is surprising, particularly as there was little effect on cell respiration. This decrease could not be accounted for by a decrease in transcription of RNA from DNA as there

was very little effect on RNA synthesis. It is possible that the bacteriocin affects the ribosomes and so stops protein synthesis but this would still not explain the lack of effect on the respiration rate. A possible explanation is that the effect observed on protein synthesis was due to a decrease in uptake of methionine and that true protein synthesis was not affected. This would have to be specific as there was no effect on uridine uptake and thymidine uptake only stops after incorporation has been affected. It is also possible that methionine is not really a suitable amino acid for following protein synthesis and so the effects observed are due to other biochemical pathways. It is known, for example, that methionine is involved in cell wall lipid synthesis in *Agrobacterium* (Law, Zalkin and Kaneshiro 1963, Goldfine and Ellis 1964).

In all cases where a comparison between pathogens and non-pathogens was made there was no detectable effect on the non-pathogen. This specificity could be due to the presence of a specific uptake mechanism or binding site in the sensitive organism, or to the presence of a sensitive biochemical pathway which is absent in the insensitive strain.

Stonier (1960b) and Kerr and Htay (1974) both report that a number of *Agrobacterium* strains produce different bacteriocins which can be distinguished on the basis of their activity against a variety of strains. Superficially at least, these all show similar properties and it is possible that they all have the same basic structure but differ in minor details such as the length or group substitution of a carbon chain.

It is possible that such a molecule may be split by the bacteria to release an active molecule.

Although these experiments yielded considerable information about the mechanism and mode of bacteriocin action they did not reveal the basis of the close correlation between pathogenicity and sensitivity to bacteriocin. There appears to be two main hypotheses: (1) that sensitivity to bacteriocin is determined by genes which are closely linked to those that determine pathogenicity such that loss of pathogenicity almost always entails loss of bacteriocin sensitivity, or (2) that sensitivity to bacteriocin is determined by some mechanism that is intimately involved in crown-gall induction so that loss of this mechanism would simultaneously cause loss of both pathogenicity and sensitivity to bacteriocin. On the available evidence it is impossible to decide between these two possibilities, although it appears to favour the first hypothesis. For example, although the correlation between pathogenicity and sensitivity to bacteriocin is very close in all Australian isolates of *Agrobacterium* there are, however, a number of exceptions where bacteria are non-sensitive to bacteriocin and are still pathogenic or they are sensitive to bacteriocin but non-pathogenic (Kerr and Htay 1974, Roberts and Kerr 1974). Roberts and Kerr (1974) also reported that about half of the overseas pathogenic strains of *Agrobacterium* tested were not sensitive to bacteriocin. On the basis of the first hypothesis these results could be explained by the possibility that the genetic linkage between pathogenicity and bacteriocin is close

but that it can still be broken. Kerr (1975) suggested that the close correlation between octopine or nopaline utilization and pathogenicity could also be explained on a similar basis and proposed a genetic model that accounts for this and the bacteriocin results.

However, if these exceptions were explained on the basis of the second hypothesis then it would be necessary to postulate that the mechanism for bacteriocin sensitivity and pathogenicity could be changed so that the bacteria could still induce galls but were no longer sensitive to bacteriocin. Such changes are not without precedent as mutants in which the bacteriocin receptor is changed in other bacteria are well known (Reeves 1972). If such a receptor site on *Agrobacterium* was involved in crown-gall induction and bacteriocin sensitivity it is not impossible to envisage changes in the receptor site which would prevent it binding bacteriocin but still allow crown gall induction. However, confirmation of either of these hypotheses will have to await further study of the bacteriocin and detailed genetic analysis of the genus *Agrobacterium*.

# APPENDICES

*Appendix 1.* Composition of media.

*Appendix 2.* Culture collection numbers and experiment numbers of bacterial strains.

*Appendix 3.* Composition of buffers.

*Appendix 4.* Isoenzyme staining methods.

*Appendix 5.* Publications.

*Appendix 1.* Composition of Media.

Yeast-mannitol agar

| | |
|---|---|
| $K_2HPO_4$ | 0.5 g |
| $MgSO_4.7H_2O$ | 0.2 g |
| NaCl | 0.2 g |
| $CaCl_2$ | 0.2 g |
| $FeCl_3$ | 0.01 g |
| Yeast extract | 1.0 g |
| Mannitol | 10.0 g |
| Agar | 15.0 g |
| Distilled water | 1 litre |

Glucose-nutrient broth

| | |
|---|---|
| Nutrient broth (Difco) | 8 g |
| Glucose (autoclaved separately) | 10 g |
| Distilled water | 1 litre |

Mannitol-glutamic acid medium

| | |
|---|---|
| Mannitol | 10 g |
| Glutamic acid | 2 g |
| $K_2HPO_4$ | 0.5 g |
| $MgSO_4.7H_2O$ | 0.2 g |
| NaCl | 0.2 g |
| Biotin | 2 μg |
| Distilled water | 1 litre |

Adjusted to pH 7.0 with 1M NaOH before autoclaving.
15 g agar added for solid medium.

*Appendix 1.* (continued)

Nutrient broth (DeLey *et al.* 1972)

| | |
|---|---|
| Beef extract | 5 g |
| Yeast extract | 1 g |
| Peptone | 5 g |
| Sucrose | 5 g |
| Distilled water | 1 litre |

Peptone medium (Zaenen *et al.* 1974)

| | |
|---|---|
| Peptone | 4 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| Distilled water | 1 litre |

Stonier's medium (Stonier 1960b)

| | |
|---|---|
| $CaSO_4$ | 0.1 g |
| $MgSO_4.7H_2O$ | 0.2 g |
| NaCl | 0.2 g |
| $NH_4NO_3$ | 2.7 g |
| Potassium citrate | 10.0 g |
| Glutamic acid | 2.0 g |
| $NaH_2PO_4$ | 0.3 g |
| $K_2HPO_4$ | 0.9 g |
| $Fe(NO_3)_3$ | 5.0 mg |
| $MnCl_2$ | 0.1 mg |
| $ZnCl_2$ | 0.1 mg |
| Biotin | 2 µg |
| Distilled water | 1 litre |

Adjusted to pH 7.0 with 1M NaOH before autoclaving.
15 g agar added for solid medium.

*Appendix 2.* Culture collection numbers and experiment numbers of bacterial strains.

| Strain No. (Table 1) | Waite Institute Plant Pathology Collection number | Experimental number |
|---|---|---|
| 57 | 57 | 57 |
| 57A | 198 | 57/19 |
| 200 | 200 | E28/9Y |
| 200A | 204 | E28/9Y-30 |
| 146 | 146 | E68/A5 |
| 146A | 150 | E68/A5-9 |
| 147 | 147 | E68/B-13 |
| 147A | 152 | B13/2 |
| 27 | 27 | G31 |
| 24 | 24 | G28/6/10 |
| 84 | 84 | 209 |

*Appendix 3.* Composition of buffers

Extraction buffer (Staples and Stahman 1962)

| | |
|---|---|
| Tris (Tris(hydroxymethyl)aminomethane) | 1.21 g |
| Sucrose | 17.0 g |
| Ascorbic acid | 0.1 g |
| Cysteine hydrochloride | 0.1 g |
| 1M HCl to pH 8.0 | |
| Distilled water | to 100 ml |

P.E.S.T. (Schilperoort 1969)

| | |
|---|---|
| $Na_2HPO_4$ | 0.84 g |
| $NaH_2PO_4$ | 0.32 g |
| EDTA | 0.37 g |
| NaCl | 10.40 g |
| Tris | 1.21 g |
| Distilled water | 1 litre |

1SSC

| | |
|---|---|
| NaCl | 8.7 g |
| Tri-sodium citrate | 4.41 g |
| Distilled water | 1 litre |

0.1SSC is 1SSC diluted 10 times.

1SSC-ET (Schilperoort 1969)

1SSC with 0.37 g EDTA and 0.12 g Tris added per litre.

*Appendix 3.* (continued)

Tris-citrate paper electrophoresis buffer, pH 7.5

| | |
|---|---|
| Tris | 4.7 g |
| Tri-sodium citrate | 3.2 g |
| Distilled water | 1 litre |

Citrate paper electrophoresis buffer, pH 5.0

| | |
|---|---|
| Tri-sodium citrate | 19.1 g |
| Citric acid | 7.4 g |
| Distilled water | 1 litre |

Formic acid-acetic acid paper electrophoresis buffer, pH 2.4

| | |
|---|---|
| Glacial acetic acid | 58 ml |
| 98% Formic acid | 6.25 ml |
| Distilled water | to 2 litres |

Oxalate paper electrophoresis buffer, pH 1.5

| | |
|---|---|
| Oxalic acid | 40.48 g |
| Sodium oxalate | 10.58 g |
| Distilled water | to 4 litres |

## *Appendix 4.* Isoenzyme staining methods

Total proteins (Chrambach *et al.* 1967)

Gels were fixed for 30 min in 12.5% TCA then stained for 30 min in a freshly prepared solution of 0.05% Coomassie blue. Gels are destained in 10% TCA.

Acid phosphatases (Choudhury and Lundy 1970)

Gels were washed a number of times in 0.1M acetate buffer, pH 5.3, then stained for 1 h at 37$^{\circ}$C with a solution of sodium α-napthyl phosphate (1 μg/ml) and fast red violet LB (1 mg/ml) in 0.1M acetate buffer.

Alkaline phosphatases (Weber *et al.* 1967)

Gels were incubated with sodium α-napthyl phosphate (1 mg/ml) and diazo blue B (1 mg/ml) in 0.1M Tris buffer, pH 9.5, for 1 h.

Esterases (Weber *et al.* 1967)

Gels were incubated with α-napthyl acetate (0.5 mg/ml) and fast blue RR (0.5 mg/ml) in 0.2M Tris buffer, pH 7.4, for 1 h.

Malate dehydrogenases (Gilbert and Goldberg 1966)

Gels were incubated in a solution of 0.1M Tris containing 1.3 mg/ml nicotinomide adenine dinucleotide (NAD), nitro blue tetrazolium (0.45 mg/ml), 0.05M malic acid and phenazine methosulphate 0.14 mg/ml, all adjusted to pH 8.3, for 20 min.

*Appendix 4.* (continued)

Catalases (Woodbury *et al.* 1971)

Gels were washed in distilled water for 45 min then incubated in 0.0033% $H_2O_2$ for 10 min and rinsed in distilled water. Gels were stained for 10 min in a 1% solution of ferric chloride and potassium ferricyanide freshly prepared from 2% stock solutions.

Peroxidases (Weber *et al.* 1967)

Gels were incubated in 0.01M guaiacol for 15 min, rinsed in distilled water and then placed in a 0.3% hydrogen peroxide solution until bands appeared.

*Appendix 5.* Publications.

Roberts, W.P. and Kerr, A. (1974). Crown gall induction: serological reactions, isozyme patterns and sensitivity to mitomycin C and to bacteriocin, of pathogenic and non-pathogenic strains of *Agrobacterium radiobacter*.
*Physiological Plant Pathology 4*, 81-92.

Roberts, W.P. (1975). The effect of bacteriocin on sensitive and insensitive agrobacteria.
*Journal of General Microbiology* (In Press).

Reprinted from *Physiological Plant Pathology* (1974) 4, 81–92

# Crown gall induction: serological reactions, isozyme patterns and sensitivity to mitomycin C and to bacteriocin, of pathogenic and non-pathogenic strains of *Agrobacterium radiobacter*

W. P. Roberts and A. Kerr

*Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064*

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# Crown gall induction: serological reactions, isozyme patterns and sensitivity to mitomycin C and to bacteriocin, of pathogenic and non-pathogenic strains of *Agrobacterium radiobacter*

W. P. Roberts and A. Kerr

*Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064*

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Several non-pathogenic strains of *Agrobacterium radiobacter* were compared with the same strains after conversion to pathogenicity. The characters studied were: serology using gel diffusion and immunoelectrophoresis, enzyme patterns following electrophoresis, reaction to mitomycin C and sensitivity to bacteriocin 84. The only consistent difference between a converted pathogen and its non-pathogenic parent was sensitivity to bacteriocin 84. All 25 pathogenic strains tested were sensitive; 9 out of 10 non-pathogenic strains were resistant. When a non-pathogen, resistant to bacteriocin 84, is converted into a pathogen, it becomes sensitive to bacteriocin 84. As bacteriocins operate through attachment to receptor sites, it is postulated that a specific molecular configuration on the bacterial surface is necessary for pathogenesis and that this configuration is the receptor site for bacteriocin 84. The possibility is discussed that conjugation of bacterial and plant cells is involved in tumour induction.

## INTRODUCTION

One method of investigating the problem of crown gall induction is to compare pathogenic and non-pathogenic strains of the causal organism, *Agrobacterium radiobacter* [*25*]. If a consistent difference between pathogens and non-pathogens could be established, this, at least, would be a useful marker making pathogenicity testing unnecessary and, very likely, would lead to a deeper understanding of the process of crown gall induction. There have been many such studies [*4, 8, 23, 31, 35, 37, 39, 40, 47, 59*] but none has demonstrated a consistent difference between pathogenic and non-pathogenic strains. Recently Kerr [*26, 27*] established that some non-pathogens can be converted to pathogens. These pathogens should be genetically identical with, or very similar to, the non-pathogenic parents except for the factor determining pathogenicity. This seemed to offer an excellent tool for investigating the problem of pathogenicity in *Agrobacterium.*

In this paper several non-pathogenic strains have been compared with the same strains after conversion to pathogenicity. One of the characters studied was the sensitivity of strains to bacteriocin 84, a highly specific antibiotic produced by strain 84 which was isolated in this laboratory in 1969. This aspect of the work was initiated because of the findings of Kerr & Htay [*28*] that there is a close correlation between pathogenicity and sensitivity to bacteriocin 84.

## MATERIALS AND METHODS

### *Strains of* Agrobacterium radiobacter *used*

The strains are listed in Table 1. The non-pathogenic strains are numbered; the converted pathogens are indicated by the number of the parent non-pathogen and a letter. With one exception, all converted pathogens acquired their pathogenicity from strain 27, a biotype 2 [*25*] pathogenic field isolate; the exception is strain 57D which acquired pathogenicity from strain 108, another biotype 2 pathogen. All converted pathogens and their non-pathogenic parents belong to biotype 1 [*25*].

All strains were freeze dried and stored at 5 °C.

Table 1

*The source of cultures used*

| Strain | Accession No. | Pathogenicity | Source |
|---|---|---|---|
| 18 | 18 | – | Soil, Balhannah, S. Australia |
| 18A | 172 | + | Strain 18 converted to pathogen (donor strain 27) |
| 21 | 21 | – | Pear gall, Balhannah, S. Australia |
| 21A | 171 | + | Strain 21 converted to pathogen (donor strain 27) |
| 23 | 23 | – | Peach gall, Aldgate, S. Australia |
| 23A | 170 | + | Strain 23 converted to pathogen (donor strain 27) |
| 27 | 27 | + | Peach gall, Loveday, S. Australia |
| 44 | 44 | – | Soil, Waikerie, S. Australia |
| 44A | 173 | + | Strain 44 converted to pathogen (donor strain 27) |
| 54 | 54 | – | John Innes Potting Compost |
| 54A | 76 | + | Strain 54 converted to pathogen (donor strain 27) |
| 54B | 77 | + | |
| 55 | 55 | – | John Innes Potting Compost |
| 55A | 72 | + | Strain 55 converted to pathogen (donor strain 27) |
| 55B | 78 | + | |
| 55C | 79 | + | |
| 57 | 57 | – | John Innes Potting Compost |
| 57A | 198 | + | Strain 57 converted to pathogen (donor strain 27) |
| 57B | 64 | + | |
| 57C | 74 | + | |
| 57D | 192 | + | Strain 57 converted to pathogen (donor strain 108) |
| 84 | 84 | – | Soil, Balhannah, S. Australia |
| 146 | 146 | – | John Innes Potting Compost |
| 146A | 150 | + | Strain 146 converted to pathogen (donor strain 27) |
| 146B | 148 | + | |
| 146C | 149 | + | |
| 146D | 151 | + | |
| 147 | 147 | – | Soil, Gawler, S. Australia |
| 147A | 152 | + | Strain 147 converted to pathogen (donor strain 27) |
| 147B | 153 | + | |
| 147C | 154 | + | |
| 147D | 155 | + | |
| 200 | 200 | – | John Innes Potting Compost |
| 200A | 204 | + | Strain 200 converted to pathogen (donor strain 27) |
| 200B | 201 | + | |
| 200C | 202 | + | |
| 200D | 203 | + | |

*Production of antisera*

Bacteria were grown for 24 h in mannitol–glutamic acid broth consisting of distilled water, 1 litre; mannitol, 10 g; L-glutamic acid, 2 g; $KH_2PO_4$, 0·5 g; NaCl, 0·2 g; $MgSO_4.7H_2O$, 0·2 g; biotin, 2 μg. The medium was adjusted to pH 7·0 with 1M NaOH. The bacteria were harvested by centrifugation, washed twice in physiological saline and taken up in buffered saline (pH 7·2) to give a suspension of approximately $10^8$ cells/ml. The isolates were injected into separate rabbits, each being given four injections at approximately 10-day intervals. The first consisted of 0·5 ml suspension of whole bacteria and the second of 0·5 ml suspension of bacteria which had been disrupted for 5 min in a MSE ultrasonic disintegrator. In both cases the bacterial suspensions were emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously. The third injection consisted of 1 ml suspension of whole bacteria and the fourth of 2 ml suspension of disrupted bacteria, both injected intravenously. The rabbits were bled approximately 10 days after the last injection, the blood allowed to clot and the collected serum centrifuged.

*Production of antigens*

Bacteria were grown in mannitol–glutamic acid broth, collected by centrifugation after 24 h, washed and resuspended in buffered saline (pH 7·2) to give approximately 0·5 g wet weight per ml. The bacteria were then disrupted by sonicating for 5 min.

*Gel diffusion*

The method of Ouchterlony [*42*] was used.

*Microimmunoelectrophoresis*

This was carried out on microscope slides using the method of Ouchterlony [*43*].

*Electrophoresis*

Bacteria were harvested from 24-h mannitol–glutamic acid broth cultures, suspended in extraction buffer [*50*] and disrupted by sonication for 5 min. The extract was centrifuged to remove cell debris and the protein content of the supernatant determined [*24*].

Polyacrylamide gels were cast and run using the method of Davis [*15*] except that extracts were layered on top of the gels. Different gels were stained for acid phosphatases [*13*], alkaline phosphatases [*56*], catalases [*57*], esterases [*56*], malate dehydrogenases [*17*] and peroxidases [*56*].

*Reaction to mitomycin C*

Inoculated flasks of nutrient broth [*16*] were placed on a rotary shaker overnight at 25 °C. Each culture was then adjusted to an optical density of 40 (EEL colorimeter) with fresh medium and 0·1 ml added to fresh broth in flasks which were placed on the shaker overnight. Mitomycin C was then added to give a concentration of 1·0 μg/ml and the flasks returned to the shaker for 1 h. The cultures were then centrifuged and the bacterial pellet resuspended in fresh medium in a side-arm flask. The optical density of each culture was measured every hour for a period of at least 9 h.

*Bacteriocin sensitivity*

The method of Stonier [*51*] was used. A brief summary of the method was given by Kerr & Htay [*28*]. Strain 84 was used as the bacteriocin producer; it produces bacteriocin 84.

## RESULTS

*Serology*

Antisera of the converted pathogens 57A, 146A, 147A and 200A were prepared and tested by gel diffusion against homologous antigens and also against antigens of the respective non-pathogenic parent strains 57, 146, 147 and 200. No difference in the reactions of the pathogens and non-pathogens could be detected (Plate 1). As all four pathogens had acquired pathogenicity from strain 27, it seemed possible that they might have antigens in common with this strain. Antiserum of strain 27 was prepared and tested against antigens of all four pathogens and their non-pathogenic parents. A very weak precipitation band was formed between strain 27 antiserum and all antigens tested, except the homologous antigen which gave strong precipitation bands (Plate 1).

Many of the antiserum–antigen reactions in the gel diffusion tests gave several precipitation bands which could not be clearly distinguished. Further resolution

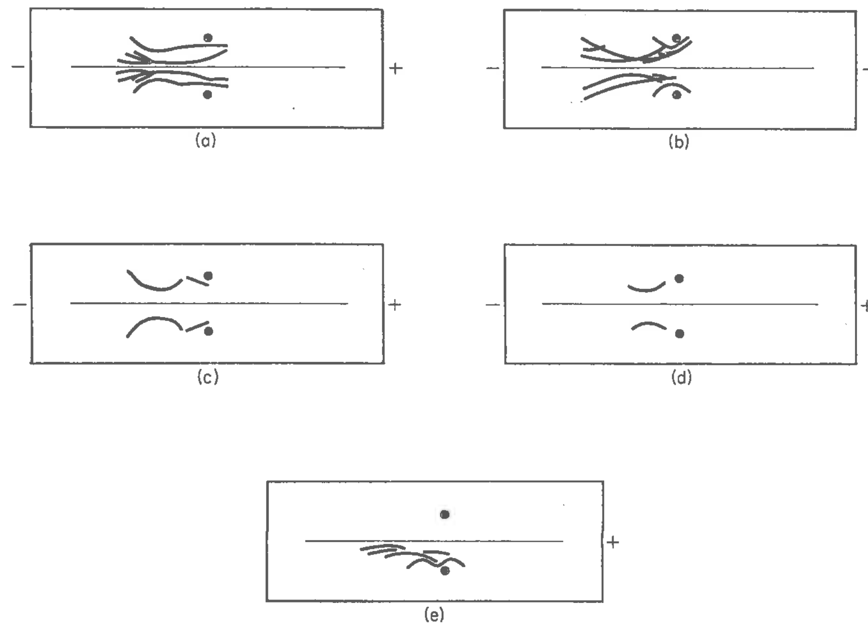


Fig. 1. Immunoelectrophoresis. (a) Antigens 200 and 200A with 200A antiserum. (b) Antigens 57 and 57A with antiserum 57A. (c) Antigens 147 and 147A with antiserum 147A. (d) Antigens 146 and 146A with antiserum 146A. Antigens to pathogens were loaded in top wells, antigens to non-pathogens in bottom wells. Antiserum to pathogen was placed in a slot cut down the centre of the slide after electrophoresis. (e) Typical reaction against antiserum 27. Antigen 27 in bottom well, antigen 200 in top well with antiserum 27 loaded in centre slot after electrophoresis.

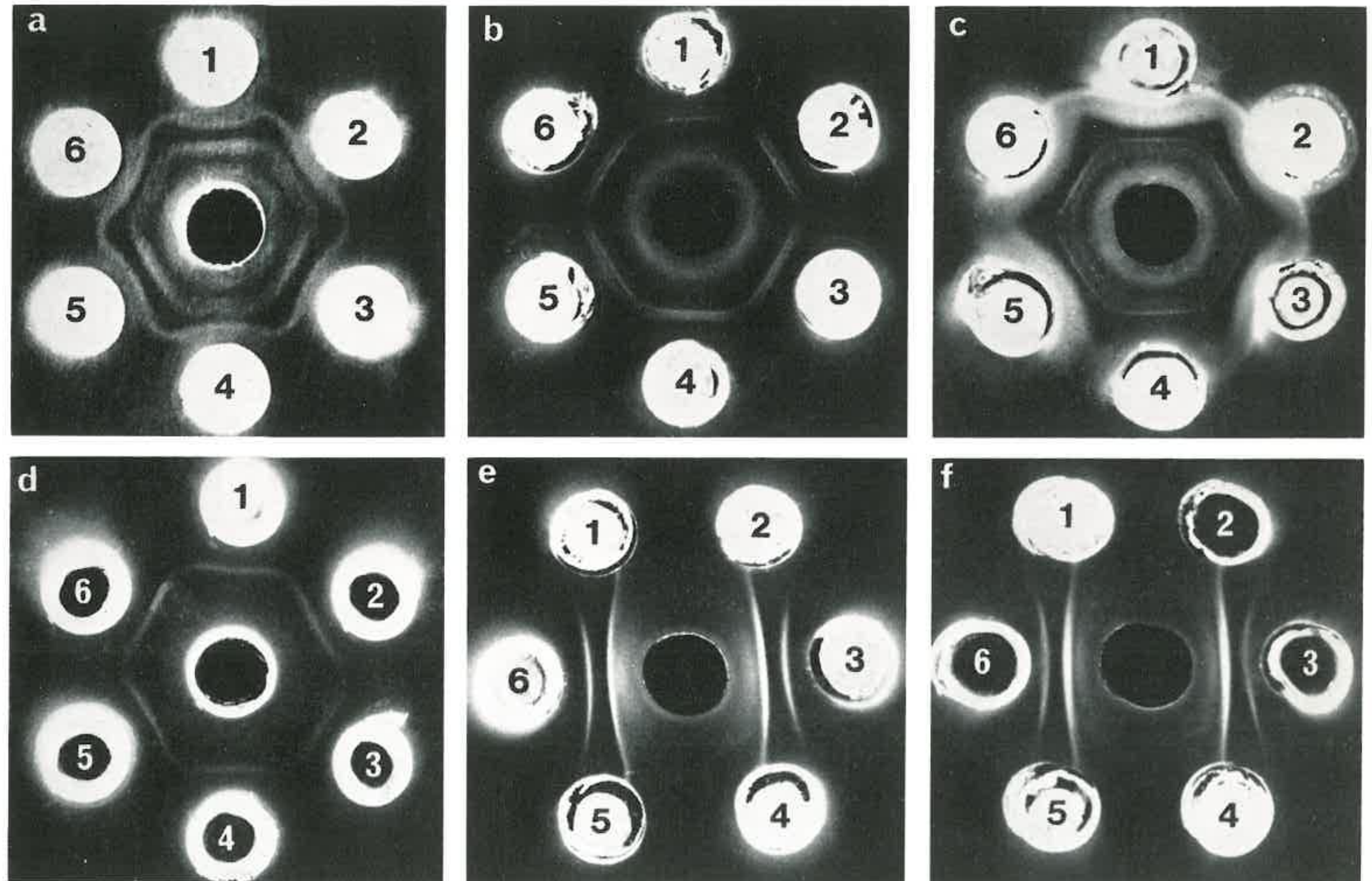


PLATE 1. Gel diffusion test. (a) Isolates 57 and 57A. (b) Isolates 200 and 200A. (c) Isolates 147 and 147A. (d) Isolates 146 and 146A. Peripheral wells 1, 3 and 5 contain antigen to non-pathogen; peripheral wells 2, 4 and 6 contain antigen of pathogen. Centre well contains antiserum to pathogen. (e) Isolate 27 antiserum against antigens 57, 57A, 200 and 200A. Peripheral wells contain antigens 57 (1), 57A (2), 27 (3 and 6) 200 (4) and 200A (5). (f) Isolate 27 antiserum against 147, 147A, 146 and 146A, Peripheral wells contain antigen 147 (1), 147A (2), 27 (3 and 6) 146 (4) and 146A (5).

was attempted by subjecting the antigens to electrophoresis before exposing them to antisera. Results are presented in Fig. 1. Although there were minor antigenic differences between some of the parent–pathogen pairs, there was no band consistently present in the pathogens and absent from the non-pathogens.

*Enzyme patterns following electrophoresis*

The pathogenic strains 57A, 146A, 147A and 200A and the respective non-pathogenic parent strains 57, 146, 147 and 200 were tested for activity of the enzymes acid phosphatase, alkaline phosphatase and malate dehydrogenase following electrophoresis. Results are presented in Fig. 2. There was a slight variation in mobility of enzymes in different gels making a simple comparison between individual strains unreliable. For this reason, each strain was tested by itself and also mixed with its

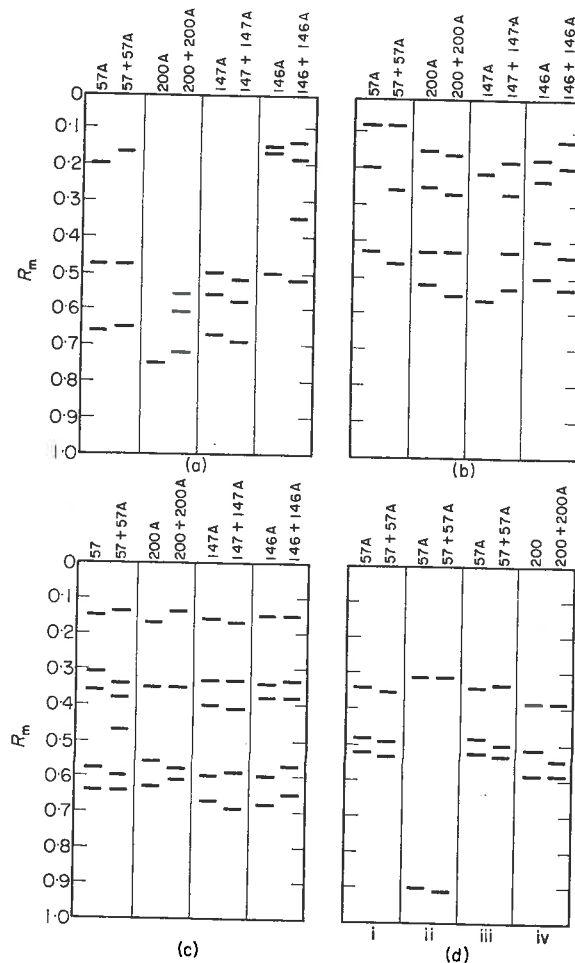


FIG. 2. Electrophoresis. (a) Acid phosphatases. (b) Alkaline phosphatases. (c) Malate dehydrogenases. (d) i, peroxidases; ii, esterases; iii and iv, catalases. Left column: one isolate run alone; right column: both non-pathogen and pathogen run mixed.

related strain. If different isozymes were present in two related strains, they would show up as distinct bands in the mixture.

There were definite differences in isozyme composition between some non-pathogenic parents and their pathogenic progeny but these differences were not consistent for all four pairs. For example, strain 146 had one more acid phosphatase isozyme than strain 146A, and strain 200 had two more than strain 200A but there was no difference between strains 57 and 57A or between strains 147 and 147A. Similar inconsistent differences occurred in isozymes of alkaline phosphatase and malate dehydrogenase.

Strains 57 and 57A were also tested for catalases, esterases and peroxidases and strains 200 and 200A for catalases only. Pathogens and non-pathogens could not be distinguished (Fig. 2).

It would seem that neither serological reactions nor isozyme patterns are promising approaches to elucidating the problem of pathogenicity in *Agrobacterium*.

*Reaction to mitomycin C*

Several workers [*1, 2, 16, 18, 31, 36, 40, 44, 54, 55, 59*] have suggested that a temperate phage of *A. radiobacter* might be involved in tumour induction. Although the claim that DNA of the phage PS 8 could induce tumours [*36*] has recently been seriously queried [*5*] the involvement of phage would certainly help to explain many of the perplexing aspects of tumour induction.

Mitomycin C is an efficient inducing agent for temperate phage [*11*] and it was decided to test the eight strains studied in the previous section for reaction to mitomycin C. Results are given in Fig. 3. A 1-h exposure to 1 μg/ml mitomycin C had little effect on strains 57, 57A, 200 or 200A, producing only a slight flattening out of the growth curve. Strains 147 and 147A were more strongly inhibited and strains 146 and 146A showed a marked drop in optical density shortly after exposure to mitomycin C, followed by recovery after about 7 or 8 h. This reaction suggests that strains 146 and 146A carry an inducible temperate phage [*1*]. However, the important point is that, within each pathogen–non-pathogen pair, reaction to mitomycin C was essentially the same. So it does not appear that the strains have acquired a temperate phage in their conversion from non-pathogen to pathogen.

*Sensitivity to bacteriocin 84*

Because Kerr & Htay [*28*] reported that there was a very high correlation between pathogenicity and sensitivity to bacteriocin, all parent non-pathogens and converted pathogens were tested for sensitivity. Results are given in Table 2. Nine out of ten non-pathogens were resistant; all 25 converted pathogens were sensitive.

## DISCUSSION

In the earlier papers on acquisition of virulence by non-pathogens, Kerr [*26, 27*] reported that the non-pathogenic parents used by him were labelled with antibiotic resistance markers and that the converted pathogens retained these markers, indicating that there was a true parent–progeny relationship. In the present work, further evidence for this relationship was obtained, particularly from the reaction of strains to mitomycin C in which each parent reacted in the same way as its progeny

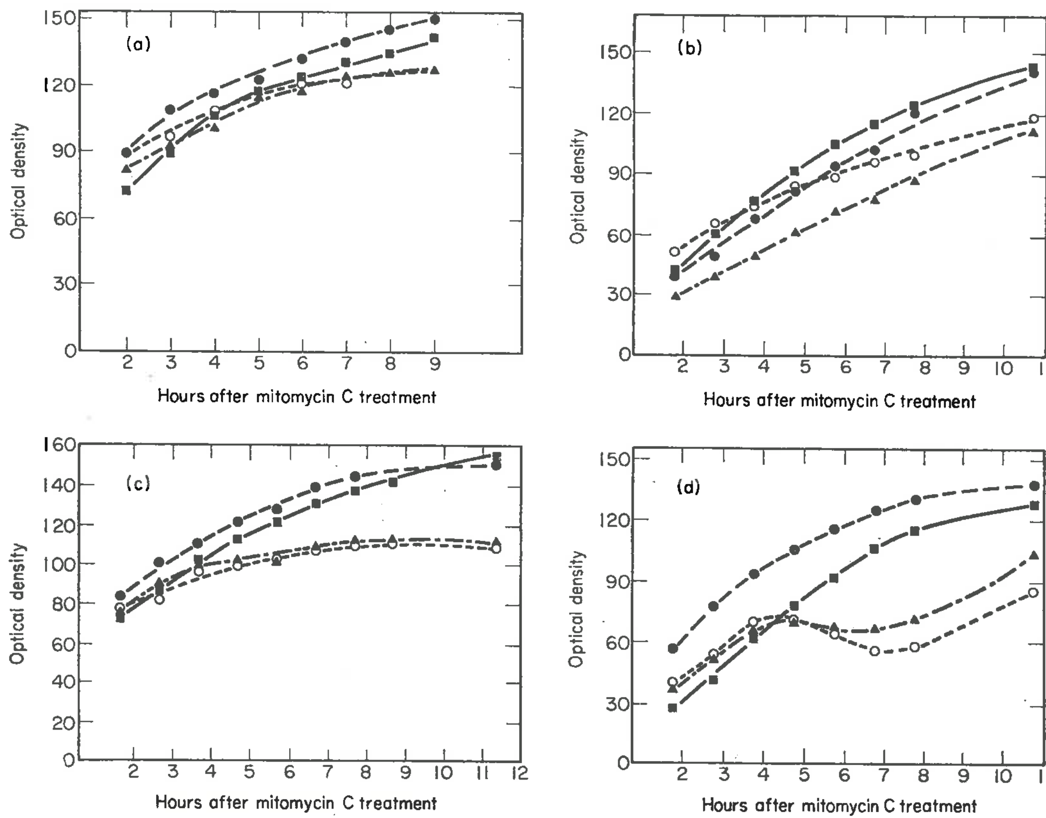


Fig. 3. The effect of mitomycin C. (a) Isolates 200 and 200A. (b) Isolates 57 and 57A. (c) Isolates 147 and 147A. (d) Isolates 146 and 146A. Non-pathogen control (● — — — ●); pathogen control (■ ——— ■); non-pathogen treated with mitomycin C (○ - - - - - ○); pathogen treated with mitomycin C (▲ — - — ▲).

Table 2

*Sensitivity of parent non-pathogens and converted pathogens to bacteriocin 84*

| Strain: | Sensitivity to bacteriocin 84 | Strain[a] | Sensitivity to bacteriocin 84 | Strain[a] | Sensitivity to bacteriocin 84 |
|---|---|---|---|---|---|
| 18 | − | 55 | − | 146C | + |
| 18A | + | 55A | + | 146D | + |
| 21 | − | 55B | + | 147 | − |
| 21A | + | 55C | + | 147A | + |
| 23 | + | 57 | − | 147B | + |
| 23A | + | 57A | + | 147C | + |
| 44 | − | 57B | + | 147D | + |
| 44A | + | 57C | + | 200 | − |
| 54 | − | 57D | + | 200A | + |
| 54A | + | 146 | − | 200B | + |
| 54B | + | 146A | + | 200C | + |
| | | 146B | + | 200D | + |

[a] Non-pathogenic parents are indicated by a number; converted pathogens are indicated by the number of their non-pathogenic parent and by a letter.
+ = sensitive; − = resistant.

but, in most cases, differently from other parents and their progeny. Serological reactions and enzyme patterns following electrophoresis also indicated that there was a very close relationship within each parent–progeny pair. None of these tests was able to distinguish all parents from all pathogens and it was concluded that they were unlikely to be useful in studying the basis of pathogenicity in *Agrobacterium*.

The high correlation between pathogenicity and sensitivity to bacteriocin 84 observed by Kerr & Htay [*28*] was confirmed. They noted that a change from bacteriocin sensitivity to resistance entailed a change from pathogenicity to non-pathogenicity. The converse was established in this paper, namely that a change from non-pathogenicity to pathogenicity entailed a change from bacteriocin resistance to sensitivity. It would seem that this correlation between pathogenicity and sensitivity to bacteriocin 84 is unlikely to be a fortuitous relationship. All bacteriocins that have been studied in depth are proteins and operate through attachment to receptor sites [*46*] and it would seem reasonable to assume that the same mechanism applies to bacteriocin 84. This would mean that tumour-inducing bacteria must have a specific molecular configuration on the cell surface acting as the receptor site for bacteriocin 84. When this configuration is changed, pathogenicity is lost [*28*]. Attenuation of pathogens by glycine, which modifies the cell wall [*33*, *34*], supports this conclusion. Treatments which increase infectivity, such as ultraviolet light [*21*], mitomycin C [*22*] and chlorpromazine hydrochloride [*20*] could operate through promoting the formation of receptor sites.

Considerable evidence is accumulating that crown gall induction involves the transfer of bacterial DNA into the plant cell [*12*, *41*, *45*, *47–49*, *52*, *53*, *58*]. However, there is also strong evidence that tumours cannot be induced by naked DNA extracted from pathogenic bacteria [*7*, *10*, *53*, *58*], despite two recent claims to the contrary [*6*, *30*]. This indicates that release of DNA by agrobacteria and its uptake by plants is not the process of tumour induction. From the mitomycin C data presented in this paper and from other data [*3*, *4*, *5*, *11*, *14*, *29*, *31*, *32*, *40*, *59*], the implication of bacteriophage in the process must now be seriously doubted. From our knowledge of DNA transfer between bacteria [*19*], it would seem that the only other likely method of DNA transfer is through conjugation. In crown gall the conjugation would have to be between bacterial and plant cells. The first prerequisite for conjugation between two bacterial cells is a specific molecular configuration on the cell surface of the donor cell in the form of sex pili [*19*] whose presence can be readily demonstrated because they act as receptor sites for male-specific. phages [*19*]. If our interpretation of data is correct, pathogenic strains of *A. radiobacter* have a specific molecular configuration on the cell surface which acts as a receptor site for bacteriocin 84. So it would appear that the first prerequisite for conjugation has been satisfied. Reciprocal sites on the surface of a dividing plant cell would also be required and these are likely to be the infection sites proposed by Lippincott & Lippincott [*38*] and by Schilperoort [*47*] to explain their data on inhibition of tumour induction by non-pathogens. Further evidence for the presence of infection sites on plant cells has been provided by Yajko & Hegeman [*58*]. Conjugation could explain the step-wise process in crown gall induction reported by Braun [*9*] because DNA transfer through conjugation between bacterial cells is a step-wise, or more accurately, a linear process [*19*].

Before conjugation between bacterial and plant cells is accepted as the process of crown gall induction, some anomalous results would have to be explained. In this paper, one non-pathogen, strain 23, is sensitive to bacteriocin 84 and, according to our argument, should be able to conjugate with plant cells. If so, transfer of DNA to the plant cells must be inhibited. A strain that behaved similarly was noted by Kerr & Htay [*28*]. These strains would occupy conjugation sites and compete for them with pathogens as proposed by Lippincott & Lippincott [*38*] and Schilperoort [*47*]. Kerr & Htay [*28*] also noted that some pathogens are resistant to bacteriocin 84. It would seem unlikely that two entirely different mechanisms are involved in crown gall induction by two closely related pathogens. Those resistant to bacteriocin 84 must have a different cell surface molecular configuration which also promotes conjugation in much the same way as two kinds of sex pili can promote conjugation in *Escherichia coli* [*19*]. Alternatively, they have the same molecular configuration as the bacteriocin-sensitive pathogens but are immune, rather than resistant to bacteriocin 84 [*46*].

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