

MORPHOLOGY, MEMBRANE CHARACTERIZATION AND DETECTION OF A BACTERIUM  
ASSOCIATED WITH RATOON STUNTING DISEASE OF SUGARCANE

by

DORSAMY PILLAY

Submitted in partial fulfilment of the requirements for the Degree of  
Master of Science in the Department of Microbiology and Hygiene in the  
Faculty of Science at the University of Durban-Westville

Supervisor : Professor R.A. Oellermann

Co-supervisor: Professor G. Roth

Date submitted: March 1984

For my late parents and Loshni

## ACKNOWLEDGEMENTS

The author would like to record his appreciation to the following:

Professor R.A. Oellermann, Associate Professor and Senior Lecturer, Department of Microbiology, University of Durban-Westville, whose meticulous direction, interest, encouragement, invaluable criticisms and suggestions has made possible the successful completion of this research programme.

His late parents and members of his family, who have afforded him the opportunity of pursuing post-graduate studies, and for their patience, encouragement and moral support.

Loshni, for her patience and constant encouragement.

Members of staff and post-graduate students of the Department of Microbiology, University of Durban-Westville, for their advice and encouragement.

Dr J.D. Conradie and Mr M. Govender of the Natal Institute of Immunology, Sarnia, for their advice and assistance with the development of the ELISA test.

The South African Medical Research Council for making his studies financially possible.

Mrs E.L. van Hooff for assistance with the photography.

Mrs P.V. Subramony for typing of the dissertation.

## CONTENTS

	PAGE
INTRODUCTION	1
I. <u>LITERATURE REVIEW</u>	3
A) RATOON STUNTING DISEASE	3
1. History, economic importance and distribution	3
2. Causal organism	5
3. Symptoms and diagnosis	11
(a) Chemical assays	13
(b) Indicator plants	15
(c) Microscopy	16
4. Control	20
B) SEROLOGY IN PLANT PATHOLOGY	24
II. <u>MATERIALS AND METHODS</u>	29
A) ISOLATION OF BACTERIUM FROM SUGARCANE	29
B) PREPARATION OF MEMBRANES	31
C) PROTEIN DETERMINATION	32
D) IMMUNIZATION	33
E) IMMUNOLOGICAL ASSAYS	35
1. Microagglutination	35
2. Immunofluorescence	36
3. Enzyme-linked immunosorbent assay	37
4. Immunodiffusion	40
F) COMPOSITION AND STRUCTURE	41
1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis	41
2. Electron microscopy	42

	PAGE
(a) Negative staining	42
(b) Ultramicrotomy	43
 III. <u>RESULTS</u>	 45
A) ISOLATION OF BACTERIUM FROM SUGARCANE	45
B) PREPARATION OF MEMBRANES	46
C) PROTEIN DETERMINATION	63
D) IMMUNOLOGICAL ASSAYS	65
1. Whole bacterium	65
(a) Microagglutination	65
(b) Immunofluorescence	68
(c) Enzyme-linked immunosorbent assay	69
2. Membranes	84
(a) Microagglutination	84
(b) Immunofluorescence	87
(c) Immunodiffusion	89
E) MORPHOLOGY AND COMPOSITION	91
1. Whole bacterium	91
(a) Electron microscopy	91
2. Membranes	93
(a) Electron microscopy	93
(b) Sodium dodecyl sulphate polyacrylamide gel electrophoresis	 95
 DISCUSSION	 96
 SUMMARY	 114
 REFERENCES	 117

## INTRODUCTION

Ratoon stunting disease (RSD) of sugarcane was first recognized in 1944 in Queensland, Australia (Steindl, 1961). The disease occurs worldwide and causes significant yield losses, especially during drought. RSD produces no external symptoms except a non-specific stunting (Steindl, 1961).

RSD, which was first recorded in South Africa in 1953 (Anon., 1960), causes a greater overall loss in yield than any other sugarcane disease in South Africa. Yields of sugarcane are reduced by 20% to 40% and the harvest of affected fields declines progressively with successive ratoons (Anon., 1980b).

A virus was originally thought to cause RSD, but in 1973, a coryneform bacterium was implicated as the causal agent (Gillaspie *et al.*, 1973; Teakle *et al.*, 1973). In 1980, our laboratory reported the successful isolation and culture of a coryneform bacterium associated with RSD of sugarcane and was indicated to be the causal agent (Nayiager *et al.*, 1980).

The lack of a rapid diagnostic technique applicable to mass screening of sugarcane has hindered progress in the control of the disease. There are two types of commonly used diagnostic tests. One test depends on the evaluation of internal stalk symptoms which may require from two to twenty six weeks to develop (Gillaspie *et al.*, 1966; Matsuoka, 1971; Schexnayder, 1960; Singh, 1969). However, these symptoms are not always present in RSD affected plants and similar symptoms can sometimes result from other causes (Steindl, 1961). The other test involves establishing the presence of the coryneform bacterium associated with

diseased plants. The bacterium is visible under high magnification by phase-contrast microscopy (Gillaspie *et al.*, 1973) or by electron microscopy (Teakle *et al.*, 1973). Although identification by the latter methods requires little time, the technology involved severely limits the number of samples that can be examined. Recently, serological techniques have been used (Brlansky *et al.*, 1982; Damann *et al.*, 1977; Davis *et al.*, 1980; Gillaspie, 1978b; Gillaspie *et al.*, 1979; Harris and Gillaspie, 1978) but their success has been limited. Besides problems with diagnosis of the disease, the precise morphology and taxonomy of the causal organism is unclarified.

The objectives of this research programme were, firstly, to characterize the cultured intact bacterium and its constituent membranes both ultra-structurally and immunologically, and secondly, to evaluate various immunological methods for detection of the bacterium. This study should contribute to enhancing the taxonomic status of the bacterium and to the use of a rapid diagnostic technique applicable to mass screening of sugarcane. Such a technique should eventually contribute to effective control of RSD.

## I. LITERATURE REVIEW

### A) RATOON STUNTING DISEASE

#### 1. History, economic importance and distribution

Sugar is unique as an important article of commerce in being produced both in temperate and tropical regions of the world. It is unique in that two quite unrelated plants provide its source: the sugarbeet in temperate countries and the sugarcane in the tropics and sub-tropics. Beet comes from seed, is an annual crop and stores its sugar in a swollen underground root. In contrast, cane is propagated by lengths of stalk or setts, gives a plant crop and a number of ratoons, and stores its sugar in aerial stalks (Hughes, 1976).

Sugarcane supplies over sixty per cent of the world's commercial sugar (Hughes, 1976). Technologically the industry is highly organized but diseases are a recurrent problem. RSD is one of the most important disease problems with the world cane industry (Abbot, 1953; King, 1953; Thomson, 1958). This view is clearly supported by Steindl (1961) who wrote: "Ratoon stunting disease has probably caused greater yield losses throughout the sugarcane growing countries of the world during recent years than any other diseases. This has been due largely to its insidious nature, which has allowed it to build up high degrees of infection and cause continuous losses without detection. It has undoubtedly been responsible for the gradual yield decline or so-called "running-out" of many varieties, and has been responsible for the discarding of many promising seedlings which have out-yielded standard canes in early trials, but have lost vigour before reaching commercial status". It was first recognized in Queensland,



Australia as a specific disorder of sugarcane during the summer of 1944-1945 when a number of fields of the variety Q28 produced extremely poor ratoon crops compared with adjacent ratoons of the same variety. All Q28 plants distributed from certain farms had produced these characteristically poor ratoon crops. The only obvious symptom of RSD was a marked reduction in growth, particularly in the ratoon crop (Steindl, 1950; Hughes and Steindl, 1955).

RSD which was first recorded in South Africa in 1953 (Anon., 1960) constitutes twenty to fifty per cent crop losses per annum (Anon., 1977; 1980b) and is probably the country's most important disease of sugarcane. In fact, RSD has been recorded in most sugar-producing countries including Antigua, Argentina, Australia, Bangladesh, Barbados, Brazil, British Honduras, Ceylon, Colombia, Cuba, Dominican Republic, Egypt, Ethiopia, Fiji, Guyana, Hawaii, India, Indonesia, Ivory Coast, Jamaica, Japan, Kenya, Malagasy Republic, Malawi, Malaysia, Mali, Mauritius, Mexico, Mozambique, Nicaragua, Peru, Philippines, Puerto Rico, Reunion, San Cristobal, St. Kitts and Nevis, South Africa, Taiwan, Tanzania, Thailand, Trinidad, Tobago, Uganda, United States of America, Venezuela, Zaire and Zimbabwe (Ahmed *et al.*, 1979; Anon., 1972; Lo, 1954; Navarette, 1960; Orian, 1954; Santos, 1972; Steib, 1972; Steindl, 1974; Todd, 1956; Wehlburg, 1956). The absence of records on sugar yield losses in many countries is often more a reflection on local staff work than any indication that the disease is not present (Hughes, 1976). However, the effect of RSD on yield losses of some sugarcane varieties have been consistently recorded (Abbot and Zummo, 1962; King and Steindl, 1953; Koike, 1974; 1977; 1980a; 1980b; Lopez-Roia and Andusar, 1970; Steib and Chilton, 1956; 1957; Steib and Forbes, 1960; Steib *et al.*, 1967). A major problem encountered in studies on the distribution of RSD has been the difficulty in diagnosing the disease (Bechet, 1976). It is likely in

view of the difficulty of identification, that it occurs everywhere that sugarcane is grown. Difficulty in diagnosis and continued failure to isolate the causal agent have contributed to its spread to most sugarcane growing areas (Early, 1975).

## 2. Causal organism

The basic pre-requisite for control of any disease is isolation and correct characterization of the causal organism(s). Such information is also a pre-requisite for the selection of disease resistant sugarcane.

When RSD was first detected in Queensland neither the causal agent of the disease had been isolated nor had it been observed in microscopic examinations of diseased plants (Mungomery, 1949; Steindl, 1949; 1950). In 1953, Steindl and Hughes, despite their negative results obtained with the electron microscope and based on their crude positive results obtained from immunizing experimental rabbits, concluded that RSD was caused by a virus. However, all attempts, to detect and characterize the virus of RSD by ultraviolet absorption, electrophoresis and serological and staining tests were unsuccessful, as were procedures to concentrate and purify it by differential centrifugation (Steindl, 1957). Despite the obscure identity of the causal agent, it was generally presumed to be caused by a virus or virus-like particles (Antoine, 1960; Edgerton, 1958; Forbes and Ling, 1960; Gillaspie, 1970; 1971a; 1971b; 1974; Gillaspie *et al.*, 1966; Hughes, 1960; 1962; 1964; Hughes and Steindl, 1956; Hughes *et al.*, 1961; 1967; Liu, 1963; Matsuoka, 1971; 1972; Roth and Whitehead, 1965; Singh, 1969; Steindl and Hughes, 1953; Wismer, 1971).

There were two definite claims of virus-like particles being associated with RSD. Firstly, Forbes and Ling (1960) detected particles of two average sizes, 31,9 nm and 73,3 nm diameter, in shadow-cast preparations of droplets of leaf juice from diseased cane. These particles were absent in healthy plants. The larger particles were thought to be by-products of the disease, since they were also present in tomato plants infected with tobacco mosaic virus. The smaller particles were considered to be virus particles, since they approximated the size of several spherical viruses. However, electron micrographs of the particles published by Forbes and Ling (1960) did not clearly reveal virus particles and no attempt was made to show their infectivity (Teakle, 1974). Secondly, Gillaspie *et al.* (1966) presented evidence of a partially purified preparation containing spherical particles of a viral nature from RSD-infected sugarcane. They clarified and centrifuged stalk juice of sugarcane, and carried out density gradient centrifugation on the resuspended pellets. A density gradient band containing most of the surviving infectivity also contained the highest concentration of spherical particles and small contaminants. They concluded that they had isolated the infectious agent of RSD, which appeared to be a spherical virus of high nucleic acid content and undetermined size.

In 1963, Liu reported the purification by differential centrifugation of a nucleoprotein associated with RSD of sugarcane. However, when inoculated into the cut ends of heat-treated healthy seed setts, this substance did not show infectivity. Therefore it was still uncertain whether or not the preparation was a virus. Similarly, Hughes and Steindl (1955) found numerous small, roughly spherical particles in the juice of diseased stalks, but similar particles were also present in healthy stalks.

Further evidence implicating a virus as the causal agent of RSD of sugarcane included:

- (i) The report by Steindl and Teakle (1974) on the apparent ability of infectivity to pass through a bacteriological filter of pore size  $0,1 \mu\text{m}$  in one out of three tests. The claim by El-Banna *et al.* (1967) implicating a virus seems unjustified since their pore size of  $1,3 \mu\text{m}$  was greater than the dimensions recorded for the RSD-associated agent;
- (ii) The insensitivity of the agent to a number of antibiotic treatments (Gillaspie, 1970; Steib and Tantera, 1970; Steindl and Teakle, 1974); and
- (iii) The inability to culture the agent in artificial media (Hughes and Steindl, 1955).

The possibility that RSD was caused by a viroid, an uncoated ribonucleic acid 'virus' had been raised by Maramorosch *et al.* (1973). Although both viroids and the RSD agent were transmitted by knife wound and are readily sedimented during low speed centrifugation, they have many distinct differences, viz.:

- (i) Either  $0,3 \text{ M}$  sodium sulphite or  $0,5 \text{ M}$  dipotassium hydrogen phosphate was efficient in releasing viroids from association with host material, but these were ineffective with the RSD agent;
- (ii) Solvents such as chloroform did not affect the infectivity of viroids but eliminated or greatly reduced the infectivity of the RSD agent; and

- (iii) The sensitivity of the RSD agent to phenol extraction and insensitivity to RNase were other reactions which were different in viroids.

Teakle (1974) therefore concluded that it was unlikely for the RSD agent to be a viroid.

In 1972, Khurana suggested that RSD might be caused by a complex of at least two pathogens, a virus and a mycoplasma-like organism. However, he advanced no evidence supporting this possibility. At the same time Plasvic-Banjac and Maramorosch (1972), using the electron microscope, reported the presence of very small bacteria or mycoplasma-like bodies in sections of the xylem of internodes of old infected plants from Puerto-Rico, but not in those of healthy or young diseased plants. Following this report, Maramorosch *et al.* (1973) suggested that the xylem-invading microorganisms in the vessels resembled bacteria or rickettsiae. The microorganisms were reported to have a thin capsule, a distinct membrane, a ribosome-rich dense cytoplasm, and a central area of low intensity suggesting DNA filaments. The microorganisms in cross-section appeared to be rounded and of irregular size. The presence of a thin capsule or cell wall excluded the possibility that the RSD agent was a mycoplasma-like organism. Previous evidence (Gillaspie, 1970; Steib and Tantera, 1970) on the ineffectiveness of tetracycline compounds on the infectious agent and on symptom expression indicated that a mycoplasma was not the causal agent. In addition, the presence of the RSD-associated microorganisms in the xylem only (Maramorosch *et al.*, 1973) and their ready mechanical transmission (Davis *et al.*, 1981) made it unlikely that a mycoplasma-like agent was responsible for RSD.

In 1973, Teakle *et al.* were able, by use of the electron microscope, to beam into focus cells of a small bacterium consistently present in negatively stained fibrovascular sap extracts of diseased cane, but not of healthy cane. The bacterium was distinguished readily from other bacteria present by its small size (usually 1,0-2,5  $\mu\text{m}$  long by 0,15-0,32  $\mu\text{m}$  wide), the coryneform (club-shaped) morphology of some cells, and its permeability to negative stain revealing a thin cell wall surrounding a cytoplasmic membrane and coiled mesosomes. Since the small bacterium was never observed in fibrovascular extracts of RSD-free sugarcane plants, it was suggested to be a possible cause of RSD.

Other evidence consistent with the hypothesis that the agent causing RSD was a bacterium, and not a virus, was the ready sedimentation of RSD infectivity by low speed centrifugation, the sensitivity of the RSD agent to organic solvents such as chloroform and butanol, and to metabolic poisons such as sodium azide, and the ready elimination of RSD by hot water treatment of sugarcane stalks for 2 to 3 hours at 50C (Gillaspie, 1971b; Steindl, 1961).

Concurrent with the reports of Teakle *et al.* (1973) and Maramorosch *et al.* (1973), Gillaspie *et al.* (1973) reported that the RSD of sugarcane could be diagnosed by examining xylem exudates or juice extracts by phase-contrast or dark-field microscopy for the presence of a small, thin, rod-shaped, sometimes bent bacterium (5-10  $\mu\text{m}$  by 0,3-0,5  $\mu\text{m}$ ) that was consistently associated with the disease. However, this bacterium was much larger than that reported by Teakle *et al.* (1973).

These investigations were followed by numerous reports implicating a bacterium as the causal agent of RSD. Damann (1975) reported the presence of a small (1,3  $\mu\text{m}$  by 0,25  $\mu\text{m}$ ), mesosome containing, rod-shaped

bacterium as the possible causal organism of RSD. One series of reports culminated in assigning *Xanthomonas vasculorum* as the causal agent of RSD (Liu *et al.*, 1974b). Another group implicated *Xanthomonas albineans* as the possible causal agent of RSD (Tokeshi *et al.*, 1974). Other reports implicating simply a bacterium as the causal agent included those by Anon. (1975; 1980a), Bailey (1976; 1979), Damann (1975; 1980), Damann *et al.* (1975), Gillaspie and Blizzard (1976), Hughes (1976), Kao and Damann (1978), Kao *et al.* (1980), Ricaud *et al.* (1977), Steib (1974) and Tejada and Damann (1978). Reports of a coryneform bacterium as the probable causal agent included publications by Chen *et al.* (1975), Damann and Derrick (1975), Gillaspie and Harris (1978), Gillaspie *et al.* (1976a; 1976b; 1981a; 1981b), Liu (1978), Liu *et al.* (1979), Ricaud *et al.* (1976), Rishi and Nath (1978), Teakle (1974), Teakle *et al.* (1979), Weaver *et al.* (1977) and Worley and Gillaspie (1975). That the RSD-associated bacterium was the causal agent soon became widely accepted. The finding of the coryneform organism was regarded as a standard diagnostic test (Hughes, 1976) even though the bacterium had not been isolated in axenic culture. However, Koch's postulates for the organism as the causal agent remained unfulfilled.

In 1980, Nayiager *et al.* reported that sugarcane inoculated with one of their cultured bacteria produced symptoms characteristic of RSD. In culture the bacterium had all the morphological features so far described for the "RSD-associated bacterium". They suggested that the unidentified bacterium reported to be consistently present in the vascular fluids of infected cane may be the causal agent of RSD. Concurrent with the aforementioned investigation a coryneform bacterium from diseased sugarcane was isolated in axenic culture by Davis *et al.* (1980) using sugarcane (SC) medium which was specifically developed for the RSD bacterium. They claimed that it was the causal agent. The SC medium was used to

isolate the RSD-associated bacterium from sugarcane from the United States, Brazil, South Africa and Japan, and the isolates apparently readily incited RSD symptoms in susceptible sugarcane cultivars after inoculation. In addition, a bacterium from Bermuda grass from Taiwan, which morphologically, ultrastructurally, and serologically resembled the RSD bacterium, was isolated on the SC medium. The Bermuda grass bacterium incited stunting of Bermuda grass but not of sugarcane (Davis *et al.*, 1981).

Liao and Chen (1981a; 1981b) described a method and medium for isolating and culturing the bacterium associated with RSD of sugarcane and Bermuda grass. The pathogenic role of cultured organisms in Sudan grass was demonstrated. Based on the *in situ* formation of microcolonies and the branching of cells, Kao and Damann (1978; 1980) proposed that the RSD bacterium was an actinomycete. Branching had also been occasionally observed by Nayiager *et al.* (1980), Ricaud and Autrey (1978) and Teakle *et al.* (1975a). Despite the steady stream of information that has accumulated over the last few years, the exact taxonomic status of the RSD coryneform bacterium has yet to be assigned.

### 3. Symptoms and diagnosis

Knowledge of the specific symptoms associated with RSD on different varieties of sugarcane is a pre-requisite for accurate diagnosis of the disease. Diagnostic methods for RSD have been considered unsatisfactory by most pathologists.

The disease does not show any external symptoms except a non-specific stunting (Anon., 1977; 1980b; Edgerton, 1958; Hughes, 1976; Hughes and Steindl, 1955; Irvine, 1976; Kao and Damann, 1978; Liao and Chen,



1981a; Mayeux *et al.*, 1979; Ricaud, 1974; Roth and Whitehead, 1965; Steindl, 1950; Steindl and Hughes, 1953; Teakle *et al.*, 1973; Wiehe, 1954) which is particularly marked under conditions of water stress (Hughes, 1976; Ricaud, 1974; Rossler, 1974; Roth and Whitehead, 1965; Steindl, 1950; 1961; Steindl and Hughes, 1953) but may be only slight if irrigation is adequate (Thomson, 1960a; 1960b). The lack of visible external symptoms undoubtedly explains why the disease was not recognized in earlier years (Edgerton, 1958). In some countries, climatic conditions causing severe water stress, are highly conducive to symptom expression and accentuate the effects of the disease. In such cases diagnosis may be easier. In others, where such marked effects are not encountered, the disease can be very insidious (Ricaud, 1974). The effects of RSD have been reported as greater in varieties with slower rates of water flow through the stalk, possibly because such varieties possessed fewer large xylem vessels than did resistant varieties (Teakle *et al.*, 1975b). Greater numbers of bacteria in sensitive varieties might, therefore, be expected to aggravate further the effects of the disease. It should be noted that stunting effects may also be caused by a number of other disorders, such as nematode infestation, or inadequate and uneven plant nutrition (Bechet, 1976).

RSD has been considered for some time among the symptomless diseases until internal symptoms, which are of two types, were described (Anon., 1960; 1972; 1975; 1977; 1980b; Antoine, 1956; Artschwager, 1960; Bechet, 1976; Early, 1975; Gillaspie, 1970; Hughes, 1956; 1960; 1964; 1976; Hughes and Steindl, 1955; 1956; Liao and Chen 1981a; Maramorosch *et al.*, 1973; Ricaud, 1974; Roth and Whitehead, 1965; Schexnayder, 1960; Steib *et al.*, 1956; Steindl, 1950; 1961; Steindl and Hughes, 1953; Teakle *et al.*, 1973; Wiehe, 1954). One type consisted of a nodal discolouration (which varied through yellow, pink,

orange, red to reddish brown in different varieties) of individual vascular bundles in mature cane. The other was a general pink, or pale strawberry-icing colour, throughout the upper nodes of very young cane.

According to Hughes (1976) internal discolourations were not entirely satisfactory for diagnosis since bundles in mature nodes may be discoloured nonspecifically from other causes. Similar symptoms may result from a number of other diseases such as gumming, leaf scald and chlorotic streak. Symptoms of *Fusarium* stem rot, red rot or borer damage may obscure RSD symptoms normally seen in mature cane (Ricaud, 1974). Absence of the symptoms is no proof of absence of the disease (Abbot, 1960).

It would appear that several factors may affect the expression of macroscopic symptoms of the disease, for example, varietal characteristics, climatic conditions, physiological status of the plant and the presence of other diseases (Ricaud, 1974). For these reasons macroscopic symptoms in the diagnosis of RSD are quite unreliable under certain conditions and special methods have been developed. These include chemical assays, the use of indicator plants, microscopical examinations and serological approaches. The use of serology, which constitutes an integral part of this dissertation, shall be considered in the next section.

(a) Chemical assays

Numerous attempts to develop chemical tests for the precise and accurate diagnosis of RSD have been made. In 1956 Parish found differences between healthy and diseased plants in investigations involving paper chromatographic analysis of amino acids. This study was followed by

Fife and Stokes (1959) who found that the concentration of aspartic acid, glutamic acid, serine, glycine, threonine, citrulline and at least one other amino acid as yet unidentified, was higher in the leaves of RSD infected plants than in the leaves of healthy plants. However, further studies were necessary to evaluate the efficiency of paper chromatography as a means of identifying sugarcane plants that were infected with RSD.

In 1957, Farrar found that slices of cane tissue from the periphery of the mature basal nodes of healthy plants developed a blue-green colour in the parenchyma around vascular bundles when treated with hydrogen peroxide and hydrochloric acid. This colour did not develop in diseased canes.

The development of a histochemical test involving the reduction of 2,3,5-triphenyl tetrazolium chloride showed some promise in the diagnosis of RSD (Antoine, 1958; 1960). Cane discs were sampled in a standardised way from nodal tissues and placed in a 0,5% solution of salt at 35C. The red colour, developing as a result of reduction of the salt to formazan, was extracted from the discs with acetone for colorimetric determination. Diseased canes were found to consistently develop a deeper red colour than healthy ones. This test was in agreement with the observation of mature stalk symptoms. However, the method had certain limitations in that it was applicable only to mature cane, it did not work with certain varieties and no absolute values could be obtained for healthy and diseased canes.

In 1965, Roth and Whitehead reported a test involving silver nitrate and ammonia or potassium hydroxide, in which fibrovascular bundles in diseased stalks were stained red. However, the physiological status of

the plant interfered with the test which was found unreliable with young cane.

All chemical assays described were lacking in reliability, accuracy and reproducibility. A basic difficulty in the acceptance of chemical tests as aids in diagnosis was that they could not be any more accurate than the criterion of performance upon which they were based.

(b) Indicator plants

The lack of a suitable diagnostic method has been a serious handicap in determining the occurrence of RSD as well as in the advancement of related phytopathological studies. Investigators have, over the decades, attempted the traditional method of inoculating indicator plants for the diagnosis of RSD. Certain investigators have attempted to discover suitable indicator plants other than sugarcane varieties (Hughes and Steindl, 1956; Steib and Forbes, 1957; Steindl, 1957; Wehlburg, 1956). These included tomato, zinnia, *Nicotiana glutinosa*, eggplant, maize, sorghum, sweet Sudan grass, Johnson grass, corn and various other gramineae.

Until recently, the best indicator plant has been sugarcane itself. Inoculation followed by observation of symptoms in the young shoots that develop from them, has been used successfully by many workers. Certain varieties have been especially preferred for this technique, for example Q28 (Hughes and Steindl, 1955; Steindl, 1961; Steindl and Teakle, 1974), CP 36-105 (Schexnayder, 1960), CP 44-101 (Gillaspie *et al.*, 1966) and CO 421 (Singh, 1969). The difficulty of this method was that some plants inoculated with a low dose of bacteria may not show symptoms despite successful infection (Ricaud, 1974).

In 1960, Schexnayder described the most refined technique for the above method of diagnosis. One-eyed buds of variety CP 36-105, from which RSD had been eliminated after four cycles of hot air treatment, were inoculated by dipping in infected juice. Symptoms were examined ten weeks after germination, followed by growth under conditions of low fertility, low water and restricted root development in steamed soil.

Inoculation was successful in 98,5 per cent of cuttings and symptoms were shown by 100 per cent of the infected ones. In 1966, Gillaspie *et al.* who used variety CP 44-101 and grew inoculated cuttings in vermiculite, found that symptoms developed in four to six weeks with the above technique. In 1976 Bechet found that of seven varieties tested, only CP 44-101 gave fairly good results. However, this occurred only when it was grown outside the glasshouse, in the shade and particularly during the winter months. Although juvenile symptoms were sometimes clear, they were not suitable for rapid and reliable diagnosis of RSD because they were unpredictable and transitory (Bechet, 1976).

Studies in Brazil (Matsuoka, 1971; 1972) have yielded successful results with varieties and hybrids of elephant grass (*Pennisetum purpureum*) when used as uprights (Benda, 1969; 1971). The shoots were inoculated by cutting the spindle with a knife dipped in infected juice, some of which was poured over the cut surface. The technique had the following advantages:

- (i) Elephant grass plants could be raised from seeds, thus facilitating the supply of disease (virus)-free plants;
- (ii) They could also be propagated by cutting with the advantage of permitting clonal selection of types more suitable for the test;

- (iii) The plants were easier to grow than sugarcane;
- (iv) They required less glasshouse space; and
- (v) Elephant grass was very susceptible to the causal agent and the symptoms shown by indicator plants were conspicuous, easy to recognize and appeared within a short while.

✦ In Australia good results were obtained with bana grass, hybrids of *P. purpureum* and *P. americanum* (Steindl and Teakle, 1974). Inoculation of napier grass (*P. purpureum*) in South Africa has been unsuccessful, only inconspicuous and very pale symptoms being observed (Bechet, 1976). However, results obtained from a series of tests with clones of bana grass used as uprights proved very promising (Bechet, 1976).

Recently Gillaspie *et al.* (1981a) reported the results of their pathogenicity testing by inoculating diagnostic hosts of RSD with their bacterium. Suspensions of bacteria from cultures were adjusted to similar concentrations in infected cane based on turbidity measurements and were used as inoculum. No indication of bacterial concentrations were given. Pathogenicity was established by the development of juvenile and mature nodal symptoms in sugarcane variety CP 44-101 and the characteristic wilting of sorghum-sudan grass hybrid NB 280S uprights. All isolates produced symptoms in CP 44-101 and in NB 280S, and all attempts at reisolation from these plants were successful.

### (c) Microscopy

One of the problems associated with the control of RSD has been the time involved in making a positive identification of affected cane at an early stage. Identification by symptoms in affected cane may be unreli-

able, but takes only about one minute. The other methods are usually reliable, but may take several months for the indicator cane Q28, and several weeks for the elephant grass.

The use of light, phase contrast, dark-field, scanning electron and transmission electron microscopy has proved very useful for positive diagnosis and may take up to one hour only. Growing evidence of the consistency of the association of a bacterium with RSD strongly indicates a bacterial etiology of RSD, and also indicates the possibility of rapid RSD diagnosis (Gillaspie *et al.*, 1973; Teakle *et al.*, 1975a).

Light microscopic examinations of concentrated stalk extracts, exudates and tissue diffusates have revealed a bacterium *in situ* within the xylem elements of sugarcane (Bailey, 1976; Gillaspie *et al.*, 1973; Ricaud *et al.*, 1975; 1976). However, careful examination of samples under the microscope was required even though the bacterium was easily distinguished from other contaminants. If none of the diagnostic bacteria were found, it was necessary to examine several samples of juice before diagnosing the cane as free of RSD. Apart from problems due to low numbers of bacteria in some varieties, and in young tissues, two other sources of error in identification of the RSD bacterium by light microscopy appeared possible. Contamination of tissue diffusates by saprophytic bacteria occurred rapidly but could largely be prevented by aseptic technique, the maintenance of low temperatures during processing and storage of preparations, and the avoidance of any delay before observation. The diffusate technique also readily extracted other vascular bacterial inhabitants, such as *Xanthomonas* spp., and any saprophytic colonising bacteria. Care in the selection of material for processing and the use of fresh samples aided in preventing confusion with other bacterial and fungal inhabitants. However, with experience the

slender form of the RSD bacterium could, with little difficulty, be distinguished from other bacteria that were likely to be present (Bailey, 1976).

Diagnosis by means of dark-field microscopy (Chen *et al.*, 1975; Gillaspie *et al.*, 1973; 1974a; 1974b; Liao and Chen, 1981b; Ricaud *et al.*, 1976) and phase contrast microscopy (Anon., 1979a; Bailey, 1976; 1977; Chen *et al.*, 1975; Gillaspie *et al.*, 1973; Kao and Damann, 1978; Ricaud *et al.*, 1976; Rishi and Nath, 1978; Steindl, 1976) has proved useful as confirmatory aids, but cannot confidently be used as the sole methods of diagnosis in very young cane. The RSD-associated microorganism can be distinguished easily from other bacterial contaminants by dark-field microscopy because of its characteristic shape and filiform morphology. Bailey (1976) found that the associated organism was observed with ease by phase contrast microscopy in diffusate preparations from mature stalk tissues of a wide range of varieties affected by RSD, provided that the preparations were adequately concentrated before examination. Phase contrast microscopy has also proved to be potentially useful as an improved method for screening sugarcane for resistance to RSD injury. The method was found to be rapid and yielded bacterial counts which correlated with clones resistant or susceptible to RSD injury (Gillaspie *et al.*, 1976a). Steindl (1976) found that phase contrast microscopy provided a rapid and satisfactory diagnosis for RSD in mature cane stalks when field identification was in doubt. In 1973, Gillaspie *et al.* found that phase contrast and dark-field microscopy were generally more accessible than electron microscopy, as well as being faster and easier. However, some limitations in the use of phase contrast examination of diffusates from young cane were apparent, as preparations from very young tissue were found to contain only a few bacteria in a confusing multiparticulate background (Bailey, 1976).



The electron microscope, which has a far greater magnifying and resolving power than the light microscope, has recently been used for the detection of the RSD-associated organism in sugarcane. In 1960, Forbes and Ling were able, by use of the electron microscope, to detect definite particles in juice from plants, affected by RSD. In 1972, Plasvic-Banjac and Maramorosch, on electron microscopical examination of sections of ratoon stunted cane, observed the presence of pleomorphic bodies which resembled small bacteria in the xylem. This finding was subsequently followed by numerous investigations in which the electron microscope provided the probe for detection and diagnosis (Bailey, 1977; Chen *et al.*, 1978; Kaiser and Ramos, 1980; Liu *et al.*, 1974a; Maramorosch *et al.*, 1973; Nayiager *et al.*, 1980; Ricaud *et al.*, 1975; 1976; Rishi and Nath, 1978; Teakle *et al.*, 1973; 1975a; 1979). Scanning electron microscopy was shown to be an extraordinary tool for the observation of the RSD-associated bacterium *in situ*, primarily because of its utility in viewing large areas of the specimen and revealing the three dimensional structure (Kao and Damann, 1980). The previous use of transmission electron microscopy (Kamiunten and Wakimoto, 1976; Maramorosch *et al.*, 1973; Weaver *et al.*, 1977; Worley and Gillaspie, 1975) for the study of this organism apparently did not allow observation of microcolonies and long branching filaments *in situ*. In general diagnosis by electron microscopy is relatively rapid but requires expensive equipment and highly skilled technicians (Steindl and Teakle, 1974).

#### 4. Control

In view of the economic importance of sugarcane, effective control of the causative agent of RSD is necessary. Although there are numerous gaps in our knowledge of RSD, sufficient information is available for recommendations to be made for its control in commercial cane plantings.

Over the past three decades, a number of control procedures were advocated in an attempt to reduce crop losses.

The most important control measures involved the use of disease-free planting material (Egan, 1980; Steindl, 1950; Steindl and Hughes, 1953) and the production of healthy seed cane (Anon., 1977; 1980b; Hughes and Steindl, 1955). The sterilization of cane knives and harvester blades was considered particularly important as a means of preventing mechanical transmission of the organism when cutting seed-cane fields or nurseries (Anon., 1977; 1980b; Early, 1975; Hughes and Steindl, 1955; Martin and Conant, 1939; Steindl, 1950; Steindl and Hughes, 1953).

Ideally, effective control of any disease would involve the substitution of susceptible varieties by resistant ones (Martin and Conant, 1939; Roth and Whitehead, 1965). The use of resistant varieties for the control of RSD does not offer as much hope of success as it did with such diseases as gumming and downy mildew (Steindl and Hughes, 1953). In 1971, Wismer reported the production of resistant clones, but these were not suitable for commercial use, and the author was not aware of the existence of any resistant commercial variety. There was considerable variation in the tolerance of commercial clones, some of which suffered only small losses with the expression of obscure symptoms. Such varieties may have some merit, but on the other hand, they could be a source of infection for more susceptible varieties, since growers would not realise that they were diseased and would disregard the necessary control measures. Therefore, the control of the disease by resistant varieties should not be encouraged. According to Hughes (1976) it appeared to be impossible to breed resistance to ratoon stunting into commercial cane.

Thermotherapy, using hot water (Anon., 1960; 1968; 1975; 1977; 1979a; 1980a; Antoine, 1956; Benda, 1972; 1974; 1975; Bourne, 1963; Cifuentes *et al.*, 1975; da Silva, 1974; Edgerton, 1958; Egan, 1980; Hoarau, 1969; Hughes *et al.*, 1967; Hughes and Steindl, 1956; Liu *et al.*, 1963; Martin and Conant, 1939; Mungomery, 1953; Pollock, 1955; Schexnayder, 1956a; 1956b; Steindl, 1950; Steib and Chilton, 1960; Thomson, 1960a; Wiehe, 1954) and hot air (Anon., 1980c; Antoine, 1956; Chu *et al.*, 1960; Chu and Lee, 1969; Cifuentes *et al.*, 1975; Edgerton, 1958; Hughes and Steindl, 1955; Robinson, 1960; Schexnayder, 1956a; 1956b; Steib *et al.*, 1956; Steib and Chilton, 1960; Steindl, 1950; Tantera and Steib, 1972; Thomson, 1971) has been the standard treatment of controlling RSD for the past three decades. The low thermal capacity of air makes it difficult to get a quick temperature rise and efficient heat transfer. Hot water is effective but has a debilitating effect on germination of young cane setts (Mayeux *et al.*, 1979). Hot-air treatment was used extensively in Louisiana and to a lesser extent in other sugar producing countries. It gave excellent results when properly controlled, but the accurate control of temperature in a hot-air oven was more difficult than in a water tank, and, in addition, the procedure was time consuming. However, better germination was obtained with succulent immature cane than with the hot water treatment. The hot water treatment is used in a majority of countries where a very cold winter does not necessitate the use of immature cane for treatment. In Australia the standard practice is to subject whole stalks to a hot water treatment of 50C for three hours. Various combinations of time and temperature within the range of one to three hours at 50C to 52C have been used in a number of countries usually with a high degree of control (Steindl, 1974). Whether whole stalks or setts are treated depended on the method of planting (Benda, 1975).

According to Antoine (1956) the hot water treatment was preferred as it appeared that it was easier in practice to obtain a good temperature distribution in water than in air. Furthermore, stabilization of the medium at the treatment temperature was reached in water almost immediately after contact with the cane mass. This temperature control led to a more uniform treatment of all the setts.

It has been reported that aerated steam could be effectively used for the control of RSD (Cifuentes *et al.*, 1975; Cochran *et al.*, 1975a; 1975b; Damann and Derrick, 1976; Mayeux *et al.*, 1979; Shukla *et al.*, 1974; 1979; Steib and Cifuentes, 1976). Aerated steam is defined as a mixture of air and steam. An appropriate mixture is obtained by adding steam to recirculating air to bring it up to the desired temperature. Aerated steam has heat transfer properties approaching that of water, yet does not exhibit the harmful effect of tissue softening. It has the simplicity of hot air without its harmful effects - slow heat rise and desiccation of setts. The success of thermotherapy in sugarcane is dependent upon maintaining a precise temperature. To maintain a temperature sufficient to inactivate the bacteria yet not lower the germination percentage requires a precision of 0,55C (Mayeux *et al.*, 1979).

Field tests showed that germination of cane treated in the commercial design by Mayeux *et al.* (1979) was not adversely affected and RSD was controlled by a four hour treatment at 52C. Compared to hot air, treatment time and cost was reduced by as much as 50 per cent and 75 per cent respectively. The large cost reduction was attributed to mechanical handling of the sugarcane and to the fact that there was no need to detrash the cane before treatment. In comparison to hot water, aerated steam had the big advantage of not requiring a large quantity of steam or electricity, and it did not injure young cane as severely as hot

water.

Some measure of success in the control of RSD stems from the fact that clean seed can be obtained, aerated steam is highly effective, and that natural spread from field to field, or even within a field is comparatively slow.

#### B) SEROLOGY IN PLANT PATHOLOGY

Problems in the control of plant bacterial diseases are largely due to difficulties in identifying the pathogens. Therefore, there is a need for the development of effective and rapid methods for identification of plant pathogenic bacteria. Much of this increased need for rapid identification is a result of an increased international trade and movement of plant propagative materials coupled with the economic importance of certain crops. According to Schaad (1979), biochemical and physiological tests which were routinely used to identify plant pathogenic bacteria were not entirely satisfactory since they were often complicated, difficult to interpret, and required weeks or months to complete. In addition, not all strains of each organism gave the same results.

The use of serology for identifying bacteria is almost as old as the science of plant pathology itself (Schaad, 1979). Many medically important bacteria are routinely identified by serological tests. The ability of medical bacteriologists to provide quick and accurate serological identification of bacteria indicated a similar potential for identification of plant pathogenic bacteria. However, an understanding of the mechanisms of serology (antigen-antibody interaction) is necessary for an adequate interpretation of serological tests.

Serology is an extremely valuable tool in molecular biology and is the science of reactions, preparations and use of sera. Serological techniques are presently used for purification, determination of structure and function, and identification of specific compounds. Studies of antigen-antibody reactions constitute the methods of serology. An antigen is a substance that elicits a specific immune response when introduced into the tissues of an animal. The response may consist of immunoglobulin (Ig) production, cell mediated immunity or immunologic tolerance. Antiserum is the fundamental reagent of serology and provides great versatility and high specificity. The antigen-antibody reaction results in the formation of a three-dimensional lattice in which the antigen and antibody alternate. This reaction can be observed microscopically by immunofluorescence or visually as precipitations which require several hours, or even days, depending upon the conditions. Precipitation is the final visible result of a serologic reaction. Agglutination is similar to precipitation except that the antigen is particulate.

The need for a rapid and accurate technique applicable to mass screening of sugarcane propagating material cannot be overemphasized. As already mentioned, the use of indicator plants is relatively accurate but requires three to six weeks. Although identification by phase contrast and electron microscopy requires little time and is rapid and sensitive, the technology involved severely limits the number of samples that can be examined, and the expensive equipment limits the usefulness of these methods of screening at most research stations.

The successful application of a serological method for RSD would simplify diagnosis. Numerous attempts in the past to obtain an antiserum to the RSD agent with infected sugarcane as a source of antigen have failed (Steindl, 1961). In an attempt to permit utilization of rapid

and inexpensive serological relationships between the bacterium and other organisms an antiserum has eventually been produced (Brlansky *et al.*, 1982; Gillaspie, 1978a; 1978b; Harris and Gillaspie, 1978; Oellermann *et al.*, 1981).

Gillaspie (1978b) found that the microagglutination test was not applicable for the diagnosis of RSD in raw sugarcane juice from diseased plants because of the low bacterial concentration. However, the method could be used for screening samples if they were concentrated, but the centrifugation required would limit the number of samples handled at any one time. The microagglutination test used for the Mauritian isolate M438/59 against the antiserum produced by Gillaspie (1978b) was also found to be negative (Anon., 1979b). Gillaspie *et al.* (1979) showed by use of microagglutination tests that the RSD-associated bacteria found in Australia, Brazil, Japan, South Africa and the United States of America were closely related serologically.

In 1977 Damann *et al.* found that serologically specific electron microscopy (SSEM) was a technique that did not require concentration of the RSD bacterium for bacterial detection. The technique was reported to be more powerful than quick dip electron microscopy and was used to diagnose RSD. The quantitative aspects of the SSEM assay for the RSD bacterium have not yet been determined. However, this technique has already been used, in conjunction with symptoms, to evaluate a number of aerated-steam heat-treatment regimes for RSD control. The SSEM assay was also reported to have potential in screening progeny for resistance in a breeding program. The technique could also be used to recognize the RSD bacterium in culture. According to Gillaspie (1978b), it is conceivable that the morphology of the bacterium changes or that pathogenicity may be lost in culture leaving only SSEM or other serological

methods for recognition. However, the crucial limitation of SSEM is that it is an expensive serodiagnostic method for RSD (Derrick and Brlansky, 1976).

Failure to find a method for lysing the RSD-associated bacterium prevented the use of immunodiffusion tests. The bacterium was not degraded by sonication, pyrrolidine or enzyme treatments (Gillaspie, 1978a). In 1980, Davis *et al.* demonstrated serological reactions of sugarcane and Bermuda grass isolates in gel double diffusion tests using cells of isolates disrupted by a French press as antigen against antisera to the intact cells.

The indirect fluorescent antibody technique, using antiserum specific for the RSD-associated bacterium, was used to diagnose RSD of sugarcane (Harris and Gillaspie, 1978). This test was found to be 100-fold more sensitive as a diagnostic technique than phase contrast microscopy and was an excellent supplemental method. However, success in application of the indirect method of staining depended greatly on the specificity of the antisera produced. Gillaspie and Harris (1978), also tested microagglutination and bentonite flocculation as possible methods for diagnosis, but neither of these was sensitive enough to show a positive reaction with infectious crude juice, even with the presence of a relatively high concentration of bacteria ( $2 \times 10^7$  bacteria.cm<sup>-3</sup>). In 1979, Gillaspie *et al.* found that isolates from Egypt, Japan, Pakistan, South Africa and the United States of America gave a positive reaction with their antiserum by the indirect fluorescent antibody method. This antiserum was also used in an indirect fluorescent antibody staining test to identify isolates (Davis *et al.*, 1980). In 1982, Brlansky *et al.* found that the RSD-associated bacterium could be detected by immunofluorescence using tetramethylrhodamine isothiocyanate (TRITC) for con-



jugation instead of the conventional fluorescein isothiocyanate (FITC) labelled antiserum. They reported that FITC labelled antiserum was not useful since xylem vessels autofluoresced at the same wavelength as fluorescein. This technique was reported as being potentially useful in locating bacteria in various areas of plants, screening tissue for the presence of bacteria prior to preparation for electron microscopy, and in following the movement of bacteria within a plant.

The enzyme-linked immunosorbent assay (ELISA) has been successfully used since 1971 for the detection and study of several different pathogens causing diseases of humans and animals and since 1976 in the detection of plant pathogens (Clark and Adams, 1977; Voller *et al.*, 1976). In plants emphasis has been on virus detection. Recently, ELISA has been reported for the detection of bacteria (Cambra and Lopez, 1977; Claflin and Uyemoto, 1978; Kishinevsky and Bar-Joseph, 1978; Kishinevsky and Gurful, 1980; Morley and Jones, 1980; Nomé *et al.*, 1980; Olsen *et al.*, 1983; Weaver and Guthrie, 1978) and more recently on the xylem-limited fastidious bacteria of ratoon stunted sugarcane (Gillaspie *et al.*, 1979; Gillaspie and Harris, 1979; Oellermann and Pillay, 1983). Gillaspie and Harris (1979), found that ELISA had several limitations. These included low sensitivity, high background absorption and inconsistent results. In addition they found that neither alkaline phosphatase nor horse-radish peroxidase was suitable for conjugation.

However, such difficulties have prompted the author of this dissertation to pursue this "elusive creature". Focus on the morphology of the RSD-associated organism, attempts to characterize its constituent membranes and various methods of detection of the organism were examined.

## II. MATERIALS AND METHODS

### A) ISOLATION OF BACTERIUM FROM SUGARCANE

Healthy and naturally diseased sugarcane of the varieties N53/216 and N55/205 were provided by the South African Sugar Association Experiment Station, Mt. Edgecombe. The sugarcane had been established in 1976 and was in its third ratoon year. All sugarcane with RSD showed typical reddening of the vascular bundles at the nodal regions.

The bacteria were isolated from the fibrovascular sap extracts and from stem diffusates. The fibrovascular sap extract was obtained using the suction method described by Teakle *et al.* (1973). The extract was centrifuged at 14 000 g for 30 min. The supernatant was decanted except for a few drops which were used to resuspend the pellet for examination by light and electron microscopy, and for culturing.

Stem diffusates were obtained by transferring aseptically, pieces of sugarcane stem approximately 10 mm long and 2-3 mm wide, to test tubes containing modified White's (1963) tissue culture medium (Table 1). The tubes were incubated at 26C under aerobic conditions, until growth, indicated by turbidity, was visible. Turbidity was usually observed after 36-48 h incubation. Subcultures were made onto agar medium using the pour-plate and streak-plate techniques to obtain pure cultures. Healthy and diseased sugarcane were subjected to identical procedures.

The isolated bacteria were routinely inoculated into 2-litre Erlenmeyer flasks containing 1 litre modified White's tissue culture medium and incubated in a Gallenkamp orbital incubator at 26C for 48 h at a shaking speed of 5. Smears were then prepared for light microscopy by heat

fixing followed by staining with crystal violet for 30 seconds before being examined for contaminants at a magnification of 400x using a Nikon light microscope. The bacteria were pelleted at 10 000 g for 10 min and were used for membrane isolation.

TABLE 1. Modified White's tissue culture medium (1963)

Mineral stock solution*	100	ml
Vitamin stock solution**	10	ml
FeSO <sub>4</sub> .7H <sub>2</sub> O	0,0025	g
H <sub>3</sub> BO <sub>3</sub>	0,0015	g
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0,0015	g
MnSO <sub>4</sub> .4H <sub>2</sub> O	0,0045	g
Yeast extract	0,25	g
Malt extract	0,5	g
Sucrose	5,0	g
Arginine	20	mg
Bacto agar	15	g
Made up to one litre with distilled water and filter sterilized - pH 6,8		

\*Mineral stock solution: Ca(NO<sub>3</sub>)<sub>2</sub>.4H<sub>2</sub>O 2g; MgSO<sub>4</sub>.7H<sub>2</sub>O 2g; Na<sub>2</sub>SO<sub>4</sub> 2g; KNO<sub>3</sub> 0,8g; NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O 0,165g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 5g; Na<sub>2</sub>MO<sub>4</sub>.2H<sub>2</sub>O 0,02 g; dissolved in one litre distilled water.

\*\*Vitamin stock solution: Pyridoxine 10mg; biotin 0,1mg; thiamine 50mg; calcium pantothenate 50mg; vitamin B<sub>12</sub> 0,2mg; nicotinic acid 50mg; dissolved in one litre distilled water.

## B) PREPARATION OF MEMBRANES

Two day old cultures were pelleted by centrifugation in a Sorvall SS-3 automatic superspeed centrifuge in a GSA rotor at 10 000 g for 10 min.

The pellet was resuspended in 30% (w/w) sucrose-10 mM Tris-HCl buffer pH 8 at 4C. Three volumes 2,5 mM EDTA-10 mM Tris-HCl buffer pH 8 and  $10^{-2}$  volumes of aqueous lysozyme (final concentration of  $100 \mu\text{g}\cdot\text{cm}^{-3}$ ) were added while shaking at 4C.

The suspension was warmed at 40C for 2 min and then transferred to a shaking waterbath at 30C where plasmolysis was completed after an incubation period of at least 30 min.  $\text{MgSO}_4$ , DNase and RNase were added to final concentrations of 20 mM,  $100 \mu\text{g}\cdot\text{ml}^{-1}$  and  $50 \mu\text{g}\cdot\text{ml}^{-1}$  respectively and the suspension incubated at 37C for 15 min in a Memmert waterbath. The suspension was then sonicated using the DAWE soniprobe (Type 1130 A) at 6 amperes for 60 seconds at 4C.

After centrifugation at 10 000 g for 10 min to remove remaining intact bacteria (pellet), the supernatant was centrifuged in a Beckman model L5-50 preparative ultracentrifuge using the type 65 rotor at 140 000 g (av) for 1 h at 4C. The supernatant was discarded and the crude membrane pellet was suspended in 1 to 2 ml (depending on the size of the pellet) 25% (w/w) sucrose-10 mM Tris-HCl buffer pH 8 and layered on to a 30-60% (w/w) sucrose gradient. The tubes were then centrifuged at 144 000 g (av) for 16 h at 4C using a Beckman SW 40 Ti rotor.

The gradient was fractionated into 0,3 ml fractions using an ISCO model 640 density gradient fractionator and model UA-5 absorbance monitor.

The densities of the recovered fractions were determined refractometric-

ally using an Atago refractometer. Membrane fractions were pooled and pelleted at 140 000 g (av) for 1 h at 4C using the type 65 rotor. The pellets were resuspended in a minimal volume of 10 mM Tris-HCl buffer, pH 8 and stored at -20C.

Further purification of the membrane fractions for analytical investigations was achieved by rerunning the pelleted fractions on 30-60% (w/w) sucrose density gradients after resuspension in 2,5 mM EDTA-10 mM Tris-HCl buffer, pH 8, digestion with  $MgSO_4$ , DNase and RNase and centrifugation at 140 000 g (av) for 1 h at 4C.

The following parameters were monitored to optimize conditions for maximum recovery of membrane fractions:

EDTA absence,  
lysozyme concentration,  
incubation temperature,  
incubation time, and  
refrigeration effects.

#### C) PROTEIN DETERMINATION

Protein was determined by the modified Lowry method described by Markwell *et al.* (1978) with bovine serum albumin (BSA) as standard. For the modification of the original procedure, stock solutions of reagent A contained 2,0%  $Na_2CO_3$ , 0,4% NaOH, 0,16% sodium tartrate and 1,0% sodium dodecyl sulphate (SDS), and reagent B was 4,0%  $CuSO_4 \cdot 5H_2O$ . These solutions were stable indefinitely when stored at room temperature. On the day of use, Folin-Ciocalteu phenol reagent was diluted 1:1 with distilled water.

In the modified procedure, 100 parts of reagent A were mixed with 1 part of reagent B to form the alkaline copper reagent C. A sample volume of 200  $\mu$ l (diluted, if necessary, with distilled water) containing protein, sucrose and EDTA was added to 600  $\mu$ l reagent C and incubated at room temperature for 10-20 min without change in the final absorbance. The samples were then mixed vigorously upon addition of 60  $\mu$ l of diluted phenol reagent and incubated for 45 min at room temperature. The absorbance of the samples were read at 660 nm using the Beckman DU-8 spectrophotometer. A standard curve was constructed using BSA.

#### D) IMMUNIZATION

A total of 28 black male virgin rabbits, obtained from the Natal Institute of Immunology, Sarnia, were bled and selected for immunization as follows:

Sera obtained from the collected blood were heated at 56C for 30 min to inactivate the normal complement present. Heat-inactivated serum from each rabbit was diluted in 10 mM Tris-HCl buffer, pH 8 (1:2) and was then tested with the intact RSD-bacteria for visible agglutination. Sera from three of the 28 rabbits exhibited visible agglutination on slides. These rabbits were excluded from the immunization programmes (Tables 2 and 3).

Six of the selected rabbits were immunized according to the schedule set out in Table 2. Three rabbits were injected with whole RSD-bacteria ( $1 \times 10^9$  bacteria.cm<sup>-3</sup>) without an oil adjuvant and three rabbits were injected with whole RSD-bacteria ( $1 \times 10^9$  bacteria.cm<sup>-3</sup>) in Freund's incomplete oil adjuvant (Difco Laboratories, Michigan, U.S.A.).

TABLE 2. Rabbit immunization schedule with whole RSD-bacteria

DAY	ANTIGEN DOSE (ml)	ROUTE OF IMMUNIZATION
1	1,0	I.M.
15	1,0	I.M.
21	1,0	I.M.
22	0,5	I.M.
24	0,5	I.M.
26	0,5	I.V.
29	1,0	I.M.

Control bleeding (zero) was carried out on all 6 rabbits prior to immunization. Secondary bleeding was carried out 7 days and 14 days after the final immunization. The collected blood was processed and the sera obtained were frozen in 1 ml aliquots for use in the immunological assays.

Twelve of the selected rabbits were immunized according to the schedule set out in Table 3. Four groups each comprising three rabbits were injected with membrane fractions (I-IV) obtained from the RSD-bacteria with respective densities of  $1,15 \text{ g.cm}^{-3}$ ,  $1,18 \text{ g.cm}^{-3}$ ,  $1,21 \text{ g.cm}^{-3}$  and  $1,24 \text{ g.cm}^{-3}$ . The fractions were emulsified in Freund's incomplete oil adjuvant. Control bleeding (zero) was carried out on all 12 rabbits prior to immunization. Secondary bleeding was carried out 7 days and 14 days after the final immunization. The collected blood was processed and the sera obtained were frozen in 1 ml aliquots for later use.

TABLE 3. Rabbit immunization schedule with membrane fractions (I-IV) obtained from RSD-bacteria

DAY	ANTIGEN DOSE (ml)	[PROTEIN].RABBIT <sup>-1</sup> (µg)	ROUTE OF IMMUNIZATION	ADJUVANT
1	1,0	100	I.M.	YES
15	1,0	100	I.M.	YES
21	1,0	100	I.M.	YES
22	1,0	100	I.M.	YES
24	1,0	150	I.M.	YES
26	1,0	150	I.V.	NO
29	1,0	100	I.M.	YES

E) IMMUNOLOGICAL ASSAYS

1. Microagglutination

The microagglutination test for whole decomplexed serum was conducted by a standard procedure using viable suspensions of bacteria, at various concentrations, in physiological saline (0,85% NaCl). Serial dilutions of serum in saline were made for determination of the agglutination titres. An aliquot (50 µl) of each of the serial dilutions of serum was mixed with 50 µl of the live bacterial suspension and incubated at room temperature (20-23C) for 30 min.

Routine light microscope examination of a drop of the mixed test or control serum with antigen on slides was carried out on a Zeiss photomicroscope using phase contrast illumination at a magnification of 100x.



Bacterial membrane fractions were similarly assessed. Appropriate controls were included in each assay.

## 2. Immunofluorescence

Intact bacteria and bacterial membrane fractions were prepared for immunofluorescence as follows: 0,1 ml of a predetermined concentration of intact bacteria or 0,1 ml of a predetermined protein concentration of the membrane fractions (I-IV) were each washed twice with phosphate buffered saline (pH 7,4) by centrifugation at 10 000 g for 15 min and 140 000 g for 45 min respectively. A volume of 0,1 ml 1:5 diluted rabbit antiserum specific for the test organism or its membrane fractions or 0,1 ml control rabbit sera (1:5) was added to the appropriate tubes and vortexed. The tubes were incubated at 37C for 30 min. The mixtures were then washed twice with 5 ml PBS (pH 7,4) by centrifugation at 20 000 g for 15 min to remove excess rabbit serum.

The fluorescent probe was goat-antirabbit serum conjugated with fluorescein isothiocyanate (FITC; Miles-Yeda Ltd., Rehovot, Israel). The FITC conjugate was diluted (1:50) and 0,5 ml were applied to each tube. The mixtures were incubated at 37C for 30 min and then washed five times with PBS (pH 7,4) by centrifugation at 20 000 g for 10 min to remove unbound conjugate. Two ml glycerol - PBS (2:1) were added to each tube, the samples placed on clean slides, air-dried and fixed in methanol for 2 min. Care was taken to prevent exposure of the FITC labelled material to light to reduce photodecomposition. A Nikon fluorescence microscope equipped with an FITC interference filter was used to examine the slides. An interference filter was necessary for unequivocally distinguishing scattered from emitted light. The samples were photographed at a magnification of 400x.

### 3. Enzyme-linked immunosorbent assay (ELISA)

#### *Isolation of IgG from antiserum against the intact RSD-B*

IgG was prepared from antiserum against the intact RSD-B by precipitation at room temperature (20-23C) with  $\text{Na}_2\text{SO}_4$ , made to 15% (w/v), for 20 min. After centrifugation at 1 500 g for 10 min the precipitate was redissolved in a volume of distilled water equal to the initial volume of serum and precipitation repeated twice. The final precipitate was dissolved in a minimal volume of distilled water and dialysed extensively (2 changes in 48 h) against distilled water at 4C. The resulting euglobulin precipitate was removed by centrifugation (1 500 g for 10 min) and the clear supernatant was lyophilised, its protein content determined and the lyophilisate stored at 4C.

#### *Production of horseradish peroxidase (HRPO) - IgG conjugates*

Highly purified HRPO (EC 1.11.1.7; Sigma Chemical Co., St. Louis, Mo., U.S.A.) with  $\text{RZ} \geq 3,0$  was linked to rabbit anti-RSD-B IgG as follows:

- (i) Ten mg HRPO was dissolved in 0,5 ml distilled water.
- (ii) Fifty microlitres freshly dissolved 0,2 M  $\text{NaIO}_4$  were added, vortexed and left to stand for 20 min at room temperature.
- (iii) The HRPO-aldehyde solution was desalted as follows:

A Sephadex G-25 M Pharmacia Column PD-10 was equilibrated by flushing through 25 ml 1 mM  $\text{CH}_3\text{COONa}$  (pH 4).

The activated HRPO-aldehyde solution was passed through the column, collected in a test tube, the volume measured and the concentration of the HRPO determined.

A volume containing 4 mg HRPO.cm<sup>-3</sup> was removed. Eight mg IgG isolated from antiserum against the intact RSD-B were dissolved in 0,5 ml 0,2 M Na<sub>2</sub>CO<sub>3</sub> (pH 9,6). This was then added to the HRPO solution, vortexed and left to stand for 2 h at room temperature.

Fifty ml of 50% glycerol in 0,05 M Tris-0,1 M NaCl buffer (TS), pH 8,0 were added to the HRPO-IgG conjugate and stored at 4C.

#### *Antibody coating of microtitre plates*

- (i) Polystyrene microtitre plates (Dynatech 129B; T & C Scientific Supplies, Durban) were coated with anti-RSD-B IgG dissolved in 0,5 M Na<sub>2</sub>CO<sub>3</sub> buffer, pH 9,6, by dispensing 100 µl of antibody solution into each well of the plate.
- (ii) This was incubated for 2 h at room temperature in a moist chamber.
- (iii) The coating solution was aspirated and the plate washed once with 500 ml TS containing 0,5% Tween 80 buffer (TST). Plates were emptied by flicking out the contents after soaking for 3 min.
- (iv) The TST was rinsed off with 500 ml distilled water, flicked out, blotted and aspirated to remove any water remaining in the wells. The final drying was

done under vacuum.

- (v) Coated plates were individually sealed in plastic bags and stored at 4C.

*ELISA technique*

Purified intact RSD-B was used throughout the experiment as the standard reference material. Serial dilutions of the reference material were made in TS and the antibody-HRPO conjugate was diluted in TS containing neutral (non-immunized) 5% (v/v) sheep and neutral 5% (v/v) rabbit sera before being assayed; the animal sera were necessary to neutralize heterophile antibovine IgG antibodies which are present in 70% of normal human sera (Foucard *et al.*, 1975).

- (i) Fifty microlitres of standard or sample were added to the wells of a plate coated with anti-RSD-B. All standards and samples were assayed in triplicate.
- (ii) The plate was incubated in a moist chamber at 45C for 1 h.
- (iii) The contents of each well were flicked off, aspirated (avoiding carryover between wells), washed once with 500 ml TST and left to soak for 3 min, flicked off again, blotted and then aspirated once more.
- (iv) Fifty microlitres of anti-RSD-B-HRPO conjugate were added to the wells at timed intervals to complete the immune complex.
- (v) The plate was then incubated at 45C for 1 h, washed with TST as in (iii) above and dried.
- (vi) Fifty microlitres chromogenic substrate were added to each well at timed intervals. The colour was allowed

to develop in the dark for 30 min at room temperature (20-23C).

- (vii) The enzymic reaction was stopped by the addition of 100  $\mu$ l 1,5 N HCl to each well at timed intervals.
- (viii) The intensity of colour developed in each well was measured at 492 nm using a colorimeter (Vitatron; T & C Scientific Supplies, Durban) with an 80  $\mu$ l flow through cell (1 cm light path length).

Chromogenic substrate was prepared from Borax ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) (18,4 mM) 0,70 g; succinic acid (30,5 mM) 40 mg; urea hydrogen peroxide (40 mM) 40 mg; distilled water 100 ml. This ratio of borax and succinic acid should yield a pH of 5,0. Fine white particles present in the solution originate in the urea hydrogen peroxide and need not be removed. Because of the catalytic breakdown of  $\text{H}_2\text{O}_2$  by metals it is important that contact with metals be completely avoided during all steps of the assay and buffer salts used in the substrate should be of sufficient purity to avoid the development of high background colour. The purity of the hydrochloric acid used is particularly important because acid of low quality will also cause spurious colour development.

NOTE: The ELISA technique consisted of several sequential steps, all of which were necessary for the formation of an enzymatically active immune complex. Optimal conditions of conjugate and coating antibody concentrations were determined for each step of each assay.

#### 4. Immunodiffusion

Ouchterlony (1948) double immunodiffusion was performed on 50 mm petri dishes (Sterilin Ltd., Middlesex, England) containing 5 ml 0,5% Agarose

(Miles Laboratories Pty. Ltd.) in Barbitone buffer (0,058 M Sodium Barbitone - 0,03 M Barbituric acid, pH 8,2).

Thirty microlitres antisera (zero or secondary bleedings) obtained against the intact bacterium or 30  $\mu$ l each of the four membrane fractions were placed in the centre wells (3 mm in diameter). An equal volume of the crude membrane or the antisera (zero or secondary bleedings) against the four membrane fractions was placed in the surrounding wells.

Although some precipitin bands appeared overnight, the gels were kept at room temperature in a humidity chamber (Frost Instruments, Ltd.) for 3-10 days for complete precipitin arc development. The gels were viewed on a light box.

After 10 days the gels were washed in 0,01 M saline solution for 1 h, soaked overnight in saline and then washed twice using distilled water. The gels were then stained in 0,003% Coomassie blue solution for 10 h and destained in a 10% glacial acetic acid solution. The destained gels were photographed using a Nikon camera.

## F) COMPOSITION AND STRUCTURE

### 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Protein composition of the crude membrane and membrane fractions was determined on 12,5% gels according to Laemmli and Favre (1973) with an acrylamide / *N*, *N*'-methylenebisacrylamide ratio of 7,8:1.

Samples for electrophoresis were first treated at 100°C for 5 min in a buffer containing 62,5 mM Tris-hydrochloride pH 6,8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol.

Electrophoresis was carried out in a buffer consisting of 0,025 M Tris-hydrochloride pH 8,3, 0,192 M glycine and 0,1% SDS at 100 mA for approximately 4 h with a Desaga (Heidelberg, Germany) vertical slab gel electrophoresis unit.

Gels were stained in 0,003% Coomassie blue over a period of 16 h and destained in a 10% glacial acetic acid solution. Protein molecular weight standards were phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and  $\alpha$ -lactalbumin (14 400).

The destained gels were scanned on a Beckman DU-8 spectrophotometer and the molecular weights of the samples determined.

## 2. Electron microscopy

### (a) Negative staining

Electron microscopic observations were carried out on a Phillips EM Model 301. Negative staining was carried out by mixing approximately 0,1 ml of the bacterial suspension with an equal volume of a 0,5% solution of sodium phosphotungstate, pH 6,5-7,0, for 10-40 seconds. A drop of this suspension was then placed on a carbon coated grid and the excess moisture was removed by touching the edge of the grid with filter paper.

(b) Ultramicrotomy

Membrane samples were spun on a Sorvall SS-3 automatic superspeed centrifuge using the SS-34 rotor at 20 000 g for 10 min. The samples were washed twice with phosphate buffer (ph 7,2) and the supernatant decanted.

The membrane pellets were fixed overnight in 3% glutaraldehyde in phosphate buffer (ph 7,2) at 4C and then washed twice in phosphate buffer (ph 7,2) by centrifugation at 20 000 g for 10 min.

Pellets were then post-fixed in 2% OsO<sub>4</sub> in phosphate buffer (pH 7,2) and twice in distilled water at 20 000 g for 10 min. The pellets were then embedded in ionagar and carefully cut into 1 mm<sup>3</sup> blocks which were stained in 2% uranyl acetate (aqueous) for 20 min.

Dehydration was effected through a graded series of alcohol as follows:

25% : 2 x 15 min  
50% : 2 x 15 min  
75% : 2 x 15 min .... left overnight  
100% : 2 x 30 min

An equal quantity of resin and 100% alcohol was added and this was left to stand at room temperature for 6 h. Resin, equal in volume to the contents of the tube was added, covered and left at 4C for 48 h. The resin was then removed, fresh resin added and left at 4C for 4 h after which the resin was once again removed and the samples orientated in the beam capsules. Fresh resin was added to the capsules which were labelled.



Polymerization was carried out at 37C overnight. Thin sectioning was performed using the Reichert Om U3 ultramicrotome and the sections were stained in a saturated solution of uranyl acetate in 5% ethanol for 5 min followed by staining in lead citrate for 15 min. The stained sections were observed using the Phillips Electron Microscope Model 301.

### III. RESULTS

#### A) ISOLATION OF BACTERIUM FROM SUGARCANE

The coryneform bacterium was isolated from sugarcane with RSD only and not from healthy cane. The modified White's medium supported growth of the RSD-associated bacteria for a sufficient length of time for the bacteria to be obtained in pure culture. Viability of the isolates on modified White's agar medium was maintained by subculturing the bacteria every four to seven days. In modified White's broth medium, however, the organisms remained viable for periods of up to 14 days as observed by Nanyiager *et al.* (1980).

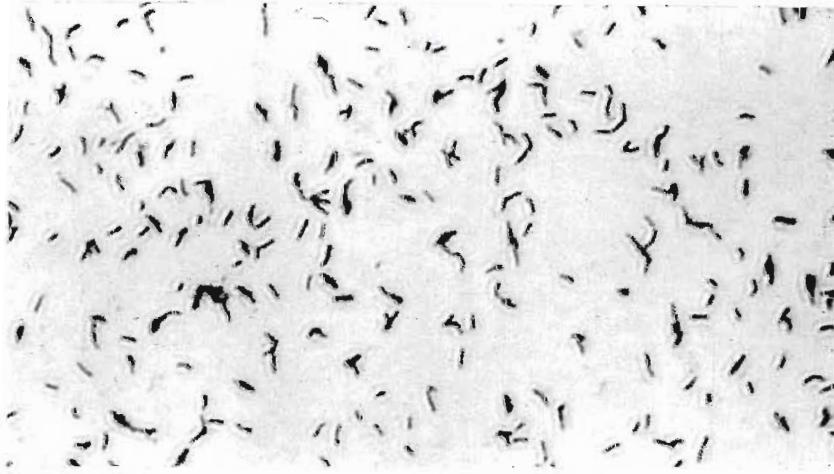


Fig. 1. Light micrograph showing the morphology of the isolated bacterium in culture (x 1250).

The bacterium was Gram-positive. The rods showed unevenly stained areas. In culture these rods were usually arranged in filaments and V, S and L

forms (Fig. 1). Club-like swellings, mesosomes and septa were produced by this organism as described by Nayiager *et al.* (1980). Branched forms similar to those reported by Kao and Damann (1978) were also produced. The cultured bacterium measured 1,25-3,50  $\mu\text{m}$  in length by 0,25-0,45  $\mu\text{m}$  in diameter.

#### B) PREPARATION OF MEMBRANES

Serological identification is clearly moving from an era of establishing the usefulness and value of serology for rapid identification to an era of establishing the antigenic structure of plant pathogenic bacteria. The preparation of immunologically cross reacting membrane fractions displaying antigens found on the surface of the intact bacterium will facilitate a detailed characterization of the RSD-bacterial surface antigens. This would consequently enhance the determination of the taxonomic status of the bacterium.

Membranes were reproducibly isolated from the pelleted bacteria. A standard sucrose density curve using sucrose concentrations from 20 to 70% (w/w) was constructed to determine the peak densities of the membrane bands (Fig. 2).

Upon isopycnic ultracentrifugation in sucrose gradients four membrane bands (I-IV) with peak densities of 1,15, 1,18, 1,21 and 1,25  $\text{g}\cdot\text{cm}^{-3}$  respectively were obtained (Fig. 3). In addition, a peak of soluble material (S) was observed at the top of the gradient. The highest yield was recorded for I followed by II, IV, III and S. Recentrifugation of these membrane bands on sucrose density gradients resulted in all fractions rebanding at their respective densities. Band II tended to produce small amounts of I, whilst bands III and IV produced small amounts

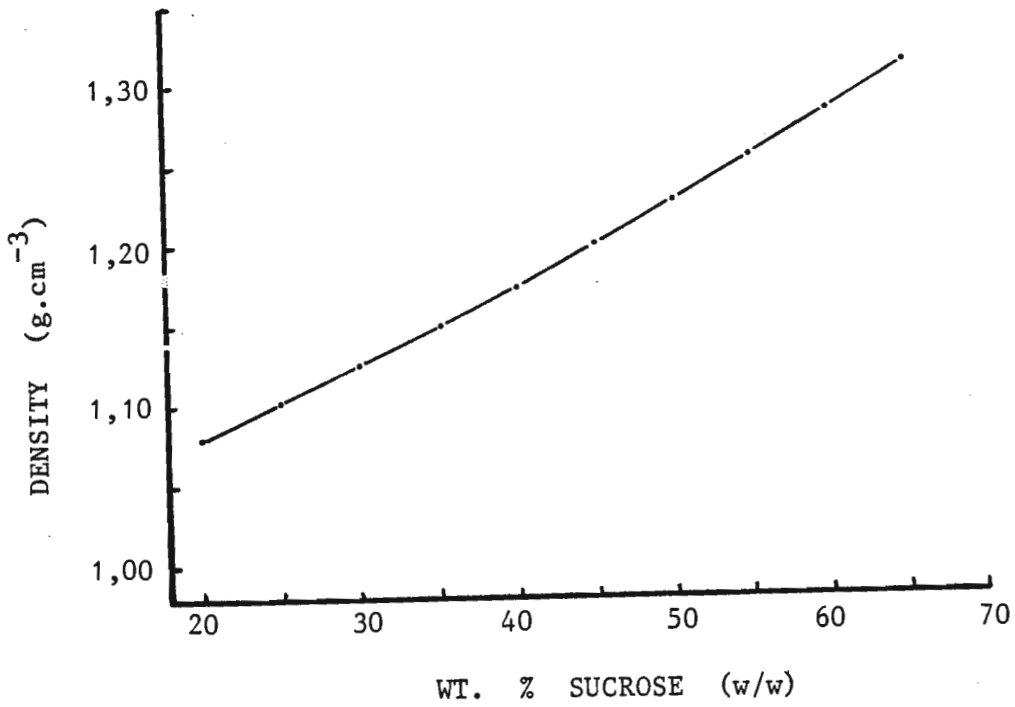


Fig. 2. Standard sucrose density curve of various sucrose concentrations at 25C.

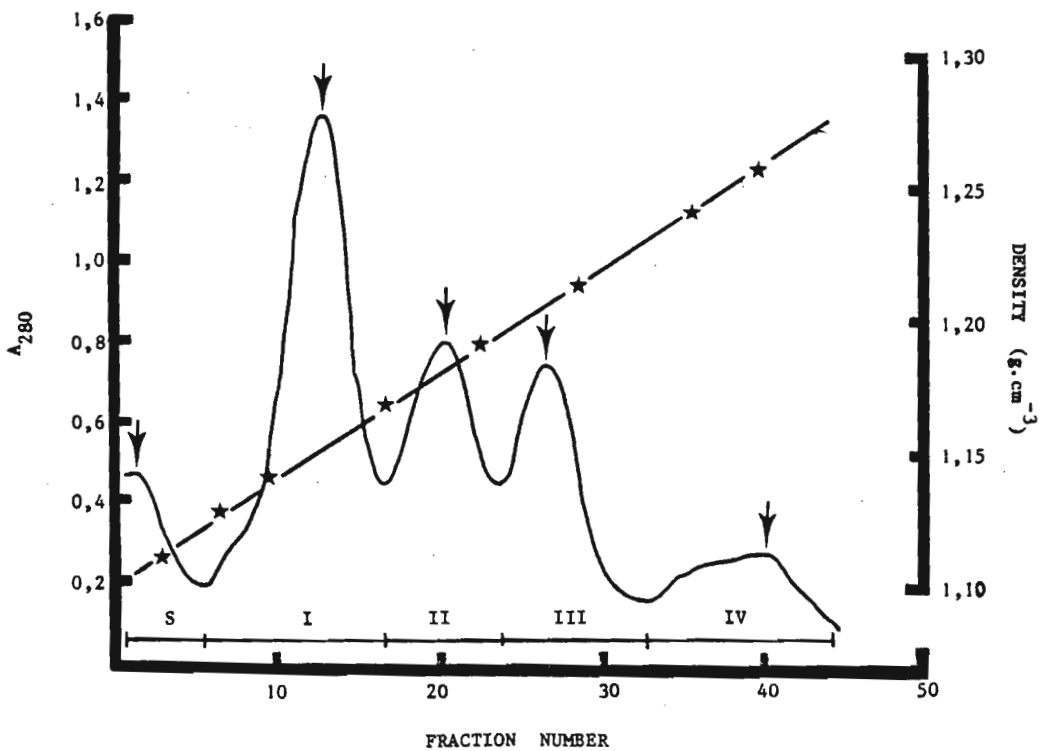


Fig. 3. Isopycnic sucrose density gradient fractionation profile of ...

of I and II (Fig. 4).

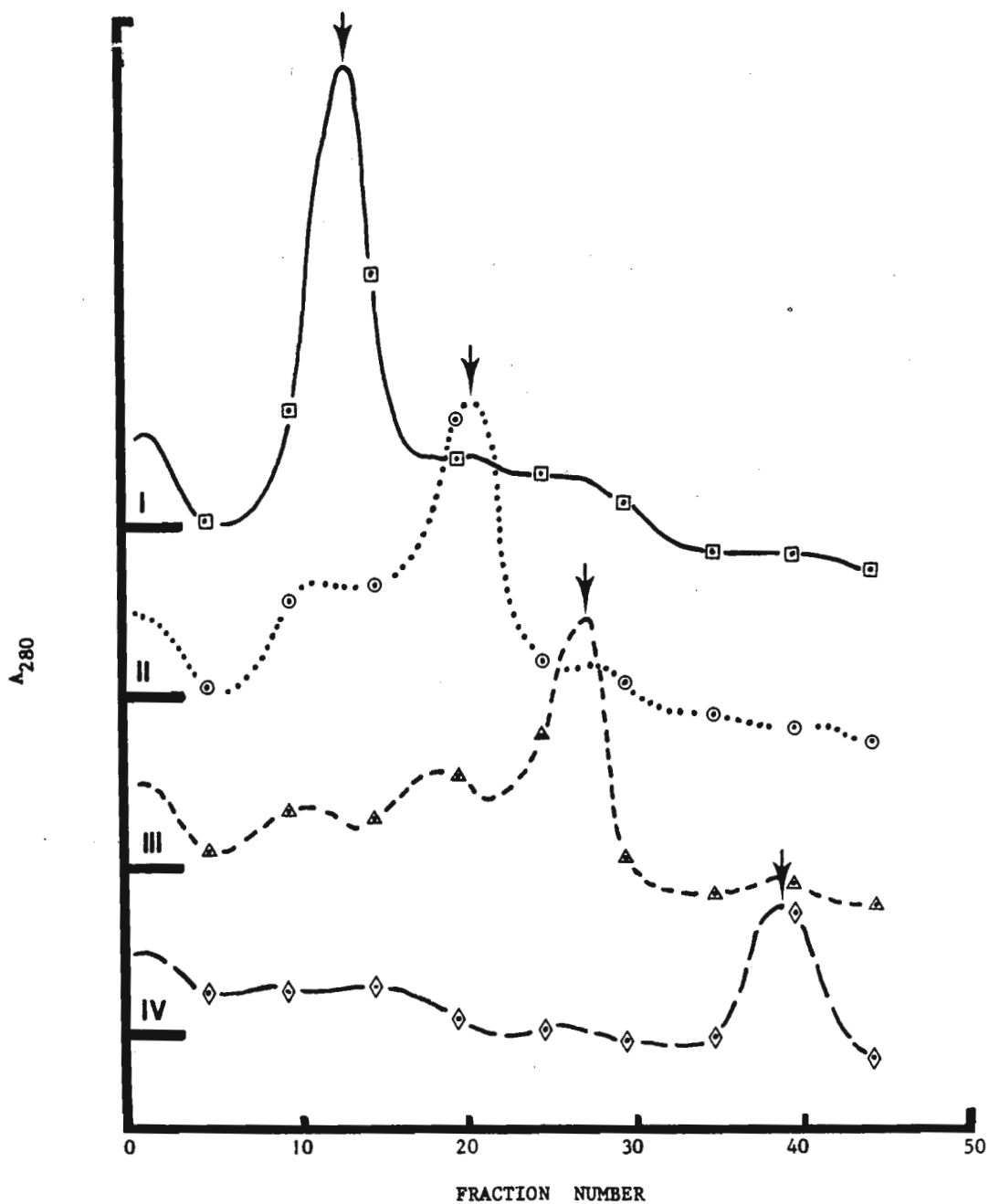


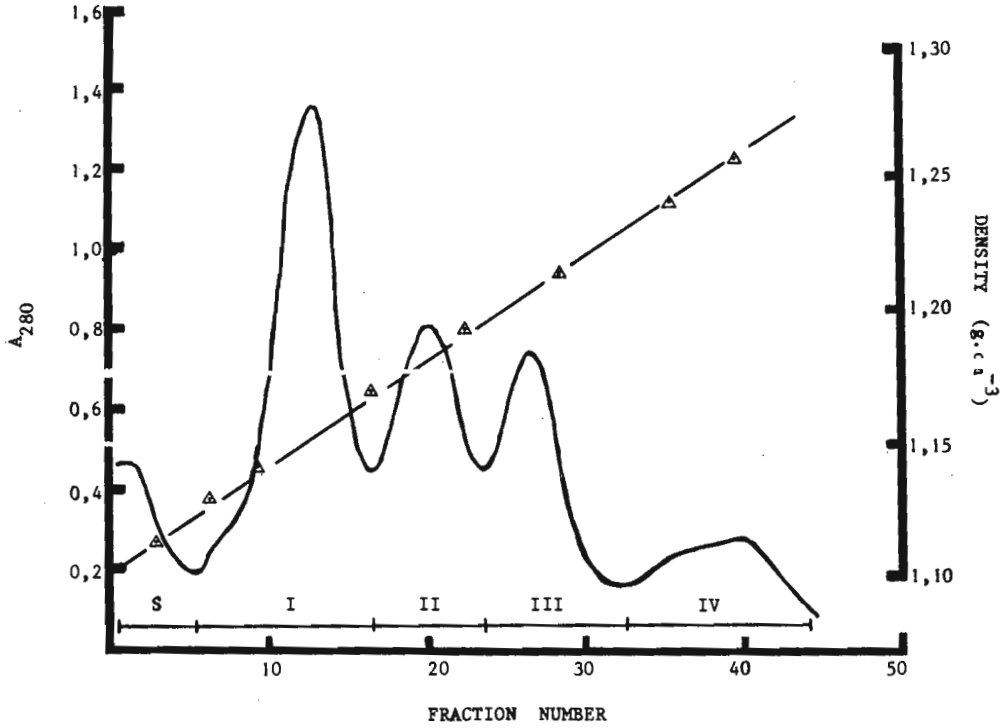
Fig. 4. Isopycnic sucrose density gradient fractionation profiles of membrane bands I-IV after recentrifugation.

To establish optimum conditions for the isolation of membranes, the influence of variation of the different parameters on relative membrane yields was examined. The membrane fractionation patterns were observed in the absence and presence of EDTA (Table 4; Fig. 5). Relative yields of the membrane bands were poor in the absence of EDTA. The highest yield was recorded for I followed by S, II, IV and III. The yield for the S fraction in the absence of EDTA was greater than in the presence of EDTA. Peak densities of all bands isolated under both conditions remained unchanged.

TABLE 4. Peak densities of membrane bands in the presence and absence of EDTA

MEMBRANE FRACTION	PEAK DENSITY	
	+ EDTA	- EDTA
S	1,108	1,109
I	1,154	1,158
II	1,184	1,186
III	1,208	1,209
IV	1,254	1,257

(i)



(ii)

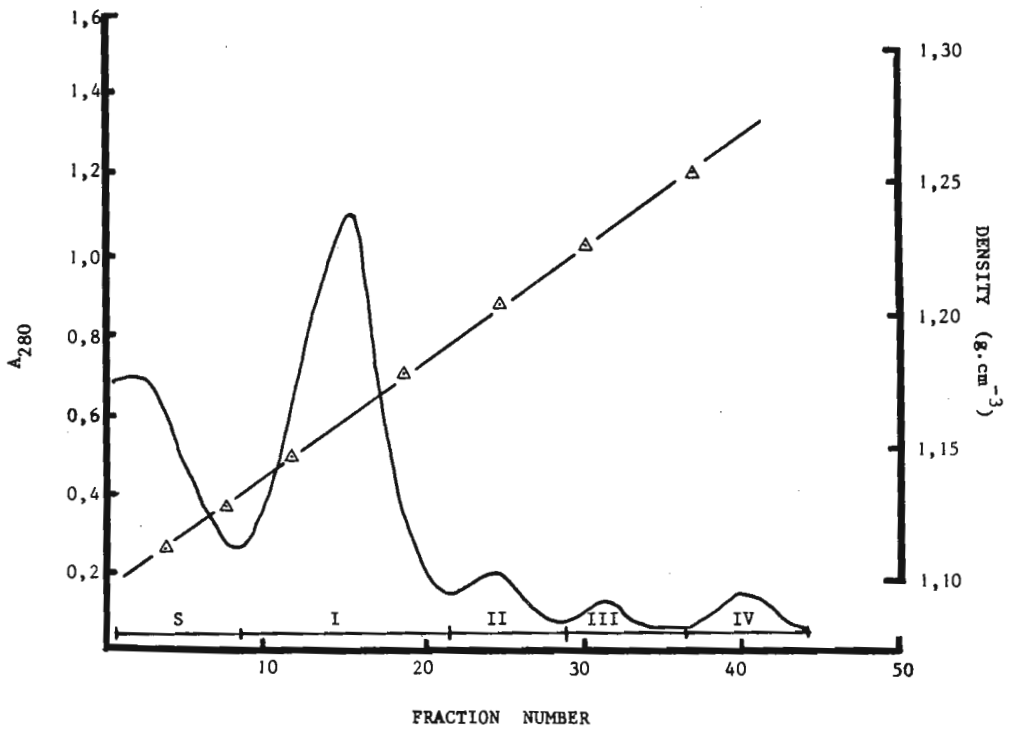


Fig. 5. Isopycnic sucrose density gradient fractionation profiles of RSD-B crude membranes in the (i) presence and (ii) absence of EDTA ( $\triangle$ — $\triangle$  = density of sucrose gradient).

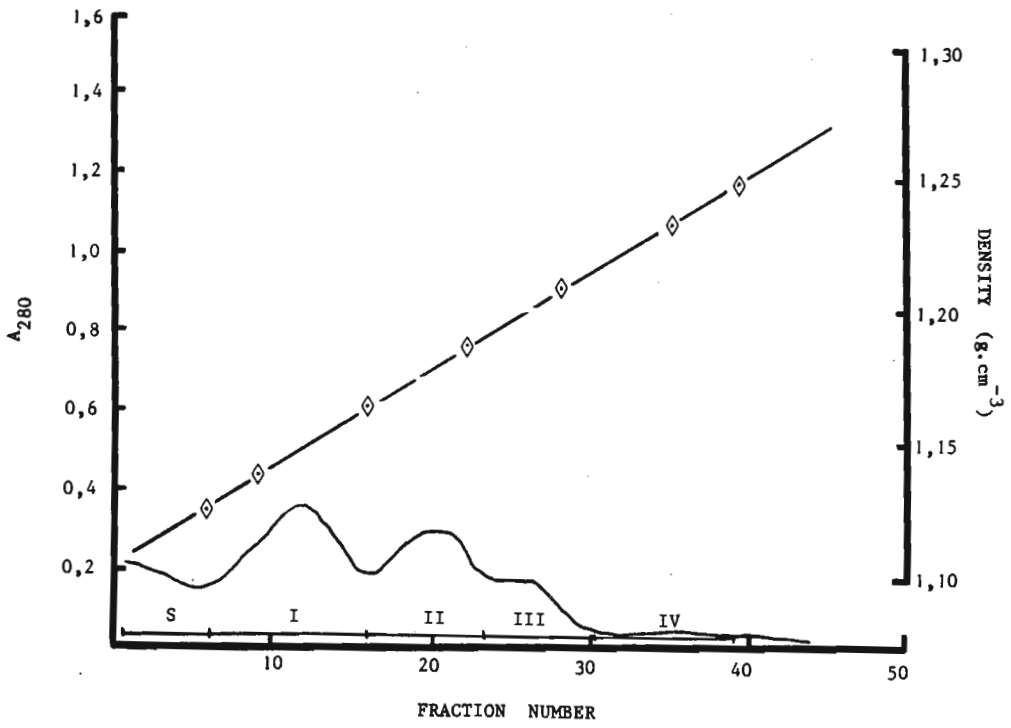
The effect of varying lysozyme concentrations on membrane isolation was investigated (Table 5; Fig. 6). Yields of membrane bands I and II were satisfactory in the absence of lysozyme. The yield of band III was poor whilst band IV was almost absent. However, membrane yields improved when the lysozyme concentration was increased to  $100 \mu\text{g.ml}^{-1}$  but steadily decreased as lysozyme concentrations were further increased to  $400 \mu\text{g.ml}^{-1}$ . Optimum yields of membrane bands were recorded when the lysozyme concentration was  $100 \mu\text{g.ml}^{-1}$ .

TABLE 5. Peak densities of membrane fractions isolated after incubation with different lysozyme concentrations

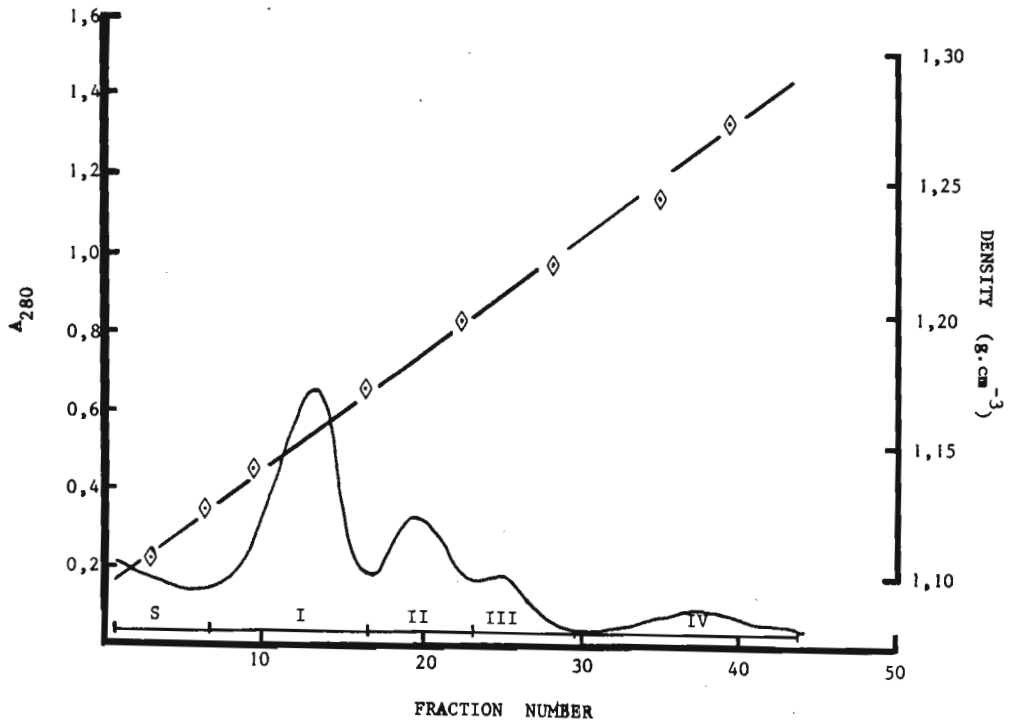
MEMBRANE FRACTION	PEAK DENSITY				
	LYSOZYME CONCENTRATION ( $\mu\text{g.ml}^{-1}$ )				
	0	50	100	200	400
I	1,154	1,155	1,156	1,148	1,154
II	1,183	1,184	1,184	1,178	1,184
III	1,206	1,207	1,207	1,202	1,208
IV	-	1,244	1,245	1,241	1,243



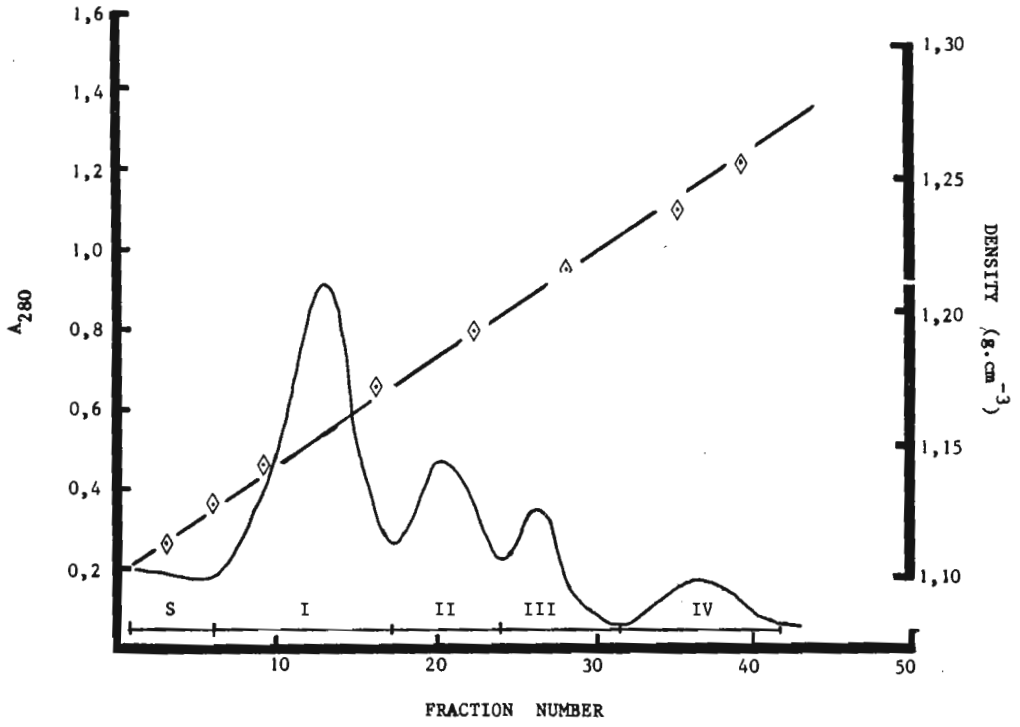
(i)



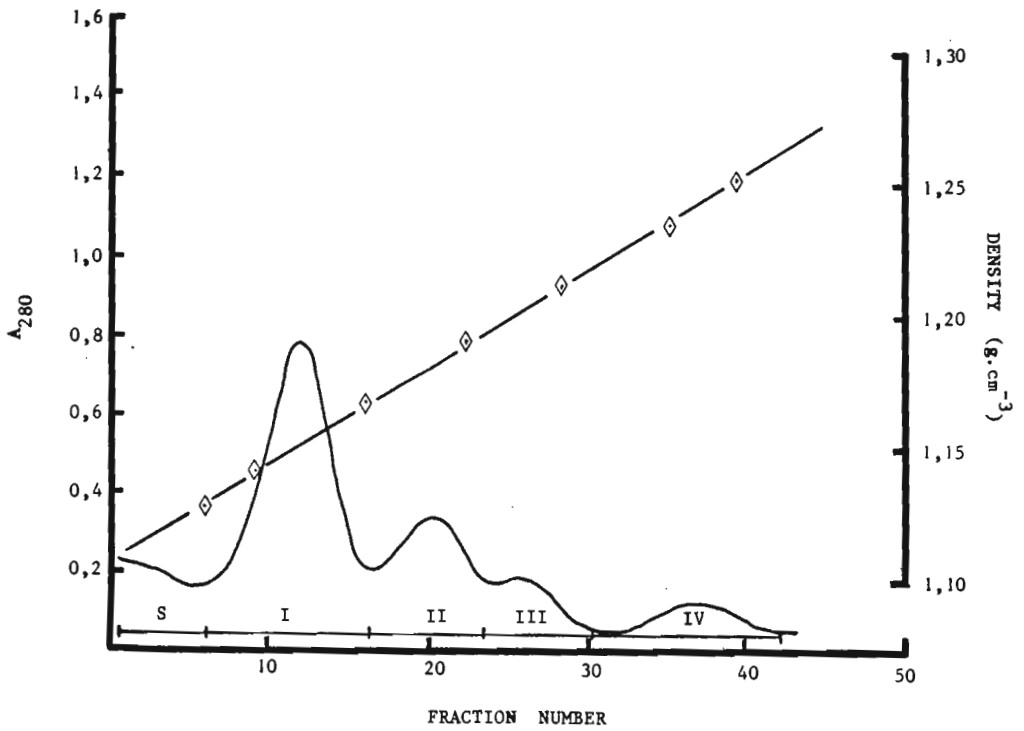
(ii)



(iii)



(iv)



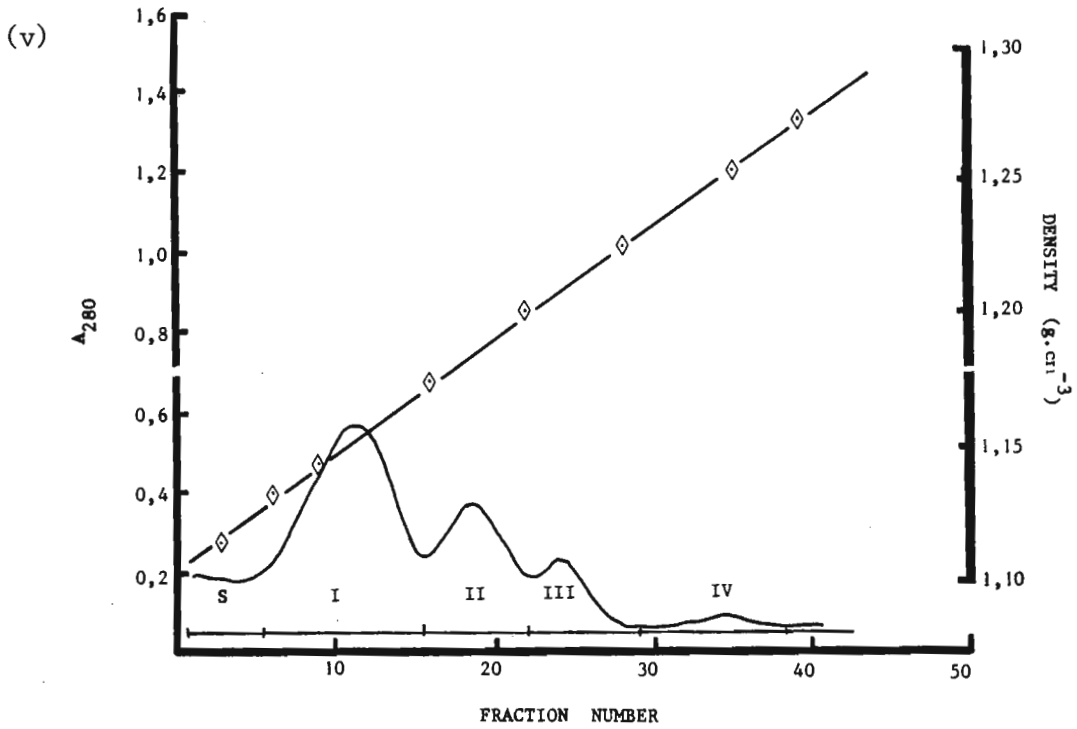
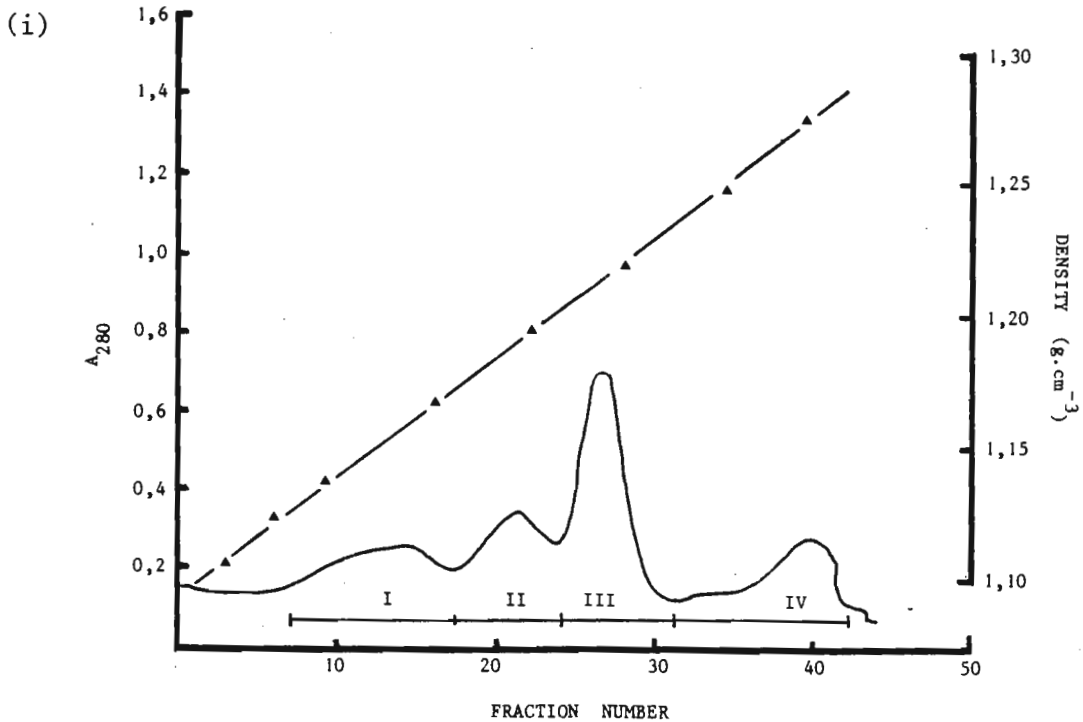


Fig. 6. Isopycnic sucrose density gradient fractionation profiles of RSD-B crude membranes at lysozyme concentrations of (i)  $0 \mu\text{g}\cdot\text{ml}^{-1}$ , (ii)  $50 \mu\text{g}\cdot\text{ml}^{-1}$ , (iii)  $100 \mu\text{g}\cdot\text{ml}^{-1}$ , (iv)  $200 \mu\text{g}\cdot\text{ml}^{-1}$  and (v)  $400 \mu\text{g}\cdot\text{ml}^{-1}$  ( $\diamond$  —  $\diamond$  = density of sucrose gradient).

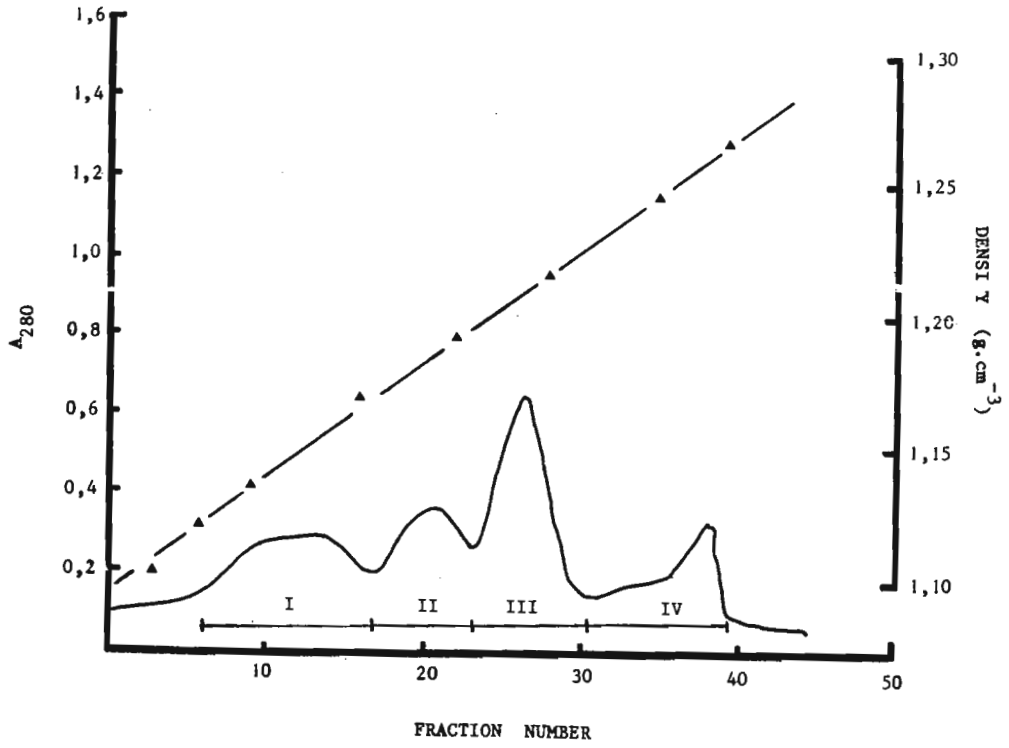
Table 6 and Fig. 7 indicate the effect of varying incubation periods on membrane isolation after lysozyme addition. Lengthening the period of incubation appeared to improve membrane yields. The yield of band III increased as the incubation period increased from 15 min to 120 min. Optimum membrane yields were recorded when the incubation period was 30 min. The peak densities of all bands remained unchanged after the different incubation periods.

TABLE 6. Peak densities of membrane bands after different periods of incubation

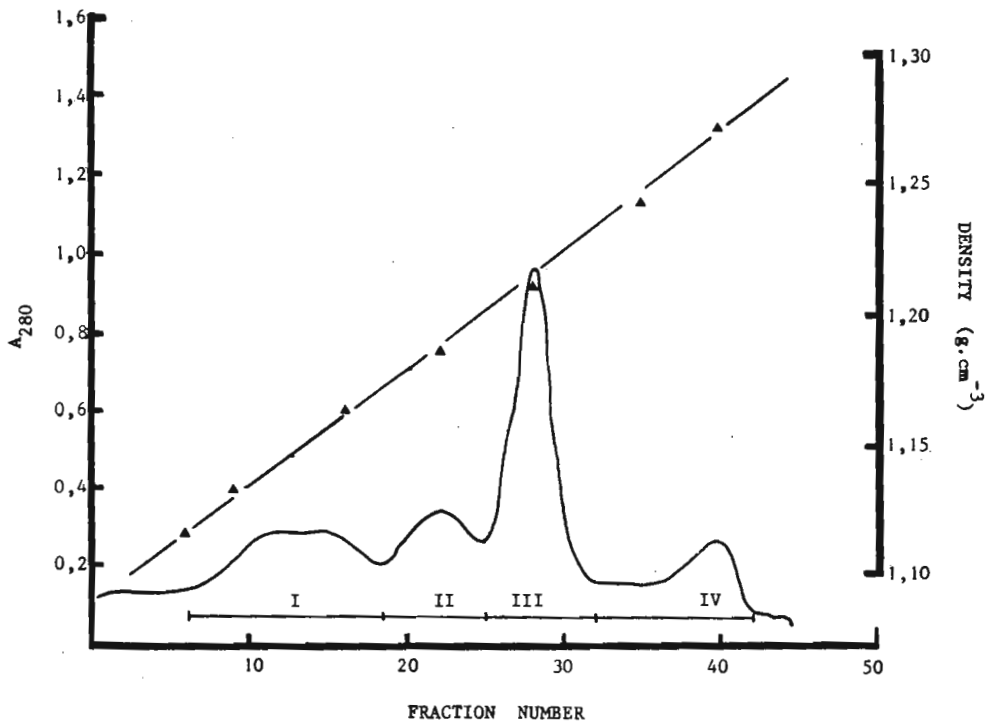
MEMBRANE FRACTION	PEAK DENSITY			
	INCUBATION PERIOD (min)			
	15	30	60	120
S	1,101	1,107	1,104	1,103
I	1,150	1,153	1,152	1,151
II	1,179	1,182	1,181	1,180
III	1,203	1,207	1,205	1,204
IV	1,250	1,255	1,253	1,252



(ii)



(iii)



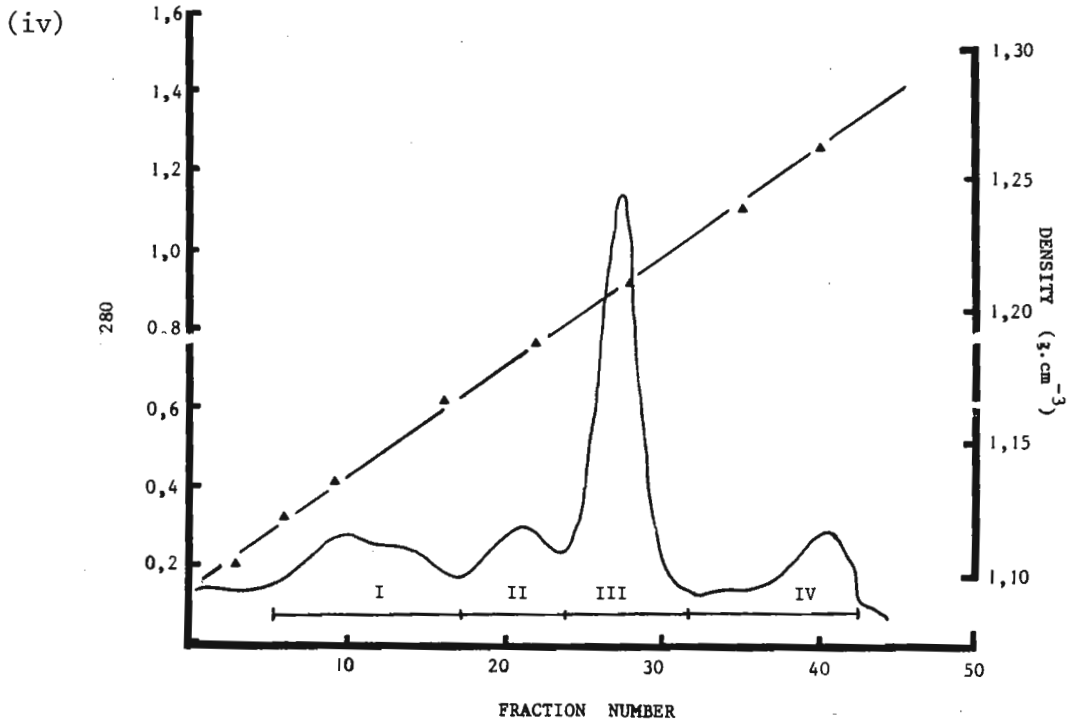


Fig. 7. Isopycnic sucrose density gradient fractionation profiles of RSD-B crude membranes after incubation periods of (i) 15 min, (ii) 30 min, (iii) 60 min and (iv) 120 min ( $\blacktriangle$ — $\blacktriangle$  = density of sucrose gradient).

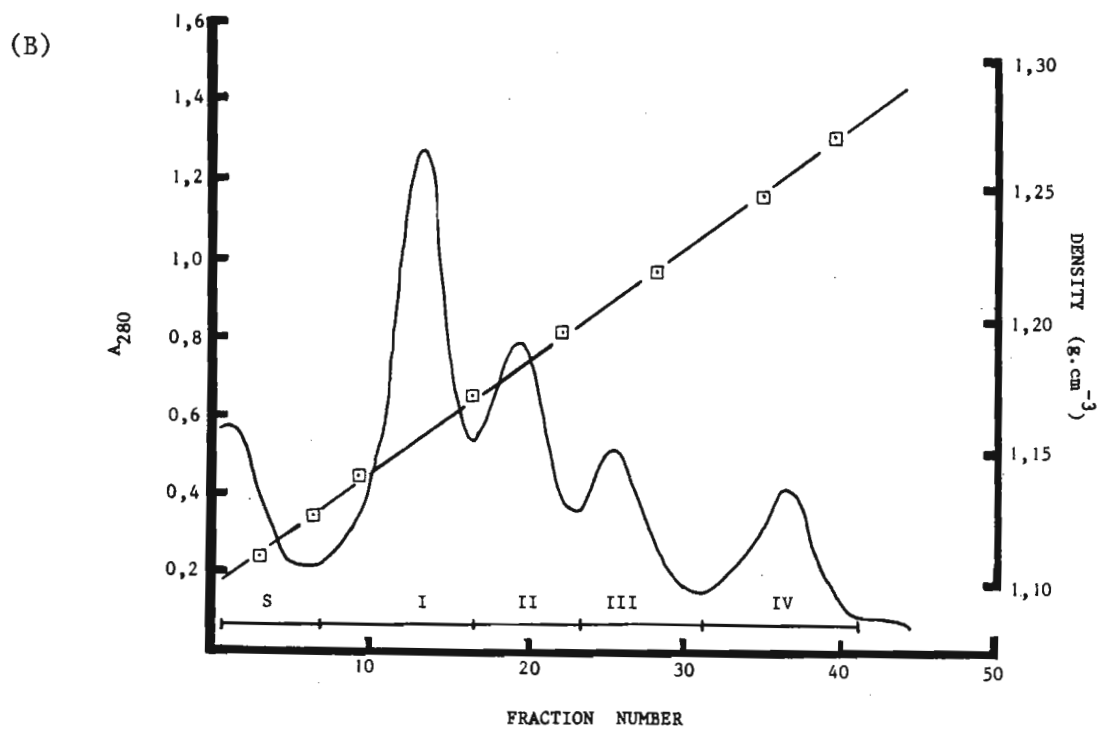
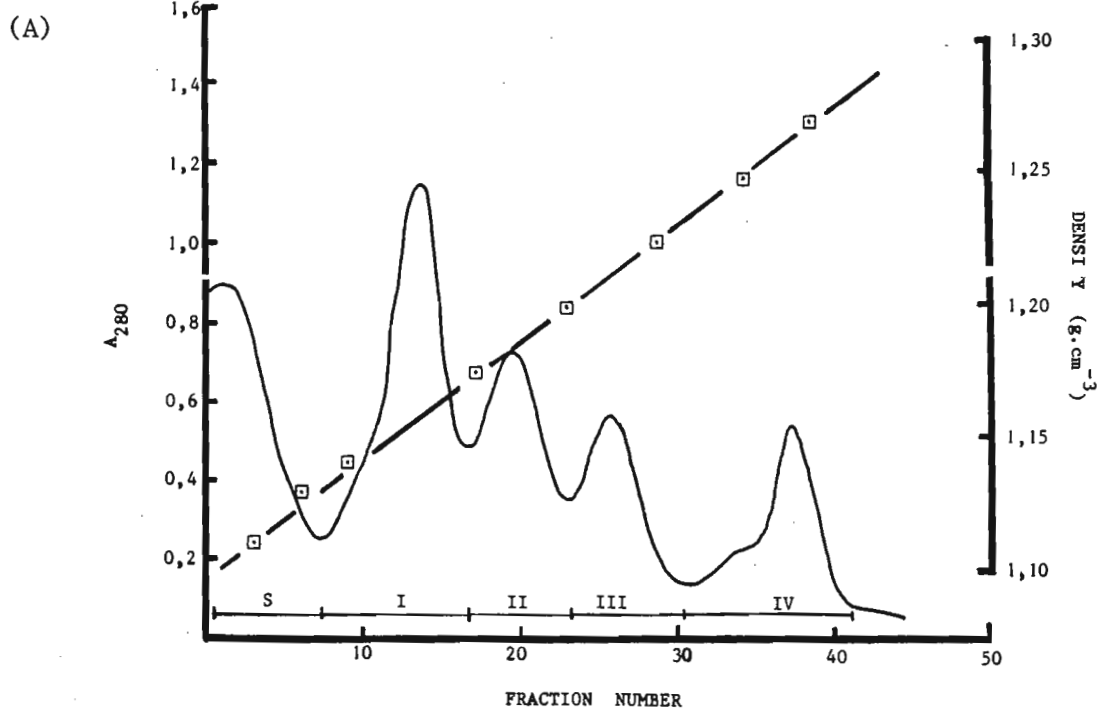
Various combinations of incubation temperatures after EDTA and lysozyme addition as well as after DNase, RNase and  $\text{MgSO}_4$  addition were monitored to determine the optimal incubation temperatures for RSD-B membrane isolation (Tables 7 and 8; Fig. 8). The relative yields of the S fraction and IV at the lower incubation temperatures were high and decreased as temperature increased whilst the relative yield of I improved as the incubation temperature was raised. Optimum yields of all bands were achieved using the 30-37°C incubation temperature combination (Fig. 8D). Recovery of I was poor at 0°C whilst the S peak was almost non-existent.

TABLE 7. Optimization of incubation temperature on membrane isolation

MEMBRANE SAMPLE	INCUBATION TEMPERATURE (C)	
	AFTER EDTA AND LYSOZYME ADDITION	AFTER DNase, RNase AND MgSO <sub>4</sub> ADDITION
A	20	20
B	20	37
C	30	30
D	30	37
E	37	37
F	0	37

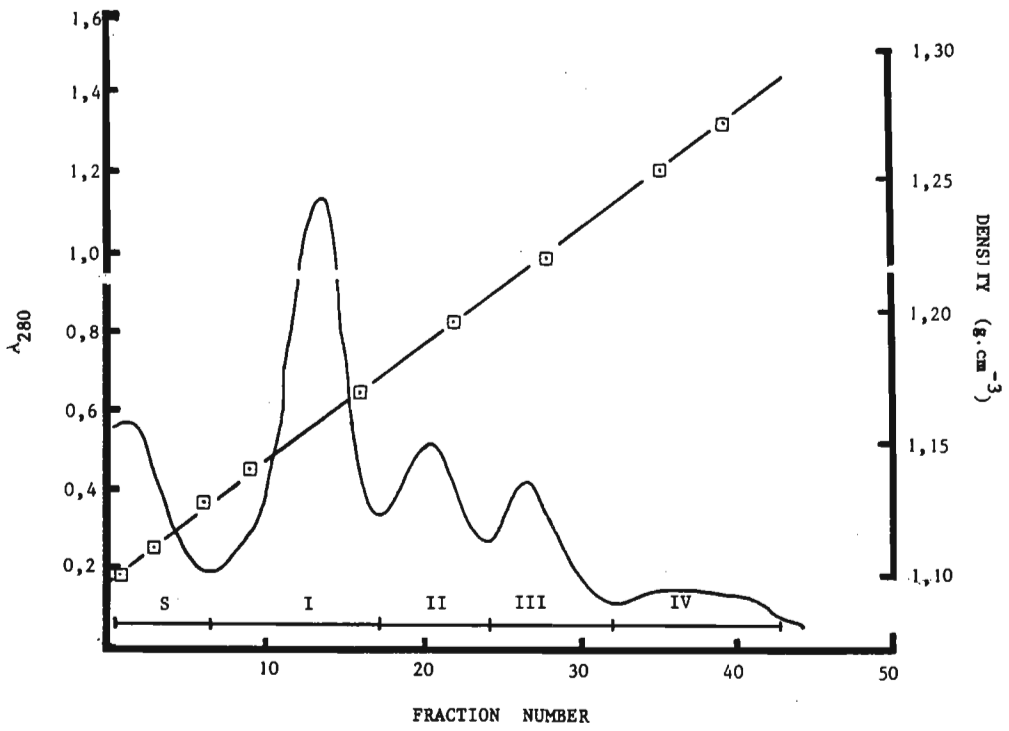
TABLE 8. Peak densities of membrane bands at different incubation temperatures

MEMBRANE FRACTION	PEAK DENSITY OF MEMBRANE SAMPLES					
	A	B	C	D	E	F
S	1,103	1,103	1,104	1,108	-	-
I	1,160	1,154	1,158	1,154	1,148	1,152
II	1,184	1,184	1,188	1,184	1,180	1,176
III	1,210	1,210	1,215	1,208	1,200	1,204
IV	1,262	1,254	1,256	1,256	1,240	1,244

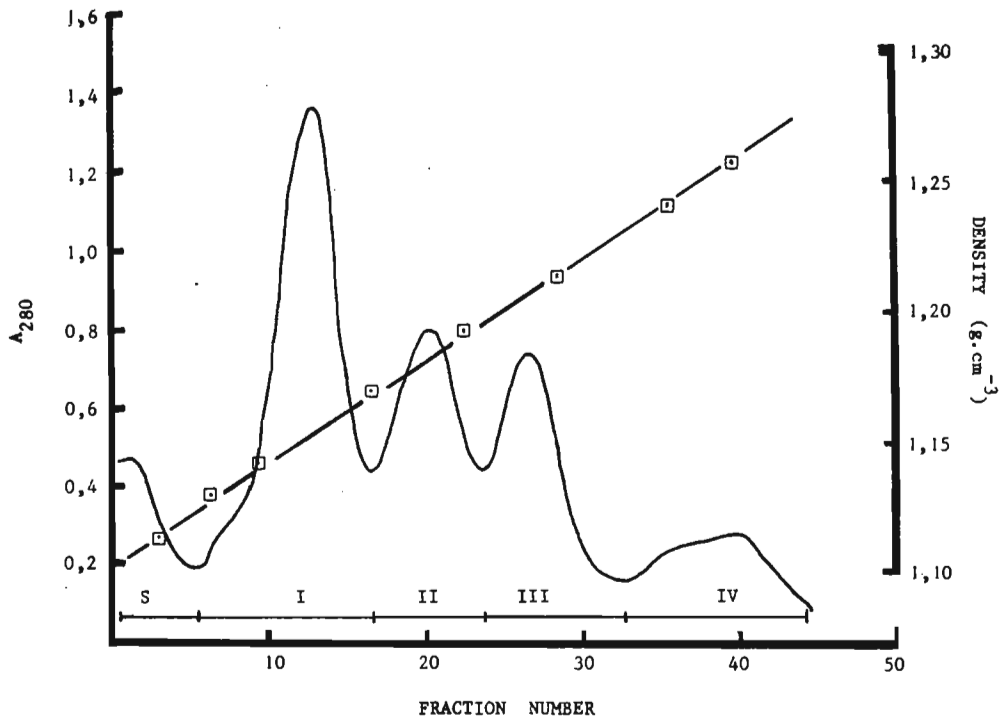




(C)



(D)



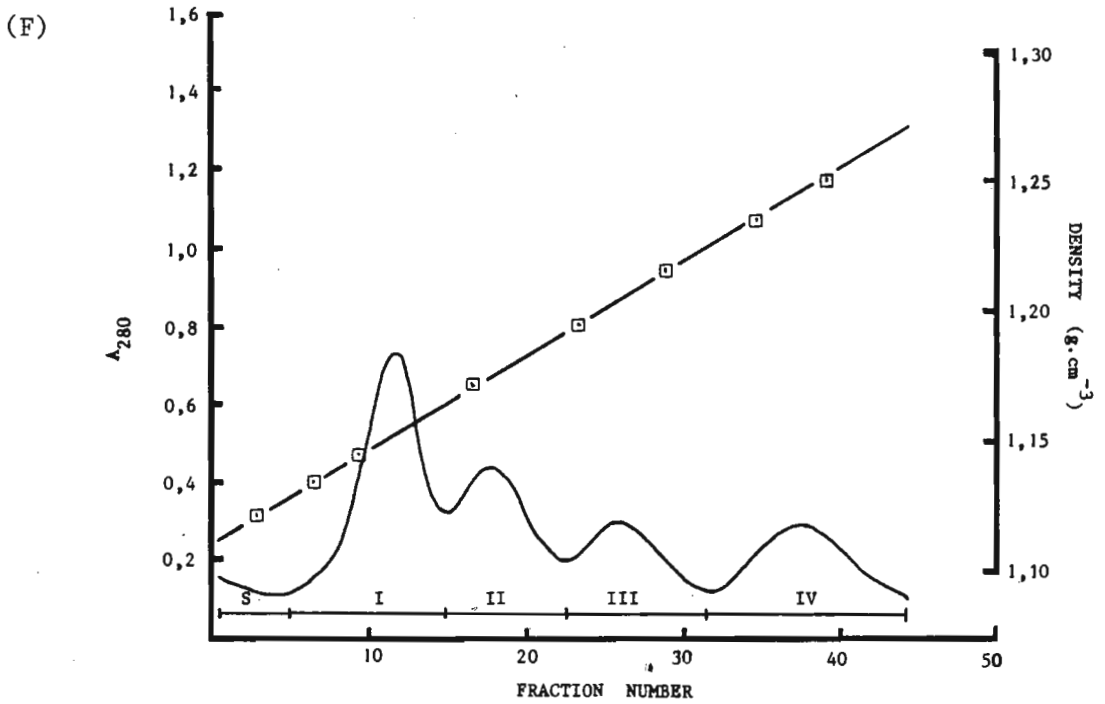
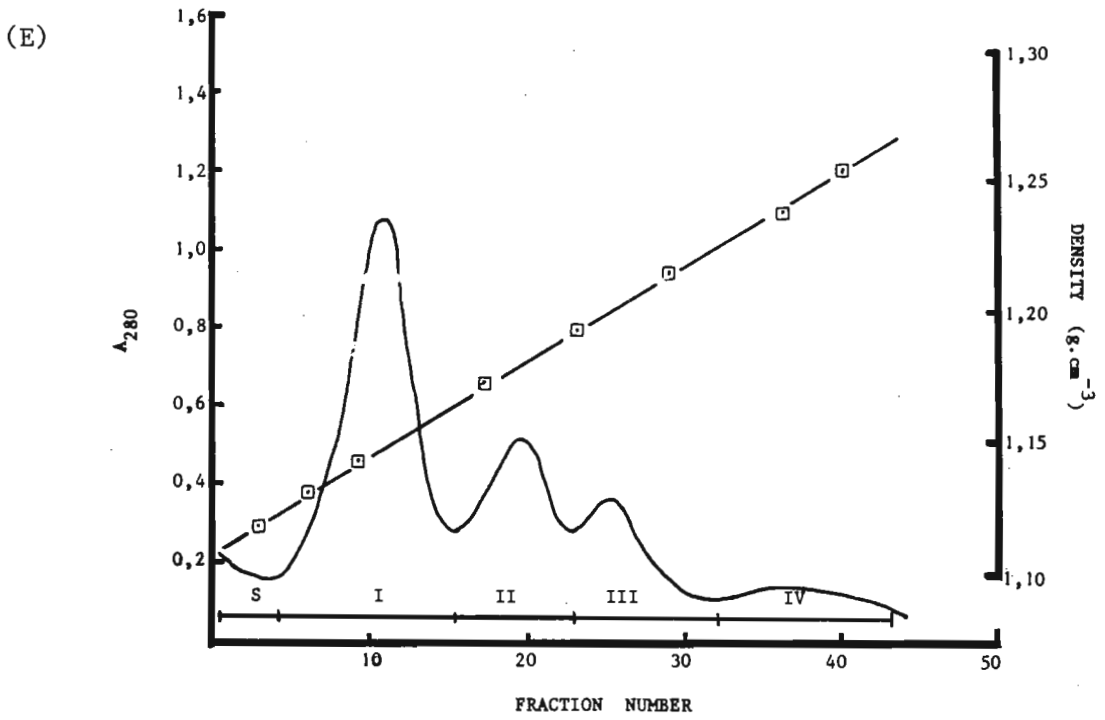
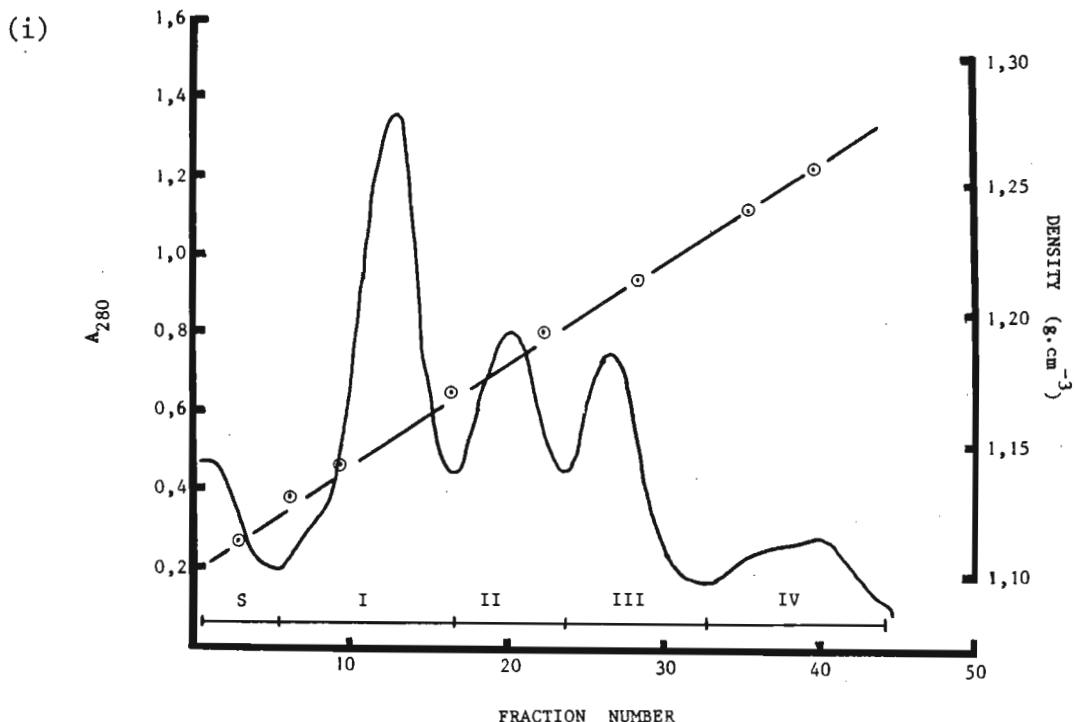


Fig. 8. Isopycnic sucrose density gradient fractionation profiles of RSD-B crude membranes at varying incubation temperature combinations as indicated in Table 7 ( $\square$ — $\square$  = density of sucrose gradient).

The effect of refrigeration on RSD-B crude membrane samples before sucrose density gradient centrifugation was also investigated (Table 9; Fig. 9). It was found that refrigeration did not affect the yields of the membrane bands upon isolation. In fact, the yields of membrane bands II and III were improved.

TABLE 9. Peak density of membrane fractions isolated without refrigeration and after refrigeration (4C, 96 h)

MEMBRANE FRACTION	PEAK DENSITY	
	- REFRIGERATION	+ REFRIGERATION
S	1,108	1,113
I	1,154	1,155
II	1,184	1,181
III	1,208	1,201
IV	1,254	1,262



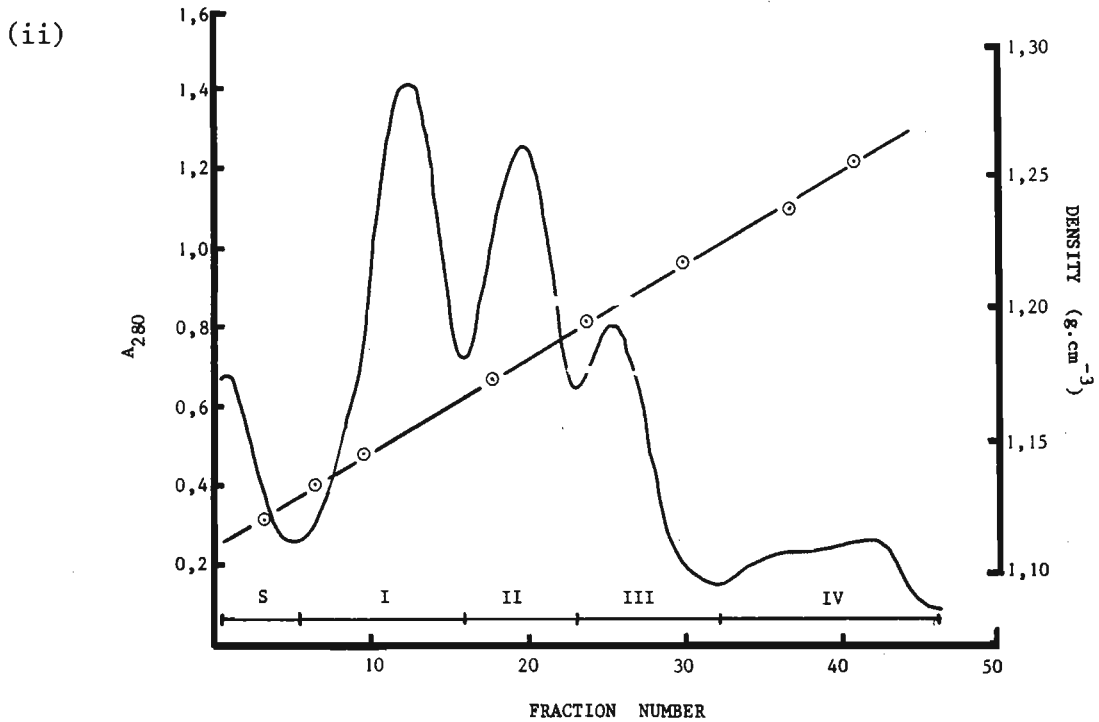


Fig. 9. Isopycnic sucrose density gradient fractionation profiles of RSD-B crude membranes (i) without refrigeration and (ii) after refrigeration at 4C for 96 h (⊙—⊙ = density of sucrose gradient).

### C) PROTEIN DETERMINATION

Determination of the protein content was necessary in order to establish the volumes of membrane fractions required for immunization, analysis by SDS-PAGE and for serological analysis. The standard curve for protein determination using the modified Lowry procedure (Markwell *et al.*, 1978) is indicated in Fig. 10 in which the absorbance values of varying BSA concentrations at 660 nm are shown. Protein determination of the membrane bands was carried out using the linear portion of the graph only. Protein concentrations of up to  $16 \mu\text{g} \cdot \mu\text{l}^{-1}$  could thus be accurately determined. The protein content of the membrane samples assayed is indicated in Table 10.

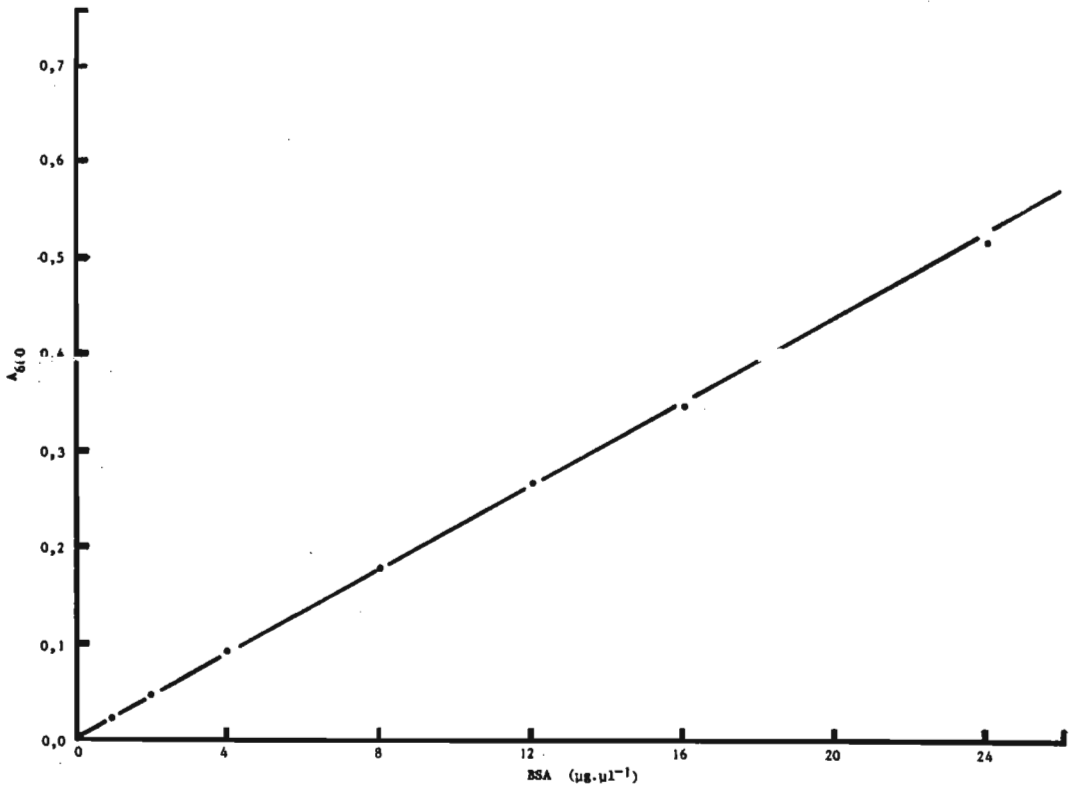


Fig. 10. Standard protein curve at 660 nm for varying BSA concentrations.

TABLE 10. Protein concentration of membrane samples assayed at 660 nm

MEMBRANE SAMPLE	[PROTEIN] (µg·µl <sup>-1</sup> )
S	0,67
I	3,20
II	2,86
III	1,51
IV	2,47
CRUDE	5,94

Using the results in Table 10, the protein concentration for immunization was adjusted to 100  $\mu\text{g}$  or 150  $\mu\text{g}$  per volume injected per rabbit. The samples prepared for SDS-PAGE were also adjusted to equate the protein concentrations of all the membrane bands assayed so that the volumes applied to the gel bed were the same.

#### D) IMMUNOLOGICAL ASSAYS

Assays using the intact cultured RSD-associated bacterium and the membrane bands I-IV included microagglutination, immunofluorescence, ELISA and immunodiffusion. Each assay was evaluated for its simplicity, speed, sensitivity and application.

##### 1. Whole bacterium

###### (a) Microagglutination

The serum from the rabbits immunized with the intact RSD-B was tested for antibody activity using the slide agglutination test. The titre of the antiserum to the RSD-associated bacterium is indicated in Table 11.

No agglutination was detected for the serum obtained from the zero bleeding for all the rabbits except one. Slight agglutination was observed for a serum dilution of 1:2 and an antigen concentration of  $1 \times 10^{10}$  cells.ml<sup>-1</sup> for this rabbit. The agglutination titre (850), as detected by phase contrast microscopy, was reasonably high for the antiserum produced against the intact bacterium injected with an adjuvant. When the dilution of antiserum was increased further, agglutination was no longer evident. The agglutinating strength (indicated by 0 to 5+) of the antiserum decreased progressively as the antigen dilution increased.

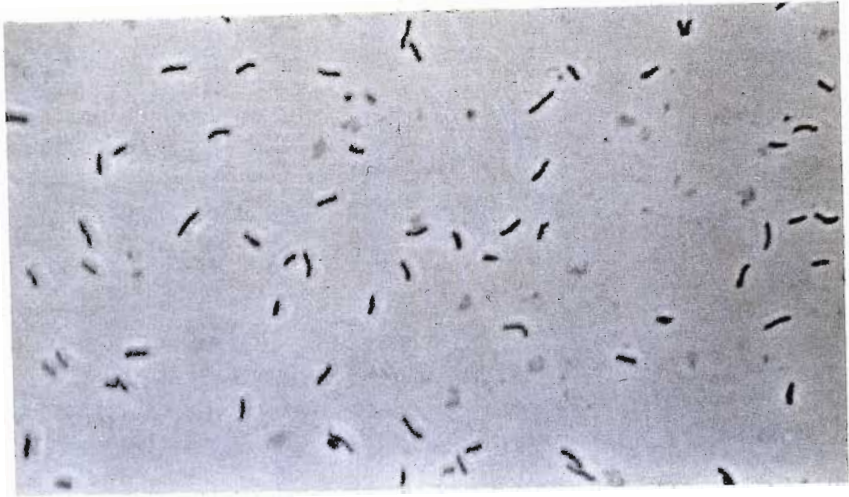
TABLE 11. Slide agglutination test for antibodies in anti-whole RSD-B rabbit serum

[ANTIGEN] (cells.ml <sup>-1</sup> )	RECIPROCAL OF ANTISERUM DILUTION								CONTROL SERUM DILUTION
	2	128	256	512	750	800	850	1000	UNDIL. TO 1000
10 <sup>10</sup>	5+*	5+	4+	4+	3+	2+	1+	0	0
10 <sup>9</sup>	5+	4+	3+	3+	2+	1+	0	0	0
10 <sup>8</sup>	4+	4+	3+	2+	1+	1+	0	0	0
10 <sup>7</sup>	3+	3+	2+	1+	1+	0	0	0	0
10 <sup>6</sup>	2+	2+	1+	1+	0	0	0	0	0
10 <sup>5</sup>	2+	1+	0	0	0	0	0	0	0
10 <sup>4</sup>	1+	0	0	0	0	0	0	0	0
10 <sup>3</sup>	0	0	0	0	0	0	0	0	0

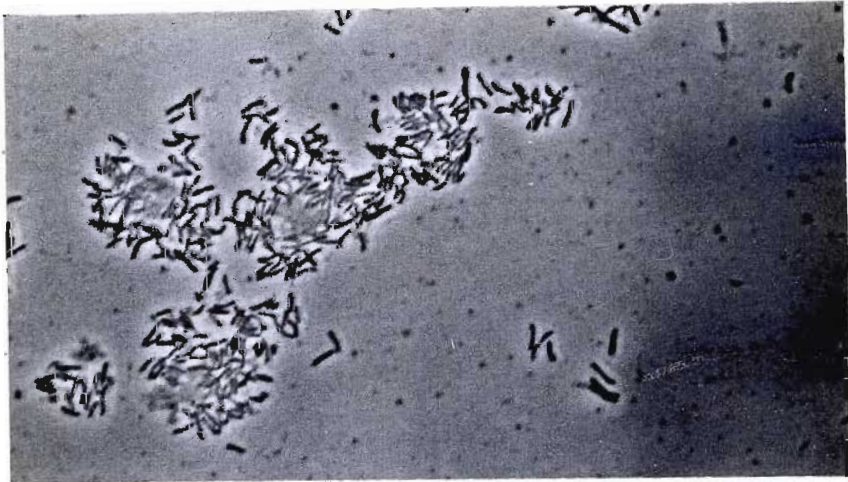
\*0-5+: indicates strength of agglutination.

Agglutination was recorded as positive (+) when 50% or more of the cells in groups of 10 or more were observed (Fig. 11). The lowest antigen concentration at which agglutination was detected was  $1 \times 10^4$  cells.ml<sup>-1</sup>. An agglutination titre of 1:512 was recorded for the antigen produced against the intact bacterium injected without an adjuvant. The former antiserum was employed for the serological assays.

(i)



(ii)



(iii)

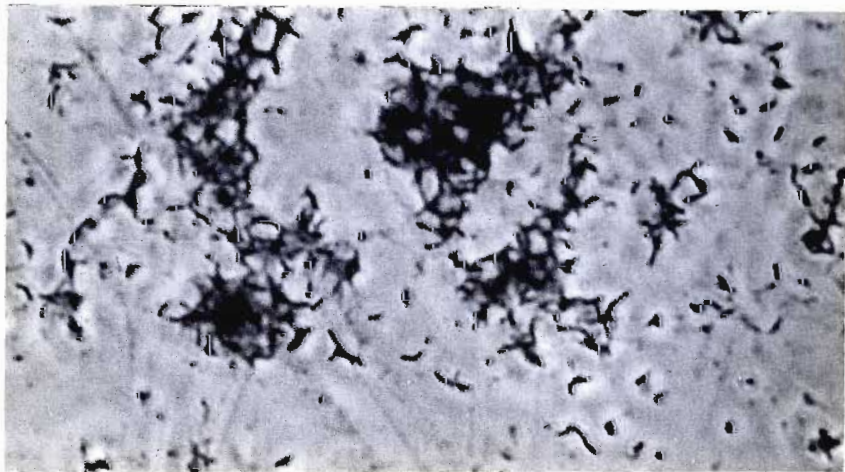


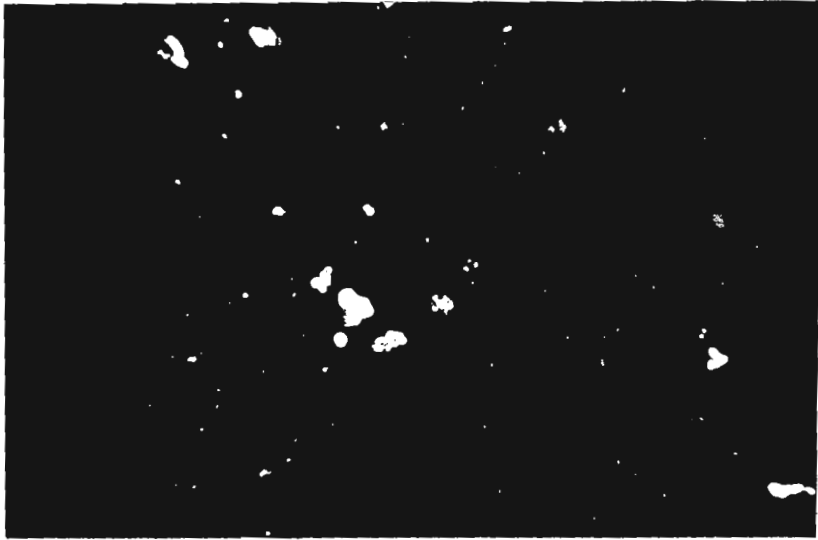
Fig. 11. Agglutination of RSD-B by (i) negative control serum and antiserum as shown by (ii) unstained test preparation and (iii) test preparation stained with crystal violet.



(b) Immunofluorescence

Specific fluorescence of the RSD-B was detected with its antiserum (Fig. 12). The control was negative.

(i)



(ii)

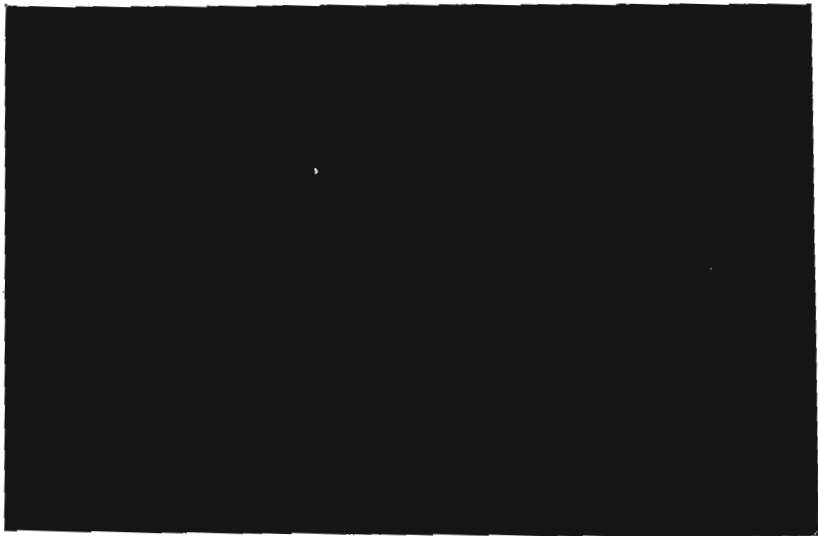


Fig. 12. Immunofluorescence of RSD-B adsorbed to (i) its antiserum and (ii) the control serum. The fluorescent probe was FITC (Miles Yeda Ltd., Rehovot, Israel).

TABLE 12. Degree of immunofluorescence of RSD-B ( $1 \times 10^8$  cells.ml<sup>-1</sup>) at varying antiserum and FITC-conjugate dilutions

FITC CONJUGATE DILUTION (RECIPROCAL)	RECIPROCAL TITRE OF ANTISERUM							CONTROL SERUM DILUTION
	2	5	10	512	1024	2048	4096	UNDIL. TO 1000
50	5+*	5+	4+	3+	2+	1+	0	0
100	4+	3+	3+	2+	1+	0	0	0
200	2+	1+	1+	1+	0	0	0	0
400	1+	1+	0	0	0	0	0	0
800	0	0	0	0	0	0	0	0

\*0-5+: indicates intensity of immunofluorescence.

Immunofluorescence of the bacterium was best observed at corresponding dilutions of 1:5 for the RSD-B antiserum and 1:50 for the FITC conjugate. The lowest antiserum concentration at which immunofluorescence was detected was 1:2048 at a corresponding FITC conjugate dilution of 1:50. Varying degrees of immunofluorescence (indicated by 0-5+) were recorded for different antiserum dilutions (Table 12).

(c) Enzyme-linked immunosorbent assay

The sensitivity of ELISA is influenced by antibody coating concentration, conjugate concentration, conjugate diluent, antigen diluent, the washing procedure, incubation temperature for sample and conjugate, pH and time. The negative employed may influence the P/N\* ratio. These variables need

to be carefully controlled at each stage of this immunoassay. The following sets of results indicate attempts to optimize these variables for the RSD-associated bacterium.

Coating of plates with anti-RSD-B antibody: A concentration of  $100 \mu\text{g}.\text{ml}^{-1}$  of rabbit antibody was found to give an optimal coating of 'capture' antibody (Tables 13 and 14). Plates coated at this antibody concentration retained full activity after three months at  $-20\text{C}$ .

TABLE 13. Determination of optimum antibody coating concentration at varying antigen dilutions

[ANTIGEN] (cells.ml <sup>-1</sup> )	ABSORBANCE UNITS AT VARYING ANTIBODY CONCENTRATIONS ( $\mu\text{g}.\text{ml}^{-1}$ )											
	0	1	2	4	8	16	32	64	125	250	500	1000
$10^8$	161	1248	1291	1367	1393	1402	1465	1468	1492	1480	1478	1479
$10^4$	20	27	31	33	34	39	42	43	43	43	42	42
$10^2$	13	21	29	31	33	34	34	34	35	35	34	34
TS negative	22	26	27	27	26	26	25	23	23	23	24	24

TABLE 14. P/N ratios for different antibody coating concentrations at varying antigen dilutions

[ANTIGEN] (cells.ml <sup>-1</sup> )	P/N RATIOS AT VARYING ANTIBODY COATING CONCENTRATIONS (µg.ml <sup>-1</sup> )											
	0	1	2	4	8	16	32	64	128	250	500	1000
10 <sup>8</sup>	7,3	48,0	47,8	50,6	53,6	53,9	58,6	63,8	64,9	64,4	61,6	61,6
10 <sup>4</sup>	0,9	1,0	1,2	1,2	1,3	1,5	1,7	1,9	1,9	1,9	1,8	1,8
10 <sup>2</sup>	0,6	0,8	1,1	1,1	1,3	1,3	1,4	1,5	1,5	1,5	1,4	1,4

\*P/N = positive (P) absorbance units divided by absorbance units obtained for the negative (N).

- NOTE:
- (i) The conjugate dilution was 1/500 in 5% S + R\* in TS;
  - (ii) The antigen diluent was TS; and
  - (iii) The plates were washed with TST.

\*S = neutral sheep serum; R = neutral rabbit serum.

Conjugate concentration: The optimal conjugate concentration was defined as that which would yield the highest final colour development at an antibody coating concentration of 100 µg.ml<sup>-1</sup>. A conjugate concentration of 1/500 was found to be the optimum (Tables 15 and 16; Fig. 13).

TABLE 15. Determination of optimum conjugate concentration at varying antigen dilutions

[ANTIGEN] (cells.ml <sup>-1</sup> )	ABSORBANCE UNITS AT VARYING CONJUGATE CONCENTRATIONS				
	1/250	1/500	1/1000	1/1500	1/2000
10 <sup>9</sup>	1580	1472	1179	526	469
10 <sup>8</sup>	1584	1476	1186	528	476
10 <sup>7</sup>	604	592	420	213	199
10 <sup>6</sup>	288	280	187	163	150
10 <sup>5</sup>	128	114	94	85	79
10 <sup>4</sup>	64	58	38	33	30
10 <sup>3</sup>	36	30	26	24	23
TS negative	40	33	31	29	28

TABLE 16. P/N ratios for different conjugate concentrations at varying antigen dilutions

[ANTIGEN] (cells.ml <sup>-1</sup> )	P/N RATIOS AT VARYING CONJUGATE CONCENTRATIONS				
	1/250	1/500	1/1000	1/1500	1/2000
10 <sup>9</sup>	39,5	44,6	38,0	18,1	16,8
10 <sup>8</sup>	39,6	44,7	38,3	18,2	17,0
10 <sup>7</sup>	15,1	17,9	13,6	7,4	7,1
10 <sup>6</sup>	7,2	8,5	6,0	5,6	5,4
10 <sup>5</sup>	3,2	3,5	3,0	2,9	2,8
10 <sup>4</sup>	1,6	1,8	1,2	1,1	1,1
10 <sup>3</sup>	0,9	0,9	0,8	0,8	0,8

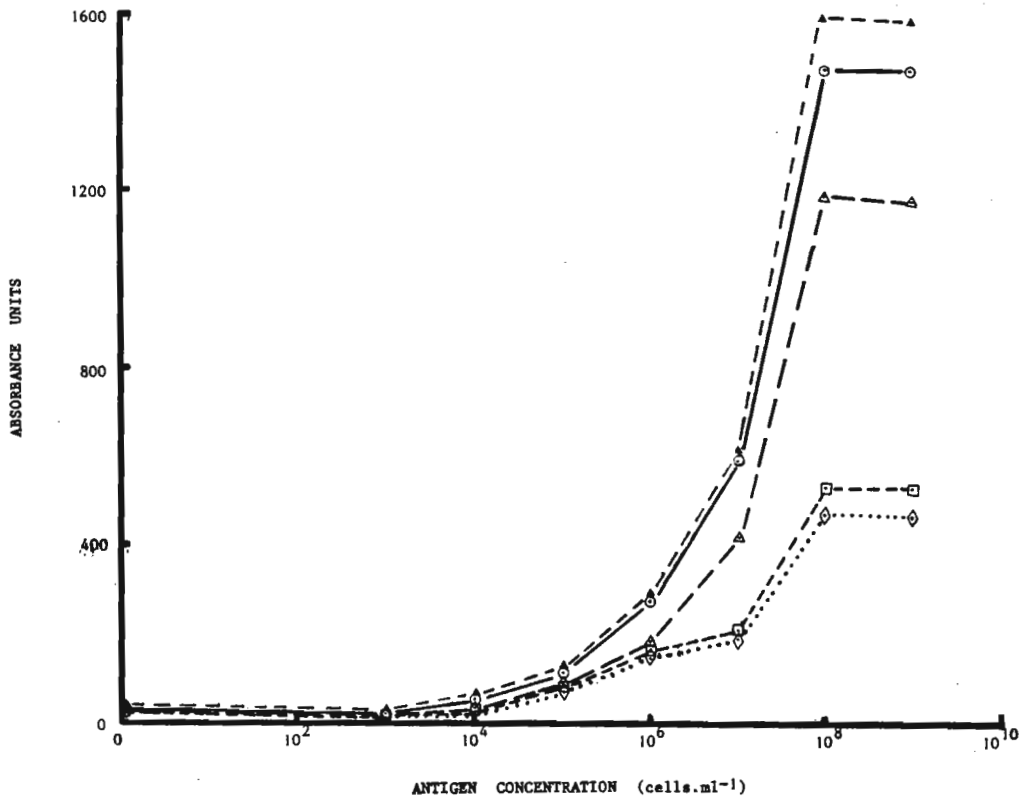


Fig. 13. Absorbance profile of varying concentrations of RSD-B at conjugate dilutions of 1/250 (▲--▲), 1/500 (⊙—⊙), 1/1000 (△--△), 1/1500 (□---□), and 1/2000 (◇····◇).

- NOTE:
- (i) The antibody coating concentration was 100  $\mu\text{g.ml}^{-1}$  anti-RSD-B;
  - (ii) The conjugate diluent was 5% S + R in TS;
  - (iii) The antigen diluent was TS; and
  - (iv) The plates were washed with TST.

Tables 17 and 18 show the results obtained for determination of the best conjugate and antigen diluents and the most suitable negative. The best conjugate diluent was S + R whilst the best antigen diluent was TS. TS also proved to be the most suitable negative.

TABLE 17. Determination of best conjugate and antigen diluents and best negative at varying concentrations of antigen

[ANTIGEN] (cells.ml <sup>-1</sup> ) and NEGATIVES	ABSORBANCE UNITS											
	CONJUGATE IN 5% R				CONJUGATE IN 5% S				CONJUGATE IN 5% S+R			
	ANTIGEN DILUENT				ANTIGEN DILUENT				ANTIGEN DILUENT			
	TS	R	S	S+R	TS	R	S	S+R*	TS*	R	S	S+R
10 <sup>9</sup>	1198	847	876	985	1154	1039	962	764	1399	807	729	684
10 <sup>8</sup>	1216	804	883	997	1169	1041	956	761	1406	822	722	637
10 <sup>7</sup>	490	258	249	129	301	175	241	129	1392	97	159	142
10 <sup>6</sup>	111	32	41	28	84	63	51	40	257	28	20	19
10 <sup>5</sup>	28	23	34	24	39	40	39	39	43	18	19	17
10 <sup>4</sup>	18	13	21	15	33	23	29	29	27	13	18	15
10 <sup>3</sup>	10	3	10	8	25	16	19	20	8	4	10	5
TS	14	-	-	-	27	-	-	-	9	-	-	-
5% R in TS	-	8	-	-	-	20	-	-	-	8	-	-
5% S in TS	-	-	11	-	-	-	30	-	-	-	11	-
5% S+R in TS	-	-	-	10	-	-	-	28	-	-	-	14

\*Absorbance profiles are illustrated for these sets of results (Fig. 14).

TABLE 18. P/N ratios for varying antigen concentrations subjected to different antigen and conjugate diluents

[ANTIGEN] (cells.ml <sup>-1</sup> )	P/N RATIOS											
	CONJUGATE IN 5% R				CONJUGATE IN 5% S				CONJUGATE IN 5% S+R			
	ANTIGEN DILUENT				ANTIGEN DILUENT				ANTIGEN DILUENT			
	TS	R	S	S+R	TS	R	S	S+R	TS	R	S	S+R
10 <sup>9</sup>	85,6	105,9	79,6	98,5	42,7	52,0	32,1	27,3	155,4	100,9	66,3	48,9
10 <sup>8</sup>	86,9	100,5	80,3	99,7	43,3	52,1	31,9	27,2	156,2	102,8	65,6	45,5
10 <sup>7</sup>	35,0	32,3	22,6	12,9	11,2	8,8	8,0	4,6	154,7	12,1	14,5	10,1
10 <sup>6</sup>	7,9	4,0	3,7	2,8	3,1	3,2	1,7	1,4	28,6	3,5	1,8	1,4
10 <sup>5</sup>	2,0	2,9	3,1	2,4	1,4	2,0	1,3	1,4	4,8	2,3	1,7	1,2
10 <sup>4</sup>	1,3	1,6	1,9	1,5	1,2	1,2	1,0	1,0	3,0	1,6	1,6	1,1
10 <sup>3</sup>	0,7	0,4	0,9	0,8	0,9	0,8	0,6	0,7	0,9	0,5	0,9	0,4

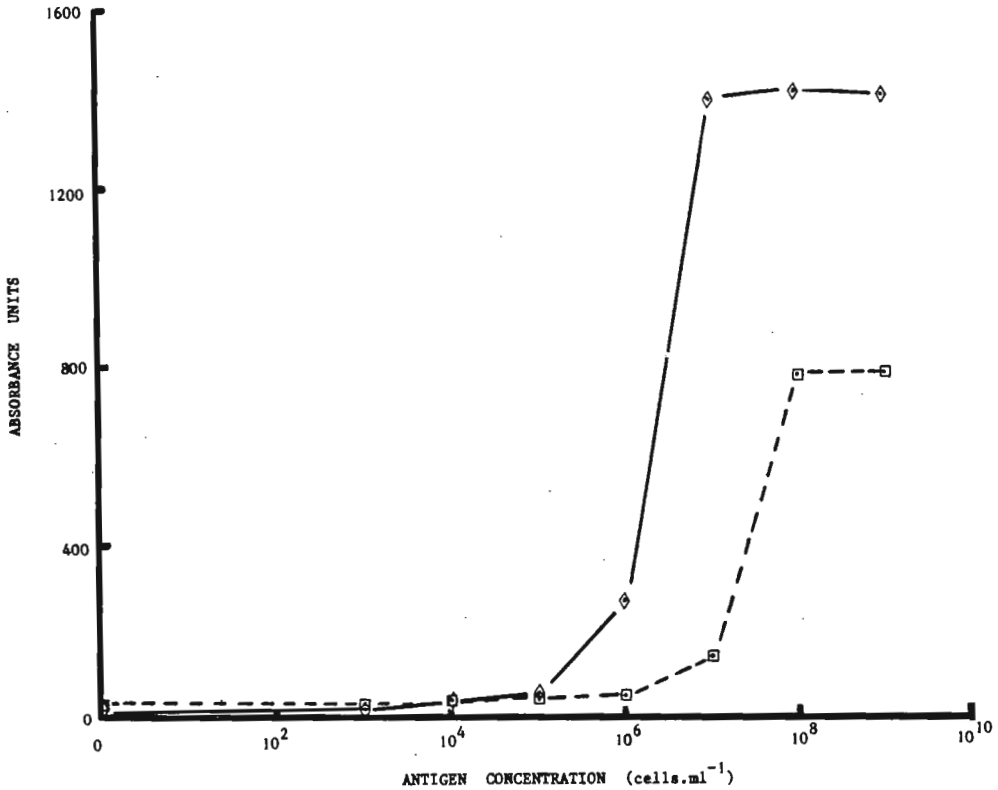


Fig. 14. Absorbance profiles of varying concentrations of RSD-B subjected to S + R conjugate diluent, TS antigen diluent and TS negative ( $\diamond$ - $\diamond$ ) and S conjugate diluent, S + R antigen diluent and S + R-negative ( $\square$ --- $\square$ ).

- NOTE:
- (i) The antibody coating concentration was  $100 \mu\text{g.ml}^{-1}$  anti-RSD-B;
  - (ii) The conjugate dilution was 1/500; and
  - (iii) The plates were washed with TST.

The effects of overcoating coated plates was investigated (Tables 19 and 20; Fig. 15). Coating of plates was achieved by applying  $100 \mu\text{g.ml}^{-1}$  anti-RSD-B IgG whilst overcoating was achieved by applying  $100 \mu\text{g.ml}^{-1}$  BSA on the coated plate for 1 h at RT in a moist chamber. Uncoated plates were also included. Results indicate that the need for coating was essential. Overcoating of coated plates did not improve the sensitivity of the assay.



TABLE 19. Absorbance values for different antigen concentrations on plates not coated, coated and overcoated

[ANTIGEN] (cells.ml <sup>-1</sup> )	ABSORBANCE UNITS		
	UNCOATED PLATES	COATED PLATES	OVERCOATED PLATES
10 <sup>9</sup>	68	1421	1437
10 <sup>8</sup>	70	1423	1441
10 <sup>7</sup>	35	1418	1440
10 <sup>6</sup>	18	292	288
10 <sup>5</sup>	18	41	33
10 <sup>4</sup>	17	28	32
10 <sup>3</sup>	15	20	25
TS negative	16	21	27

TABLE 20. P/N ratios for different antigen concentrations on plates not coated, coated and overcoated

[ANTIGEN] (cells.ml <sup>-1</sup> )	P/N RATIOS		
	UNCOATED PLATES	COATED PLATES	OVERCOATED PLATES
10 <sup>9</sup>	4,3	67,7	53,2
10 <sup>8</sup>	4,4	67,8	53,4
10 <sup>7</sup>	2,2	67,5	53,3
10 <sup>6</sup>	1,1	13,9	10,7
10 <sup>5</sup>	1,1	2,0	1,2
10 <sup>4</sup>	1,1	1,3	1,2
10 <sup>3</sup>	0,9	1,0	0,9

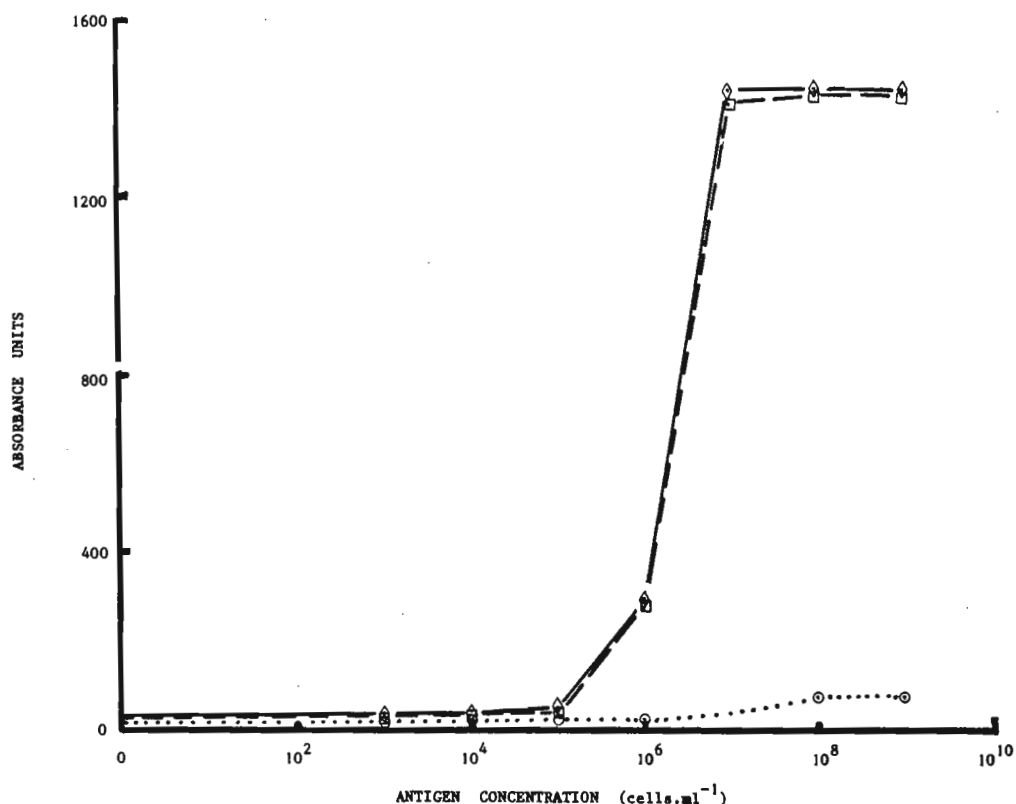


Fig. 15. Absorbance profile for different antigen concentrations on plates not coated (○.....○), coated (◇-◇) and overcoated (◻--◻).

- NOTE:
- (i) The conjugate dilution was 1/500 in 5% S + R in TS;
  - (ii) The antigen diluent was TS;
  - (iii) All the plates were washed with TST.

The washing procedure adopted influenced the sensitivity of the assay. Procedures employed included washing with TS, TST and centrifugation of coated plates followed by washing with TST. These procedures were tested on coated plates (Tables 21 and 22; Fig. 16) as well as overcoated plates (Tables 23 and 24; Fig. 17). On coated plates, the ELISA test was more sensitive when the plates were washed with TST as opposed to TS alone. Centrifugation followed by washing with TST yielded the lowest P/N ratios on both coated and overcoated plates. The sensitivity of the assay on overcoated plates washed with either TS or TST was the same.

TABLE 21. Absorbance values at varying antigen concentrations subjected to different washing procedures

[ANTIGEN] (cells.ml <sup>-1</sup> )	ABSORBANCE UNITS		
	WASHING PROCEDURE		
	TS	TST	CENTRIFUGATION + TST
10 <sup>9</sup>	1304	1421	1302
10 <sup>8</sup>	1312	1423	1310
10 <sup>7</sup>	1300	1418	1305
10 <sup>6</sup>	223	292	246
10 <sup>5</sup>	51	41	41
10 <sup>4</sup>	42	28	29
10 <sup>3</sup>	31	20	25
TS negative	30	21	37

TABLE 22. P/N ratios of varying antigen concentrations subjected to different washing procedures

[ANTIGEN] (cells.ml <sup>-1</sup> )	P/N RATIOS		
	WASHING PROCEDURE		
	TS	TST	CENTRIFUGATION + TST
10 <sup>9</sup>	43,5	67,7	35,2
10 <sup>8</sup>	43,7	67,8	35,4
10 <sup>7</sup>	43,3	67,5	35,3
10 <sup>6</sup>	7,4	13,9	6,7
10 <sup>5</sup>	1,7	2,0	1,1
10 <sup>4</sup>	1,4	1,3	0,8
10 <sup>3</sup>	1,0	1,0	0,7

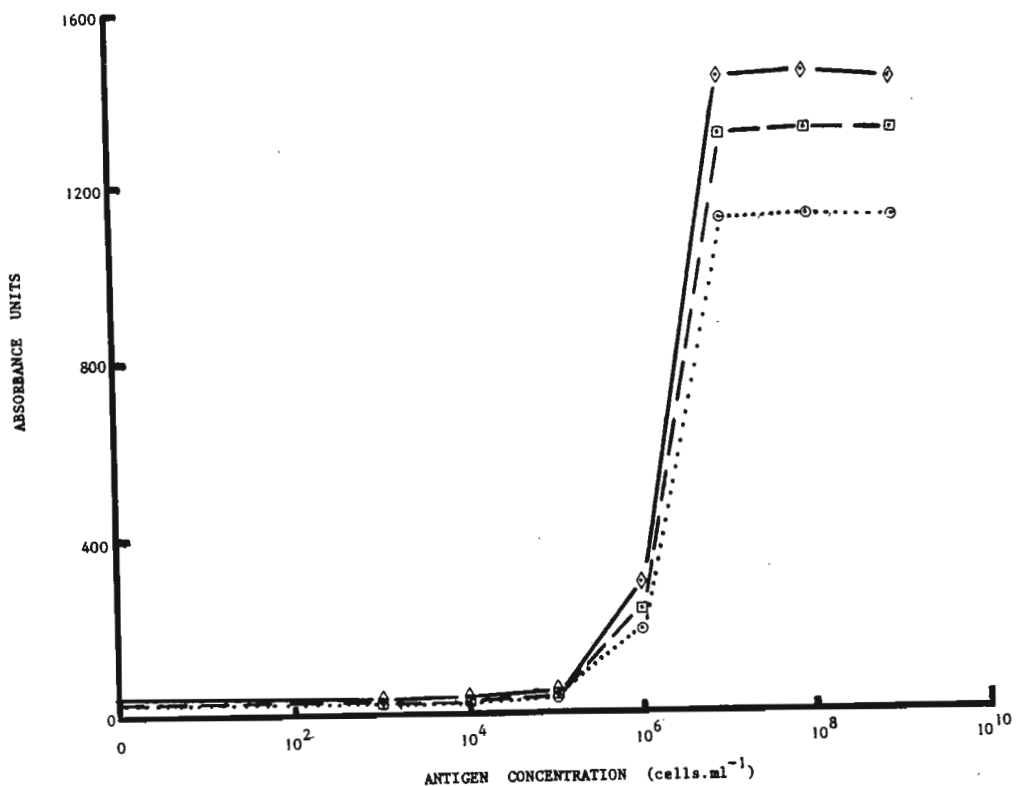


Fig. 16. Absorbance profiles of varying antigen concentrations in plates coated with  $100 \mu\text{g.ml}^{-1}$  anti-RSD-B IgG and washed with TS ( $\square$ — $\square$ ), TST ( $\diamond$ — $\diamond$ ) and centrifugation + TST ( $\odot$ ..... $\odot$ ).

NOTE: (i) The conjugate dilution was 1/500 in 5% S + R in TS; and  
(ii) The antigen diluent was TS.

TABLE 23. Absorbance values of varying antigen concentrations subjected to different washing procedures on plates overcoated with BSA

[ANTIGEN] (cells.ml <sup>-1</sup> )	ABSORBANCE UNITS		
	WASHING PROCEDURE		
	TS	TST	CENTRIFUGATION + TST
10 <sup>9</sup>	1119	1437	1309
10 <sup>8</sup>	1120	1441	1311
10 <sup>7</sup>	1115	1440	1307
10 <sup>6</sup>	185	288	239
10 <sup>5</sup>	51	53	41
10 <sup>4</sup>	37	32	38
10 <sup>3</sup>	20	25	35
TS negative	21	27	42

TABLE 24. P/N ratios of varying antigen concentrations subjected to different washing procedures on plates overcoated with BSA

[ANTIGEN] (cells.ml <sup>-1</sup> )	P/N RATIOS		
	WASHING PROCEDURE		
	TS	TST	CENTRIFUGATION + TST
10 <sup>9</sup>	53,3	53,2	31,2
10 <sup>8</sup>	53,3	53,4	31,2
10 <sup>7</sup>	53,1	53,3	31,1
10 <sup>6</sup>	8,8	10,7	5,7
10 <sup>5</sup>	2,4	2,0	1,0
10 <sup>4</sup>	1,8	1,2	0,9
10 <sup>3</sup>	1,0	0,9	0,8

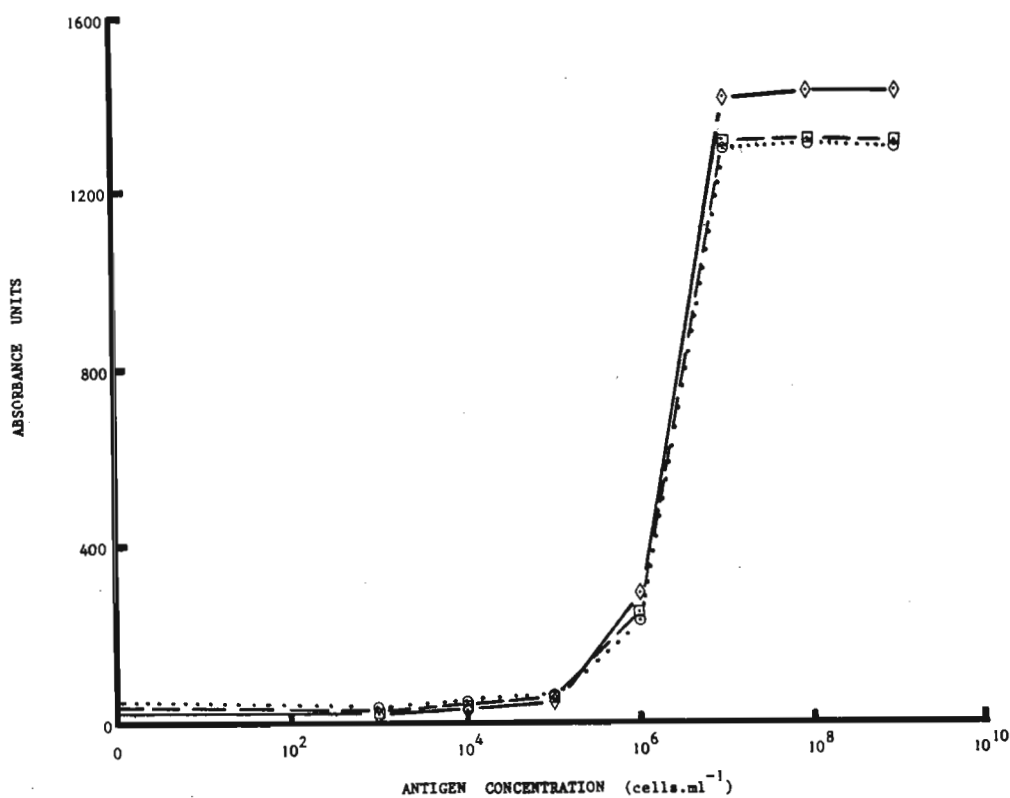


Fig. 17. Absorbance profiles of varying antigen concentrations on plates overcoated with BSA and washed with TST (◇—◇) TS (⊙.....⊙) and centrifugation + TST (□- -□).

NOTE: (i) The conjugate dilution was 1/500 in 5% S + R in TS; and  
(ii) The antigen diluent was TS.

The effect of an antibody coating period of 2 days opposed to 1 h for the adopted procedure was also investigated (Tables 25 and 26; Fig. 18). There was no decrease in absorbance when compared to the negative at an antigen concentration of  $1 \times 10^3$  cells.ml<sup>-1</sup> as was evident in all the other ELISA investigations (Tables 15, 17, 19, 21 and 23). The assay was very sensitive at the lower antigen concentrations.

TABLE 25. Absorbance values of varying antigen concentrations on plates subjected to a coating period of 2 days at RT

[ANTIGEN] (cells.ml <sup>-1</sup> )	ABSORBANCE UNITS
10 <sup>9</sup>	1341
10 <sup>8</sup>	1348
10 <sup>7</sup>	1356
10 <sup>6</sup>	323
10 <sup>5</sup>	50
10 <sup>4</sup>	21
10 <sup>3</sup>	16
TS negative	11

TABLE 26. P/N ratios of varying antigen concentrations on plates subjected to a coating period of 2 days at RT

[ANTIGEN] (cells.ml <sup>-1</sup> )	P/N RATIOS
10 <sup>9</sup>	121,9
10 <sup>8</sup>	122,5
10 <sup>7</sup>	123,3
10 <sup>6</sup>	29,4
10 <sup>5</sup>	4,6
10 <sup>4</sup>	1,9
10 <sup>3</sup>	1,5

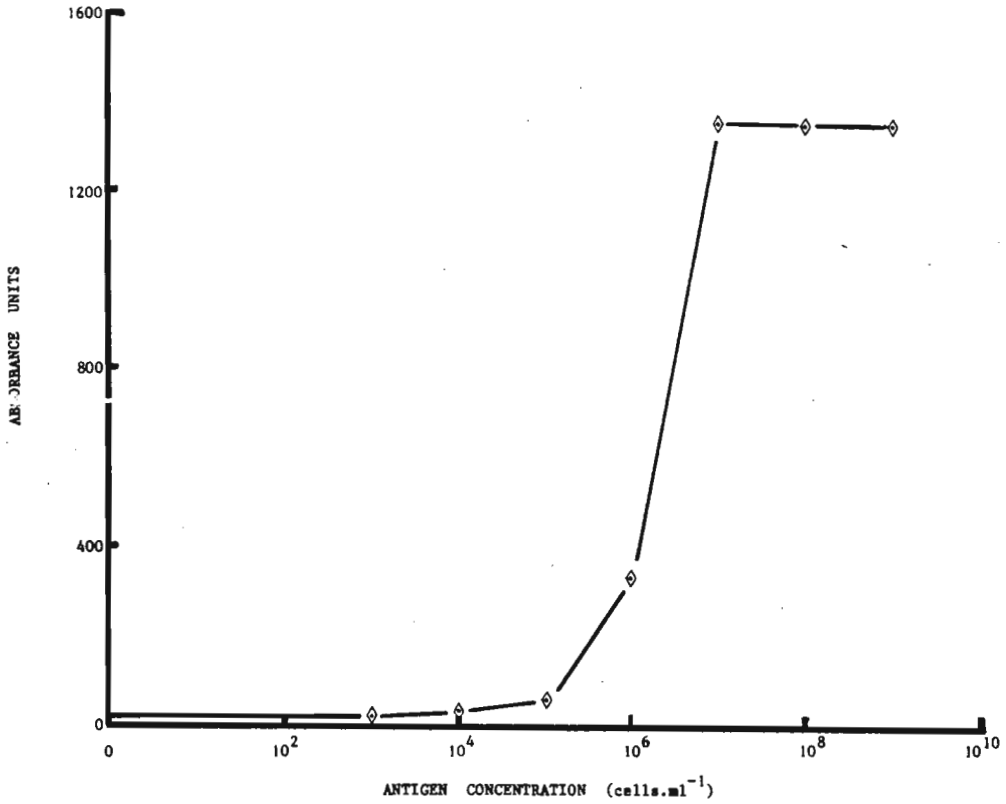


Fig. 18. Absorbance profile of varying antigen concentrations on plates subjected to a prolonged coating period.

- NOTE:
- (i) The plate was coated with  $100 \mu\text{g.ml}^{-1}$  anti-RSD-B and kept at RT in a moist chamber for 2 days;
  - (ii) The conjugate dilution was 1/500 in 5% S + R in TS;
  - (iii) The antigen diluent was TS; and
  - (iv) The plates were washed with TST.

Colour development: The final intensity of colour developed in this assay was found to be a function of the time and temperature at which the chromogenic substrate was allowed to react with the immobilized enzyme. Colour intensity was also controlled by conjugate concentration. In the assay reported here colour development was terminated after 30 min in a dark container at RT.



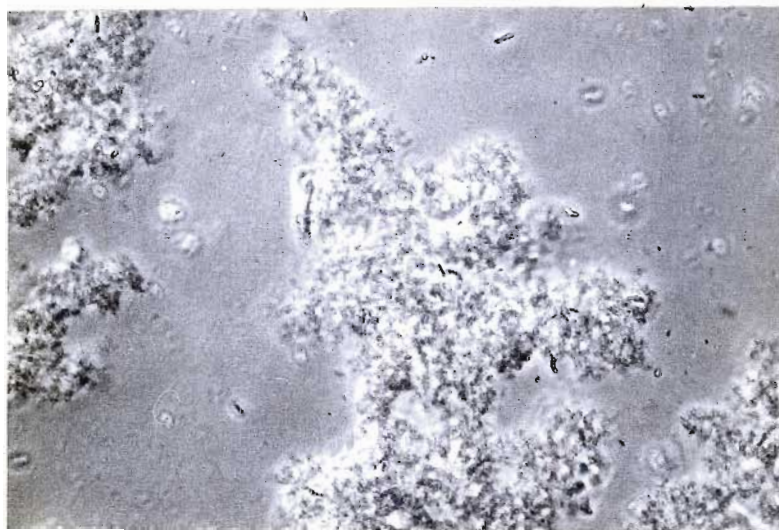
## 2. Membranes

Membrane bands I to IV were used in microagglutination, immunofluorescence and immunodiffusion tests. Each test was evaluated and the serological relatedness of the membrane fractions was established.

### (a) Microagglutination

Positive agglutination was recorded for all four membrane bands tested with the antiserum prepared against the intact bacterium (Fig. 19). The appropriate controls were negative.

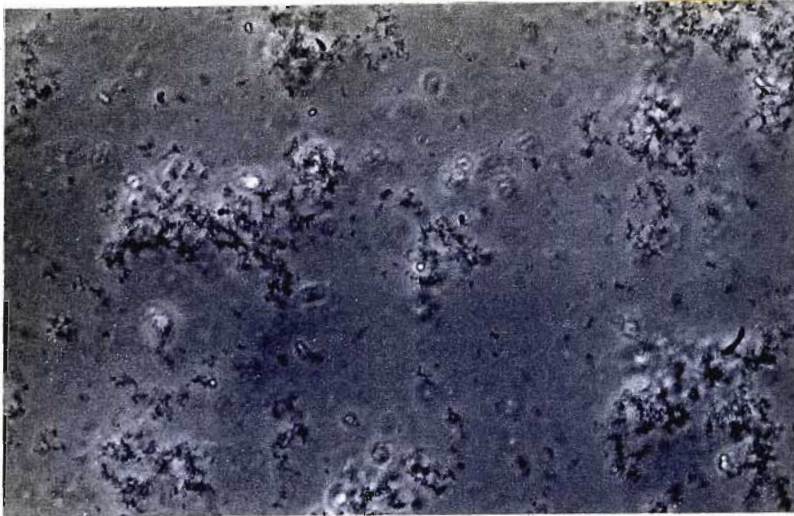
(i)



(ii)



(iii)



(iv)

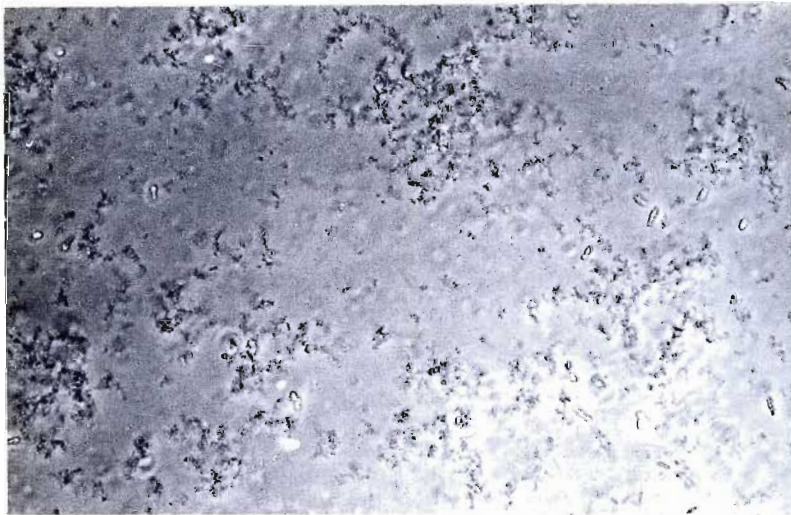


Fig. 19. Slide agglutination tests of membrane fractions (i) I, (ii) II, (iii) III and (iv) IV tested with antiserum prepared against RSD-B.

In addition, the agglutination pattern (indicated by 1+ to 4+) for each antigen by the antisera to membrane bands I ( $A_I$ ), II ( $A_{II}$ ), III ( $A_{III}$ ), IV ( $A_{IV}$ ) and RSD-B ( $A_B$ ) were tested (Table 27). No agglutination was observed with the control sera. The agglutination titres recorded were relatively high when observed by phase contrast microscopy. Titres recorded were 1:1024 for membrane fraction I, 1:1000 for membrane fractions II and IV and 1:750 for membrane fraction III when the protein content of the membranes was adjusted to  $3 \mu\text{g.ml}^{-1}$ .

TABLE 27. Agglutination pattern of membrane and bacterial antigens by antisera to the respective membrane bands and the intact bacterium

ANTIGEN	ANTISERUM				
	$A_B$	$A_I$	$A_{II}$	$A_{III}$	$A_{IV}$
I	3+*	4+	2+	2+	1+
II	2+	2+	3+	1+	1+
III	2+	1+	1+	3+	1+
IV	2+	2+	2+	1+	3+
RSD-B ( $1 \times 10^9 \text{ cells.ml}^{-1}$ )	3+	2+	2+	1+	1+

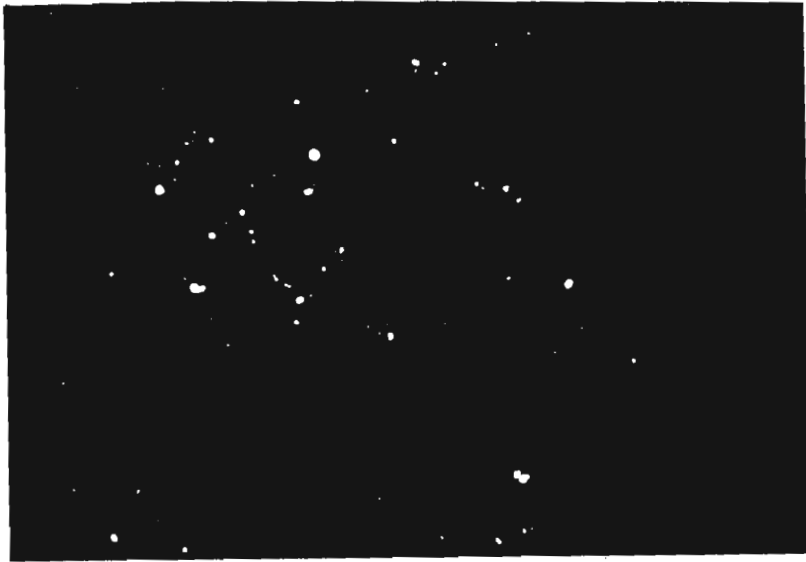
\*1+ to 4+ indicates strength of agglutination.

Each membrane fraction was best agglutinated by its own antiserum and was stronger than against the intact bacterium.

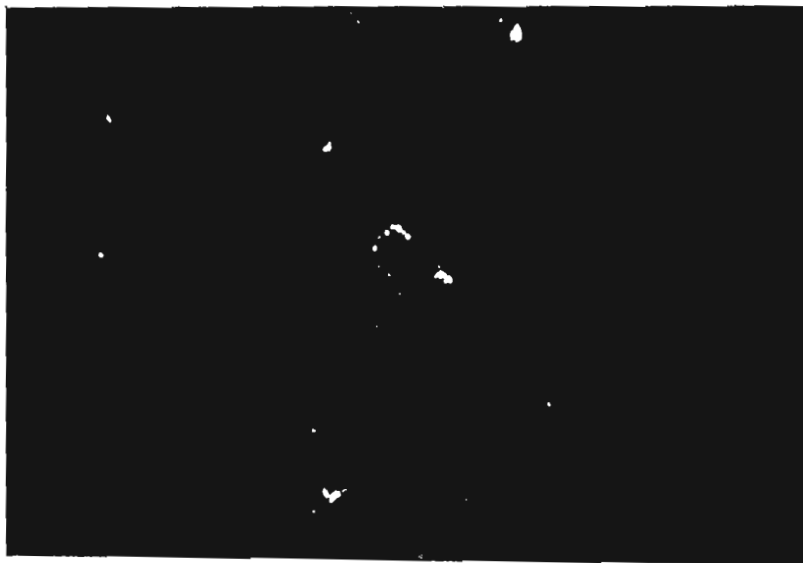
(b) Immunofluorescence

All membrane fractions tested against their own antiserum exhibited specific immunofluorescence (Fig. 20). The appropriate controls were negative.

(i)



(ii)



(iii)



(iv)

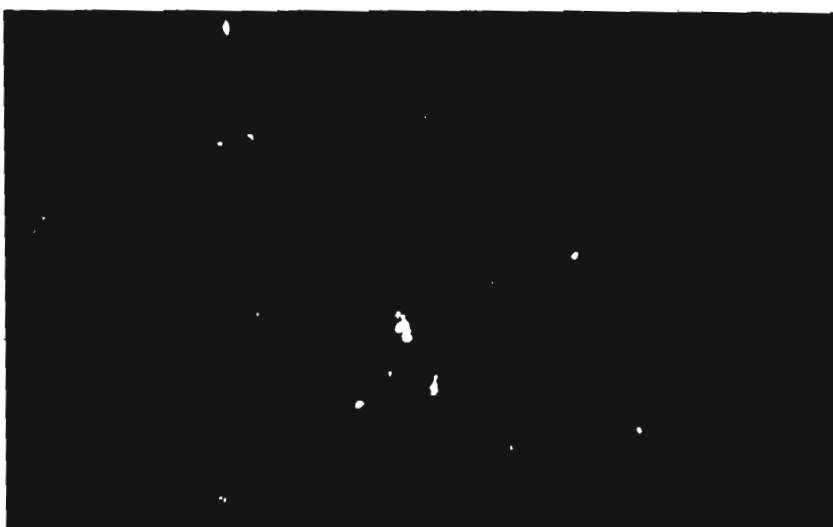


Fig. 20. Immunofluorescent stain of membranes of RSD-B tested with (i)  $A_I$ , (ii)  $A_{II}$ , (iii)  $A_{III}$  and (iv)  $A_{IV}$ .

Cross reactions of membrane bands tested by immunofluorescence showed all bands to be immunologically related (Table 28). The degrees of immunofluorescence of the membranes were found to be, in general, more intense than the immunofluorescence observed for the intact bacterium. Non-specific fluorescence detected in some of the controls appeared dull and was easily distinguished from the test.

TABLE 28. Immunofluorescence intensities of RSD-B and membrane bands (I-IV) tested against all antisera produced

ANTIGEN	ANTISERUM				
	A <sub>B</sub>	A <sub>I</sub>	A <sub>II</sub>	A <sub>III</sub>	A <sub>IV</sub>
RSD-B	3+*	2+	2+	1+	2+
I	3+	5+	4+	2+	4+
II	3+	4+	5+	2+	3+
III	2+	1+	2+	4+	1+
IV	2+	2+	3+	1+	5+

\*1+ to 5+ indicates intensity of immunofluorescence.

(c) Immunodiffusion

The results of the Ouchterlony immunodiffusion test are illustrated in Fig. 21.

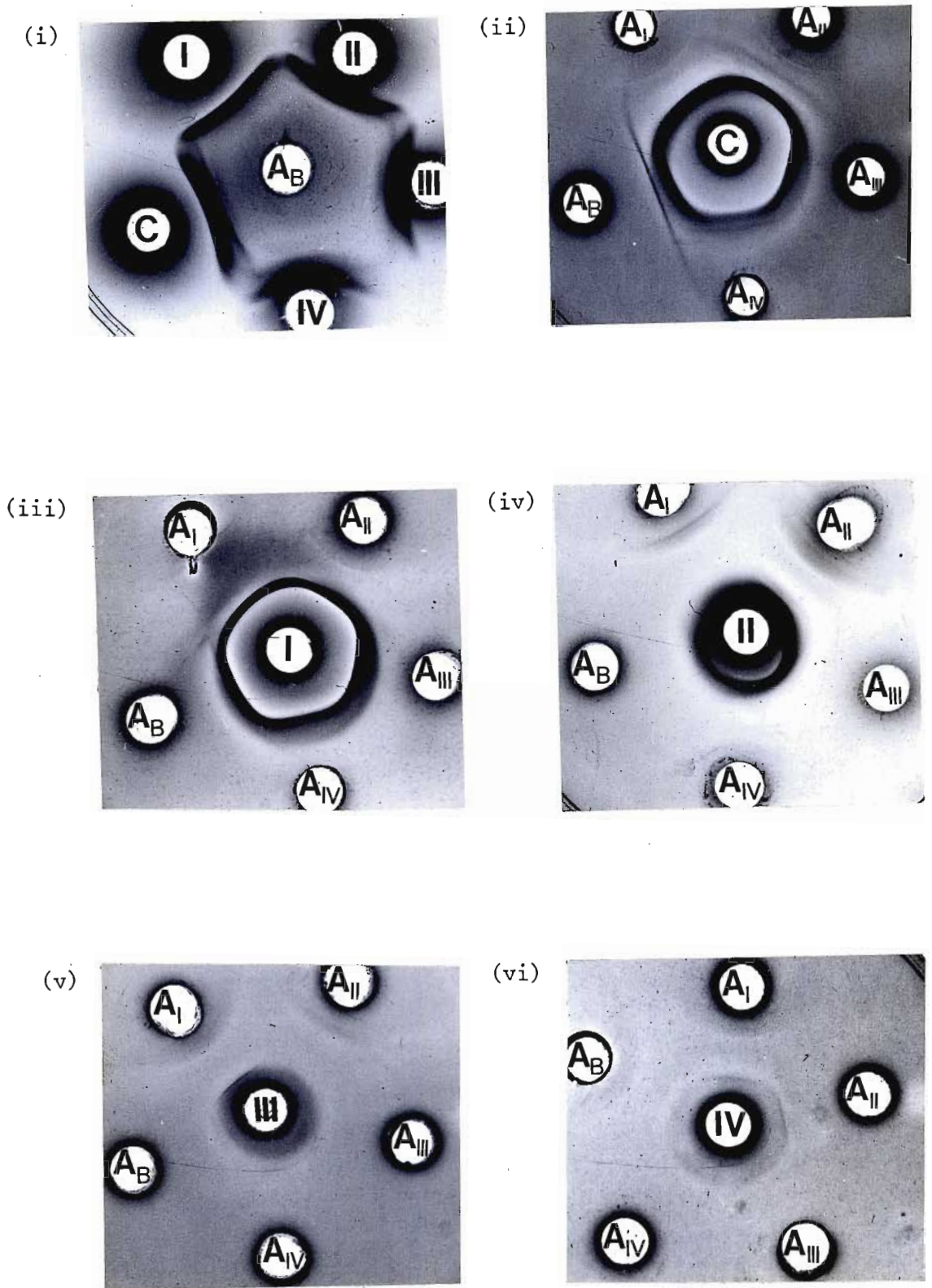


Fig. 21. Immunodiffusion tests of (i) membrane fractions I to IV and crude membranes (C) tested against  $A_B$ ; and antisera  $A_I$ ,  $A_{II}$ ,  $A_{III}$ ,  $A_{IV}$  and  $A_B$  tested against (ii) C, (iii) I, (iv) II, (v) III and (vi) IV.

From Fig. 21(i) it is clearly evident that immunologically active membrane fractions (C, I-IV), which react with  $A_B$  were obtained. The antigenic integrity of the bacterium is therefore maintained during the process of membrane isolation. In addition, the crude membrane (C) as well as the membrane fractions I-IV all reacted with the antisera produced against the individual membrane fractions ( $A_I$ - $A_{IV}$ ). Positive results were not obtained for the appropriate controls included in the tests.

#### E) MORPHOLOGY AND COMPOSITION

##### 1. Whole bacterium

##### (a) Electron microscopy

Electron micrographs of the intact bacterium, negatively stained for electron microscopy, revealed pleomorphic bacteria with mesosomes, septa and branching. The ends of the rod shaped bacteria were swollen (Fig. 22).

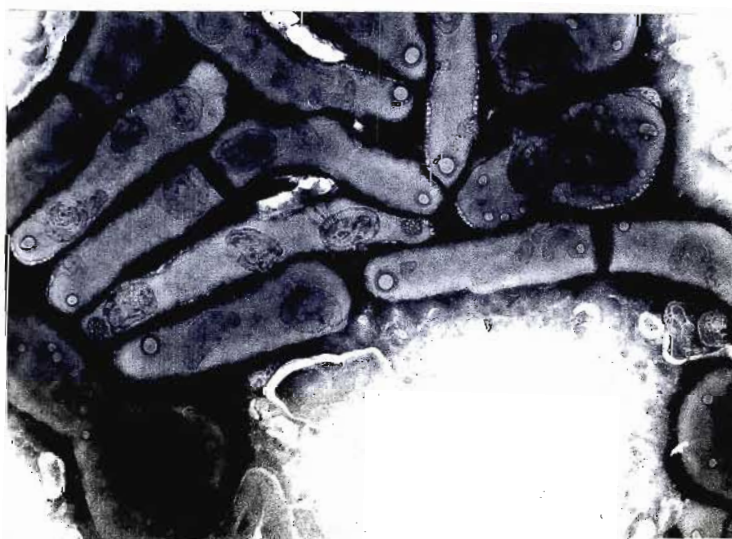


Fig. 22. Electron micrograph of negatively stained bacteria showing mesosomes, septa, branching and swollen ends (x 45000).



Thin sections of the bacterium showed the presence of a distinct cell wall and cytoplasmic membrane (Fig. 23).

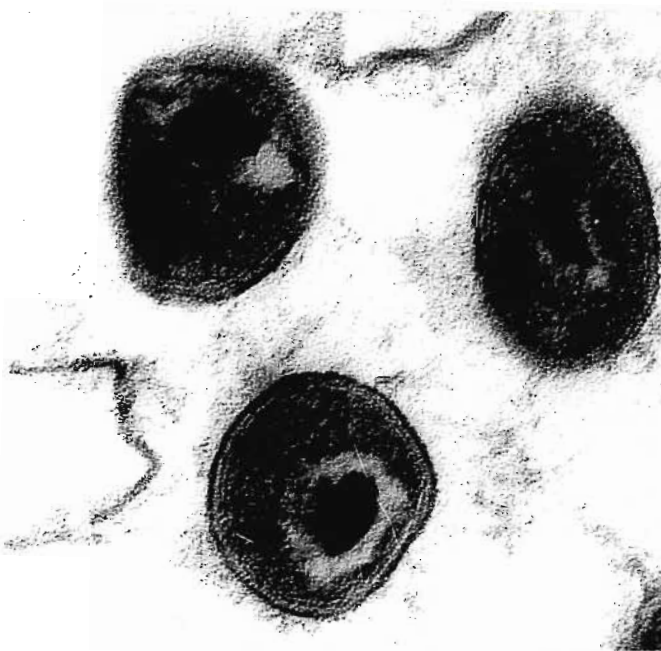
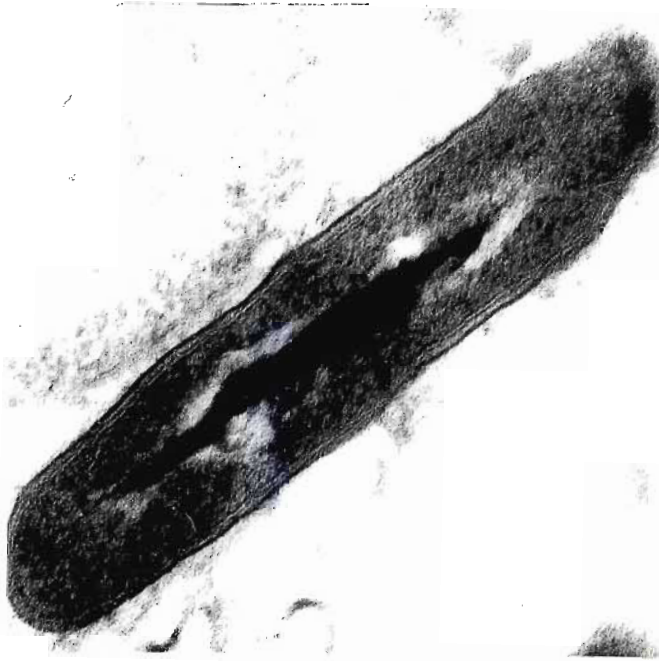


Fig. 23. Electron micrograph of thin sections of RSD-B showing typical double-layered membrane structures (x 88000).

## 2. Membranes

### (a) Electron microscopy

All membrane bands, negatively stained for electron microscopy, revealed morphologically similar vesicles (Fig. 24). Thus the electron micrograph for band I is shown only.

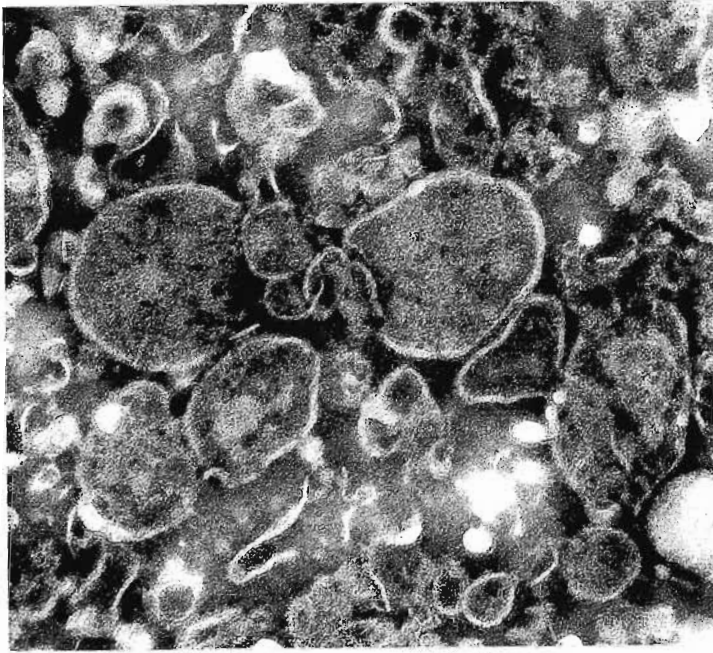


Fig. 24. Vesicles of membrane band I negatively stained for electron microscopy (x 65000).

Thin sections of the membranes revealed relatively pure membrane bands (Fig. 25).

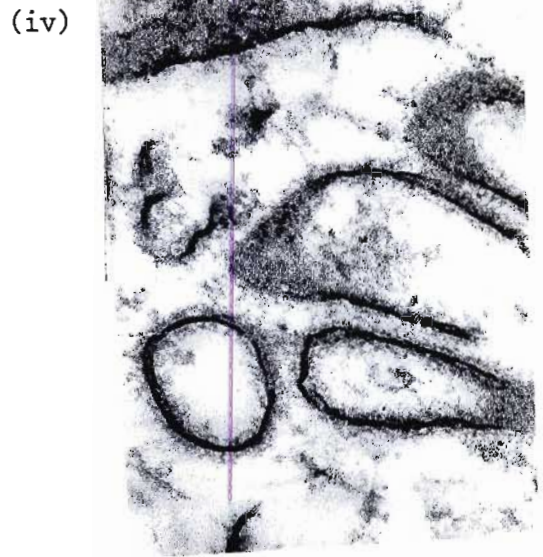
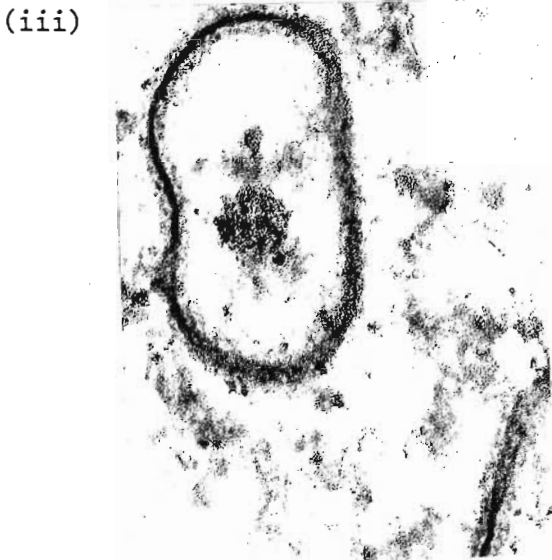
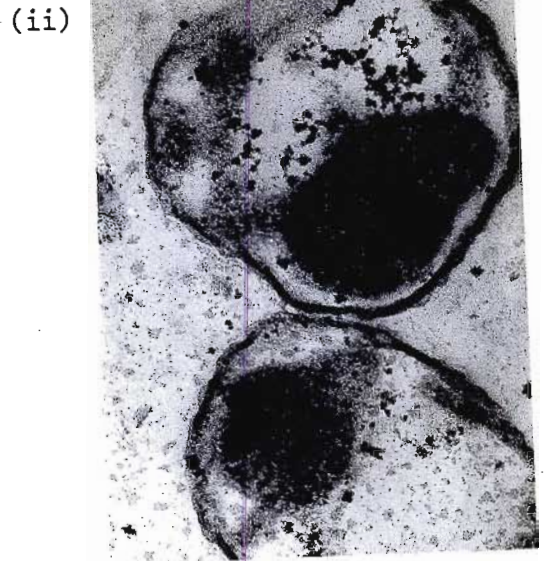
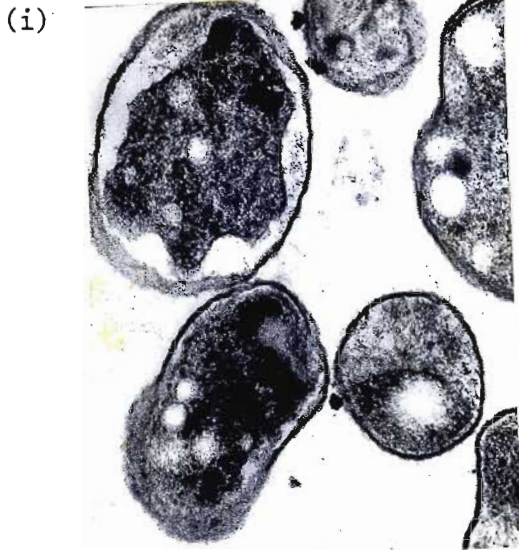


Fig. 25. Electron micrographs of thin sections of membrane bands (i) I (x 46700), (ii) II (x 81400), (iii) III (x 111600) and (iv) (x 75000).

(b) Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The protein components of crude membranes and sucrose density gradient bands I-IV are illustrated in Fig. 26. The protein composition appeared remarkably similar for all membrane bands. A component of 146 kilodaltons (K) was evident in all fractions but was found to be a major component in fraction IV and also in the crude membranes. All fractions contained a number of similar components in the 40 K to 60 K size range and a small component of 15,76 K.

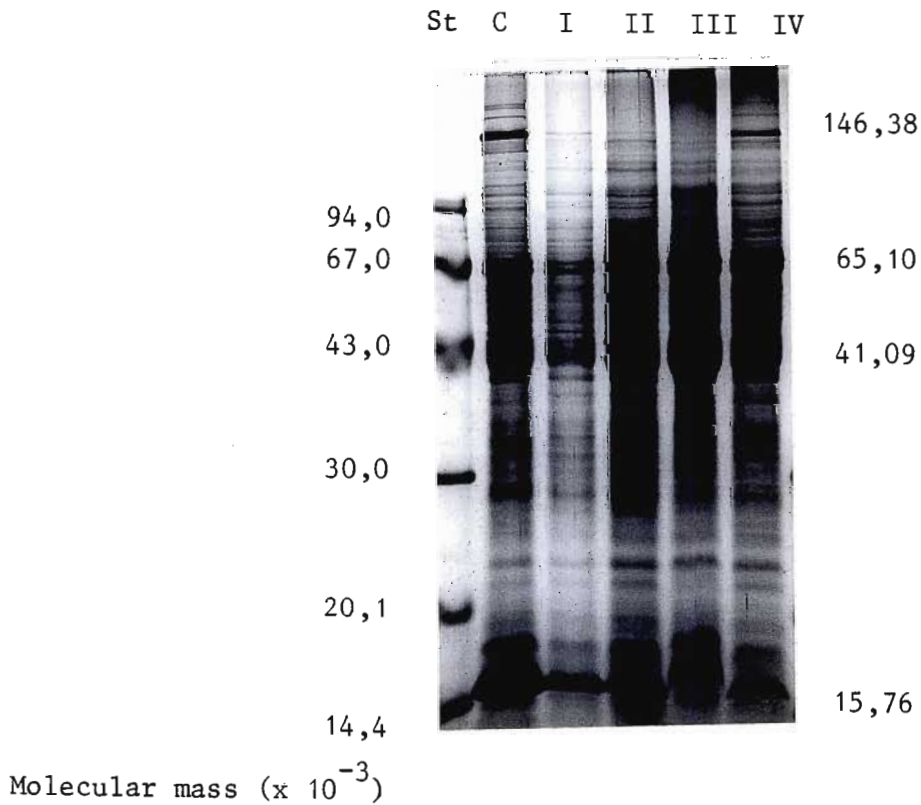


Fig. 26. Protein components of membrane bands I-IV, crude membrane (C) and protein standards (St).

## DISCUSSION

The severity of RSD and its economic implications require no further emphasis. Uncertainty governing the identity of the causal agent may be attributed to difficulty in identification owing to one or more of the following reasons:

- (i) Diagnosis is often difficult, either because symptoms are not clear or because RSD-like symptoms result from other causes;
- (ii) The long incubation periods in sugarcane makes research slow and tedious; and
- (iii) The inability to demonstrate readily the causal agent has probably led to a conceptual error that a virus is involved.

Reports since 1973 favour a bacterial etiology for RSD (Table 29). However, the exact taxonomic status of the bacterium remains unclarified.

The cultured, slender, pleomorphic, rod shaped bacteria containing septa and mesosomes that we observed were consistently present in the vascular fluids of RSD-infected sugarcane and correspond closely to the descriptions of the "RSD-associated" bacterium reported by various workers (Bailey, 1976; Chen *et al.*, 1975; Damann and Derrick, 1976; Gillaspie *et al.*, 1976a; 1981a; 1981b; Kaiser and Ramos, 1980; Liao and Chen, 1981a; Nayiager *et al.*, 1980; Ricaud *et al.*, 1976; Rishi and Nath, 1978). Morphological characteristics of our isolate in culture suggest their close relationship to the coryneform group of bacteria to which the RSD-associated bacterium has already been related (Table 29).

TABLE 29. Nature and dimensions of the organism associated with ratoon stunting disease of sugarcane

NATURE OF AGENT	DIMENSIONS (µm)	REFERENCE
Bacterium/rickettsia-like	not reported	Maramorosch <i>et al.</i> , 1973
Actinomycete	not reported	Kao and Damann, 1978; 1980
<i>Xanthomonas albinoans</i>	0,6 - 1,0 x 0,25-0,30	Tokeshi <i>et al.</i> , 1974
<i>Xanthomonas vasculorum</i>	1,0 - 1,5 x 0,4 -0,5	Liu <i>et al.</i> , 1974a; 1974b
Bacterium	1,3 x 0,25	Damann, 1975
Bacterium	1,0 - 3,5 x 0,25	Damann and Derrick, 1976
Bacterium	1,0 - 2,6 x 0,13-0,26	Ricaud <i>et al.</i> , 1976
Coryneform bacterium	1,2 - 3,0 x 0,12-0,30	Chen <i>et al.</i> , 1975
Coryneform bacterium	1 - 4 x 0,25-0,50	Gillaspie <i>et al.</i> , 1981a; 1981b
Coryneform bacterium	3 -10 x 0,3 -0,4	Gillaspie <i>et al.</i> , 1976a
Coryneform bacterium	5,0 -10,0 x 0,3 -0,5	Gillaspie <i>et al.</i> , 1973; 1974b Worley and Gillaspie, 1975
Coryneform bacterium	1,0 - 3,0 x 0,1 -0,3	Kaiser and Ramos, 1980
Coryneform bacterium	2 - 5 x 0,25-0,35	Liao and Chen, 1981a
Coryneform bacterium	1,43- 1,83 x 0,14-0,17	Liu, 1978
Coryneform bacterium	1,0 - 6,0 x 0,2 -0,6	Nayliger <i>et al.</i> , 1980
Coryneform bacterium	1,9 - 3,2 x 0,15-0,30	Rishi and Nath, 1978
Coryneform bacterium	1,0 - 2,5 x 0,15-0,32	Teakle <i>et al.</i> , 1973

Apparent size differences of the coryneform bacterium (Table 29) may be attributed to one or more of the following reasons. It is possible that extreme pleomorphism in length exists with this bacterium and that the growth conditions of the bacterium *in vivo* as well as *in vitro* may contribute to differences in size. Although the methods of preparing materials and the calibration of light and electron microscopes were not considered, they may contribute to discrepancies in size measurements. The possibility that differences could exist between organisms from different parts of the world should not be overlooked.

The problems connected with the diagnosis of RSD indicate that accurate diagnosis can be very difficult and necessitates a careful consideration of all factors involved (Ricaud, 1974). Although electron microscopy may serve a role in diagnosis, its degree of accuracy with respect to dimensions of the bacterium remains dubious. This may be attributed to the methods used for observing and measuring the RSD-associated bacterium. Although the method of electron microscopy employed by us may be used diagnostically, we make no claims that it indicates the true size of the RSD-associated bacterium.

Isolation of the causal bacterium (Davis *et al.*, 1980; Liao and Chen, 1981a; 1981b; Nayiager *et al.*, 1980) has made the production of relatively large quantities of bacteria possible. The successful production of an antiserum against the causal bacterium (Brlansky *et al.*, 1982; Gillaspie, 1978a; 1978b; Harris and Gillaspie, 1978; Oellermann *et al.*, 1981) has been the first step towards a serological approach to diagnosis of RSD. We also produced an antiserum to the bacterium and went a step further by isolating the bacterial membranes determining their protein content and producing an antiserum against each membrane fraction. Electron micrographs of membrane fractions that we prepared revealed relatively pure membrane preparations as shown in Figs. 24 and 25. No morphological differences could be observed among these membranes. Negatively stained electron microscopy of membranes cannot be used diagnostically for RSD-B. It was observed that thin sections of these membranes possessed a distinct double layered structure typical of membranes. Other ultrastructural details were not obvious. One of the limiting factors governing electron microscopy as a tool for diagnosis is the expensive equipment required and the need for

highly skilled technicians (Steindl and Teakle, 1974).

We found the immunological constitution of the causal bacterium to be important and that successful diagnosis could be achieved using an immunological approach. However, each immunological technique had its inherent advantages and disadvantages. Some methods were found to be advantageous over other methods in terms of specificity, speed, simplicity and sensitivity. A careful consideration of all factors governing a technique was necessary in order to successfully optimise the assay conditions.

Membrane proteins have been reported to be highly immunogenic (Schaad, 1979). The use of membranes in serological investigations should eventually show whether or not different strains of the coryneform bacterium causing RSD exist. Furthermore, the characterization of common antigens on isolates from the various sugarcane producing countries should facilitate the development of accurate, rapid and simple procedures for the early diagnosis of RSD. It is highly probable that these studies will eventually enhance investigations on the taxonomic status of this bacterium.

The need for a gentle method to lyse a bacterium is common to many areas of research in microbiology (Chassy and Giuffrida, 1980). According to reports published by Gillaspie (1978a) and Gillaspie *et al.* (1979) it was not possible to degrade the bacterium by sonication, pyrrolidine or enzyme treatments. No attempts were made to optimise buffer, lysozyme or SDS concentrations to achieve lysis. Neither was there any variation in incubation times nor temperatures. Following this Davis *et al.* (1980) published serological reactions of sugarcane and Bermuda grass isolates in gel double-diffusion tests using cells of isolates that had



been disrupted by passage through a French press. Details of this procedure remain unpublished. The method of membrane isolation for *Escherichia coli* used by Mizushima and Yamada (1975) was adapted for our bacterium. Lysis of the bacterium was easily achieved. Four immunologically cross reacting membrane bands (I-IV) bearing antigens found on the surface of the intact bacterium were reproducibly obtained.

The choice of buffers has probably been a major factor in determining the success of many attempted lysis experiments (Chassy and Giuffrida, 1980). We employed a dilute (10 mM) concentration of Tris buffer which has been reported to be superior to the buffer systems most commonly employed in published muramidase-based lysis techniques (Chassy and Giuffrida, 1980). Based on experience with Gram-negative organisms, we have included EDTA (2,5 mM) in our lysozyme incubation buffer to weaken the cell wall to a point where it became susceptible to mechanical damage by lysozyme. It has been observed that the absence of EDTA resulted in satisfactory yields of the four bands (Fig. 5ii). However, the presence of EDTA improved yields of the bands and was necessary for the effective action of lysozyme.

We found that increasing the concentration of lysozyme was not particularly effective in increasing the extent of lysis (Table 5; Fig. 6). However, the presence of lysozyme was found to be absolutely necessary for effective lysis. Isolation of membranes in the absence of lysozyme (Fig. 6i) was poor. Although increase in the amount of lysozyme did not proportionately increase the extent of lysis, lengthening the period of incubation improved lysis (Table 6; Fig. 7). In particular, the percentage recovery of band III increased. However, there was no change in the isolation profile and bands I to IV were always obtained. Caution should be exercised in exposure of cells to prolonged incuba-

tion at temperatures greater than 30C because intracellular degradative changes are possible (Chassy and Giuffrida, 1980).

Incubation of cells after EDTA and lysozyme addition at 30C, and after DNase, RNase and MgSO<sub>4</sub> addition at 37C (Fig. 8D), proved to be the optimum temperatures for successful isolation of membranes. Although other combinations of temperatures (Figs. 8A, 8B, 8C, 8E and 8F) indicate reasonable isolation patterns, the 30C-37C combination was best. It was observed that incubation in ice after EDTA and lysozyme addition (Fig. 8F) and the 37C-37C combinations (Fig. 8E) provided reasonable yields of the membrane bands. However, the soluble band was absent in both these combinations. Refrigeration of the crude membranes at 4C for 96 h did not alter the immunological integrity of the membranes. The isolation profile appeared normal (Fig. 9ii) and the percentage recovery of the fractions was good.

The modified Lowry procedure (Markwell *et al.*, 1978) of protein determination was used for the assay of membrane preparations. The application of the Lowry procedure (Lowry *et al.*, 1951), as routinely used for the assay of soluble proteins, to samples containing membranes or lipoprotein requires extensive pretreatment of the sample for solubilization before analysis. In addition, sucrose and EDTA, common components of media used to isolate membrane fractions, interfere with protein determination by the Lowry procedure. The method of Markwell *et al.* (1978) which was modified by the addition of SDS in the alkali reagent and an increase in the amount of copper tartrate reagent allowed the method to be used with membrane preparations without prior solubilization and with samples containing sucrose and EDTA.

SDS-PAGE was used for the compositional analysis of the membrane proteins. The resolving power of electrophoresis in polyacrylamide gels was found to be considerably superior to that achieved with any other support medium including agar (Anderton and Thorpe, 1980). The essential physical property which makes polyacrylamide the medium of choice was the limiting pore size of the gel. Its sieving effect on the movement of macromolecules and the reduction of diffusion together resulted in increased sharpness of bands.

The protein composition was almost similar for all fractions separated with a few minor differences (Fig. 26). Four main components were evident in all fractions, having molecular masses of  $146 \times 10^3$  d,  $65 \times 10^3$  d,  $41 \times 10^3$  d and  $15,70 \times 10^3$  d. The highest molecular mass component was found to be in high percentage in fraction IV. The significance of these results requires further investigation.

A number of factors may influence the qualitative results obtained in the compositional analysis of membrane proteins, viz.:

- (i) The method of isolating the membranes;
- (ii) The degree of cross linkage of the polyacrylamide;
- (iii) The duration of storage of the membrane preparation before disaggregation by SDS; proteolytic enzymes present in the preparations can hydrolyse the proteins during storage; and
- (iv) The temperature at which the membranes are treated with SDS solution to disaggregate them.

An obvious drawback is the unknown effects of SDS on antigenic determinants (Anderton and Thorpe, 1980). However, SDS-PAGE may provide a valuable tool for the production of monospecific antigens. The major individual components can be cut out from the gel and injected to raise "monospecific" antibodies.

The successful production of an antiserum was an important pre-requisite for our serological assays. The suspensions of whole bacteria injected produced antibodies against the mosaic of immunogenic determinants making up the bacterial cell surface. Thus the antiserum produced was not monospecific but rather it was specific for the multi-determinant RSD-B used in this study. In an attempt to raise a more specific antiserum we raised antibodies to the membrane fractions. A major advantage of using an antiserum of higher specificity is that crude extracts of cells or untreated cells can be used as test organisms in diagnosis of the disease.

It is difficult to make any direct comparisons between our results and the results of other workers (Davis *et al.*, 1980; Gillaspie, 1978a; Harris and Gillaspie, 1978) with respect to antiserum production because of the numerous variables involved. Our investigations indicated that an immunization schedule of 29 days followed by secondary bleedings 7 and 14 days after the final injection to be a satisfactory period in which antisera of reasonable specificity for both the intact bacterium as well as the membrane fractions were obtained. The bacteria were injected at a concentration of  $1 \times 10^9$  cells.ml<sup>-1</sup>. The agglutination titres as detected by phase-contrast microscopy were 1:850 for the intact bacterium and 1:1024 for membrane band I, 1:1000 for II and IV and 1:750 for III. The immunization schedule employed was decided upon after failure by D. Naraidu (1978, unpublished observations) to elicit an immune response within 14 days at an antigen concentration of  $1 \times 10^8$  cells.ml<sup>-1</sup>. From

our results and those of Harris and Gillaspie (1978) who used an immunization period of 23 days, it is clear that a prolonged immunization period of 3-5 months, as used by Gillaspie (1978a; 1978b), was unnecessary. The bacteria used by Gillaspie (1978a) were injected at a concentration of  $1,5 \times 10^8$  cells.ml<sup>-1</sup>. The low antigen concentration used by Gillaspie (1978a) may account for the low titres obtained and the necessity for the prolonged immunization period adopted by them. All workers (Gillaspie, 1978a; Harris and Gillaspie, 1978) found the use of an adjuvant necessary. We found that the titre of the antiserum obtained after the final bleed from rabbits injected with antigen plus adjuvant was 1:850 compared to 1:512 for rabbits injected with an antigen only.

Factors such as the species of animal injected and the presence of contaminating substances affect the specificity of an antiserum (Schaad, 1979). In all immunization programmes thus far reported for RSD-B rabbits were used (Davis *et al.*, 1980; Gillaspie, 1978a; Harris and Gillaspie, 1978; D. Naraidu, 1978 - unpublished observations; Oellermann *et al.*, 1981). The antigen that we used, compared to the antigen used by Gillaspie (1978a) and Harris and Gillaspie (1978), contained no host material since the antigen was a cultured bacterium. Consequently, there was no danger of causing anaphylactic shock and death as was evident in Gillaspie's (1978a) investigation. The use of one experimental animal per immunization treatment, as used by Gillaspie (1978a), is unscientific. This opinion is clearly underlined by the fact that in his investigation, one of the rabbits (the only one for a particular treatment) died after 121 days. We used 3 rabbits per immunization treatment.

The source of our bacterium was sugarcane whilst the source of the bacterium used by Gillaspie (1978a; 1978b) and Harris and Gillaspie (1978) was sudangrass and sorghum-sudangrass hybrid NB280S respectively.

In 1980 Davis *et al.* used an antiserum produced against the intact bacterium in gel double diffusion tests. No details of their immunization period, antigen concentration, routes of injection or antisera titres were published. From the above contrasting results recorded for the antisera produced against the intact bacterium it is clear that there is a need for some standardised procedures such as methods of preparing antigens for immunization, protein content of antigens injected, immunization periods, methods of injection, animals used and type of test to establish titres. Because serological tests are very sensitive, small changes in methods can result in significant changes in the results (Schaad, 1979). This makes it difficult to decide from published results whether or not a certain serological method can be used successfully for identification.

We found the microagglutination test to be a reasonably sensitive test for detection of the RSD-associated bacterium (Table 11). The titre obtained for the intact bacterium was reasonably high. However, the microagglutination test was not applicable to the diagnosis of RSD in raw sugarcane juice from diseased plants because of the low numbers of bacteria per millilitre. It is evident that a simple method for the selective concentration of bacteria from diseased cane needs to be formulated. Gillaspie *et al.* (1981a) found that in microagglutination tests the bacterium in culture was indistinguishable from the bacteria selectively concentrated from diseased plant extracts. Despite inherent setbacks of the microagglutination test, we found it to be simple, rapid and it saved on antigen and antibody. In addition, valuable information on serological relationships among bacteria from different sugar producing countries may be assessed. Using microagglutination tests it was reported that the RSD-associated bacterium found in Australia, Japan, South Africa and the United States of America

were serologically closely related (Gillaspie *et al.*, 1979) whereas a Mauritian isolate tested with the antiserum produced against the USA isolate was negative (Anon., 1979b).

Results of the microagglutination tests for the membrane bands produced were very encouraging (Table 27; Fig 19). The strength of agglutination was determined by the size of the clumps. As expected, the antiserum produced against each fraction agglutinated its fraction strongest. Cross reactions indicated that all fractions were related serologically. Thus each fraction may possess one or more common antigenic determinants. Fractions I and II agglutinated the antiserum produced against the intact bacterium more strongly than fractions III and IV. This might be indicative of the occurrence of more receptor sites in fractions I and II which consequently resulted in greater affinity of the fractions for the antiserum.

From a diagnostic viewpoint, we found the immunofluorescent technique to be rapid and specific for the RSD-associated bacterium and its constituent membranes (Figs. 12 and 20). All membrane bands were found to be immunologically related. The dull background fluorescence seen in some of the controls may be due to autofluorescence or non-specific staining which could easily be distinguished from the distinctive green fluorescence of the fluorescein-stained RSD-B and its membrane bands.

Success in application of the immunofluorescence method of staining depended greatly on the specificity of the antiserum produced (Harris and Gillaspie, 1978). This was clearly evident with the bacterial membrane bands. The degrees of immunofluorescence of the membranes which were tested against their own antiserum were found to be, in general, more intense than the immunofluorescence observed for the intact

bacterium. The intensity of immunofluorescence was stronger in bands I, II and IV than in band III.

The immunofluorescence staining technique cannot be considered as a quantitative method because of the uneven distribution of bacteria in the dry smear and the possibility of loss during rinses. Our antigen preparation, compared to the preparation used by Harris and Gillaspie (1978), was free of contaminants since we used a cultured RSD-B. Brlansky *et al.* (1982) found that tetramethylrhodamine isothiocyanate (TRITC) was superior to FITC as a fluorescent probe because there was less autofluorescence of the xylem vessels. We did not find the use of TRITC necessary since we did not apply the technique directly to diseased plants. However, the usefulness of this observation is not unnoticed. The TRITC procedure could be useful in direct detection of bacteria in the xylem vessels of affected plants. In addition, this technique could be very useful in locating bacteria in various areas of plants, screening tissue for the presence of bacteria prior to preparation for electron microscopy, and in following the movement of bacteria within a plant.

Proof of the serological relatedness of our membrane bands was strengthened by the appearance of precipitin lines in immunodiffusion tests. Membrane band I produced two precipitin arcs, bands II and III each produced three arcs whilst bands IV and the crude membrane fraction obtained before sucrose gradient fractionation each produced four arcs. The major precipitin arc was produced by all bands. The significance of this result warrants further investigation. Comparisons with the results of Gillaspie *et al.*, (1981a) and Davis *et al.* (1980) would be futile since details of their methods employed for obtaining membranes are lacking.



The use of membranes in immunodiffusion tests can be used to establish serological relationships among strains from other countries which are suspected of causing RSD. Serological methods, like immunodiffusion, provide a valuable probe for detecting serological relatedness which may not be evident morphologically or ultrastructurally.

Our results indicate that a successful ELISA test can be and has been developed for the RSD-associated bacterium. This is contrary to the report filed by Gillaspie and Harris (1979) that the RSD-ELISA test was unsatisfactory. They reported that neither alkaline phosphatase (ALP) nor HRPO was satisfactory and that modifications of incubation times and the use of agents to reduce nonspecific reactions yielded no improvements. In addition, they found that the number of "false positives" occurring in these tests with ALP or HRPO made the ELISA method especially undesirable for use in RSD screening. They suggest that the results of other workers (Claflin and Uyemoto, 1978; Weaver and Guthrie, 1978) attempting to detect plant pathogenic bacteria by ELISA have also met with similar problems. These workers reported low sensitivity, high background absorption and inconsistent results. However, the reports by most investigators working on plant pathogenic bacteria indicate that the ELISA technique can be used successfully (Cambra and Lopez, 1977; Kishinevsky and Bar-Joseph, 1978; Kishinevsky and Gurfel, 1980; Morley and Jones, 1980; Nomé *et al.*, 1980).

As with any type of immunoassay the theoretical basis of ELISA is simple but much work is needed to determine the optimum conditions for a successful routine diagnostic test (Voller, 1978). We found that a concentration of  $100 \mu\text{g}.\text{ml}^{-1}$  anti-RSD-B IgG provided an optimal coating of capture antibody (Tables 13 and 14). As the concentration of antibodies was increased, the P/N ratios also increased until a plateau was

reached between 64 to 125  $\mu\text{g.ml}^{-1}$  anti-RSD-B IgG. This could be due to the fact that all attachment sites for the antibody on the microtitre plate were occupied. Subsequently, the unbound antigen at the higher antibody concentrations was washed out. It should be noted that one of the biggest problems encountered in the ELISA test is the inconsistency of protein adsorption to the plastic of the microtitre trays. Preliminary experiments using Dynatech 129B plates indicated a coefficient of variation of approximately 11%. We used plates from the same production batch for all our investigations. Another drawback of ELISA is that the bound protein may also desorb (Conradie *et al.*, 1981). According to Conradie *et al.* (1981), if protein desorbs during the process of immunoassay it can compete with the solid phase for its binding partner (antigen or antibody) leading to a decrease in sensitivity of the assay.

We observed that a conjugate concentration of 1/500 was the optimum (Tables 15 and 16; Fig. 13). At the higher conjugate concentration (1/250), all available antibody sites were occupied. However, at a conjugate concentration of 1/500 optimal attachment of conjugate to antibody occurred. At the lower conjugate concentrations not all available sites were taken which resulted in lower absorbance values and subsequently lower P/N ratios. Thus the level of detectability at the higher antigen concentrations was lower at the conjugate concentrations of 1/1000, 1/1500 and 1/2000. The levels of detectability at the lower antigen concentrations for the different conjugate concentrations were the same.

According to Gillaspie and Harris (1978) only values twice the value of the control can be accepted as significant in ELISA tests (i.e.,  $P/N \geq 2$ ). The level of detectability of the cultured bacterium in our ELISA tests was between  $1 \times 10^4$  to  $1 \times 10^5$   $\text{cells.ml}^{-1}$  with P/N values ranging

from 1,8-4,8. Such levels of detectability makes the ELISA test an attractive qualitative and quantitative method compared to agglutination or immunofluorescence. According to Cambra and Lopez (1977) sensitivity should be the most important characteristic of any antibody titration method.

Determination of the best negative implicated tris saline. The absorbance recorded for this negative was low when the antigen diluent was tris saline and the conjugate diluent was 5% sheep plus rabbit neutral serum (Table 17; Fig. 14). Lower negative values consequently increased the P/N ratios which indicated an increased sensitivity of the assay. The combination of using sheep plus rabbit neutral serum negative in conjunction with neutral sheep serum as conjugate diluent, and sheep plus rabbit neutral serum antigen diluent was found to be least sensitive (Fig. 14). The reason for this is unknown. With reference to field application, controls in the ELISA system are intrinsically different from those in agglutination testing, since a negative control for each isolate is not required (Olsen *et al.*, 1983). A single positive and a single negative are required per plate allowing 94 isolates and both controls to be tested per plate per antiserum.

It was observed that overcoating of coated plates did not improve the sensitivity of the ELISA test and the additional time required did not warrant further investigation in this regard. However, the need for coating was essential (Tables 19 and 29; Fig. 15). The observation by Catt and Tregear (1967) that proteins bind tightly to plastic surfaces prompted us to attempt the assay on uncoated plates. Although the sensitivity of the assay on uncoated plates was the same at the lower antigen concentrations ( $1 \times 10^3$  to  $1 \times 10^5$  cells.ml<sup>-1</sup>) on coated and overcoated plates, sensitivity was poor at the higher antigen concentrations. It

is obvious that coating leads to a more effective solid phase by presenting a specific attachment site for the antigen. The limiting factor determining the sensitivity of most immunoassays is the affinity of the antibody for the antigen to be assayed (Conradie *et al.*, 1980).

The sensitivity of the RSD-ELISA test was affected by the washing procedure which included washing with TS, TST, and centrifugation followed by washing with TS. Since we were dealing with a bacterium, which is a relatively large particle compared to a virus, we attempted to enhance adsorption of the bacteria to the plastic microtitre plate by centrifugation. On coated plates (Tables 21 and 22; Fig. 16), the sensitivity of the assay improved when the plates were washed with TST as opposed to TS alone or centrifugation followed by washing with TST. However, the levels of sensitivity at the lower antigen concentrations were the same for TS and TST. On overcoated plates (Tables 23 and 24; Fig. 17) the sensitivity of the assay was the same for both plates washed with either TS or TST. The sensitivity of the assay on plates washed with TST was greater on coated plates than on overcoated plates. Tween 80, the detergent in the TST wash solution, may be responsible for contributing to an increased sensitivity of the ELISA test. It is possible that this detergent may have a mild denaturing effect on the coated antibody. It has been shown that partial denaturation of the coating antibody yields a more efficient solid phase (Conradie *et al.*, 1983). Consequently, this could contribute to an enhanced colour development in our ELISA test. Conditions such as pre-exposure of antibody to low pH, high temperatures and urea which reportedly perturb the structure of the antibody molecule (Conradie *et al.*, 1983) needs to be investigated for our bacterium. It is also possible that the inclusion of a detergent in the washing solution resulted in the removal of loosely bound antibodies and antigens resulting in specific colour reactions.

The reason for the decrease in absorbance at an antigen concentration of  $1 \times 10^3$  cells.ml<sup>-1</sup> in most investigations (Tables 15, 17, 19, 21 and 23) is not known. However, the consistency of this occurrence leaves cause for further investigation. Observations that a longer antibody coating period alleviated this decrease is interesting (Fig. 18). The longer antibody coating period also improved the sensitivity of the assay especially at the higher antigen concentrations.

What becomes clear is that, compared to microagglutination and immunofluorescence, the ELISA test is a rapid, sensitive and accurate qualitative and quantitative method that could easily be adopted for field application. Based on the level of detection, the ELISA test offers a method for detecting the presence of the RSD-associated bacterium in plants that do not yet show disease symptoms. Early detection of the disease has numerous positive economic advantages. The ELISA test could also be used to identify the pathogen in the tissues of alternate host species, making the test very useful for epidemiological studies of RSD. ELISA could also be used as a tool to map distribution of the pathogen within the host. Mapping such distribution has been very difficult with the older detection methods (Nomé *et al.*, 1980). In addition, ELISA could be used to identify specific strains while shortening the time of test (Olsen *et al.*, 1983).

Other attributes of the ELISA test include the following:

- (i) It is technically easy to perform as it employs simple incubation and wash protocols;
- (ii) They are primary binding assays and do not require the participation of secondary events (e.g., complement fixation, agglutination, etc.)

to obtain a readable result;

- (iii) The enzyme conjugates are easily prepared, are stable for prolonged periods (usually indefinitely) and are relatively inexpensive;
- (iv) The results can be read with simple, inexpensive colorimeters or spectrophotometers;
- (v) The use of a 96 well microtitre plate, because of its low cost, may well be applied to mass screening of RSD-B of sugarcane;
- (vi) The method can be easily automated to a considerable degree by means of existing dilutors, dispensers and automatic plate washing machines. By using such a system and the incorporation of a small desk-top computer, a single technician should be capable of assaying several hundred isolates per day (Conradie and Mbhele, 1980).

However, ELISA has the inherent disadvantage that accurate quantitation of enzyme activity requires precise control of buffer composition, pH, reaction time and temperature (Conradie, 1980). Because the enzyme label in the solid-phase immune complex can only be detected by its biological activity, an additional source of experimental error is introduced. Despite the inherent disadvantages associated with the ELISA test, the advantages of this test for detection of the RSD-associated bacterium is far greater compared to other serological methods.

The assays which we used for detection of the cultured bacterium needs to be evaluated for their field application. It is highly probable that immunoassays would eventually replace the conventional methods of diagnosis of RSD.

## SUMMARY

The production of relatively large quantities of bacteria associated with RSD of sugarcane was made possible by the successful isolation and growth of the bacterium. The cultured, slender, pleomorphic, rod shaped bacterium containing septa and mesosomes were consistently present in the vascular fluids of RSD infected sugarcane. Thin sections of the bacterium viewed electron microscopically revealed a distinct cell wall and cytoplasmic membrane.

Membranes were reproducibly isolated from the pelleted bacteria. Conditions for the isolation of membranes were optimised. Upon isopycnic ultracentrifugation in sucrose gradients four membrane bands (I-IV) with peak densities of 1,15, 1,18, 1,21 and 1,25 g.cm<sup>-3</sup> respectively were obtained. A peak of soluble (S) material was observed at the top of the gradient. Recentrifugation of these membrane bands on sucrose density gradients resulted in all fractions rebanding at their respective densities. Band II tended to produce small amounts of I, whilst bands III and IV produced small amounts of I and II. All membrane bands negatively stained for electron microscopy revealed pure membrane vesicles which appeared morphologically similar. Thin sections showed a distinct double layered structure typical of membranes.

The protein components of crude membranes and sucrose density gradient bands I-IV was determined by SDS-PAGE and was found to be remarkably similar for all bands with a few minor differences. All fractions contained a number of similar components in the 40 K to 60 K size range and a small component of 15,76 K. A component of 146 K which was present in all fractions was found to be a major component in fraction IV and in the crude membranes.

The successful production of antisera for the intact bacterium and its constituent membranes enabled serological analysis of the bacterium as well as the membrane bands. Immunological assays for the intact bacterium included microagglutination, immunofluorescence and ELISA. The microagglutination test was found to be a reasonably sensitive test for the detection of the cultured RSD-associated bacterium. It was simple, rapid and saved on antigen and antibody. However, the test was not applicable to the diagnosis of RSD in raw sugarcane juice from diseased plants because of the low numbers of bacteria per millilitre. The immunofluorescent test was found to be rapid and specific for the RSD-associated bacterium. The technique cannot be considered as a quantitative method because of the uneven distribution of bacteria in the dry smear and the possibility of loss during rinses. Compared to microagglutination and immunofluorescence, the ELISA test was found to be a rapid, sensitive and accurate qualitative and quantitative method that could easily be adopted for field application. Parameters governing the RSD-ELISA test were optimised. Based on the level of detection, the ELISA test offers a method for detecting the presence of the causal bacterium in plant materials that do not yet show disease symptoms. The numerous positive attributes of the ELISA test makes it the method of choice for diagnosis of RSD.

As indicated above, membrane bands I-IV were also used in microagglutination, immunofluorescence and immunodiffusion tests. Positive agglutination was recorded for all membrane bands tested with the antisera prepared against the intact bacterium and against the membrane fractions. All membrane fractions tested against their own antiserum exhibited specific immunofluorescence. Immunodiffusion tests revealed the presence of a major precipitin arc produced by all bands. In addition, membrane band I produced two precipitin arcs, bands II and III each



produced three arcs whilst bands IV and the crude membrane fraction obtained before sucrose gradient fractionation each produced four arcs.

This work contributed to knowledge on the morphology and composition of the RSD-associated bacterium and to the development of assays for diagnosis of the cultured causal organism.

REFERENCES

- ABBOT, E.V. 1953. The occurrence of a hitherto unrecognized growth retarding disease of sugarcane in Louisiana. Sugarcane Pathologists' Newsletter 31: 116-120.
- ABBOT, E.V. 1960. Relation of ratoon stunting disease to varietal yield decline in Louisiana. Proceedings of the International Society of Sugar Cane Technologists 10: 66-71.
- ABBOT, E.V. & N. ZUMMO. 1962. Effects of ratoon stunting disease on yields of some sugarcane varieties in Louisiana. Sugar Bulletin 40: 226-229.
- AHMED, H.U., M.A. KHAN & M.H. RAHMAN. 1979. Ratoon stunting disease - a new record in Bangladesh. Sugarcane Pathologists' Newsletter 22: 23-24.
- ANDERTON, B.H. & R.C. THORPE. 1980. New methods of analysing for antigens and glycoproteins in complex mixtures. Immunology Today 1: 122-127.
- ANONYMOUS. 1960. Ratoon stunting disease. South African Sugar Journal 44: 674-675.
- ANONYMOUS. 1968. Ratoon stunting disease - heat treatment and germination. Annual Report of the South African Sugar Association Experiment Station 1967-68: 37-40.

- ANONYMOUS. 1972. Report of the Standing Committee on sugarcane diseases. Proceedings of the International Society of Sugar Cane Technologists 14: 1655-1681.
- ANONYMOUS. 1975. Causative agent of RSD. Annual Report of the South African Sugar Association Experiment Station 1974-75: 56-60.
- ANONYMOUS. 1977. Sugarcane diseases in South Africa. Ratoon stunting disease. Bulletin of the South African Sugar Association Experiment Station 1: 29.
- ANONYMOUS. 1979a. Ratoon stunting disease. Annual Report of the Bureau of Sugar Experiment Stations, Queensland 79: 47-48.
- ANONYMOUS. 1979b. Ratoon stunting disease. Annual Report of the Mauritius Sugar Industry Research Institute 79: 44-46.
- ANONYMOUS. 1980a. Ratoon stunting disease. Annual Report of the South African Sugar Association Experiment Station 1979-80: 68-69.
- ANONYMOUS. 1980b. Sugarcane diseases in South Africa. Ratoon stunting disease. Bulletin of the South African Sugar Association Experiment Station 9: 5.
- ANONYMOUS. 1980c. How to control sugarcane diseases. Maharashtra Sugar 5: 55-57.
- ANTOINE, R. 1956. Cane diseases. 2. Ratoon stunting disease. Report of the Mauritius Sugar Industry Research Institute 3: 57-59.

- ANTOINE, R. 1958. A staining technique for detecting ratoon stunting disease of sugarcane. *Nature* 181: 276-277.
- ANTOINE, R. 1960. Note on the tetrazolium test for detecting ratoon stunting disease in sugarcane. *Proceedings of the International Society of Sugar Cane Technologists* 10: 1042-1045.
- ARTSCHWAGER, E. 1960. Sieve tube lignification in sugarcane and its significance in relation to ratoon stunting disease. *Proceedings of the International Society of Sugar Cane Technologists* 10: 1077-1082.
- BAILEY, R.A. 1976. Some observations on the bacterium associated with ratoon stunting disease of sugarcane. *Proceedings of the South African Sugar Technologists' Association Annual Congress* 50: 60-64.
- BAILEY, R.A. 1977. The systemic distribution and relative occurrence of bacteria in sugarcane varieties affected by ratoon stunting disease. *Proceedings of the South African Sugar Technologists' Association Annual Congress* 51: 1-2.
- BAILEY, R.A. 1979. The disease situation of the South African sugar industry. *South African Sugar Journal* 63: 509-513.
- BECHET, G.R. 1976. Ratoon stunting disease and rapid diagnostic techniques. *Proceedings of the South African Sugar Technologists' Association Annual Congress* 50: 65-68.

- BENDA, G.T.A. 1969. The upright: an experimental form of sugarcane. Sugarcane Pathologists' Newsletter 2: 34-37.
- BENDA, G.T.A. 1971. Wilting and death in the ratoon stunting disease of sudangrass hybrid uprights. Proceedings of the American Society of Sugar Cane Technologists 1: 39-47.
- BENDA, G.T.A. 1972. Hot water treatment for mosaic and ratoon stunting disease control. Proceedings of the American Society of Sugar Cane Technologists 2: 211-218.
- BENDA, G.T.A. 1974. Field planting of sugarcane hot-water treated for sugarcane mosaic and ratoon stunting disease. Proceedings of the American Society of Sugar Cane Technologists 4: 102.
- BENDA, G.T.A. 1975. Short hot-water treatment for the control of the ratoon stunting disease of sugarcane. Proceedings of the American Society of Sugar Cane Technologists 5: 119-122.
- BOURNE, B.A. 1963. Studies on the heat treatment for the control of ratoon stunting disease of sugarcane in Florida. Proceedings of the International Society of Sugar Cane Technologists 11: 776-787.
- BRLANSKY, R.H., R.F. LEE, L.W. TIMMER, D.E. PURCIFULL & B.C. RAJU. 1982. Immunofluorescent detection of xylem-limited bacteria *in situ*. Phytopathology 72: 1444-1448.
- CAMBRA, M. & M.M. LOPEZ. 1977. Titration of *Agrobacterium radiobacter* var *tumefaciens* antibodies by using enzyme-labelled anti-rabbit  $\gamma$ -globulins (ELISA indirect method). Proceedings of the International Conference on Plant Pathology and Bacteriology 4: 327-330.

- CATT, K. & G.W. TREGGAR. 1967. Solid-phase radioimmunoassay in antibody-coated tubes. *Science* 158: 1570-1571.
- CHASSY, B.M. & A. GIUFFRIDA. 1980. Method for the lysis of Gram-positive, asporogenous rods with lysozyme. *Applied and Environmental Microbiology* 39: 153-158.
- CHEN, C.T., M.J. CHEN & S.M. LEE. 1978. Studies on sugarcane ratoon stunting disease in Taiwan. 1. Phase contrast and electron microscopy of the small coryneform bacteria in the diseased sugarcane. *Report of the Taiwan Sugar Research Institute* 80: 13-20.
- CHEN, C.T., S.M. LEE & M.J. CHEN. 1975. Small coryneform bacteria in ratoon-stunted sugarcane. *Sugarcane Pathologists' Newsletter* 13/14: 6-8.
- CHU, H.T. & S.M. LEE. 1969. RSD control in Taiwan. *Proceedings of the International Society of Sugar Cane Technologists* 13: 1124-1130.
- CHU, H.T., K.C. LING & S.M. LEE. 1960. Ratoon stunting disease control in Taiwan. *Proceedings of the International Society of Sugar Cane Technologists* 10: 1072-1077.
- CIFUENTES, O.M., R.J. STEIB, B.J. COCHRAN & M.M. MAYEUX. 1975. The use of aerated steam for the control of ratoon stunting disease in Louisiana. *Proceedings of the American Society of Sugar Cane Technologists* 5: 127-129.
- CLAFLIN, L.E. & J.K. UYEMOTO. 1978. Serodiagnosis of *Corynebacterium sependonicum* by enzyme-linked immunosorbent assay. *Phytopathology News* 12: 156.

- CLARK, M.F. & A.N. ADAMS. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475-483.
- COCHRAN, B.J., M.M. MAYEUX & R.J. STEIB. 1975a. Development of an aerated steam system for the control of ratoon stunting disease in sugar cane. *Proceedings of the American Society of Sugar Cane Technologists* 5: 123-126.
- COCHRAN, B.J., M.M. MAYEUX, R.J. STEIB & O.M. CIFUENTES. 1975b. Development and use of an aerated steam system for the control of ratoon stunting disease in sugarcane. *Sugar y Azúcar* 70: 19-23.
- CONRADIE, J.D. 1980. Recent developments in blood transfusion. *South African Journal of Science* 76: 149-150.
- CONRADIE, J.D., M. GOVENDER & L. VISSER. 1983. ELISA solid phase: Partial denaturation of coating antibody yields a more efficient solid phase. *Journal of Immunological Methods* 59: 289-299.
- CONRADIE, J.D., R. GRAY & B.E.L. MBHELE. 1980. Serum alphafetoprotein determination by enzyme-linked immunosorbent assay. *South African Medical Journal* 58: 169-171.
- CONRADIE, J.D. & B.E.L. MBHELE. 1980. Quantitation of serum ferritin by enzyme-linked immunosorbent assay (ELISA). *South African Medical Journal* 57: 282-287.
- CONRADIE, J.D., B.J. VORSTER & R. KIRK. 1981. A simple and rapid method of washing and drying micro-titre plates used in ELISA. *Journal of Immunoassay* 2: 109-116.

- DAMANN, K.E., Jr. 1975. A possible causal organism of ratoon stunting disease. Proceedings of the American Society of Sugar Cane Technologists 5: 53-55.
- DAMANN, K.E. Jr. 1980. Attachment to glass of the bacterium associated with ratoon stunting disease of sugarcane. Phytopathology 70: 566.
- DAMANN, K.E. Jr. & K.S. DERRICK. 1975. Association of a distinctive bacterium with ratoon stunting disease of sugarcane. Proceedings of the American Phytopathological Society 2: 133.
- DAMANN, K.E. Jr. & K.S. DERRICK. 1976. Bacterium associated with ratoon stunting disease in Louisiana. Sugarcane Pathologists' Newsletter 15/16: 20-22.
- DAMANN, K.E. Jr., K.S. DERRICK, A.G. GILLASPIE, Jr., B.F. FONTENOT & J. KAO. 1977. Detection of the BSD-associated bacterium by serologically specific electron microscopy. Proceedings of the International Society of Sugar Cane Technologists 16: 433-437.
- DAMANN, K.E. Jr., R.J. STEIB & O. CIFUENTES. 1975. Occurrence of the bacterium associated with ratoon stunting disease in varieties of sugarcane. Proceedings of the American Phytopathological Society 2: 67.
- da SILVA, W.M. 1974. Hot water treatment of single buds for ratoon stunting disease control. Sugarcane Pathologists' Newsletter 11/12: 32.



- DAVIS, M.J., A.G. GILLASPIE, Jr., R.W. HARRIS & R.H. LAWSON. 1980. Ratoon stunting disease of sugarcane: Isolation of the causal bacterium. *Science* 210: 1365-1367.
- DAVIS, M.J., R.F. WHITCOMB & A.G. GILLASPIE, Jr. 1981. Fastidious bacteria of plant vascular tissue and invertebrates (including so-called rickettsia-like bacteria), p. 2172-2188. *In* M.P. Starr, H. Stolp, H.G. Truper, A. Balows & H.G. Schlegel (Eds.), *The Prokaryotes Volume II*. Springer Verlag, New York.
- DERRICK, K.S. & R.H. BRLANSKY. 1976. Assay for viruses and mycoplasmas using serologically specific electron microscopy. *Phytopathology* 66: 815-820.
- EARLY, M.P. 1975. Ratoon stunting disease of sugarcane in Kenya. *East African Agricultural and Forestry Journal* 41: 106-109.
- EDGERTON, C.W. 1958. Sugar and its diseases. *Second Annual Review, Louisiana State University Press* 51-53.
- EGAN, B.T. 1980. Methods used to improve the health status of plant sources during the 1970's. *Proceedings of the Australian Society of Sugar Cane Technologists'* 83-87.
- EL-BANNA, M.T., M.A. MOURSI & F. NOUR-EL-DIN. 1967. Studies on sugar cane ratoon stunting virus disease. A. Properties of the causal virus and control of the disease. *Agricultural Research Reviews* 45: 74-92.
- FARRAR, L.L. 1957. A chemical test for ratoon stunting disease of sugarcane. *Phytopathology* 47:10.

- FIFE, J.M. & I.E. STOKES. 1959. Amino acid changes identify ratoon stunting disease of sugarcane. *Sugar y Azúcar* 54: 27-28.
- FORBES, I.L. & K.C. LING. 1960. Particles associated with the ratoon stunting disease of sugarcane. *Sugar Journal* 23: 2.
- FOUCARD, T., H. BENNICH, S.G.O. JOHANSSON & U. LUNDKVIST. 1975. Human antibodies to bovine  $\gamma$ -globulin: Occurrence in immunological disorders and influence on allergy radio-immunoassays 48: 812-823.
- GILLASPIE, A.G. Jr. 1970. Evidence that ratoon stunting disease is caused by a virus and not mycoplasma. *Phytopathology* 60: 1448-1450.
- GILLASPIE, A.G. Jr. 1971a. Ratoon stunting disease virus: Problems in purification. *Proceedings of the American Society of Sugar Cane Technologists* 1: 52-56.
- GILLASPIE, A.G. Jr. 1971b. Ratoon stunting disease virus *in vitro*: Association with host material. *Sugarcane Pathologists' Newsletter* 7: 15.
- GILLASPIE, A.G. Jr. 1974. Current status of sugarcane viruses in the American Type Culture Collection. *Sugarcane Pathologists' Newsletter* 11/12: 14.
- GILLASPIE, A.G. Jr. 1978a. Ratoon stunting disease of sugarcane: Serology. *Phytopathology* 68: 529-532.
- GILLASPIE, A.G. Jr. 1978b. An antiserum to the ratoon stunting disease virus. *Sugarcane Pathologists' Newsletter* 20: 5-6.

GILLASPIE, A.G. Jr. & J.W. BLIZZARD. 1976. Some properties of the ratoon stunting agent of sugarcane. Sugarcane Pathologists' Newsletter 15/16: 34-36.

GILLASPIE, A.G. Jr., M.J. DAVIS, R.W. HARRIS & R.H. LAWSON. 1981a. Isolation and pathogenicity of the ratoon stunting disease bacterium. International Sugar Journal 83: 324-326.

GILLASPIE, A.G. Jr., M.J. DAVIS, R.W. HARRIS & R.H. LAWSON. 1981b. Cultivation of the causal agent of ratoon stunting disease of sugarcane. Sugarcane Pathologists' Newsletter 26: 6-7.

GILLASPIE, A.G. Jr., R.E. DAVIS & J.F. WORLEY. 1973. Diagnosis of ratoon stunting disease based on the presence of a specific micro-organism. Plant Disease Reporter 57: 987-990.

GILLASPIE, A.G. Jr., R.E. DAVIS & J.F. WORLEY. 1974a. A diagnostic method for ratoon stunting disease. Sugarcane Pathologists' Newsletter 11/12: 1.

GILLASPIE, A.G. Jr., R.E. DAVIS & J.F. WORLEY. 1974b. Nature of the ratoon stunting disease agent. Proceedings of the International Society of Sugar Cane Technologists 15: 218-224.

GILLASPIE, A.G. Jr., R.E. DAVIS & J.F. WORLEY. 1976a. Nature of the bacterium associated with ratoon stunting disease of sugarcane. Sugarcane Pathologists' Newsletter 15/16: 11-15.

- GILLASPIE, A.G. Jr., G. FLAX & H. KOIKE. 1976b. Relationship between numbers of diagnostic bacteria and injury by ratoon stunting disease in sugarcane. *Plant Disease Reporter* 60: 573-575.
- GILLASPIE, A.G. Jr. & R.W. HARRIS. 1978. Screening for resistance to ratoon stunting disease-injury: The enzyme-linked immunosorbent assay. *Sugar y Azúcar* 73: 42-43.
- GILLASPIE, A.G. Jr. & R.W. HARRIS. 1979. Limitations of ELISA for detection of the RSD-associated bacterium in sugarcane and sudan-grass. *Sugarcane Pathologists' Newsletter* 22: 25-28.
- GILLASPIE, A.G. Jr., R.W. HARRIS & D.S. TEAKLE. 1979. Serological comparison of RSD-associated bacteria from five sugarcane growing areas of the world. *Sugarcane Pathologists' Newsletter* 23: 23-24.
- GILLASPIE, A.G. Jr., J.E. IRVINE & R.L. STEERE. 1966. Ratoon stunting disease virus. Assay technique and partial purification. *Phytopathology* 56: 1426-1427.
- HARRIS, R.W. & A.G. GILLASPIE, Jr. 1978. Immunofluorescent diagnosis of ratoon stunting disease. *Plant Disease Reporter* 62: 193-196.
- HOARAU, M. 1969. Effects on cane of heat treatment for control of ratoon stunting disease of sugarcane. *Proceedings of the International Society of Sugar Cane Technologists* 13: 1112-1113.
- HUGHES, C.G. 1956. Disease investigations: Ratoon stunting disease. *Annual Reports of the Bureau of Sugar Experiment Stations, Queensland* 56: 81-84.

- HUGHES, C.G. 1960. Disease investigations: ratoon stunting disease. Annual Report of the Bureau of Sugar Experiment Stations, Queensland 60: 82-83.
- HUGHES, C.G. 1962. Disease investigations: ratoon stunting disease. Annual Report of the Bureau of Sugar Experiment Stations, Queensland 62: 66-67.
- HUGHES, C.G. 1964. Disease investigations: ratoon stunting disease. Annual Report of the Bureau of Sugar Experiment Stations, Queensland 64: 76-78.
- HUGHES, C.G. 1976. Diseases of sugarcane - a review. Pesticides Abstracts and News Summaries 24: 143-159.
- HUGHES, C.G. & D.R.L. STEINDL. 1955. Ratoon stunting disease of sugarcane. Technical Communication number 2, Bureau of Sugar Experiment Stations, Queensland 1-54.
- HUGHES, C.G. & D.R.L. STEINDL. 1956. Some further developments in the study of ratoon stunting disease in Queensland. Proceedings of the International Society of Sugar Cane Technologists 9: 1012-1022.
- HUGHES, C.G., D.R.L. STEINDL & B.T. EGAN. 1967. Disease investigations: ratoon stunting disease. Annual Report of the Bureau of Sugar Experiment Stations, Queensland 67: 58-59.
- HUGHES, C.G., D.R.L. STEINDL, O.W. STURGESS & B.T. EGAN. 1961. Disease investigations: ratoon stunting disease. Annual Report of the Bureau of Sugar Experiment Stations, Queensland 61: 73-74.

- IRVINE, J.E. 1976. Factors affecting the expression of juvenile symptoms of the ratoon stunting disease. Proceedings of the American Society of Sugar Cane Technologists 5: 109-113.
- KAISER, W.J. & A.H. RAMOS. 1980. Association of a bacterium-like organism with ratoon stunting disease of sugarcane in Kenya. Sugarcane Pathologists' Newsletter 24: 14-16.
- KAMIUNTEN, H. & S. WAKIMOTO. 1976. Coryneform bacterium found in the xylem of ratoon stunting diseased sugarcane. Annual Report of the Phytopathology Society, Japan 42: 500-503.
- KAO, J., E.W. BLAKENEY, M.A. GERENCSEK & K.E. DAMANN, Jr. 1980. Cell wall composition, % GC in the DNA and serotyping of the bacterium associated with ratoon stunting disease of sugarcane. Phytopathology 70: 568.
- KAO, J. & K.E. DAMANN, Jr. 1978. Microcolonies of the bacterium associated with ratoon stunting disease found in sugarcane xylem matrix. Phytopathology 68: 545-551.
- KAO, J. & K.E. DAMANN, Jr. 1980. *In situ* localization and morphology of the bacterium associated with ratoon stunting disease of sugarcane. Canadian Journal of Botany 58: 310-315.
- KHURANA, S.M.P. 1972. Causal organisms of ratoon stunting disease. Sugarcane Pathologists' Newsletter 8: 15.
- KING, N.J. 1953. The ratoon stunting disease problem. Cane Growers' Quarterly Bulletin 17: 10-13.

- KING, N.J. & D.R.L. STEINDL. 1953. The relationship between varietal yield deterioration and ratoon stunting disease. Proceedings of the International Society of Sugar Cane Technologists 8: 851-860.
- KISHINEVSKY, B. & M. BAR-JOSEPH. 1978. *Rhizobium* strain identification in *Arachis hypogaea* nodules by enzyme-linked immunosorbent assay (ELISA). Canadian Journal of Microbiology 24: 1537-1543.
- KISHINEVSKY, B. & D. GURFUL. 1980. Evaluation of enzyme-linked immunosorbent assay (ELISA) for serological identification of different *Rhizobium* strains. Journal of Applied Bacteriology 49: 517-526.
- KOIKE, H. 1974. Interaction between diseases on sugarcane: sugarcane mosaic and ratoon stunting disease. Proceedings of the International Society of Sugar Cane Technologists 15: 258-265.
- KOIKE, H. 1977. Disease as a factor influencing sugarcane yields in Louisiana during the last decade. Proceedings of the American Society of Sugar Cane Technologists 6: 178-181.
- KOIKE, H. 1980a. Ratoon stunting and mosaic diseases: Factors contributing to declining sugarcane yields in Louisiana. Sugar Bulletin 58: 12-14.
- KOIKE, H. 1980b. Yield effects of ratoon stunting and sugarcane mosaic diseases on some sugarcane varieties in Louisiana. Proceedings of the American Society of Sugar Cane Technologists 1: 47-51.

- LAEMMLI, U.K. & M. FAVRE. 1973. Maturation of the head of bacteriophage T4I. DNA packaging events. *Journal of Molecular Biology* 80: 575-599.
- LIAO, C.H. & T.A. CHEN. 1981a. Isolation, culture and pathogenicity to sudan grass of a corynebacterium associated with ratoon stunting of sugarcane and with Bermuda grass. *Phytopathology* 71: 1303-1306.
- LIAO, C.H. & T.A. CHEN. 1981b. Coryneform bacteria in ratoon stunted sugarcane and in white-leaf diseased Bermuda grass: Isolation, cultivation and pathogenic role. *Phytopathology* 71: 236.
- LIU, H.P. 1963. Studies on the ratoon stunting disease of sugarcane. Purification of a nucleoprotein associated with diseased cane. *Taiwan Sugar* 10: 9-11.
- LIU, H.P., S.M. LEE & W.S. TENG. 1963. Studies on the ratoon stunting disease of sugarcane. 1. Effect of one heat treatment on the control of RSD in seed-cane with various years of infection. *Report of Taiwan Sugar Experiment Station* 32: 131.
- LIU, L. 1978. Association of a small coryneform bacterium with RSD in Puerto Rico. *Sugarcane Pathologists' Newsletter* 20: 4.
- LIU, L., A. CORTÉS-MONLLOR, K. MARAMOROSCH, H. HIRUMI, J.E. PÉREZ & J. BIRD. 1974a. Bacteria associated with ratoon stunting disease of sugarcane in Puerto Rico. *Journal of Agriculture of the University of Puerto Rico* 58: 418-425.



- LIU, L., A. CORTÉS-MONLLOR, K. MARAMOROSCH, H. HIRUMI, J.E. PÉREZ & J. BIRD. 1974b. Isolation of an organism resembling *Xanthomonas vasculorum* from sugarcane affected by ratoon stunting disease. Proceedings of the International Society of Sugar Cane Technologists 15: 234-240.
- LIU, L., G. RAMÍREZ-OLIVERAS, J.L. SERAPIÓN & C.L. GONZÁLEZ-MOLINA. 1979. Further developments in the study of the ratoon stunting disease of sugarcane in Puerto Rico. Journal of Agriculture of the University of Puerto Rico 63: 146-161.
- LO, T.T. 1954. A survey of ratoon stunting disease of sugarcane in Taiwan. Taiwan Sugar Quarterly 5: 67.
- LÓPEZ-ROIA, J.H. & J. ANDUSAR. 1970. Effect of stunting disease on yield of some sugarcane varieties in Puerto Rico. Journal of Agriculture of the University of Puerto Rico 54: 149-160.
- LOWRY, O.H., N.J. ROSEBROUGH, A.L. FARR & R.J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-275.
- MARAMOROSCH, K., B. PLASVIC-BANJAC, J. BIRD & L.J. LIU. 1973. Electron microscopy of ratoon stunted sugarcane: Microorganisms in xylem. Phytopathologische Zeitschrift 17: 270-273.
- MARKWELL, S.M.H., L.L. BIEBER & N.Z. TOLBERT. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Analytical Biochemistry 87: 206-210.

- MARTIN, J.P. & R.K. CONANT. 1939. Disease control and stimulation of cane cuttings by the hot-water treatment. Hawaiian Plant Record 43: 277-285.
- MATSUOKA, S. 1971. Elephant-grass, an indicator plant for ratoon stunting virus of sugar cane. Plant Protection Bulletin. Food and Agricultural Organization 19: 110-115.
- MATSUOKA, S. 1972. Ratoon stunting disease diagnosis with elephant-grass as an indicator plant. Sugarcane Pathologists' Newsletter 8: 10-11.
- MAYEUX, M.M., B.J. COCHRAN & R.J. STEIB. 1979. An aerated steam system for controlling ratoon stunting disease. Transactions of the American Society of Agricultural Engineers 22: 653-656.
- MIZUSHIMA, S. & H. YAMADA. 1975. Isolation and characterization of two outer membrane preparations from *Escherichia coli*. Biochemica et Biophysica Acta 375: 44-53.
- MORLEY, S.J. & D.G. JONES. 1980. A note on the highly sensitive modified ELISA technique for *Rhizobium* strain identification. Journal of Applied Bacteriology 49: 103-107.
- MUNGOMERY, R.W. 1949. Ratoon stunting disease or Q28 disease. Annual Report of the Bureau of Sugar Experiment Stations, Queensland 49: 41-43.
- MUNGOMERY, R.W. 1953. Obstacles to be avoided in hot-water treating cane setts against ratoon stunting disease. Cane Growers' Quarterly Bulletin 17: 54-56.

- NAVARETTE, F.S. 1960. Ratoon stunting disease at San Cristobal. Proceedings of the International Society of Sugar Cane Technologists 10: 1083-1090.
- NAYIAGER, M.P., R.A. OELLERMANN & G. ROTH. 1980. The isolation, culture and morphology of the bacteria associated with ratoon stunting disease of sugarcane. Phytopathologische Zeitschrift 99: 273-281.
- NOME, S.F., B.C. RAJU, A.C. GOHEEN & D. DOCAMPO. 1980. Enzyme-linked immunosorbent assay for Pierce's disease bacteria in plant tissues. Phytopathology 70: 746-749.
- OELLERMANN, R.A., M.P. NAYIAGER & G. ROTH. 1981. Membranes from the bacterium associated with ratoon stunting disease of sugarcane. Proceedings of the South African Societies for Plant Pathology and Microbiology 19: 26.
- OELLERMANN, R.A. & D. PILLAY. 1983. Different methods for the detection of a coryneform bacterium. Proceedings of the South African Biochemical Society 7: 213.
- OLSEN, P.E., W.A. RICE, G.W. STEMKE & W.J. PAGE. 1983. Strain-specific serological techniques for the identification of *Rhizobium meliloti* in commercial alfalfa inoculants. Canadian Journal of Microbiology 29: 225-230.
- ORIAN, G. 1954. La maladie du rabougrissement de la canne á sucre. Revue Agricole et Sucriere de I'Ile Maurice 33: 275-284.

- PARISH, D.H. 1956. Cane diseases: 3. Chemical diagnosis of ratoon stunting disease. Report of the Mauritius Sugar Industry Research Institute 3: 49-51.
- PLASVIC-BANJAC, B. & K. MARAMOROSCH. 1972. Electron microscopy of the xylem of ratoon stunted sugarcane. Phytopathology 62: 498.
- POLLOCK, J.R. 1955. Hot water treatment against ratoon stunting disease. Cane Growers' Quarterly Bulletin, Queensland 19: 8-9.
- RICAUD, C. 1974. Problems in the diagnosis of ratoon stunting disease. Proceedings of the International Society of Sugar Cane Technologists 15: 241-247.
- RICAUD, C. & J.C. AUTREY. 1978. Branched forms of the organism associated with ratoon stunting disease. Sugarcane Pathologists' Newsletter 21: 19-21.
- RICAUD, C., S. SULLIVAN & J.C. AUTREY. 1975. Systemic infection of sugarcane by the bacterium associated with symptoms of ratoon stunting disease. Revue Agricole et Sucriere de I'Ile Maurice 55: 159-162.
- RICAUD, C., S. SULLIVAN & J.C. AUTREY. 1976. Presence of the RSD-associated bacterium in Mauritius. Sugarcane Pathologists' Newsletter 17: 37-39.
- RICAUD, C., S. SULLIVAN & J.C. AUTREY. 1977. Interception of the RSD-associated bacterium in quarantine. Sugarcane Pathologists' Newsletter 19: 19-20.

- RISHI, N. & P. NATH. 1978. Association of the coryneform bacterium with ratoon stunting disease of sugarcane in India. Sugarcane Pathologists' Newsletter 20: 9-10.
- ROBINSON, P.E. 1960. Discussion following paper by Steib, R.J. & S.J.P. Chilton: Control and rate of increase of ratoon stunting disease in hot air treated sugarcane in Louisiana. Proceedings of the International Society of Sugar Cane Technologists 10: 1061-1066.
- ROSSLER, L.A. 1974. The effects of ratoon stunting disease under different irrigation regimes. Proceedings of the International Society of Sugar Cane Technologists 15: 250-257.
- ROTH, G. & C. WHITEHEAD. 1965. Virus diseases and sugarcane. South African Sugar Journal 49: 675-683.
- SANTOS, M. 1972. Sugarcane diseases in Mozambique. Sugarcane Pathologists' Newsletter 8: 15.
- SCHAAD, W.W. 1979. Serological identification of plant pathogenic bacteria. Annual Review of Phytopathology 17: 123-147.
- SCHEXNAYDER, C.A. 1956a. The effect of stunting disease of sugarcane on yields of cane and sugar in Louisiana, and the use of heat treatment for control. Sugar Bulletin 34: 1-5.
- SCHEXNAYDER, C.A. 1956b. The ratoon stunting disease of sugarcane in Louisiana with notes on its control. Proceedings of the International Society of Sugar Cane Technologists 9: 1058-1065.

SCHEXNAYDER, C.A. 1960. The use of a sugarcane "test plant" as a means of detecting the presence of ratoon stunting disease in sugarcane. Proceedings of the International Society of Sugar Cane Technologists 10: 1068-1074.

SHUKLA, U.S., R.S. RAM & R.C. TRIPATHI. 1974. Effect of moist hot air treatment on the control of GSD and RSD. Annual Report of the Indian Institute of Sugarcane Research 72-74.

SHUKLA, U.S., K. SINGH, V.K. MADAN & Y.R. SAXENA. 1979. Effect of moist hot air treatment on germination and invertase activity in RSD-affected sugarcane buds. Sugarcane Pathologists' Newsletter 23: 21-22.

SINGH, G.R. 1969. An indicator sugarcane variety for ratoon stunting disease. Current Science 38: 221-222.

STEIB, R.J. 1972. Results of studies relating to the mosaic-RSD disease complex of sugarcane in Louisiana. Sugarcane Pathologists' Newsletter 8: 8-9.

STEIB, R.J. 1974. Aerated steam treatment of seed cane to control sugar cane diseases. Proceedings of the American Society of Sugar Cane Technologists 4: 87-88.

STEIB, R.J. & S.J.P. CHILTON. 1956. Recent studies conducted on the ratoon stunting disease of sugarcane in Louisiana. Sugar Bulletin 34: 238-243.

STEIB, R.J. & S.J.P. CHILTON. 1957. Interrelationship studies of mosaic and ratoon stunting disease of sugarcane in Louisiana. Proceedings of the International Society of Sugar Cane Technologists 12: 1061-1069.

STEIB, R.J. & S.J.P. CHILTON. 1960. Control and rate of increase of the ratoon stunting disease in hot-air treated sugarcane in Louisiana. Proceedings of the International Society of Sugar Cane Technologists 10: 1061-1066.

STEIB, R.J. & O.M. CIFUENTES. 1976. Control of RSD of sugarcane by use of aerated steam. Sugar Journal 39: 22-23.

STEIB, R.J., L.L. FARRAR, I.L. FORBES & S.J.P. CHILTON. 1956. Occurrence of ratoon stunting disease in Louisiana and its control by the use of hot air treatments. Sugar Bulletin 34: 22-33.

STEIB, R.J. & I.L. FORBES. 1957. Johnson grass and corn as carriers of the virus of ratoon stunting disease of sugarcane. Sugar Bulletin 35: 375-379.

STEIB, R.J. & I.L. FORBES. 1960. Effects of controlling the ratoon stunting disease on yields of present and former commercial varieties of sugarcane in Louisiana. Proceedings of the International Society of Sugar Cane Technologists 10: 1053-1060.

STEIB, R.J., J.P. HOLLIS & S.J.P. CHILTON. 1967. Effects of treating the soil with bromoethane on yield of sugarcane infected with ratoon stunting virus. Proceedings of the International Society of Sugar Cane Technologists 12: 1078-1088.

- STEIB, R.J. & D.M. TANTERA. 1970. Studies to determine the effect of tetracycline antibiotic on the ratoon stunting disease (RSD) of sugarcane. Sugar Bulletin 32: 217-219.
- STEINDL, D.R.L. 1949. Q28 disease. Cane Growers' Quarterly Bulletin 12: 191-193.
- STEINDL, D.R.L. 1950. Ratoon stunting disease. Proceedings of the International Society of Sugar Cane Technologists 7: 457-465.
- STEINDL, D.R.L. 1957. Host range of the ratoon stunting disease virus. Journal of the Australian Institute of Agricultural Science 23: 238.
- STEINDL, D.R.L. 1961. Ratoon stunting disease. p. 433-453. In J.P. Martin, E.V. Abbot & C.G. Hughes (Eds.), Sugarcane diseases of the world, Volume 1. Elsevier Publishing Company, Amsterdam.
- STEINDL, D.R.L. 1974. Ratoon stunting disease: History, distribution and control. Proceedings of the International Society of Sugar Cane Technologists 15: 210-212.
- STEINDL, D.R.L. 1976. The use of phase-contrast microscopy in the identification of ratoon stunting disease. Proceedings of the Queensland Society of Sugar Cane Technologists 43: 71-72.
- STEINDL, D.R.L. & C.G. HUGHES. 1953. Ratoon stunting disease. Cane Growers' Quarterly Bulletin 16: 79-95.



- STEINDL, D.R.L. & D.S. TEAKLE. 1974. Recent developments in the identification of ratoon stunting disease. Proceedings of the Queensland Society of Sugar Cane Technologists 41: 101-104.
- TANTERA, D.M. & R.J. STEIB. 1972. Temperatures and treatment durations with hot air for control of ratoon stunting disease in Louisiana. Proceedings of the International Society of Sugar Cane Technologists 14: 997-1008.
- TEAKLE, D.S. 1974. The causal agent of sugarcane ratoon stunting disease (RSD). Proceedings of the International Society of Sugar Cane Technologists 15: 225-233.
- TEAKLE, D.S., D. KONTZE & J.M. APPLETON. 1979. A note on the diagnosis of ratoon stunting disease of sugarcane by negative-stain electron microscopy of the associated bacterium. Journal of Applied Bacteriology 46: 279-284.
- TEAKLE, D.S., P.M. SMITH, A.C. HAYWARD & D.R.L. STEINDL. 1975a. Diagnosis of RSD by electron microscopy of sugar cane tissue diffusates. Proceedings of the Queensland Society of Sugar Cane Technologists 42: 115-116.
- TEAKLE, D.S., P.M. SMITH & D.R.L. STEINDL. 1973. Association of a small coryneform bacterium with ratoon stunting disease of sugarcane. Australian Journal of Agricultural Research 24: 869-874.
- TEAKLE, D.S., P.M. SMITH & D.R.L. STEINDL. 1975b. Ratoon stunting disease of sugarcane: Possible correlation of resistance with vascular anatomy. Phytopathology 65: 138-141.

- TEJADA, J. & K.E. DAMANN. 1978. Serologically specific attachment of the bacterium associated with ratoon stunting disease of sugarcane to plastic cover slips. *Phytopathology News* 12: 176.
- THOMSON, G.M. 1958. The diseases of sugar cane in Natal: ratoon stunting disease. *Bulletin of the South African Sugar Association Experiment Station* 9: 1-11.
- THOMSON, G.M. 1960a. Cane disease: ratoon stunting disease. *Annual Report of the South African Sugar Association Experiment Station 1959-60*: 34-38.
- THOMSON, G.M. 1960b. RSD and drought - a disastrous combination. *South African Sugar Journal* 52: 201-203.
- THOMSON, G.M. 1971. RSD in three commercial varieties. *Sugarcane Pathologists' Newsletter* 7: 16-17.
- TODD, E.H. 1956. Apparent resistance to ratoon stunting disease. *Proceedings of the International Society of Sugar Cane Technologists* 9: 1169-1173.
- TOKESHI, H., A. SANGUINO & F. AKIBA. 1974. *Xanthomonas albilineans* provavel agente causal de requeitismo da suqueira e escaldadura de cana de açúcar. *Brazil Aqucareiro* 84: 28-40.
- VOLLER, A. 1978. The enzyme linked immunosorbent assay (ELISA). *Dynasciences Corporation* 1-5.

VOLLER, A., A. BARTLETT, D.E. BIDWELL, M.F. CLARK & A.N. ADAMS. 1976.

The detection of viruses by enzyme-linked immunosorbent assay.

Journal of Virology 33: 165-167.

WEAVER, W.M. & J.M. GUTHRIE. 1978. Enzyme-linked immunospecific assay:

application to the detection of seedborne bacteria. Phytopathology

News 12: 156-157.

WEAVER, L., D.S. TEAKLE & A.C. HAYWARD. 1977. Ultrastructural studies

on the bacterium associated with the ratoon stunting disease of

sugarcane. Australian Journal of Agricultural Research 28:

843-852.

WEHLBURG, C. 1956. Ratoon stunting disease in Cuba. Sugar 51:

27-29.

WHITE, P.R. 1963. The cultivation of animal and plant cells, p. 228.

The Ronald Press Co., New York.

WIEHE, P.O. 1954. Diseases. 1. Ratoon stunting disease. Annual

Report of the Mauritius Sugar Industry Research Institute, Port

Louis, Mauritius 1: 47-48.

WISMER, C.A. 1971. A sugarcane clone apparently immune to RSD. Sugar-

cane Pathologists: Newsletter 6: 46.

WORLEY, J.F. & A.G. GILLASPIE Jr. 1975. Electron microscopy *in situ*

of the bacterium associated with RSD in sudangrass. Phytopathology

65: 287-295.