PHYSICOCHEMICAL AND IMMUNOLOGICAL STUDIES OF THERMALLY INDUCED CONFORMATIONAL CHANGES IN PROTEIN ANTIGENS OF *MYCOBACTERIUM BOVIS*, STRAIN BCG

by

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ABBREVIATIONS

BAI medium	Bureau of Animal Industries liquid medium.
BCG-CF	The non-dialysable materials of filtrates of cultures of *Mycobacterium bovis*, strain BCG 172.
BCG-CF100	A preparation of BCG-CF which has been dissolved in distilled water, heated at 100°C for 60 min, clarified and lyophilized.
BCG-CF100(BAI)	A preparation of BCG-CF which has been dissolved in BAI medium, heated at 100°C for 60 min, clarified and lyophilized.
BCG-CF(SDS)	A preparation of BCG-CF which has been dissolved at a concentration of 1 mg/ml in 1% (m/v) SDS and 0,1% 2-mercaptoethanol, dialysed against PBS and stored at -20°C.
DNA	Deoxyribonucleic acid.
DEAE	Diethylaminoethyl-
EDTA	Ethylenediaminetetraacetic acid, disodium salt.
IgG	Immunoglobulin G.
MHC	Major histocompatibility complex.
Micro-ELISA	Micro-enzyme linked immunosorbent assay.
ORD	Optical rotatory dispersion.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
SDS	Sodium dodecyl sulphate.
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.
Tris	Tris(hydroxymethyl)aminomethane.
UV	Ultraviolet.

ABSTRACT

In this study the conformational changes in heat stable and heat labile protein immunoprecipitin antigens of a typical mycobacterin, the culture filtrate of Mycobacterium bovis, strain BCG 172, during heating and cooling have been investigated and related to the more obvious manifestations of thermal denaturation which have been reported from studies of heated mycobacterins.

Filtrates of four week old BCG cultures grown on BAI (Bureau of Animal Industries) liquid medium were collected by membrane filtration, concentrated by pervaporation, dialysed against distilled water and lyophilized (BCG-CF). Analytical chemical assays, gel exclusion chromatography, polyacrylamide gel electrophoresis, immunoelectrophoresis and gel immunoprecipitation were used to analyse the composition of BCG-CF and to monitor the compositional changes which occurred during heating at 100°C and the factors which contributed to these changes. An interfacial (immunoprecipitin) ring test, a micro-enzyme linked immunosorbent (micro-ELISA) assay which was specifically developed for BCG-CF antigens, and tuberculin skin test assays in sensitized guinea pigs were used to quantitate changes in immunological properties which resulted from heating.

A combination of gel exclusion chromatography on Bio-Gel P-200, ion exchange chromatography on DEAE-cellulose, and polyacrylamide gel electrophoresis was used to fractionate BCG-CF to isolate protein immunoprecipitin antigens which were resistant to heating at 100°C for 60 min, and antigens which were inactivated under the same conditions. The conformational status of the heat stable and heat labile antigens before and after heating and the structural changes which occur during heating were studied by optical rotatory dispersion, ultraviolet absorbance difference spectroscopy and by micro-ELISA titrations.

The most obvious phenomenon associated with heating of BCG-CF (which consisted initially of 41% protein and 38% carbohydrate) was the

precipitation of protein from solution. The extent to which this occurred was dependent an a number of factors which included temperature, heating time, concentration of non-dialysable culture filtrate materials, the solvent composition and the pH of the medium. High molecular weight proteins aggregated and precipitated from solutions more readily than the lower molecular weight proteins thus confirming the various observations that the majority of proteins of heated mycobacterial culture filtrates are of low molecular weight.

Electrophoretic and immunoprecipitin studies confirmed that most BCG-CF proteins which remained in solution were denatured during heating at 100°C for 60 min. However, two immunoprecipitin protein antigens, designated EC4a and EC4b, which were resistant to this treatment were successfully isolated from unheated BCG-CF. They were shown to be single polypeptides with molecular weights of 8 700 and 12 200. Their amino acid compositions were similar, although they were antigenically distinct. Neither was represented in the United States-Japan reference system for mycobacterial antigens.

Three antigenic fractions (EC1a, EC1b and EC1c) which were inactivated by heating were also isolated from BCG-CF. Fraction EC1a contained two immunoprecipitin antigens each having a molecular weight of 54 000 and consisting of two polypeptide subunits, the molecular weights of which were 19 000, 35 000, 26 000 and 28 000.

One of the two proteins of EC1a represented antigen 10 of the reference immunoelectrophoretic system, while the other appeared to correspond with one of the unlabelled antigens described by Daniel et al. (1975). Fraction EC1b comprised a single polypeptide with a molecular weight of 40 000 and fraction EC1c contained at least five polypeptides with a range of molecular weights. Only one of these five antigens reacted with the reference immunoelectrophoretic antisera and this was identified as antigen 6.

Conformational studies showed that at elevated temperatures all the antigens investigated underwent characteristic denaturation changes which included the conversion of α-helical secondary structure via

the random coil form to β-structures. The heat sensitive fraction EC1a was irreversibly denatured by heating but retained some antigenic determinants capable of primary binding when cooled. The heat stable antigen EC4a renatured to a structure which was essentially correct but which contained some incorrectly folded regions, while the second heat stable antigen EC4b contained a population of molecules which was largely correctly folded and a population which was irreversibly denatured when cooled. It was concluded that the heat stability of the antigens EC4a and EC4b was dependent not on their secondary structures nor on the temperature at which denaturation occurred, but rather on the melting out during cooling of the β-structures which formed at high temperature.

The tuberculin skin test activity of none of the BCG-CF isolates was measurably affected by the conformational changes induced by heating. The micro-ELISA procedure which was used in this study, however, provided a sensitive measure of confirmational changes which occurred and it was concluded that this assay would also be of great benefit in studies of the specificty of crude to highly purified mycobacterial antigen preparations.

CHAPTER ONE

INTRODUCTION

1.1. DEFINITION AND DESCRIPTION

Mycobacterial culture filtrates have, since the discovery of the tuberculin reaction by Robert Koch in 1890, been widely used as skin test reagents for the diagnosis of tuberculosis and related infections. They are commonly prepared from cultures of mycobacteria grown aerobically on synthetic liquid media. Following suitable incubation periods the cultures are sterilized by heat treatment and the filtrates are harvested and processed. This processing may include concentration of the crude culture filtrates by boiling or by chemical precipitation procedures which, in addition, provide some degree of purification of active components. Tuberculin preparations which are concentrated by heat evaporation procedures are referred to as "Old Tuberculin". Those which are concentrated by chemical precipitation are referred to as "purified protein derivatives of tuberculin" (PPD).

Crude and partially purified skin test reagents have been prepared from a variety mycobacteria for the identification of specific infectious agents in epidemiological studies. The efficiency with which causative organisms may be differentiated is, however, limited by the broad specificity of the antigenic compositions of mycobacteria (Magnusson, 1961; Daniel, 1976). The emphasis of research has therefore shifted more recently to the isolation of purified, species specific antigens from unheated culture filtrates and from cell extracts of mycobacteria (Barksdale and Kim, 1977; Daniel and Janicki, 1978). In spite of some successes in this field, the use of purified antigens has been limited by the difficulties in isolating sufficient quantities of these materials (Daniel and Anderson, 1978).

With the proliferation of mycobacterial antigen preparations, a suitable nomenclature has become essential. The term "sensitin" (Magnusson, 1961) has been used to describe microbial products which are able to provoke delayed hypersensitivity skin responses in suitably sensitized individuals, but which are unable to specifically induce sensitization. Recently it has been suggested that the term "elicitin" would be more appropriate (Barksdale and Kim, 1977). Runyon (1976) has proposed a system according to which all microbial agents which elicit a delayed hypersensitivity skin reaction should be termed tuberculin-like agents or mycobacterins. The name of each mycobacterin may be derived from the specific name of the producing organism by addition of the suffix "in" to the name. Consequently, the name tuberculin refers to the products of growth and lysis of M. tuberculosis. Aviin refers to mycobacterins derived from M. avium, kansasiin to products from M. kansasii, and lepromin from M. leprae. Although the use of this system of nomenclature has not yet become common, its principles will be adhered to in this thesis.

1.2 COMPOSITION OF UNHEATED MYCOBACTERIAL CULTURE FILTRATES

Unheated mycobacterial culture filtrates are complex mixtures of proteins and polysaccharides which may contain cellular debris, lipids, nucleic acids, lipopolysaccharides, medium components, various denatured components and trace minerals, the composition and nature of which may vary according to the strain of the mycobacterium, the composition and pH of the medium and the incubation period (Magnusson and Bentzon, 1958; Kim et al., 1963; Magnusson et al., 1963; Castelnuovo et al., 1964; Turcotte and Des Ormeaux, 1972; Janicki et al., 1976). Significant variations in successive batches may occur even when prepared under identical conditions (Magnusson and Bentzon, 1958).

Proteins account for a varying proportion of the total solids of crude mycobacterial culture filtrates, from less than 10% to approximately 50% or greater (Augier et al., 1971; Turcotte and Des Ormeaux, 1972). Chemical fractionation procedures, various combinations of exclusion chromatography, ion exchange chromatography, affinity chromatography, iso-electric focussing, zone electrophoresis, polyacrylamide gel

electrophoresis and centrifugation have been used to fractionate mycobacterial culture filtrates. Many protein components have been isolated and these have varied in character from complex cellular enzymes to undefined polypeptides, denatured components and peptidoglycans (Baer and Chaparas, 1963; Chaparas and Baer, 1964a; 1964b; Yoneda and Fukui, 1965; Moulton et al., 1971; Nagai et al., 1974b; Laguerre and Turcotte, 1975; Daniel and Anderson, 1978; Matsumoto et al., 1979; Gupta and Landi, 1980; Payne and Daniel, 1980). Molecular weights have similarly been found to vary from less than 10 000 to 150 000 and greater (Yoneda and Fukui, 1965; Landi and Held, 1971; Nagai et al., 1974a; 1974b; Matsumoto et al., 1979).

The carbohydrate components consist principally of the sugars D-arabinose, D-glucose, and D-mannose in the form of glucans, mannans, arabinomannans, and arabinogalactans (Seibert, 1949; Azuma et al., 1968; Misaki et al., 1974; Daniel and Misaki, 1976).

Immunological studies of the antigenic compositions of mycobacterial culture filtrates have revealed that a number of antigens are widely shared by different mycobacteria but there are, in addition, groups of antigens and single antigens which are limited either to single species or to various groups of mycobacteria (Castelnuovo and Morellini, 1965; Turcotte, 1969; Chaparas, 1975; Kronvall et al., 1976; Daniel et al., 1979). The isolation of mycobacterial antigens has in the past been characterized by lack of correlation of results from one laboratory to another. The development of an immunoelectrophoretic reference system for mycobacterial antigens under the auspices of the United States-Japan Cooperative Medical Sciences Program (Janicki et al., 1971) has greatly improved the comparison of isolates from various laboratories. With this system eight anodic migrating protein antigens and three cathodic migrating polysaccharide antigens have been identified and catalogued with polyvalent reference goat antiserum specific for M. tuberculosis H37Rv culture filtrate antigen. Daniel et al. (1975) have extended this immunoelectrophoretic reference system to cell extract antigens of M. tuberculosis H37Rv where a similar number of immunoprecipitin arcs have been identified. Several authors have applied these reference systems successfully and have been able to correlate previously unrelated data (Daniel and Affronti, 1973; Chaparas and Hedrick, 1973; Chaparas, 1975; Daniel et al., 1979; Kolushky and Engibarov, 1980). Although the reference systems

for mycobacterial antigens have wide application, the immunoprecipitin lines defined in these systems often do not represent single antigens, but rather complex mixtures of antigens (Wright and Roberts, 1974).

1.3 BIOLOGICAL PROPERTIES OF MYCOBACTERINS

Many of the components of mycobacterins, when injected into animals will stimulate the production of antibodies and under appropriate conditions will elicit the development of delayed hypersensitivity to the specific antigen (Barksdale and Kim, 1977). In order to achieve efficient sensitization for tuberculin type delayed hypersensitivity, mycobacterial antigens must be injected with a suitable adjuvant such as certain mineral oils, various synthetic or mycobacterial lipids, or in association with live or heat killed mycobacteria (Green, 1946; Chaparas and Baer, 1966; Smith and Johnston, 1972; Daily and Hunter, 1977).

Inoculation of tuberculin into the skin of a primed individual results in the development of a characteristic skin lesion. This consists of an area of induration, with or without surrounding oedema and erythema, which appears within 6 - 12 hours, reaches a peak of intensity 24 - 72 hours after injection and slowly fades over a period which may extend over several days (American Thoracic Society, 1971; Freedman and Kongshavn, 1975). The gross morphological features of the reaction may vary somewhat from one species to another. Induration is the major feature of tuberculin reactions in humans whereas erythema is strongly evident in guinea pig skin reactions. Histological investigations have shown that induration is initially mediated by infiltration with polymorphonuclear leucocytes with a small but rapidly increasing number of mononuclear cells which transform to macrophages and histiocytes and tend to aggregate around blood vessels (Boughton and Spector, 1963; Schroff et al., 1980). The number of polymorphonuclear cells declines and significant numbers of lymphocytes appear at the reaction site. By 24 - 48 hours after inoculation infiltration around the vessels consists largely of macrophages and lymphocytes which slowly decrease in number over several days (Norton and Ziff, 1965).

These lymphocytes which are thymus-derived, or T lymphocytes, have been shown to be responsible for recognition and effector responses in delayed hypersensitivity reactions (Schwartz and Leskowitz, 1969; Seeger and Oppenheim, 1972). Following the injection of specific antigen, rapid clearance from the injection site and localization within macrophages occurs (Nossal and Ada, 1971; Landi and Held, 1974). Here antigen is processed and presented to T lymphocytes by a mechanism which requires cell contact between macrophage and lymphocyte and in which the immunogen resides predominantly on the surface of the macrophage (Rosenthal et al., 1976).

The nature of the antigenic signal to the T-lymphocyte and the nature of the lymphocyte receptor are presently incompletely understood. It has been shown that in addition to antigen, the antigen bearing macrophage must share a common histocompatibility antigen with the responding T lymphocyte (Rosenthal and Schevach, 1973). Several hypotheses have been proposed to explain this requirement and the first of these, the "altered self" hypothesis (Benacerraf, 1978) postulates that T lymphocytes bear a single surface receptor which is only capable of recognizing antigens in association with products of the major histocompatibility complex. According to this hypothesis the combination of antigen and histocompatability gene products gives rise to a new antigenic determinant which is specifically recognized by T lymphocytes. The second model which has been proposed is the "dual recognition hypothesis" according to which T lymphocytes have a receptor for the histocompatibility complex antigen carried by the macrophange and an independent receptor for the specific antigen (Rosenthal et al., 1976). A sophisticated extention of this model referred to as the "three-receptor, clonal expansion model" predicts that each T lymphocyte carries three types of receptor (Williamson, 1980). Two are non-antibody receptors, at least one of which is specific for a self-major histocompatibility complex (MHC) antigen. The second may also be a self-MHC antigen receptor or a non-self MHC species specific (alloantigen) receptor. The third receptor is proposed to be an antibody type receptor specific for the presented antigen. Proliferation and effector responses of the T lymphocyte are accordingly initiated by binding of the macrophage-presented antigen by the antibody receptor and simultaneous binding of

an MHC antigen of the stimulatory macrophage by one of the non-antibody receptors.

Effector functions of T lymphocytes consequent to antigen recognition may include cytotoxic activities, T cell helper functions and the production of lymphokines (Blanden et al., 1976; Epstein, 1976). These are non-antigen specific pharmacological agents which elicit responses from other lymphoidal cells and include the mitogenic factor which induces the proliferation of lymphocytes and the migration inhibition factor which inhibits the migration of macrophages from the site of antigenic activation (Rosenstreich and Oppenheim, 1976).

Protein fractions of mycobacterins are largely responsible for the elicitation of tuberculin delayed hypersensitivity reactions and account for the major proportion of the immunoprecipitin antigens of mycobacterial culture filtrates (Crowle, 1958; Dannenberg, 1968; Turcotte, 1975). The role of carbohydrates in tuberculin reactions has not been unequivocally defined but crude to highly purified polysaccharides and lipopolysaccharides have, in most cases, been incapable of sensitizing guinea pigs or of eliciting the tuberculin reaction in sensitized guinea pigs (Daniel and Hinz, 1974). On the other hand, some polysaccharides including arabinomannans and arabinogalactans, have been shown to be capable of eliciting antibody formation and of reacting specifically with antibody in immunoprecipitin, complement fixation and passive haemagglutination reactions (Seibert, 1949; Yamamura et al., 1968). Anaphylactic shock reactions have been reported following intravenous injection and Arthus reactions following intradermal injection (Seibert, 1949; Heilman and McFarland, 1966; Heilman, 1967; Azuma et al., 1968; Birnbaum and Affronti, 1968).

A linear model which has been proposed to explain specificity relationships of mycobacteria (Chaparas et al., 1970) provides some insight into the elicitin properties of mycobacterial cell extract and culture filtrate preparations. According to this model each species contains a complex of antigens of which the most predominant are present in sufficient concentration to produce full sensitization. Each of these antigens behaves independently in sensitization and elicitation of skin reactions but all contribute additively to the observed skin

reaction. Each species contains antigens which are unique to that species, antigens common to two or a limited number of species and antigens which are common to all mycobacteria. The complexity of the delayed hypersensitivity response to crude and partially purified mycobacterins is clearly illustrated by this model and the difficulties associated with the preparation of specific skin test reagents are evident.

1.4 EFFECT OF HEAT ON THE COMPONENTS OF MYCOBACTERIAL CULTURE FILTRATES

The heat sterilization process is the most drastic treatment to which a mycobacterin is subjected during preparation and may greatly modify its composition. There have, however, been relatively few investigations in which these changes have been detailed by the direct comparison of heated mycobacterial culture filtrates with their unheated forms. The early work of Seibert (1928) showed that precipitation of as much as 50 - 60% of the proteins of culture filtrates may accompany heating, but that both the precipitated materials and the proteins which remain in solution retain their tuberculin skin test reactivity. In later studies immunochemical procedures such as gel immunoprecipitin reactions and immunoelectrophoresis have been applied to the investigation of heat-induced changes. These investigations have shown that immunoprecipitin antigens of mycobacterins display different tolerances to heat (Castelnuovo and Morellini, 1965) and at high temperatures only a limited number remain active (Gussoni, 1962; Lind, 1965).

An indication of the changes which accompany heating may be inferred by comparing compositional investigations of various PPD preparations (which have been been heated) with work on unheated culture filtrates. From gel exclusion chromatography, ion exchange chromatography and polyacrylamide gel electrophoresis studies it is apparent that fewer protein components, many of which may be denatured, are detected in PPD (Affronti et al.,1965; Moulton et al., 1972; Glenchur et al., 1973) than in unheated culture filtrate preparations (Baer, 1965; Roszman et al., 1968; Affronti et al., 1971). The molecular weights of proteins of heated PPD preparations are generally low, in the region

of 10 000 (Green, 1946; Nagai et al., 1974a), although the presence of higher molecular weight proteins has been reported (Landi and Held, 1971; Glenchur et al., 1973).

Polysaccharides are apparently affected to a lesser extent than proteins by heating and many may remain in heated culture filtrates. Castelnuovo and Morellini (1965) found the major antigen of autoclaved mycobacterial culture filtrates to be a carbohydrate component. The polysaccharide antigens 1 and 2 of the reference immunoelectrophoretic system for mycobacterial antigens have been identified in heated PPD preparations (Daniel and Janicki, 1978).

1.5. CONFORMATION AND DENATURATION OF PROTEINS

The conformation,or three dimensional structure of a protein is described by the terms primary, secondary, tertiary and quaternary structure (Haschemeyer and Haschemeyer, 1973). The sequence of amino acids in the polypeptide chain is referred to as the primary structure. Secondary structure represents short range interactions between neighbouring amino acids in the polypeptide chain which result in regular hydrogen bonded structures. These include α-helical regions, parallel and anti-parallel β-pleated sheet structures and β-turns which are regions consisting of tetrapeptides where the direction of the polypeptide chain is reversed (Chou and Fasman, 1978). The relative proportions in which these secondary structures are present vary from one protein to another and are dependent on the amino acid sequences (Mathews, 1977).

The term tertiary structure embraces arrangements of all the atoms in space including disulphide bridges and positions of side chains so that all long range and short range interactions are considered (Chou and Fasman, 1978). Native globular proteins in which α-helical secondary structures predominate tend to assume lobular, tertiary folded structures in which side chains form a hydrophobic core with the polypeptide backbone wrapped around the outside (Mathews, 1977). This conformation is stabilized by the unfavourable thermodynamic free energy of interaction of the internalized hydrophobic side chains with water. Most

hydrophilic side chains face outwards in this arrangement and interaction of their polar groups with the solvent provides additional stabilization of the native structure (Tanford, 1970). A number of proteins have more extended structures with a large proportion of β-sheets which tend to remain internalized within the molecule and which are stabilized by hydrogen bonding. These include fibrous proteins such as silk fibroin and certain globular proteins such as carbonic anhydrase, immunoglobulins, pepsin and soybean trysin inhibitor (Mathews, 1977).

Interaction between individual polypeptide chains such as the subunit polypeptides of di- or multimeric proteins are defined by the term quaternary structure. These structures occur as a result of interchain disulphide bonds, hydrogen bonding or apolar interactions (Schulz and Schirmer, 1979).

The three dimensional spatial arrangements of native proteins are relatively stable over a wide range of conditions although small conformational alterations may occur in which the relative distances between various regions of the proteins fluctuate with small amplitudes (Gō 1975). When native proteins are heated, subjected to extremes of pH or treated with chemical denaturants, major denaturation transitions take place with disruption of organized structure and the conversion to a random coil form (Tanford, 1968). Such transitions are considered to be cooperative reactions of an all or none character and may often be reversible when the denaturing condition is removed (Privalov, 1979). In many instances, particularly at high temperature, the formation of β-pleated sheet structure from the denatured random coil form results in irreversible aggregation and precipitation (Imahori, 1961). From studies of the synthetic polypeptide poly-L-lysine a mechanism for the heat induced α-helix to β-structure transformation has been suggested (Fasman, 1967). Accordingly, mild heating to 40°C unravels or allows breaks to occur in the β-helical structure of the polypeptide. Heating is thought to provide the activation energy necessary to break the hydrogen bonds which stabilize the helical structure and initiate the reaction. On cooling the process is reversible. However, if further heating occurs, the enthalpy necessary to remove hydrocarbon side chains of the amino acids of randomized regions of the polypeptide from water is provided. The subsequent hydrophobic association of these

side chains results in the formation of foci of β-structure which grow and disrupt any remaining α-helical regions. The resultant β-structure is very stable being the conformation with the lowest free energy and is therefore not readily reversible.

The irreversibility of the denaturation of complex proteins at high temperatures may be compounded by the rupture of disulphide bonds and the subsequent incorrect reformation, or scrambling, of these links on cooling (Brandt and Andersson, 1975). Covalent bonds other than disulphide bridges may also contribute to the formation of stable aggregated structures (Tanford, 1968).

1.6 SCOPE OF THE THESIS

Much of the variability in composition, potency and specificity of mycobacterins may be attributed to the heat sterilization procedures to which they are subjected during preparation. Recent research on mycobacterins has usually involved the study of either heated or non-heated culture filtrates, providing only an indirect indication of the effect of heating. Where unheated culture filtrates have been used as starting materials for heating studies, the emphasis has been confined to general chemical changes, and to changes in immunoprecipitin, immunoelectrophoretic and skin test reactivities. As yet there has been no attempt to relate these more obvious changes (or lack of changes) to the conformational status of the molecule by more sophisticated procedures such as optical rotatory dispersion, ultraviolet difference spectroscopy and highly sensitive immunoassay methods which detect primary antigen-antibody interactions. Recently the isolation and characterization of tuberculin skin reactive proteins from autoclaved culture filtrates (Nagai et al., 1974b) and autoclaved mycobacterial cells (Kuwabara, 1975a) has been described. Although the amino acid sequence of one of these proteins has been determined (Kuwabara, 1975b), little is known of the native conformations of such proteins and to what extent conformational changes may have occurred during the heating procedures which preceed their isolation.

The objective of this study was thus to isolate from a mycobacterial culture filtrate, protein components which are overtly "heat stable" or "heat labile", examine the changes in conformation that occur through heating and cooling and relate these changes to changes of the type studied by others.

This involved the preparation of a large batch of concentrated culture filtrate of Mycobacterium bovis, strain BCG; the examination of the effects on this product of heat and factors which influence the gross manifestations of denaturation during heating; the isolation and characterization of heat stable and heat labile protein components; and the study of the conformational status of these components during heating and cooling. A number of biophysical procedures including chromatographic, analytical and preparative scale polyacrylamide gel electrophoresis, centrifugation, optical rotatory dispersion and ultraviolet absorbance spectroscopy techniques were utilized. Immunological techniques included tuberculin skin tests, immunoprecipitation, immunoelectrophoretic procedures and the adaption of a highly sensitive micro-enzyme linked immunosorbent assay (micro-ELISA) for the detection of the primary binding of mycobacterial culture filtrate antigens by specific antibody.

CHAPTER TWO

MATERIALS AND METHODS

2.1 BUFFER SOLUTIONS

2.1.1 Tris-HCl buffers

A 0,5 M stock solution was prepared by dissolving 60,6 g of Tris-(hydroxymethyl)aminomethane in 700 ml of distilled water. The pH of the solution was adjusted to 7,0 by the addition of 1 M HCl and the volume was made up to 1000 ml with distilled water. This solution was used undiluted or diluted 1/10 or 1/50 with distilled water to yield 50 mM and 10 mM Tris-HCl buffers respectively.

2.1.2 Phosphate buffers

Phosphate buffered saline (PBS)

Phosphate buffered saline consisted of a solution of NaCl (8 g/l), KH_2PO_4 (0,2 g/l), Na_2HPO_4 (1,2 g/l), KCl (0,2 g/l) and NaN_3 (0,2 g/l) in distilled water. The pH was checked and adjusted to 7,4.

0,1M Sodium phosphate buffer.

This was prepared as two stock solutions containing 0,1M $NaH_2PO_4H_2O$ (13,8 g/l) and 0,1 M Na_2HPO_4 (15,2 g/l). These two stock solutions were mixed in appropriate proportions to give a pH value of 6,8.

17,5 mM Sodium phosphate buffer

Two stock solutions of 17,5 mM Na_2HPO_4 (2,74 g/l) and 17,5 mM $NaH_2PO_4 \cdot H_2O$ (2,42 g/l) in distilled water were mixed appropriately to yield a pH of 6,3.

2.1.3 Buffered solutions used in immunological procedures.

Coating buffer

This buffer consisted of a 50 mM sodium carbonate-sodium bicarbonate solution of pH 9,6. It was prepared by dissolving 1,59 g Na_2CO_3 and 2,93 g $NaHCO_3$ in distilled water to give a volume of 1000 ml and 0,2 g of NaN_3 was added as a preservative.

Post-coating buffer

Post-coating buffer consisted of a solution of 0,5 M glycine and 3% (m/v) bovine serum albumin in coating buffer.

PBS-tween

This buffer contained 0,05% (v/v) Tween 20 (Polyoxyethylene sorbitan monolaurate, Merck) in PBS.

Micro-ELISA diluent

Micro-ELISA diluent was prepared by dissolving 3,8 g of glycine (0,5 M) and 3 g of bovine serum albumin in 100 ml of PBS-Tween.

Skin test diluent

This solution which was used for diluting antigens for tuberculin skin tests contained 0,005% (v/v) Tween 80 (Polyoxyethylene sorbitan monooleate, Merck) in 50 mM Tris-HCl buffer.

2.2 BACTERIOLOGICAL MEDIA

Several bacteriological media were used in the preparation of the mycobacterial culture filtrate material in this investigation. The first of these was Middlebrook 7H10 agar which was prepared from Bacto Middlebrook 7H10 agar base (Difco, U.S.A.) This was dissolved in distilled water (19 g/l) at 96°C and autoclaved at

120°C for 15 min. On cooling to 50°C sterile Bacto OADC enrichment medium (Difco, U.S.A.) was added to the base (100 ml/l) and agar slopes were prepared in sterile McCartney bottles.

Two liquid culture media were used. These included the Bureau of Animal Industries (BAI) medium (Green, 1946), and Sauton medium (Magnusson et al., 1963) the compositions of which are given in Appendix 1. These media were sterilized by filtration, dispensed in 500 ml volumes into sterilized Roux flasks and pre-incubated at 37°C for 14 days to ensure sterility before use.

2.3 LYOPHILIZATION

Mycobacterial culture filtrates, fractions and isolates were stored as lyophilized powders. Solutions of these products were dispensed into glass vials, frozen at -70°C and lyophilized in an Edwards EF6 freeze drier. A primary drying cycle of at least 24 hours was followed by a secondary drying cycle over phosphorous pentoxide. The vacuum obtained by this procedure ranged from 2 - 4 Pa. Vials were sealed with butyl rubber stoppers which were capped with aluminium overseals after the vacuum had been broken with dry, pure Nitrogen.

2.4 PREPARATION OF BCG CULTURE FILTRATE (BCG-CF)

Mycobacterium bovis, strain BCG 172 was obtained from the seedlot prepared on the 30th October 1960 by the Japanese BCG laboratory. The contents of an ampoule of the lyophilized seedlot were suspended in 1 ml of sterile Sauton medium and inoculated onto Middlebrook 7H10 agar slopes. Following incubation at 37°C for seven days pellicles were floated onto the surface of liquid BAI medium (500 ml) in Roux flasks. A transferring device consisting of platinum wire twisted into three planar loops of 5 mm diameter each and having a three leafed clover arrangement was used for this purpose. Flotation of the pellicles from the agar slopes was facilitated by adding a small amount of Sauton medium containing 0,01% (m/v) Triton X-100 (Merck) the previous day.

The Roux flasks were incubated at 37°C for 28 days. During the course of incubation cultures were examined regularly for the presence of bacterial or fungal contamination which was readily identified by the uncharacteristic appearance of the pellicle or cloudiness in the medium. Culture filtrates were collected by filtration through sterile muslin and clarified by filtration through a pre-filter and 0,8 µm membrane. The filtrate was sterilised by a second filtration through 0,45 µm and 0,2 µm pore size membranes. A total of 130 l of sterilized culture filtrate was obtained from 284 Roux flasks. The bulk culture filtrate was concentrated by pervaporation in dialysis tubing to approximately one fiftieth of the original volume. The dialysis tubing used (A.H. Thomas Co., U.S.A.) had a flat width of 143 mm and a specified molecular weight cut off of 8 000. During pervaporation the tubes were dialysed periodically against cold distilled water to reduce the concentration of media components. When the culture filtrate was reduced to the required volume it was pooled and extensively dialysed against distilled water to remove all salts. The concentrate was clarified by centrifugation for 30 min at 10 960 g in a Beckman JA-10 fixed angle rotor, dispensed into glass vials and lyophilized as described in Section 2.3. The total yield of this product which was labelled BCG-CF was approximately 18 g, distributed in 444 vials of 40 mg each. The vials were stored at -20°C.

2.5 HEATED BCG CULTURE FILTRATE PREPARATIONS

2.5.1 BCG-CF heated at 100°C in distilled water (BCG-CF100)

A 2 g mass of BCG-CF was dissolved in distilled water to a concentration of 5 mg/ml and heated at 100°C for 60 min in a waterbath. The solution was cooled under tapwater and precipitates were removed by centrifugation at 10 960 g for 30 min. Pellets were discarded and the clarified solution dispensed into glass vials and lyophilized. This preparation was labelled BCG-CF100.

2.5.2 BCG-CF heated at 100°C in BAI medium (BCG-CF100(BAI))

A 2 g mass of BCG-CF was dissolved in BAI medium at a concentration of 5 mg/ml and heated at 100°C for 60 min as described in Section 2.5.1. Precipitates were separated from the supernatant fluids by centrifugation and these were lyophilized in glass vials. This preparation was labelled BCG-CF100(BAI).

2.6 SODIUM DODECYL SULPHATE TREATED BCG CULTURE FILTRATE (BCG-CF(SDS)).

A 100 mg of BCG-CF was dissolved in 50 ml of PBS and mixed with an equal volume of the same buffer containing 10% (m/v) sodium dodecyl sulphate (SDS) and 0,2% (v/v) 2-mercaptoethanol. The solution was heated to 90°C for 3 minutes to ensure dissociation and cooled. Unbound SDS and 2-mercaptoethanol were removed by dialysis against several changes of PBS at room temperature. The non-dialysable residue which was labelled BCG-CF(SDS)was stored at -20°C.

2.7 ANALYTICAL TECHNIQUES.

2.7.1 Biuret protein determination

Protein concentrations were determined by the biuret method described by Garvey et al. (1977). Bovine serum albumin (Miles Laboratories) dried under vacuum over phosphorous pentoxide was used as a laboratory standard.

2.7.2 Lowry protein determination

Protein concentrations of dilute solutions were determined by the method of Lowry et al. (1951). Commercial Folin-Ciocalteu reagent was obtained from Merck and the standard described in Section 2.7.1 was used.

2.7.3 Anthrone determination of total carbohydrates

The anthrone method described by Koehler (1952) for the determination

of total hexoses and pentoses was used. Analytical grade anthrone was obtained from Merck. Carbohydrate concentration was determined by comparision with a reference standard which consisted of a mixture of arabinose, galactose, mannose and glucose in a 2/1/1/1 molar ratio.

2.7.4 N-acetylamino sugars

The method of Reissig et al. (1955) for the determination of N-acetylamino sugars was used to detect the presence of these components in BCG-CF and BCG-CF100. Ehrlich's reagent (p-dimethylaminobenzaldehyde) was obtained from Merck and N-acetylglucosamine which was obtained from the same supplier was used as a calibration standard.

2.7.5 DNA determination

The concentration of DNA was determined according to the method of Dische as described in Kabat and Mayer (1961). Diphenylamine was obtained from Merck and DNA which was used as a calibration standard was obtained from Miles Laboratories.

2.8 ABSORBANCE SPECTROSCOPY

A Zeiss PMQII spectrophotometer was used for ultraviolet light (UV) and visible light absorbance measurements. Matched Suprasil quartz cuvettes with a path length of 1 cm were used throughout.

2.9 ELECTROPHORESIS

2.9.1 Analytical polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to techniques described by Maizel (1971) for high pH disc electrophoresis (PAGE) and SDS-disc electrophoresis (SDS-PAGE). Procedures for the application of samples deviated from the protocol of Maizel (1971) in that no stacking or sample gels were used. Equivalent results were obtained by application of samples dissolved in a sample solution which consisted of stacking gel buffer (60 mM Tris-HCl, pH 6,7), 0,4 M sucrose and 75 µM bromophenol blue directly to the resolving gel surface.

BCG culture filtrate preparations, purified fractions and protein calibration standards were prepared for electrophoresis either by dialysis against PAGE sample solution or by dissolving lyophilized powder directly in PAGE sample solution. In the case of SDS-PAGE, samples were dissociated by the addition of SDS and 2-mercaptoethanol to final concentrations of 1% (m/v) and 0,1% (v/v) respectively. Complete dissociation was achieved by heating samples to 90°C for 3 minutes.

Resolving gels with acrylamide monomer concentrations of 7,5% or 10% (m/v) were prepared in 0,5 cm x 10,5 cm or 0,8 cm x 10,5 cm glass tubes. Electrophoresis proceeded under a constant current of 2 mA or 4 mA per gel. Gels were stained for protein by soaking in a 0,25% (m/v) solution of Coomassie brilliant blue R-250 (Merck) in methanol/acetic acid/water (5/1/5, v/v/v). Unbound dye was eluted by washing in several changes of the same solvent. Densitometer tracings were prepared with a Vitatron TLD100 densitometer.

Carbohydrate components in gels were visualized by the periodic acid-Schiff reaction described by Bolognesi and Bauer (1970). Gels were fixed in a 0,75 M aqueous solution of trichloroacetic acid for 30 min and incubated for 60 min in aqueous 50 mM periodic acid and 0,5 M acetic acid. Gels were washed overnight in running tapwater and stained in a freshly prepared solution of 0,5% (m/v) basic fuchsin for one hour in the dark. Stain was developed by three washings in 20 mM potassium metabisulphate followed by rinsing in tapwater.

2.9.2 Preparative polyacrylamide gel electrophoresis

PAGE and SDS-PAGE procedures described previously (Section 2.9.1) were applied to the isolation of components from partially purified BCG-CF fractions. Tube gels of 0,8 x 10,5 cm were used for preparative PAGE and purified polypeptides where excised according to their relative mobility positions and eluted in 50 mM Tris-HCl buffer.

2.10 CHROMATOGRAPHY

2.10.1 Gel exclusion chromatography

Bio-Gel P-200 (100-200 mesh) polyacrylamide gel beads (Bio Rad

Laboratories) were pre-swollen in 50 mM Tris-HCl buffer and glass chromatography columns with dimensions of 3 x 90 cm and 5 x 100 cm were packed according to procedures recommended by the manufacturers. Samples in 50 mM Tris-HCl buffer were applied to the column and eluted with the same buffer. UV absorbance of the eluates was monitored with an LKB Uvicord and chopper bar recorder. Samples were collected with a rotatory LKB fraction collector and protein and carbohydrate concentrations were determined by the Lowry and anthrone methods.

2.10.2 Ion exchange chromatography

Ion echange chromatographic fractionations were carried out on a 1,5 cm x 20 cm glass chromatography column packed with diethylaminoethyl-cellulose (DEAE-cellulose). For column preparation the required quantity of Whatman grade DE-52 microgranular DEAE-cellulose powder was stirred into 15 volumes of 0,5 M HCl. After a swelling and precycling period of 60 min, the acid was removed by filtration on a Buchner funnel fitted with a Whatman No. 54 acid hardened paper. The residue was washed thoroughly with distilled water until the pH value of the effluent was approximately 4. The swollen ion exchanger was activated by stirring it into 15 volumes of 0,5 M NaOH and equilibrated for 60 min. Alkali was filtered off and the residue washed with distilled water until the pH value of the effluent was neutral. Before use the ion exchanger was degassed by suspension in 0,5 M HCl under vacuum. The pH value was raised to 7 with 0,5 M Tris in distilled water and equilibrated by washing in several changes of 0,5 M Tris-HCl, pH7. A thick slurry was prepared by the addition of 0,15 volumes of 0,5 M Tris-HCl buffer to one volume of settled exchanger before being used for packing of the column. Once packed, the column was re-equilibrated with 50 mM Tris-HCl, pH7.

Samples for fractionation were dialysed against several changes of the starting buffer and were applied to the column. Unadsorbed components were eluted with 50 mlof starting buffer and adsorbed components were eluted by means of an ionic concentration gradient of 50 mM to 0,5 M Tris-HCl at pH7 as shown in Fig. 2.1.

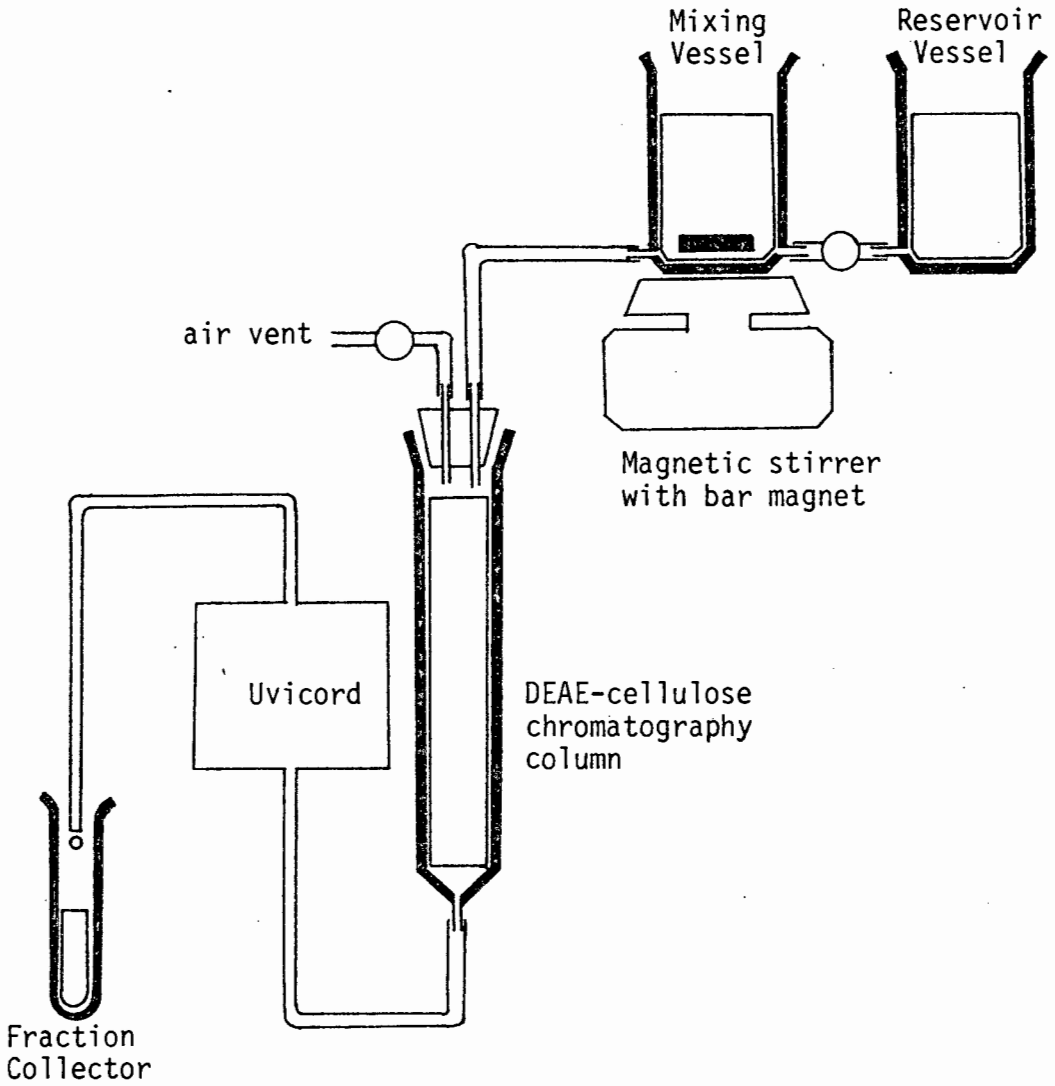


Fig. 2.1 ION EXCHANGE CHROMATOGRAPHY APPARATUS.

The ionic gradient forming system consisted of 500 ml of starting buffer (50 mM Tris-HCl, pH7) in a mixing vessel and 500 ml of elution buffer (0,5 M Tris-HCl, pH7) in an equivalent interconnected reservoir vessel. The contents of the mixing vessel were stirred with a bar magnet.

pH 7,8 was added dropwise with stirring to two volumes of serum. The resultant precipitate was stirred for two hours, collected by centrifugation at 2 000 g for 10 min, and dissolved in PBS. The precipitation procedure was repeated three times and the final precipitate was resuspended in PBS and dialysed for 18 hours against several changes of 17,5 mM sodium phosphate buffer, pH 6,3. A white precipitate which appeared during dialysis was removed by centrifugation. The clarified supernatant was applied to a 1,5 x 10 cm chromatography column containing DEAE-cellulose, precycled and equilibrated with 17,5 mM sodium phosphate buffer, pH 6,3 and eluted with the same buffer. The IgG was eluted in the wash volume leaving any remaining non-IgG serum proteins adsorbed to the column. Purified IgG was dialysed against PBS and stored at -20°C.

2.11.3 Gel immunoprecipitin tests

Gel double diffusion (Ouchterlony) tests were performed in slides prepared with 1% agarose (Miles Laboratories) containing 0,15 M NaCl, 50 mM Tris-HCl, pH 7,4 and 1,5 mM sodium azide. Slides were incubated at room temperature in a humidified chamber and optimum precipitation occurred within 24 hours. Plates were washed in several changes of PBS overnight followed by several washes in distilled water to elute proteins not participating in the immunoprecipitin reaction. Agar slides were dessicated in a 37°C incubator and stained with a 0,25% (m/v) solution of Coomassie brilliant blue R-250 in methanol/acetic acid/water solution (5/1/5, v/v/v). Slides were clarified in the same solvent and photographed.

2.11.4 Interfacial ring tests

Thin glass tubes of 3 mm internal diameter were used for this assay. Antiserum was introduced into the tubes to a height of 4-5 mm and overlayed carefully with an equal volume of antigen. Tubes were incubated at room temperature and were inspected at intervals over 24 hours.

2.11.5 Immunoelectrophoresis

Agar gel reference immunoelectrophoresis was performed as described by Janicki et al. (1971) for mycobacterial culture filtrate antigens and by Daniel et al. (1975) for mycobacterial cell extract antigens. Two ml samples of a 1% (m/v) molten agar (Ionagar No. 2, Difco) solution containing 3 mM barbital, 17 mM sodium barbital, 1,2 mM calcium lactate and 0,25 mM thiomersalate in distilled water were pipetted onto clean, 2 5 x 76 mm microscope slides. A central trough, 2,5 x 57 mm was cut into each gel and a well was punched midway on each side of the trough and 4 mm from it.

A 5 µl sample of test antigen was pipetted into one well and a 5 µl sample of reference antigen into the second well on each slide. Electrophoresis proceeded under a constant current of 1,7 mA per slide for 90 min. Thereafter, 0,15 ml of reference antiserum was pipetted into the trough and the slide was incubated in a humidified chamber at room termperature. After full development of immunoelectrophoretic patterns the agar slides were dried, stained and photographed. Reference antigens and specific reference antisera were kindly supplied by Dr. Paul D. Lambert of the National Institute for Allergy and Infectious Diseases, Bethesda, Maryland, U.S.A. These included an M. tuberculosis H37Rv cell extract polyvalent antigen (Ref-CE antigen) and an M. tuberculosis H37Rv culture filtrate polyvalent antigen (Ref-CF antigen). Reference goat antisera specific for the two polyvalent reference antigens were of batch 002A and will be referred to as Ref-CE antiserum and Ref-CF antiserum.

2.11.6 Enzyme-linked immunosorbent assay (Micro-ELISA)

Preparation of enzyme-antibody conjugate.

Anti-BCG-CF IgG (Section 2.11.2) was conjugated with horse radish peroxidase (Miles Laboratories) according to the glutaraldehyde method of Avrameas (1969). Twelve milligrams of peroxidase were dissolved in 1 ml of a solution containing 5 mg of IgG in 0,1 M sodium phosphate buffer, pH 6,8. To this solution 50 µl of 1% (m/v) aqueous glutaraldehyde was added dropwise with stirring. The reaction mixture was

gently shaken at room temperature for two hours and dialysed overnight against PBS at 4°C. The stock enzyme-antibody conjugate was stored at 4°C.

Micro-ELISA procedure

A double antibody sandwich procedure adapted from methods described by Voller et al. (1976) and Madore and Baumgarten (1979) was used for the micro-ELISA titration of BCG-CF antigens. In this procedure test antigen was incubated in the presence of immobilized antibody. Following thorough washing the bound antigen was detected by enzyme-labelled antibody. Polystyrene Linbro micro-titre plates were used for the assays and all reagents were added in 300 µl volumes. Coating buffer consisted of 50 mM sodium carbonate-sodium bicarbonate, pH 9,6. This buffer was also used as a post-coating buffer to prevent non-specific adsorption but contained in addition 3% (m/v) bovine serum albumin and 0,5 M glycine. Following each step, plates were washed three times with PBS-Tween. This buffer, with the addition of 3% (m/v) bovine serum albumin and 0,5 M glycine (micro-ELISA diluent), was used to dilute antigen and antibody-conjugate to prevent non-specific adsorption. Coating of plates with anti-BCG-CF IgG diluted in coating buffer was carried out at 37°C for 60 min, followed by 30 min incubation at 37°C with post-coating buffer.

Treatment with antigen proceeded at 37°C for 60 min and with antibody-enzyme conjugate for 120 min. Substrate was added following thorough washing. This consisted of a freshly prepared solution containing 3,7 mM o-phenylenediamine (British Drug House), 0,08% (m/v) urea hydrogen peroxide, (British Drug House), 18,4 mM di-sodium tetraborate and 30,5 mM succinic acid. Plates were incubated at room temperature in the dark for 30 min and the reaction was terminated by the addition of 50 µl of 7 M HCl. Absorbance was read at a wavelength of 492 nm.

Optimum concentrations for coating antibody and enzyme-antibody conjugate were established by block titration of these components at constant antigen concentration.

2.11.7 Tuberculin skin tests

White female guinea pigs were used for tuberculin skin testing. These animals were divided into three groups designated A, B, and C. Members of group A were sensitized by intradermal inoculation with 0,1 ml volumes of live BCG suspended in saline at a concentration of 5 mg (dry mass) of bacilli per ml. These animals were isolated in an infectious animal house to prevent infection of unsensitized control animals. Guinea pigs from group B were sensitized by intradermal inoculation with 0,1 ml volumes of an emulsion containing heat killed (100°C for 2 hours) BCG (100 mg/ml) and 50% (v/v) Freund's incomplete adjuvant (Difco). Guinea pigs allocated to group C were used as negative controls and these animals were inoculated intradermally with 0,1 ml volumes of an emulsion of saline and Freund's incomplete adjuvant (1/1, v/v).

Guinea pigs were used for skin testing 8 to 10 weeks after sensitization. Where a period of six months or more elapsed between consecutive tests on guinea pigs, tuberculin sensitivities of the group were boosted by intradermal inoculation with 1 µg of BCG-CF antigen in saline two weeks prior to testing.

For the skin test assay dilutions of test and standard preparations were coded, randomized and 0,1 ml volumes were injected intradermally into both depilated flanks of the guinea pigs. Glass tuberculin syringes fitted with 26 gauge intradermal bevel needles were used and dilutions were prepared in skin test diluent which contained Tween 80 (0,005%) to prevent adsorption of antigen to the glass. Skin injection sites were inspected 24 hours after injection although in some instances readings at 6, 24, 48 and 72 hours were made. The size of the indurated lesions was determined by two measurements with calipers at right angles along the short and long axes of the reaction. The geometric mean of the two readings was taken as the reaction diameter.

Parallel line assays.

The parallel line tuberculin potency assay is based on the observation that within certain limits the size of the skin test response is directly proportional to the logarithm of the dose of tuberculin. The linear range lies between reaction diameters of about 8-20 mm and outside this range, the dose response curve becomes sigmoidal. The principle of the parallel line assay may be seen by reference to Fig. 2.2 in which a graphical representation of the dose response relationship for two preparations, standard and sample are shown. Mean responses to high and low doses of the standard preparation are given by S_1 and S_2 and mean responses to equivalent doses of the test preparation by U_1 and U_2. The potency ratio is the ratio between the doses of the test preparation and the standard which elicit reactions of the same size which, in this case, would be c/b. Although the potency ratio may be determined graphically in this way, it is preferable to calculate it algebraically in order that confidence limits may be obtained and tests for parallelism and linearity applied.

In this investigation the design and statistical analysis of the skin test assays was done as described in the European Pharmacopoeia, using a two dose, two preparation design with 8-12 guinea pigs. Details of the computational process are shown in Appendix 3.

Point assays.

Point assays were used for the detection of gross changes in potency such as might be observed with partial or complete inactivation of skin test antigens. In this format a number of preparations were simultaneously tested at single dose levels. All assay materials were diluted to a concentration of 10 µg/ml and 0,1 ml volumes were injected intradermally in 6-8 guinea pigs. A BCG-CF standard diluted to 10 µg/ml was included in each assay and results were reported as mean reaction diameters. No dose response curves, potency ratios or confidence limits could be calculated for this assay format.

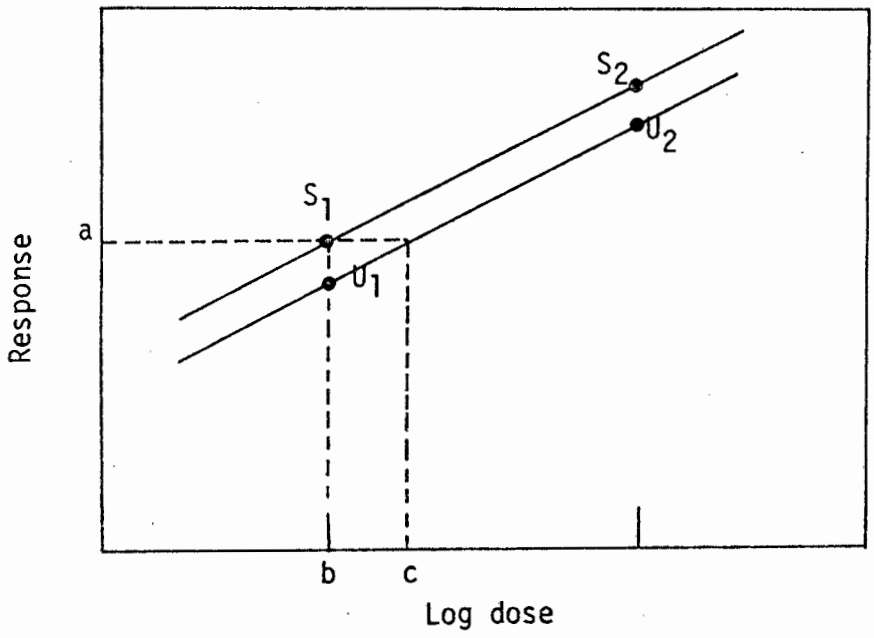


Fig.2.2 PARALLEL LINE TUBERCULIN POTENCY ASSAY

Graphical representation of the relationship between mean reaction diameter and log dose for the calculation of potency ratio in the parallel line assay. Mean responses to low and high doses of the standard and test preparations are indicated as S_1, S_2, U_1, and U_2 respectively.

CHAPTER THREE

EFFECT OF HEAT ON THE COMPOSITION OF BCG CULTURE FILTRATE (BCG-CF)

3.1 INTRODUCTION

This Chapter describes a detailed study of the effects of heat on a BCG culture filtrate preparation (BCG-CF) and the detection of protein immunoprecipitin antigens which display thermal stability. The composition of heated culture filtrate was compared directly with that of unheated culture filtrate using chemical assay procedures, gel exclusion chromatography, ion exchange chromatography, polyacrylamide gel electrophoresis, mycobacterial antigen reference immunoelectrophoresis and gel immunoprecipitation. A temperature of 100°C was used in all heating studies and the ability to display resistance of immunoprecipitin properties to heating at this temperature for 60 minutes was regarded as the criterion for heat stability. These conditions precluded all but the most heat resistant protein antigens from consideration as heat stable since a large number of mycobacterial antigens will withstand temperatures marginally less than 100°C, or shorter heating periods at this temperature (Gussoni, 1962; Moulton et al., 1972).

Analytical protein assay procedures were used to measure the protein content of BCG culture filtrate and to quantitate the extent to which precipitation of protein occurred during heating. It is known that the pH of the culture filtrate during heat sterilization may influence the yield of mycobacterin protein (Lesslie et al., 1975), but the extent to which this is due to protein precipitation is not clear. The contribution of several factors including heating time, concentration of non-dialysable culture filtrate materials and the composition and pH of the medium to the thermal precipitation of BCG culture filtrate proteins has therefore been investigated with a view to showing that heat precipitation may contribute to the reported variability in mycobacterin protein yields (Magnusson and Bentzon, 1958; Lesslie et al., 1975).

For the determination of the total carbohydrate, DNA and amino sugar contents of BCG culture filtrate, the anthrone, Dische and amino sugar assays described in Chapter 2 were used. The accuracy of the anthrone method is subject to the variability of colour production by different sugars and polysaccharides (Koehler, 1952). Therefore the customary use of glucose which reacts more strongly than most sugars for a calibration standard may lead to erroneous results when other sugars are assayed. This practice has been common in studies of mycobacterin compositions (Dietz et al., 1969; Landi and Held, 1971; Nagai et al., 1974a). Because of this limitation in the selection of a standard substance, a reference standard representative of the major sugars of mycobacterins was prepared. This consisted of a mixture of arabinose, galactose, mannose and glucose in a 2:1:1:1 molar ratio.

Gel exclusion chromatography in combination with other procedures has successfully been used for the isolation of purified antigens from mycobacterial culture filtrate and cell extract preparations (Nagai et al., 1974a; 1974b; Matsumoto et al., 1979). It has therefore been utilized in this study for the primary fractionation of unheated and heated BCG culture filtrates and for the determination of the molecular sizes and distributions of the major protein and polysaccharide components of these preparations. For the determination of molecular size, protein molecular weight markers have been used to prepare a calibration curve according to the equation

$$V_e = k \log M \qquad \text{equation 3.1}$$

where V_e represents elution volume, M, protein molecular weight and k is a constant (Andrews, 1965). A sigmoidal relationship exists between V_e and log M but within the optimum operating range for each gel bed this relationship approximates linearity.

Analytical polyacrylamide gel electrophoresis has found application in the demonstration of the heterogeneity of mycobacterial antigen preparations (Roszman et al., 1968) and for the determination of the molecular weights of isolated components (Daniel and Fergusson, 1970; Nagai et al., 1974a; 1974b; Matsumoto et al., 1979). In this study

it has been used as an indicator of protein denaturation and for the determination of the molecular weights of BCG culture filtrate components. For molecular weight determinations in the presence of SDS, a calibration graph of relative mobility, R_F, versus log molecular weight, log M, is prepared according to the equation

$$R_F = k \log M \qquad \text{equation 3.2}$$

which yields a relationship which, within limits, is linear (Weber and Osborn, 1975).

Gel immunoprecipitation and the reference immunoelectrophoretic system for mycobacterial antigens were used in this study for the detection of heat stable antigens of BCG culture filtrate. The immunoprecipitin reaction has in the past been of great use in the study of the antigenic composition and the affect of heat on mycobacterial culture filtrates, and its use has been well documented (Pickett et al., 1968; Daniel and Janicki, 1978).

3.2 MATERIALS AND METHODS

3.2.1 ANALYTICAL INVESTIGATIONS

The chemical composition of BCG-CF was determined by the analytical techniques described in Section 2.7. Stock solutions of BCG-CF in distilled water were prepared volumentrically from lyophilized powder and these solutions were appropriately diluted for the various assays. Protein, carbohydrate, DNA and amino sugar concentrations were determined and expressed as a percentage of the mass of total solids. Assays were repeated five times with freshly prepared stock solutions and mean results were calculated.

3.2.2 PROTEIN PRECIPITATION STUDIES

The heat precipitation of protein from BCG-CF solutions was investigated by the comparison of protein concentrations of the supernatant fluids of heated BCG-CF solutions with those of unheated solutions. Heating studies were carried out in a thermostatically controlled waterbath (Precision Scientific Co., Chicago) accurate to ± 1°C with a temperature range from ambient to 101°C. Samples of BCG-CF solutions were transferred to screw capped pyrex tubes or flame sealed in glass vials and immersed in water of the specified temperature. Timing was commenced when temperature, as recorded by a thermometer suspended in an equivalent control tube, equilibrated with that of the waterbath. Following heating, tubes were rapidly cooled by immersion in cold water and precipitates were removed by centrifugation at 9 000 g for 20 min. Protein concentrations were determined by the Lowry method with the inclusion of appropriate blanks and controls.

3.2.3 GEL EXCLUSION CHROMATOGRAPHY

BCG-CF, BCG-CF100 and BCG-CF100(BAI) were subjected to exclusion chromatography on Bio-Gel P-200 as described in Section 2.10.1. Samples were applied to the column and eluted with 50 mM Tris-HCl, pH7 or with the same buffer containing, in addition, 1% (m/v) EDTA. Eluates were tested for protein and polysaccharide concentrations, and also monitored at A_{260} The Bio-Gel P-200 column used in these investigations was calibrated for molecular weight determination with standard materials which included blue dextran (Pharmacia, Uppsala), human IgG (Natal Institute of Immunology), phenol red (British Drug House), haemoglobin, myoglobin, carbonic anhydrase and cytochrome C (Miles Laboratories). These materials were eluted from the column, and their mean elution volumes, V_e, were determined from five consecutive runs. A calibration graph of V_e versus log molecular weight was plotted according to equation 3.1.

3.2.4 POLYACRYLAMIDE GEL ELECTROPHORESIS

Samples of BCG-CF, BCG-CF100, BCG-CF100(BAI) and the fractions obtained in gel exclusion chromatography studies were subjected to PAGE and SDS-PAGE in 10% tube gels as described in Section 2.9.1. Gels were stained either with Coomassie brilliant blue or by the periodic acid-Schiff technique and densitometer traces were prepared. For molecular weight determination, a series of protein standards which included ribonuclease, lysozyme, haemoglobin, trypsin, IgG (L and H chains), carbonic anhydrase, ovalbumin, leucine amino peptidase and bovine serum albumin (Miles Laboratories) were used. These were subjected to SDS-PAGE simultaneously with BCG-CF and the heated BCG-CF preparations to ensure comparative electrophoretic conditions. Relative mobility (R_F) values were determined with respect to the tracking dye and these were plotted against the logarithm of molecular weight according to equation 3.2. A best fit slope was determined by the least squares method (Sokal and Rohlf, 1969), the computational procedure of which is given in Appendix A3.1. Mobility data for reference standards, determination of the regression coefficient and determination of the intercept for the molecular weight calibration graph are given in Appendix A3.2.

3.2.5 GEL IMMUNOPRECIPITIN REACTIONS

The effect of heat on gel immunoprecipitin properties of the fractions obtained by gel exclusion chromatography of BCG-CF was investigated as follows. Solutions containing 1mg/ml of each fraction in 50 mM Tris-HCl, pH7 were sealed in glass ampoules, heated at 100°C for 60 min in a waterbath, cooled and clarified by centrifugation at 9 000 g for 20 min. These solutions were subjected to a gel immunoprecipitin test with anti-BCG-CF serum.

3.2.6 IMMUNOELECTROPHORESIS

BCG-CF, BCG-CF100 and the Ref-CF antigen preparation were subjected to reference immunoelectrophoresis as described in Section 2.11.5.

Ref-CF antiserum, anti-BCG-CF serum and concanavalin-A (So and Goldstein, 1969) were used to develop immunoelectrophoresis patterns.

3.3. RESULTS

3.3.1 CHEMICAL ANAYLSIS OF BCG-CF

Lyophilized BCG-CF was a brown powder which had none of the volatile odours characteristic of BCG cultures growing on BAI medium. It dissolved readily in distilled water, saline and all other aqueous buffered solutions investigated. It did not dissolve in the presence of an excess of a number of organic solvents which included acetone, benzene, carbon tetrachloride, n-hexane, trichloromethane and the alcohols, methanol, ethanol and propanol.

Analytical investigations (Table 3.1) showed that protein was the major constituent of BCG-CF. This fraction accounted for 41,3% to 41,7% of the dry mass of BCG-CF whereas carbohydrate, DNA and amino sugars accounted for 37,9%, 5,3% and 0,6% respectively.

3.3.2 FACTORS CONTRIBUTING TO THE THERMAL PRECIPITATION OF BCG-CF PROTEINS

The coagulation of proteins of BCG-CF solutions heated at 100°C was shown to be variable and dependent on several factors. These included heating time, solvent composition, the concentration of BCG-CF and the pH of the heated solution.

3.3.2.1 Heating time

Five millilitre volumes of a 3,37 mg/ml solution of BCG-CF in 50 mM Tris-HCl, pH7 were heated at 100°C for various time intervals from 10 min to 120 min. Solutions which were heated for periods of less than

TABLE 3.1 CHEMICAL COMPOSITION OF BCG-CF

Protein, carbohydrate, DNA and amino sugar contents of BCG-CF are expressed as percentages of the mass of total solids. The figure shown for residual materials is obtained by subtraction from 100%.

Analytical procedure	content (%)	Standard deviation
Protein :		
Biuret	41,3	2,2
Lowry	41,7	0,6
Carbohydrate	37,9	1,5
DNA	5,3	0,02
Amino sugars	0,6	0,3
Residual materials	14,5 - 14,9	-

60 min showed increasing turbidity with heating time due to finely dispersed precipitates. Those heated for longer periods were not turbid but contained significant quantities of reddish brown precipitate which settled in pellet form. The percentage protein precipitated from each heated solution was calculated relative to an unheated control sample and a graph of percentage protein precipitation as a function of heating time was plotted (Fig.3.1). The rate of protein precipitation was initially rapid but stabilized following heating for 60 min, after which no further precipitation occurred. Therefore in order to allow maximum development of precipitates, a minimum heating time of 60 min was required for heat coagulation studies.

3.3.2.2. Solvent composition and BCG-CF concentration

The results in Table 3.2 and Fig.3.2 show that the heat precipitation of BCG-CF proteins was dependent on both the solvent composition and BCG-CF concentration. In this experiment BCG-CF was dissolved in either distilled water, 0,1 M NaCl (both adjusted to pH7 with 0,01 M NaOH), 10 mM Tris-HCl, pH7, Sauton medium or BAI medium to yield solutions with final concentrations within the range of 6-7 mg BCG-CF/ml. These solutions were diluted in the appropriate solvent, heated at 100°C for 60 min and the percentage protein precipitation determined.

Maximum precipitation of protein occurred when BCG-CF was heated in distilled water. Somewhat less precipitation occurred with 10 mM Tris-HCl, pH7 and with 0,1 M NaCl. A significant measure of protection against precipitation was provided by Sauton and BAI media where this effect was significantly less than in other solutions.

Precipitation, in all cases investigated, was dependent on BCG-CF concentration. At very low concentration it was insignificant but increased in an exponential fashion with increasing BCG-CF concentration until peak values, which were solvent dependent, occurred. Thereafter further increases in BCG-CF concentration were not accompanied by increases in percentage precipitation. In the presence of Sauton and BAI media no significant precipitation occurred at BCG-CF concentrations of less than 2 mg/ml.

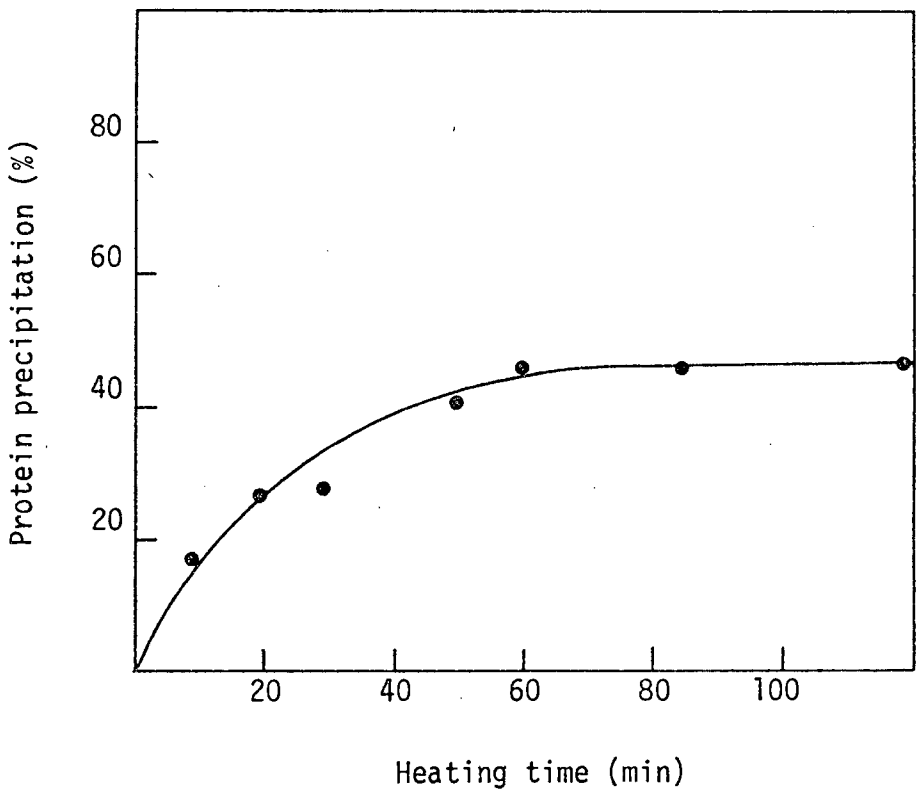


Fig. 3.1 RELATIONSHIP BETWEEN PROTEIN PRECIPITATION AND HEATING TIME

A 3,37 mg/ml solution of BCG-CF in 50 mM Tris-HCl was heated at 100°C for time intervals from 10-120 min. Protein precipitates were removed by centrifugation and the protein concentrations of the supernatant fluids were determined.

TABLE 3.2 EFFECT OF SOLVENT COMPOSITION AND BCG-CF CONCENTRATION ON THERMAL PRECIPITATION OF PROTEIN

BCG-CF at a range of concentrations in distilled water, 10 mM Tris-HCl, 0,1 M NaCl, Sauton medium and BAI medium was heated at 100°C for 60 min. Protein precipitated from solution is shown as a percentage of initial protein concentration.

Solvent	% precipitation at various BCG-CF concentrations			
	1 mg/ml	2 mg/ml	4 mg/ml	6 mg/ml
Distilled water	46,0	56,4	57,8	58,4
10mM Tris-HCl, pH7	16,0	36,6	47,0	50,7
0,1 M NaCl	0,2	3,8	24,0	41,8
Sauton medium	0,1	2,0	9,5	14,4
BAl medium	0,3	1,0	4,8	9,0

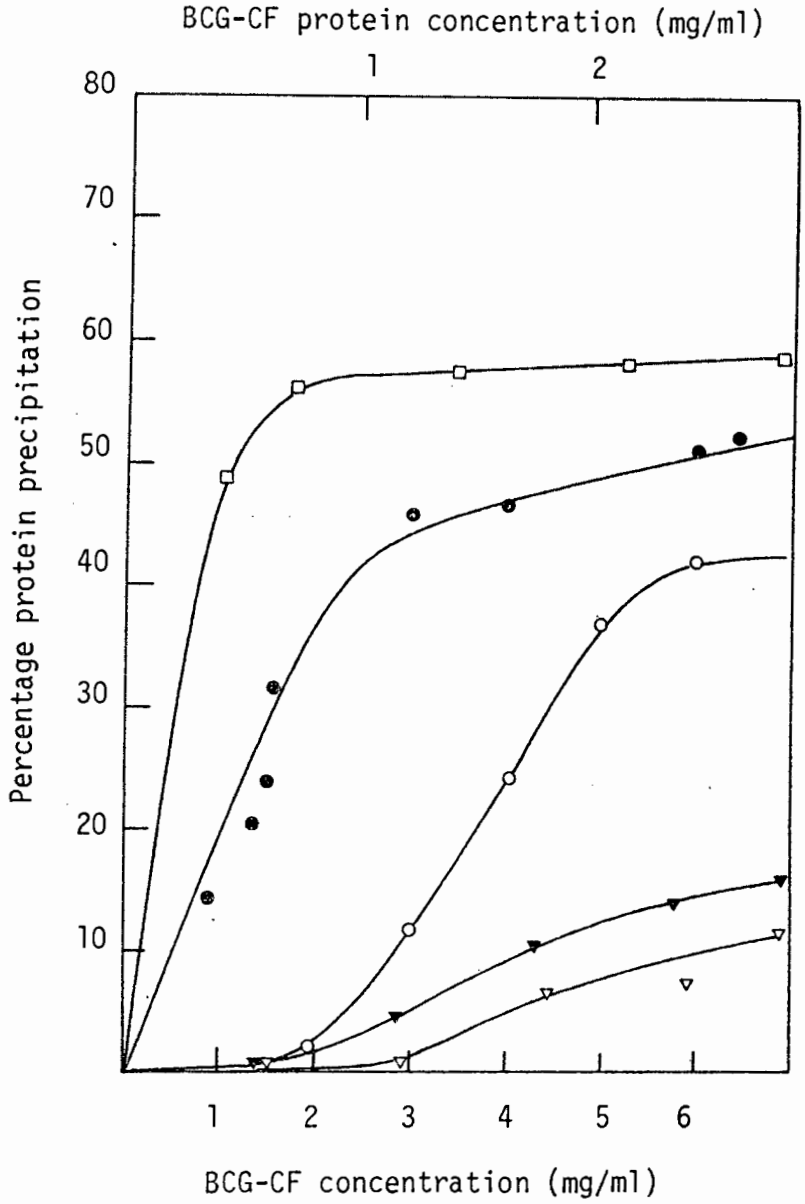


Fig. 3.2 RELATIONSHIP BETWEEN THERMAL PRECIPITATION OF BCG-CF PROTEINS, SOLVENT COMPOSITION AND THE CONCENTRATION OF BCG-CF.

Solutions of BCG-CF in distilled water, □—□; 0,1 M Nacl, ○—○; 10 mM Tris-HCl, ●—●; Sauton medium, ▼—▼; and BAI medium, ▽—▽ at a range of concentrations were heated at 100°C for 60 min. Precipitates were removed by centrifugation and protein concentrations of supernatant fluids determined. Protein precipitation calculated as percentages of protein concentrations of unheated samples are plotted against initial protein and BCG-CF concentrations.

3.3.2.3 pH of medium

Results of the investigation of the effect of the pH of the medium on the thermal precipitation of BCG-CF proteins may be seen in Fig. 3.3. Stock solutions of BCG-CF containing approximately 2 - 3 mg/ml of protein in distilled water, BAI medium and Sauton medium were prepared. The hydrogen ion concentrations of aliquots of these solutions were adjusted by addition of a few drops of phosphoric acid or tri-sodium phosphate to provide a pH range from approximately 2,5 to 11.

At pH values below 5 isoelectric precipitation of BCG-CF proteins occurred. Turbid solutions were clarified and a sample of each was heated at 100°C for 60 min. The protein concentrations of unheated and heated solutions and the percentage thermal protein precipitation are shown in Fig. 3.3. Isoelectric protein precipitation at low pH was marked in BCG-CF solutions prepared in distilled water and in Sauton medium where up to half the initial proteins precipitated when solutions were acidified. This effect was less obvious in BAI medium (Fig. 3.3B). The percentage protein precipitated from solution during heating was maximal in all three solutions within the pH range of 4 to 6 with peak values at approximately pH 5,5. In the case of distilled water (Fig. 3.3A) the percentage precipitation of protein at pH values above neutral was relatively large. This was not the case with BAI (Fig. 3. 3B) and Sauton media (Fig. 3.3C) where thermal protein precipitation was minimal at neutral and alkaline pH values.

3.3.3 EFFECT OF HEAT ON BCG-CF COMPOSITION

3.3.3.1 Gel exclusion chromatography

The results of gel exclusion chromatography studies of BCG-CF, BCG-CF100 and BCG-CF100(BAI) on Bio-Gel P-200 may be seen in Fig. 3.4. In Fig. 3.5 the molecular weights of protein standards have been plotted on log scale versus their elution volumes from Bio-Gel P-200 and the linear relationship obtained has been drawn on an elution profile of BCG-CF proteins from the same column. The proteins of BCG-CF displayed a wide

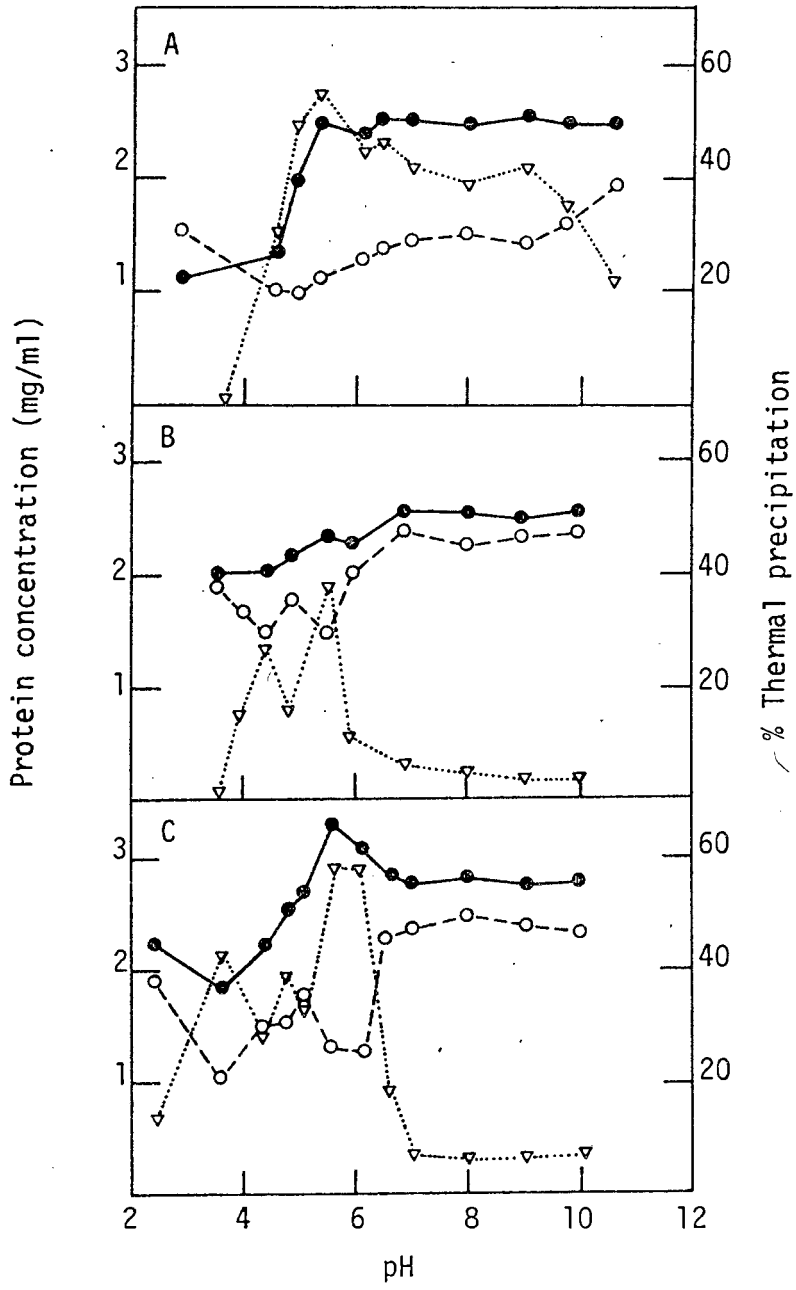


Fig. 3.3. EFFECT OF pH OF THE MEDIUM ON THERMAL PRECIPITATION OF BCG-CF PROTEINS

BCG-CF was dissolved in distilled water (Panel A), BAI medium (Panel B) and Sauton medium (Panel C) to give a protein concentration of 2-3 mg/ml. Hydrogen ion concentrations of these solutions were adjusted by addition of phosphoric acid or tri-sodium phosphate to provide a range of 2,5-11 pH units. Clarified solutions were heated at 100°C for 60 min and precipitates were removed by centrifugation. Initial protein concentrations ●——●, final protein concentrations of supernatant fluids ○---○, and precipitation as a percentage of initial protein concentration ▽·····▽, are shown.

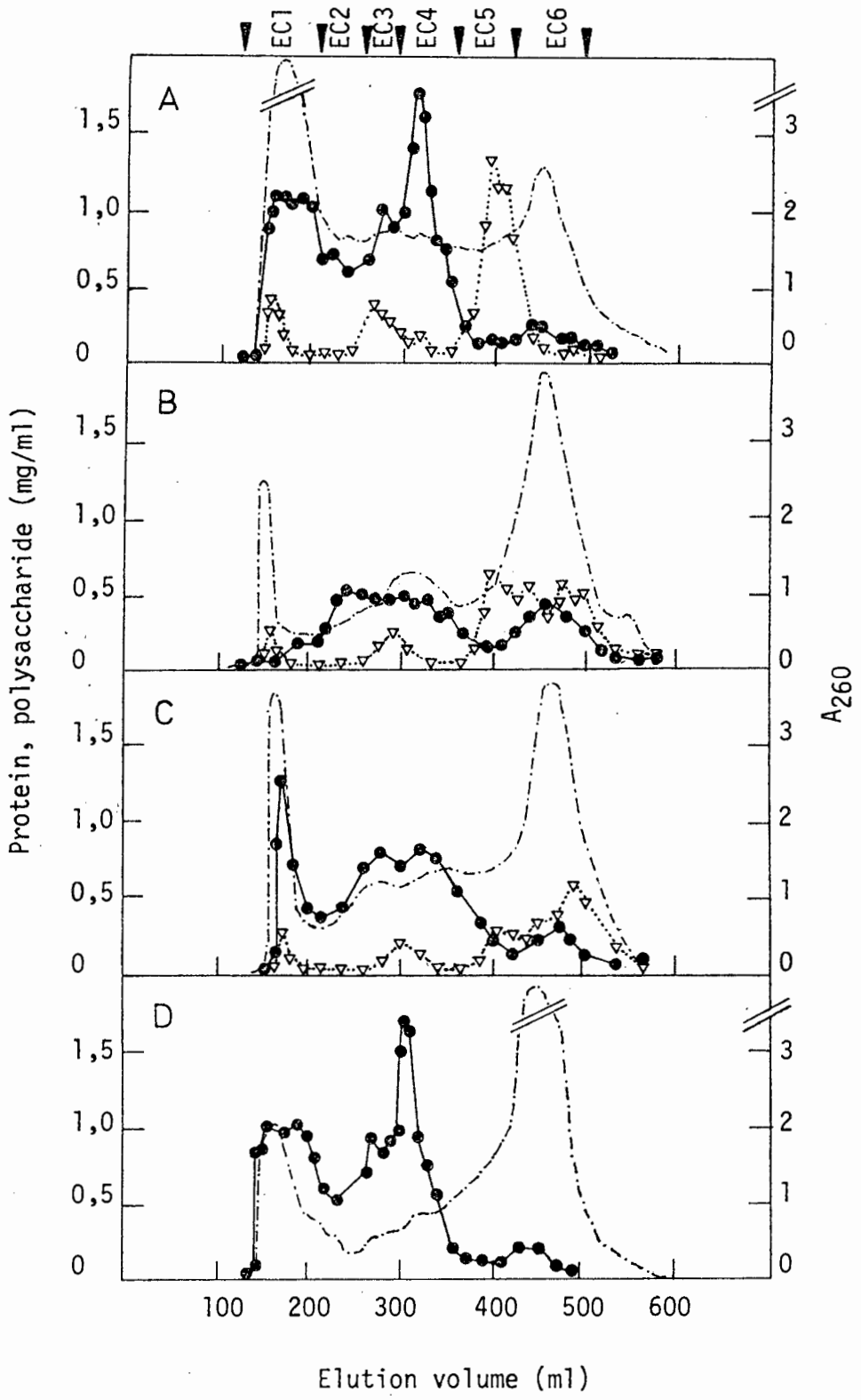


Fig. 3.4 GEL EXCLUSION CHROMATOGRAPHY OF NATIVE AND HEATED BCG-CF PREPARATIONS

Samples (0,5 g) of BCG-CF (Panel A), BCG-CF100 (Panel B) and BCG-CF100(BAI) (Panel C) were eluted from a 3 x 90 cm Bio-Gel P-200 column with 50 mM Tris-HCl. BCG-CF eluted from the same column with 50 mM Tris-HCl containing 1 % (m/v) EDTA is shown in Panel D. Eluates were monitored for A_{260}, –·–·– ; protein concentration, ●—● ; and polysaccharide concentration, ▽·····▽ .

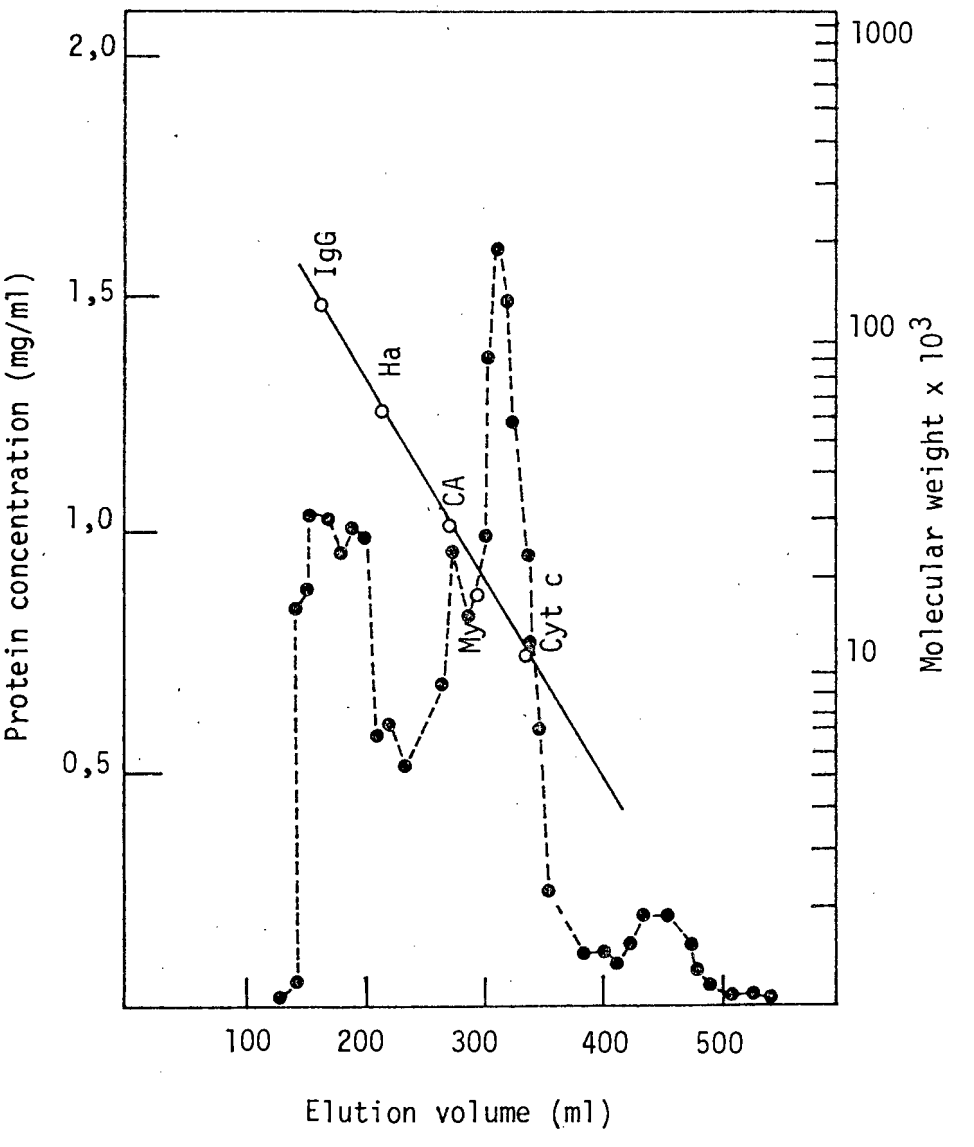


Fig. 3.5 MOLECULAR WEIGHT DETERMINATION OF BCG-CF COMPONENTS BY GEL EXCLUSION CHROMATOGRAPHY

Protein calibration standards which included human IgG, haemoglobin (Ha), carbonic anhydrase (CA), myoglobin (My) and cytochrome c (Cyt c) were eluted from a 3 x 90 cm Bio-Gel P-200 column. The molecular weights of these standards were plotted versus elution volume and a BCG-CF protein elution diagram has been drawn onto this graph.

range of molecular weights but the majority eluted in two major peaks which represented molecular weights of 70 000 - 150 000 and 8 000 - 30 000.

Comparision of the elution profiles of BCG-CF100 and BCG-CF100(BAI) with that of BCG-CF showed that heating of BCG-CF in aqueous solution (BCG-CF100) resulted in the precipitation of all high molecular weight proteins (Fig. 3.4B). The shape of the low molecular weight protein peaks was altered in a way which indicated that many low molecular weight proteins also precipitated from solution. When BCG-CF was heated in BAI medium (BCG-CF100(BAI), Fig. 3.4C) the precipitation of many high molecular weight proteins did not occur. However, these components all eluted at the void volume which indicates that aggregation had nevertheless taken place. The aggregation of low molecular weight proteins also occurred but some degree of definition of the lower molecular weight protein fractions was still retained.

Gel exclusion chromatography separated the carbohydrates of BCG-CF into a very high molecular weight fraction (VH-P), an intermediate molecular weight fraction (I-P) and a low molecular weight fraction (L-P) which contained most of the BCG-CF polysaccharides (Fig. 3.4A). Carbohydrates of BCG-CF were more resistent to heating than proteins and the high molecular weight (VH-P) and intermediate molecular weight (I-P) fractions of BCG-CF100 and BCG-CF100(BAI) demonstrated elution properties unchanged from those of BCG-CF. The patterns of elution of the low molecular weight (L-P) fractions of BCG-CF100 and BCG-CF100 (BAI) suggested that some breakdown accompanied heating.

Strong UV absorbance in the 250-280 nm wavelength range was associated with BCG-CF materials and large absorbance peaks appeared at the void volume and at the tail of the elution diagram (Fig. 3.4A). The brown colour of BCG-CF was largely concentrated into these UV absorbance peaks although some colour was also distributed throughout the elution profile. It was possible that since BAI medium was rich in ferric ammonium citrate, the brown colour could have resulted from bound iron or iron complexes. Development of the chromatogram with 50 mM Tris-HCl buffer, pH7 containing 1% (m/v) EDTA resulted in displacement of materials with strong UV

absorbance and most brown colour to the tail of the elution profile (Fig. 3.4D). This suggests that the brown colour of BCG-CF was due either to bound iron or to other divalent cation complexes.

Materials which eluted from the column were pooled into six fractions as shown in Fig. 3.4A and these were labelled EC1, EC2, EC3, EC4, EC5, and EC6.

3.3.3.2 Polyacrylamide gel electrophoresis

Electrophoretic investigations demonstrated that the protein fraction of BCG-CF contained numerous polypeptide components. In PAGE studies no clear separation of components was obtained and stain was taken up throughout the gel (Fig. 3.6A). In contrast, up to 34 individual polypeptides could be distinguished in the SDS-PAGE system by visual inspection of the original gels (Fig. 3.6B). The sum total of polypeptides of BCG-CF was probably greater than this number as overlapping of bands was to some extent apparent. A strong band was present at the migration front and it is likely that this band also contained several to many low molecular weight components.

The calibration of SDS-PAGE for molecular weight determination may be seen in Fig. 3.7 where R_F values for protein standards have been plotted against their log molecular weights. A linear relationship was obtained over the 13 000 - 70 000 molecular weight range. This molecular weight calibration confirmed the wide range of polypeptide molecular weights observed in gel exclusion chromatography studies. Here molecular weights ranged from greater than 70 000 to less than 10 000.

Electrophoretic studies of the gel exclusion chromatography fractions (Section 3.3.3.1) showed that fractions EC1, EC2 and EC3 each comprised multiple polypeptide components (Fig. 3.6). Fraction EC4 which represented the large protein peak from gel exclusion chromatography contained relatively few components of which two were present in large quantity. In 10% PAGE gels the relative mobilities of these two components were 0,52 and 0,57 (Fig. 3.6I). SDS-PAGE showed that their

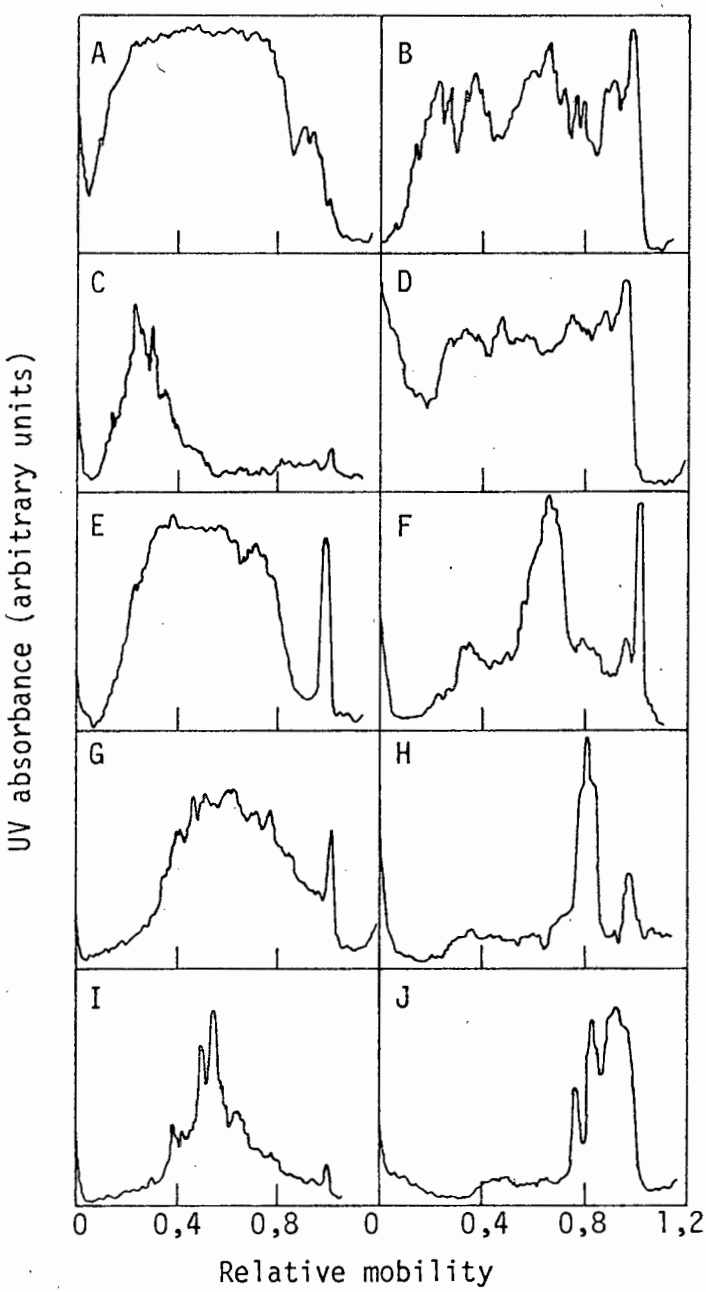


Fig. 3.6 POLYACRYLAMIDE GEL ELECTROPHORESIS OF BCG-CF AND GEL EXCLUSION CHROMATOGRAPHY FRACTIONS

BCG-CF (Panels A & B) and Fractions EC1 (Panels C & D), EC2 (Panels E & F), EC3 (Panels G & H) and EC4 (Panels I & J) were subjected to PAGE and SDS-PAGE in 10% gels. Densitometer traces of PAGE studies are shown in the panels on the left and results of SDS-PAGE investigations on the right.

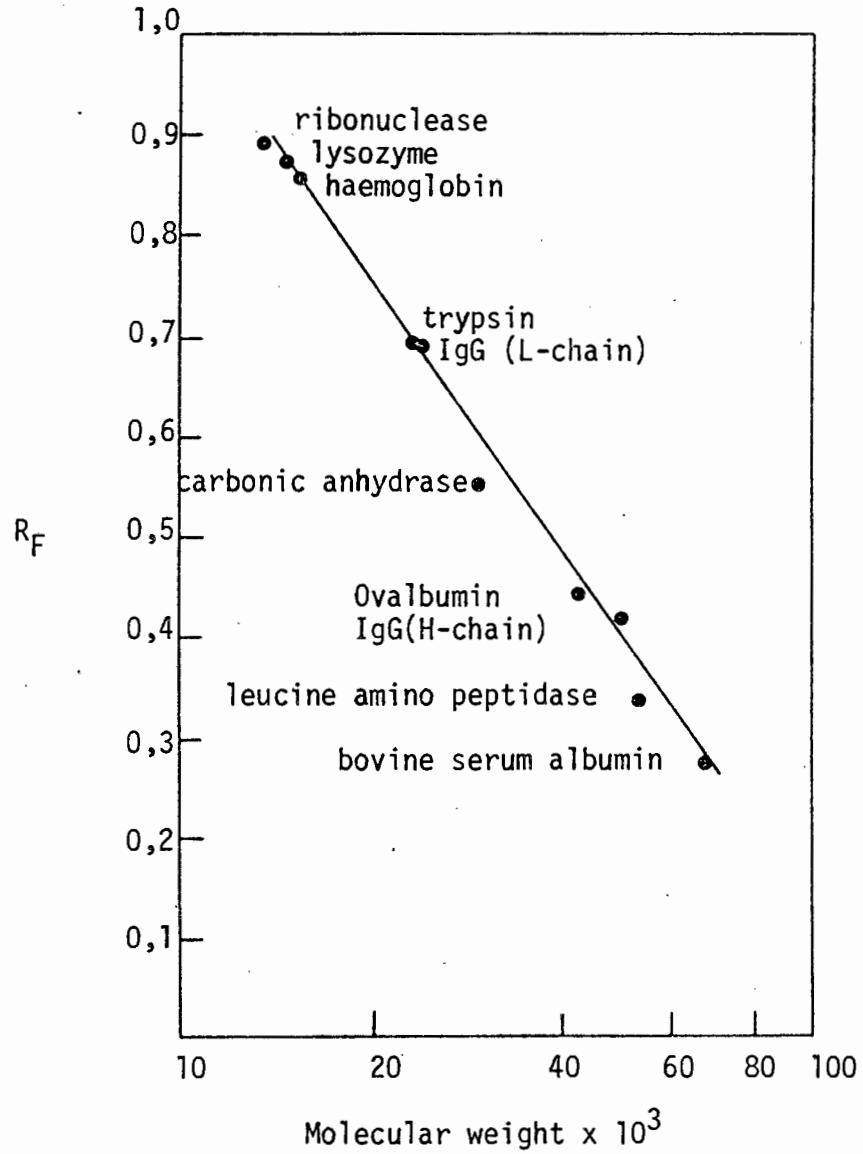


Fig. 3.7 MOLECULAR WEIGHT DETERMINATION BY SDS-PAGE

Protein calibration standards were subjected to SDS-PAGE and their relative mobilities (R_F) were plotted versus molecular weight. A best fit regression line was calculated by the least squares method. See Appendix 3.

molecular weights were approximately 10 000 (Fig. 3.6J). No protein or polysaccharide electrophoretic bands were produced by fractions EC5 and EC6.

Densitometer traces of BCG-CF100 and BCG-CF100(BAI) subjected to PAGE (Figs. 3.8C, 3.8E) showed that very few components of these heated solutions migrated in distinct bands. In both preparations two bands with R_F values of approximately 0,51 and 0,57 and a weak band which migrated with the tracking dye were the only electrophoretic components. These bands were always present in preparations heated to 100°C, R_F values were reproducible and very similar to those of the two major bands (0,52 and 0,57) of fraction EC4 (Fig. 3.6I). Electrophoresis (PAGE) of a sample of fraction EC4 heated to 100°C for 60 min confirmed that the two major proteins of fraction EC4 were in fact the two heat stable proteins of BCG-CF100 (Fig. 3.8G). The molecular weights of the proteins of BCG-CF100 and BCG-CF100(BAI) were shown by SDS-PAGE to be less than about 15 000 (Figs. 3.8D, 3.8F).

Polyacrylamide gels in which BCG-CF had been subjected to PAGE and stained for carbohydrate by the periodic acid-Schiff method produced four weak bands which were sharp but very faint. Densitometer traces of these bands have not been shown here, but one migrated at the electrophoretic boundary position and the other three bands appeared close to the origin with relative mobilities of 0,025, 0,028 and 0,056 respectively. The faintness of these bands suggested that they were not produced by the major carbohydrate components of BCG-CF but rather that they represented carbohydrate moeities of glycoproteins. Since no carbohydrate bands were noted in the carbohydrate rich fraction EC5 it is probable that electrophoretic migration of these compounds did not occur. Similar observations were made by Roszman *et al.*, (1968) who failed to obtain migration of carbohydrates of BCG culture filtrates on polyacrylamide gel electrophoresis. Two glycopolypeptide bands were present in the heated preparation BCG-CF100. These included the band which migrated with the tracking dye and one of low mobility (R_F = 0,029).

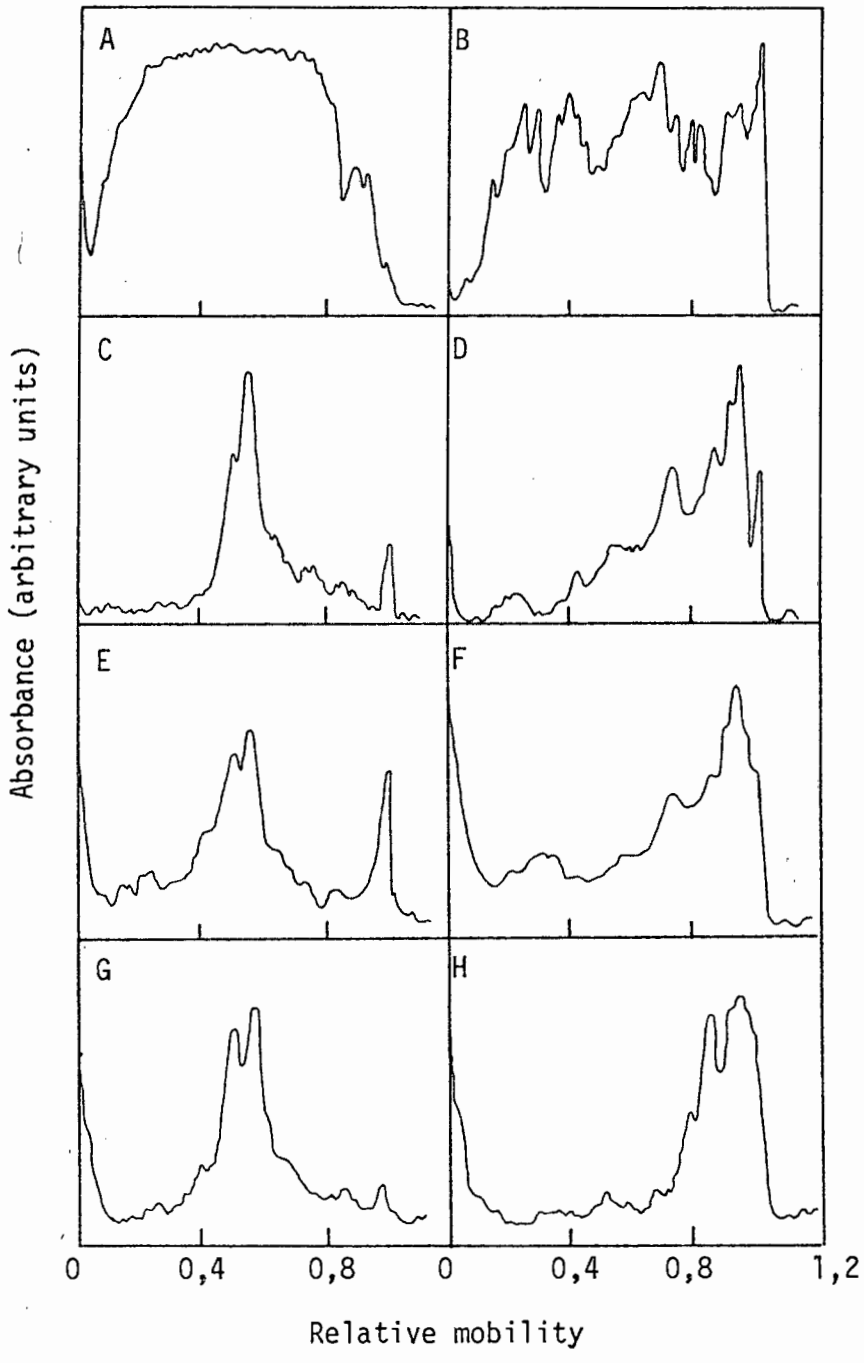


Fig. 3.8 POLYACRYLAMIDE GEL ELECTROPHORESIS OF HEATED BCG-CF PREPARATIONS

Samples of BCG-CF (Panels A & B), BCG-CF100 (Panels C & D), BCG-CF100(BAI)(Panels E & F) and fraction EC4 heated at 100°C for 60 min (Panels G & H) were subjected to PAGE and SDS-PAGE in 10% gels. Left hand panels show densitometer traces of PAGE studies and right hand panels indicate SDS-PAGE results.

3.3.3.3 Gel immunoprecipitation

In fig. 3.9 gel immunoprecipitin reactions of fractions EC1, EC2, EC3, EC4, EC5 and EC6 with anti-BCG-CF serum are shown. Numerous immunoprecipitin antigens were demonstrated in each of the first four fractions but fractions EC5 and EC6 were unreactive with the antiserum. Heating at 100°C for 60 min inactivated virtually all immunoprecipitin antigens of the high molecular weight fractions EC1 and EC2. Most antigens of fraction EC3 were similarly heat inactivated. The low molecular weight fraction EC4 contrasted with higher molecular weight fractions in that several antigens were resistant to heating. Of these, two strong lines were prominent.

3.3.3.4 Immunoelectrophoresis

Optimum immunoprecipitin band formation was obtained after incubation with antiserum for 24 hours, but the quality of the gels deteriorated during the washing, drying and staining procedures which followed. Line drawings made prior to staining and relevant photographs are shown in Fig. 3.10. These include the immunoelectrophoretic patterns of Ref-CF, BCG-CF and BCG-CF100 each developed with Ref-CF antiserum, anti-BCG-CF serum and concanavalin A. The antigens detected by Ref-CF antiserum in the Ref-CF antigen, BCG-CF and BCG-CF100 preparations are compared in Table 3.3.

3.4 DISCUSSION

Proteins and polysaccharides were the main non-dialysable components of BCG-CF, with protein accounting for approximately 41% and polysaccharide accounting for 38% of the dry mass. Results obtained in protein determinations with the biuret method, which specifically determines the peptide bond content, and the Lowry method, where both peptide bonds and aromatic amino acids contribute to the reaction (Garvey et al., 1977), correlated well. The relative proportions of proteins and carbohydrates

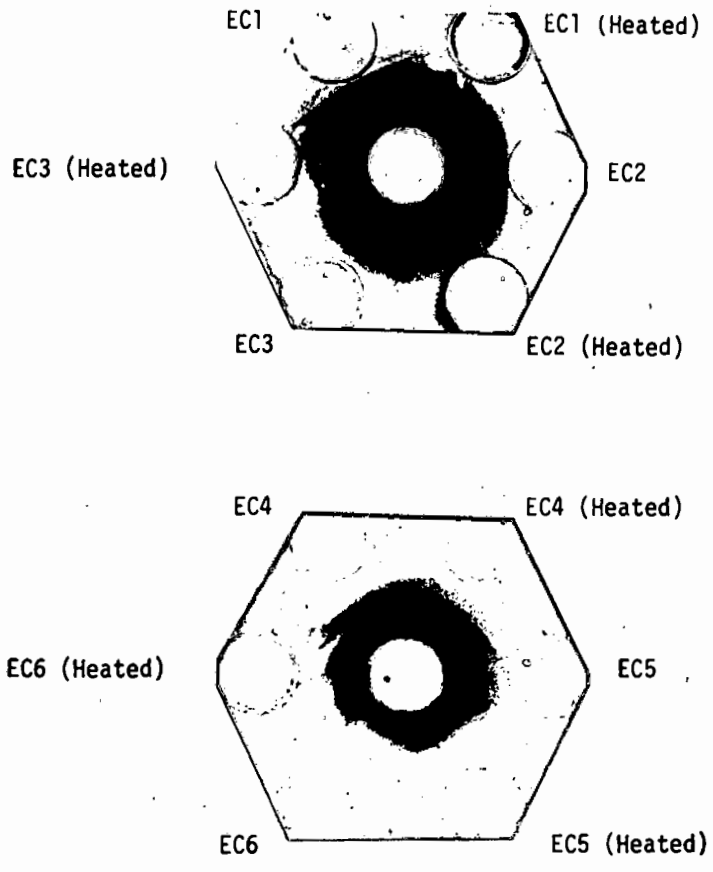


Fig 3.9 HEAT STABLE IMMUNOPRECIPITIN ANTIGENS OF BCG-CF

Samples of the gel exclusion chromatographic fractions EC1, EC2, EC3, EC4, EC5 and EC6 were heated at 100°C for 60 min. Native and heated samples of the fractions were transferred to the peripheral wells of an agarose gel plate and anti-BCG-CF serum was pipetted into the central wells.

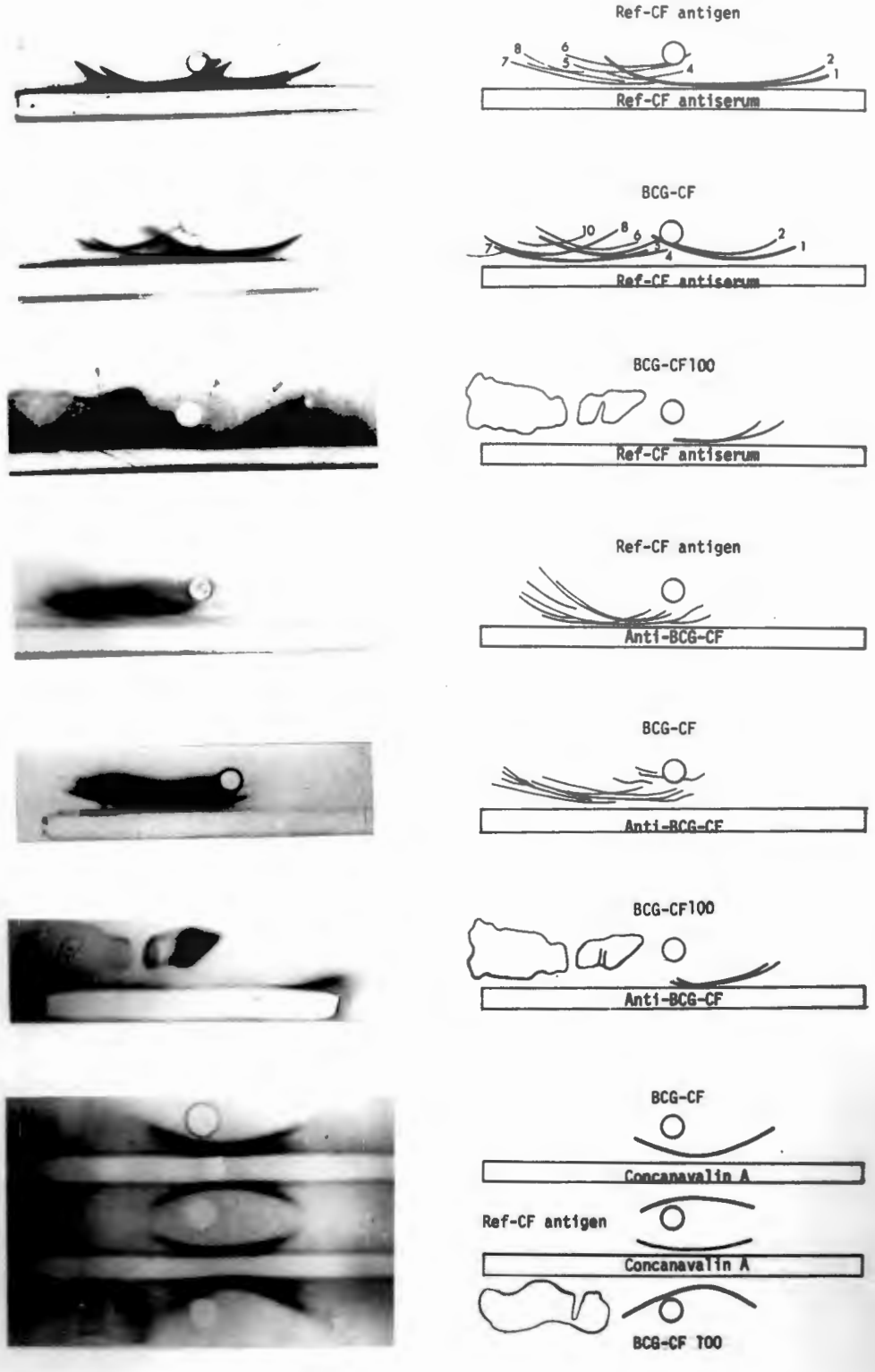


Fig. 3.10 IMMUNOELECTROPHORESIS OF BCG-CF and BCG-CF100

BCG-CF, BCG-CF100 and the Ref-CF antigen preparations were subjected to immunoelectrophoresis according to the procedure described by Janicki et al. (1971). Immunoelectrophoretic patterns were developed with Ref-CF antiserum, anti-BCG-CF and concanavalin A.

TABLE 3.3 IMMUNOELECTROPHORESIS OF BCG-CF AND BCG-CF100

Immunoelectrophoresis patterns of BCG-CF, BCG-CF100 and the Ref-CF antigen preparation were developed with Ref-CF antiserum. The immunoprecipitin bands of the reference system (Janicki et al., 1971) was identified in these preparations according to their migration positions.

Preparation	Reference band number										
	1	2	3	4	5	6	7	8	9	10	11
Ref-CF antigen	+	+	-	+	+	+	+	+	±	±	-
BCG-CF	+	+	-	+	+	+	+	+	-	+	-
BCG-CF100	+	+	-	-	-	-	-	-	-	-	-

of the non-dialysable BCG-CF components were in close agreement with the proportions found by Chaparas and Baer (1964a).

The precipitation of proteins which occurred when BCG-CF was heated to 100°C was related to heating time, the concentration of non-dialysable BCG-CF materials, solvent composition and the pH of the medium. It was shown, however, that the extent to which precipitation would occur under the conditions normally used in mycobacterin production should be very limited or non existent. This is due to the low concentration of the nondialysable materials in the unprocessed filtrate and to the presence of protective medium components. In this respect the extensive heat precipitation of tuberculin proteins reported by Seibert (1928) can be attributed to the very high concentration of the protein solute (approximately 160 mg/ml) and the use of distilled water as a solvent in that author's heating studies.

The factor which has been shown in this investigation to be the most likely to be responsible for thermal protein precipitation during mycobacterin production is the pH of the medium. The pH values of filtrates may vary widely during incubation of cultures and at harvesting may often be acidic in the region of 5 - 6 or even lower (Lesslie et al., 1975). It is therefore probable that the observed relationship between low pH and low protein yield (Kim et al., 1963) is due largely to thermal protein precipitation which is enhanced in acidic media.

The protein fraction of BCG-CF contained more than 34 individual polypeptides, a figure which is comparable with the 36 antigenic components detected by two dimensional immunoelectrophoresis procedures in culture filtrates of M. tuberculosis H37Rv (Wright and Roberts, 1974). The molecular weights of BCG-CF proteins displayed a spectrum from less than 10 000 to greater than 150 000 in accordance with the observations of Baer (1965) that molecules of mycobacterial culture filtrates fall within a wide range of molecular sizes. It was shown however that the native proteins of BCG-CF largely existed in two molecular size groupings of approximately 8 000 - 30 000 and 70 000 - 150 000. Electrophoretic studies of the high molecular weight gel exclusion

chromatography fraction EC1 showed the extent to which aggregated and multisubunit proteins were present in BCG-CF. This fraction displayed a number of discrete protein components, all of which demonstrated retarded mobility, ostensibly due to high molecular weight, when electrophoresis in 10% gels in the PAGE system (Fig. 3.6C). However, when reduced, dissociated and subjected to SDS-PAGE in 10% gels (Fig. 3.6D), many polypeptides were distributed throughout the gel indicating a molecular weight spectrum from very high to very low (less than 10 000). This liberation of polypeptides with a wide range of molecular weights from an essentially high molecular weight fraction following dissociation with SDS and reduction with 2-mercaptoethanol suggests that numerous multisubunit proteins and/or aggregated proteins were present in BCG-CF.

The proteins which remained in solution following heating of BCG-CF at 100°C for 60 min were of low molecular weight. When BCG-CF was heated in BAI medium, some aggregates which formed did not precipitate from solution. This observed stabilization against precipitation which was provided by the presence of medium components could possibly explain the reported isolations of high molecular weight proteins from heated PPD preparations (Landi and Held, 1971; Glenchur et al., 1973), when the proteins of PPD have generally been shown to be of low molecular weight (Green, 1946; Nagai et al., 1974a; Daniel and Janicki, 1978). The majority of proteins of heated BCG culture filtrates were denatured but several low molecular weight components, of which two were prominent, retained their immunoprecipitin and electrophoretic properties following heating. These polypeptides were localized in the gel exclusion chromatography fraction, EC4.

The immunoelectrophoretic studies detected all of the reference system antigens apart from antigen 3 in the Ref-CF antigen preparation. The antiserum of batch 002A used in this study has, however, previously been shown to be unreactive to this antigen (Daniel et al., 1975). The immunoelectrophoresis pattern of BCG-CF closely resembled that of the Ref-CF antigen and immunoprecipitin bands 1,2,4,5,6,7,8 and 10 could be assigned to BCG-CF according to relative migration positions (Table 3.3). The similarity in the antigenic compositions of BCG-CF and the

M. tuberculosis H37Rv Ref-CF antigen preparation is in agreement with similar observations of other workers (Chaparas and Hedrick, 1973; Chaparas, 1975).

The immunoelectrophoretic patterns of BCG-CF and the Ref-CF antigen preparation, when developed with anti-BCG-CF, could not be compared with the patterns obtained with the Ref-CF antiserum. The anti-BCG-CF serum was unreactive to antigens 1 and 2 and showed a different distribution of the anodic antigens (Fig. 3.10). The unreactivity of anti-BCG-CF serum to the polysaccharide antigens 1 and 2 could possibly indicate a difference in response of rabbits in which anti-BCG-CF serum was prepared and goats in which the reference antiserum was prepared (Janicki et al., 1971). The differences in specificity could also be due to the use of different adjuvants. Ref-CF antiserum was prepared to water in oil emulsions of antigen whereas antiserum to BCG-CF was prepared to alum precipitated antigen. It is possible that the polysaccharide fractions of BCG-CF were not precipitated with alum and therefore no exposure to these antigens occurred.

When heated BCG-CF (BCG-CF100) was subjected to immunoelectrophoresis, the anodic migrating components displayed electrophoretic properties characteristic of denatured proteins. The spontaneous precipitation of protein which occurred during electrophoretic separation (Fig. 3.10) prevented the formation of immunoprecipitin lines by any proteins which might have displayed native properties. Cathodic migrating antigens were resistant to the effects of heating and the polysaccharide antigens 1 and 2 were detected in BCG-CF100. No alteration in the distribution of the major polysaccharides of BCG-CF resulted from heating as demonstrated by the development of the immunoelectrophoresis patterns with concanavalin A (Fig. 3.10). It was, however, shown in PAGE studies that two of four protein-associated polysaccharides (glycoproteins or peptidoglycans) of BCG-CF precipitated from solution when heated. The general heat stability of the polysaccharides of BCG-CF found in this study is in agreement with the observations of other authors from studies of culture filtrate polysaccharides of M. tuberculosis (Daniel and Wisnieski, 1970) and of PPD preparations (Daniel and Janicki, 1978).

CHAPTER FOUR

IMMUNOLOGICAL PROPERTIES OF BCG-CF, BCG-CF100 AND BCG-CF(SDS)

4.1 INTRODUCTION

Previous studies of the effect of heat on the immunological properties of mycobacterins have largely been limited to investigations of immunoprecipitin and elicitin properties (Seibert, 1928; Gussoni, 1962; Lind, 1965; Castelnuovo and Morellini, 1965). The immunoprecipitin reaction is dependent on secondary interactions consequent to antigen-antibody binding for the formation of visible immunoprecipitates (Minden et al., 1969). Therefore, although it provides a good qualitative indication of the thermal denaturation of individual mycobacterial antigens, it does not necessarily provide a quantitative measure of conformational alteration.

With the development of highly sensitive immunochemical assays which measure primary binding, antigen-antibody reactions have become useful indicators of conformational alterations such as those found in denaturation reactions of proteins. Vertical and lateral shifts in antigen titration curves, two phenomena whereby such changes may be interpreted, have been demonstrated in micro-complement fixation studies of native and denatured proteins (Levine and Van Vunakis, 1967). A vertical shift is defined as a decrease in maximum antigen-antibody binding at the same antigen concentration originally required for maximum reactivity (Fig. 4.1, curve B). This is considered representative of a decrease in affinity of one or a minority of antigenic determinants for the specific antibody. A lateral shift has been defined as an increase in antigen concentration required to reach maximum reactivity (Fig. 4.1, curve C). This type of shift is indicative of generalized conformational changes marked by an overall reduction in the affinity of antigenic determinants.

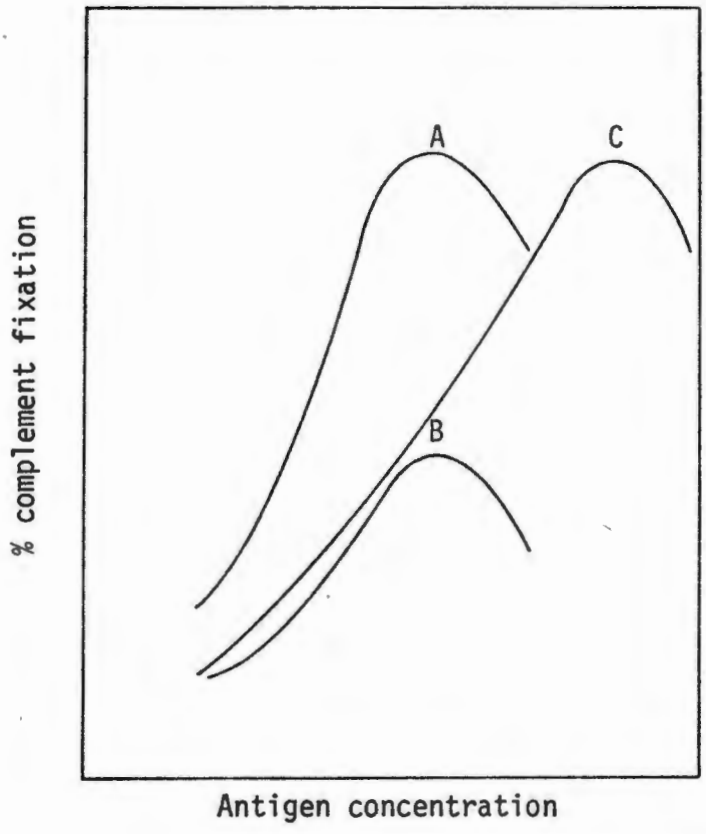


Fig. 4.1 VERTICAL AND LATERAL SHIFTS IN MICRO-COMPLEMENT FIXATION CURVES

Schematic representation of the vertical and lateral shifts in micro-complement fixation curves which accompany conformational changes in the antigen (Adapted from Levine and Van Vunakis, 1967). Curve A represents a titration curve of % complement fixation versus antigen concentration. Curve B represents the titration curve of the same antigen following alteration of the conformation of a portion of the molecule. This curve is vertically shifted in relation to A and an example is found in the hydrolysis of the two C-terminal amino acids of the β-chain of haemoglobin by carboxypeptidase A. Curve C represents the titration curve of the same antigen as in curve A following denaturation with urea which results in a generalized conformational change

The heat stability of the elicitin properties of mycobacterial proteins is not unique to these antigens but rather a property of the immune system (Gell and Benacerraf, 1961). The early work of these authors (Gell and Benacerraf, 1959) demonstrated that native forms of several serum proteins could stimulate delayed hypersensitivity to their heat denatured forms and conversely heat denatured proteins stimulated delayed hypersensitivity to their native forms. Heat denatured proteins were, however, unable to react with antibodies to their native forms in immunoprecipitin reactions and were themselves poor stimulators of antibody production. It is now well established that antibodies react with antigenic determinants having a minimum size of 9 - 12 amino acids and that the recognition of antigen is dependent on its conformation (Arnon, 1974; Ishizaka et al., 1974). The delayed hypersensitivity response requires a larger antigenic determinant with a minimum size of 12 - 18 amino acids, the conformation of which is inconsequential for recognition (Singh et al., 1980).

There is little evidence of any gross differences between the biological activities of heated and unheated mycobacterins but quantitative analyses have revealed some discrepancies. The slopes of dose-response lines of heat concentrated tuberculins (Old Tuberculin) have been shown to be greater than those of PPD-tuberculins which are often subjected to milder heating conditions during preparation (Long et al., 1954; Maxild et al., 1976). This has been a major factor in the establishment of separate international standards for PPD-tuberculins and for Old Tuberculin (Outschoorn, 1971). The nature of the sensitizing antigen may also contribute to the potency differences between preparations. The slopes of dose-response lines of preparations assayed in guinea pigs sensitized with live or heat killed virulent tubercule bacilli, have been shown to be greater than when assayed in guinea pigs sensitized with live BCG (Schneider et al., 1973; Maxild et al., 1976).

In this investigation the changes in the immunological properties of BCG-CF which accompany heat denaturation were investigated by the interfacial ring test (a representative immunoprecipitin test) and by an enzyme-linked immunosorbent assay (micro-ELISA) procedure which measures primary binding. As an additional comparision of native and denatured molecules, the immunological properties of BCG-CF denatured

with SDS and 2-mercaptoethanol were investigated. The elicitin potencies of BCG-CF, BCG-CF100 and BCG-CF(SDS) were examined in order to provide a comparision with the other tests, and proteolytic enzymes were used to confirm that the elicitin potencies of both native and heated BCG culture filtrates were due to protein components. The skin test reactivities of the fractions EC1, EC2, EC3, EC4, EC5 and EC6 which were isolated by gel exclusion chromatography in Section 3.3.3.1 were tested and their heat stability determined.

It might be expected that greater skin reactions would be elicited by antigens in the form originally used for the sensitization of test animals. Consequently BCG-CF would be expected to be more potent than BCG-CF100 in guinea pigs sensitized with live BCG and the reverse would be expected in guinea pigs sensitized with heat killed BCG. Therefore in the comparison of the elicitin potencies of BCG-CF and BCG-CF100 the nature of the sensitizing antigen has been taken into account and these preparations have been assayed in guinea pigs sensitized with live BCG and in guinea pigs sensitized with heat killed BCG.

4.2 MATERIALS AND METHODS

4.2.1 INTERFACIAL RING TEST

Solutions containing 1mg/ml of each of the preparations BCG-CF, BCG-CF100 and BCG-CF(SDS) were diluted in two-fold doubling series and the approximate interfacial ring test endpoints were determined as described in Section 2.11.4. Using a narrow range of dilutions over the endpoint zone, the immunoprecipitin titres were determined in a second titration.

4.2.2 MICRO-ELISA STUDIES

A five-fold dilution series was prepared from a 1mg/ml solution of each

of the BCG-CF, BCG-CF100 and BCG-CF(SDS) preparations, and assayed with the double-antibody sandwich micro-ELISA procedure (Section 2.11.6). As a control, the neutralization of BCG-CF polyvalent antigen by specific antibody was investigated as follows : a five-fold dilution series of BCG-CF was prepared and a sample of each dilution was incubated at 37°C for 60 min with an equal volume of 1/10 diluted anti-BCG-CF serum. Following incubation the neutralized antigen dilutions were assayed for unbound antigen by the micro-ELISA procedure. The neutralizing capacity of anti-BCG-CF was determined as follows: a series of doubling dilutions of anti-BCG-CF was prepared in ELISA diluent and a sample of each was mixed with an equal volume of diluted BCG-CF solution (1 µg/ml). Following incubation at 37°C for 60 min, the quantity of unbound antigen was determined by means of the micro-ELISA system.

4.2.3 TUBERCULIN SKIN TESTS

4.2.3.1 Assay of BCG-CF and BCG-CF100

Assay 1 : Guinea pigs sensitized with live BCG.

Solutions of BCG-CF and BCG-CF100 were each diluted in skin test diluent to a high dose (10 µg/ml) and a low dose (1 µg/ml). These preparations were coded, randomized, and assayed in 12 guinea pigs, previously sensitized with live BCG, by the parallel line assay procedure described in Section 2.11.7.

Assay 2 : Guinea pigs sensitized with heat killed BCG.

The coded and randomized preparations of BCG-CF and BCG-CF100 from Assay 1 were also assayed in eight guinea pigs sensitized with heat killed BCG. A parallel line assay format was employed (Section 2.11.7).

4.2.3.2 Elicitin potencies of BCG-CF, BCG-CF100 AND BCG-CF(SDS)

BCG-CF and BCG-CF100 solutions in skin test diluent (2mg/ml) were mixed with equal volumes of skin test diluent containing 2% (m/v) SDS and 0,2% (v/v) 2-mercaptoethanol. The resultant solutions were diluted to final concentrations of 10 µg/ml in skin test diluent which contained 0,1% (m/v) SDS. Half of each diluted sample was dialysed for 72 hours against skin test diluent to remove unbound SDS. Elicitin activities of the dialysed and the undialysed SDS-denatured samples of BCG-CF and BCG-CF100 were assayed in eight guinea pigs sensitized with heat killed BCG according to the point assay protocol (Section 2.11.7). Undenatured BCG-CF and BCG-CF100 samples (10 µg/ml) and an SDS blank (0,1% m/v) were included as standards.

4.2.3.3 Elicitin properties of thermal precipitates of BCG-CF

Lyophilized precipitates obtained from a BCG-CF solution heated at 100°C for 120 min were dispersed in skin test diluent to a final concentration of approximately 20 µg/ml and two guinea pigs previously sensitized with heat killed BCG were injected intradermally with 0,1 ml volumes. BCG-CF and BCG-CF100 control solutions (10 µg/ml) were also injected at several sites. Skin reactions were inspected at 6 hours, 24 hours and 48 hours post injection.

4.2.3.4 Enzyme digestion studies

BCG-CF and BCG-CF100 were treated with proteolytic enzymes and the skin test activities of these digests were determined. Solutions of BCG-CF and BCG-CF100 were diluted in skin test diluent to concentrations of 10 µg/ml. Samples of 1,8 ml were mixed with 0,1 ml of pronase (0,2% m/v) and incubated at 40°C for 48 hours. Trypsin solution (14 µg/ml) was added to the digests in volumes of 0,1 ml and incubation continued for a further 24 hours. In control samples of BCG-CF and BCG-CF100, enzyme solutions were replaced by skin test diluent. The digested samples and the undigested controls were assayed for skin reactivity in

eight guinea pigs sensitized with heat killed BCG. A BCG-CF standard (10 µg/ml) and an enzyme blank were included in the assay.

4.2.3.5 Elicitin properties of gel exclusion chromatography fractions

Lyophilized samples of each of the fractions EC1, EC2, EC3, EC4, EC5 and EC6 were dissolved in skin test diluent and diluted to final concentrations of 10 µg/ml. A five millilitre sample of each of the fractions was heated at 100°C for 60 min and cooled to ambient temperature. Elicitin activities of unheated and heated samples of the fractions were assayed in eight guinea pigs sensitized with heat killed BCG according to the point assay protocol (Chapter 2.11.7). A BCG-CF standard (10 µg/ml) was included in the assay and three unsensitized guinea pigs were used as negative controls. Skin reaction diameters were recorded at 4 hours, 24 hours,48 hours and 72 hours.

4.3 RESULTS

4.3.1 INTERFACIAL RING TESTS

The maximum dilutions of 1mg/ml solutions of BCG-CF, BCG-CF100 and BCG-CF(SDS) which produced visible immunoprecipitates with anti-BCG-CF serum in the interfacial ring test were 1/167, 1/3 and 1/32 respectively.

4.3.2 MICRO-ELISA STUDIES

Micro-ELISA titration curves obtained with BCG-CF, BCG-CF100, BCG-CF(SDS) and BCG-CF neutralized with antiserum are shown in Fig. 4.2. The lower limit of antigen resolution was of the order of 1 - 10 ng/ml and the absorbance increased exponentially with increasing antigen concentration. At concentrations of greater than 10-100 µg/ml, maximum absorbance occurred as a plateau, indicating antigen excess. No absorbance was

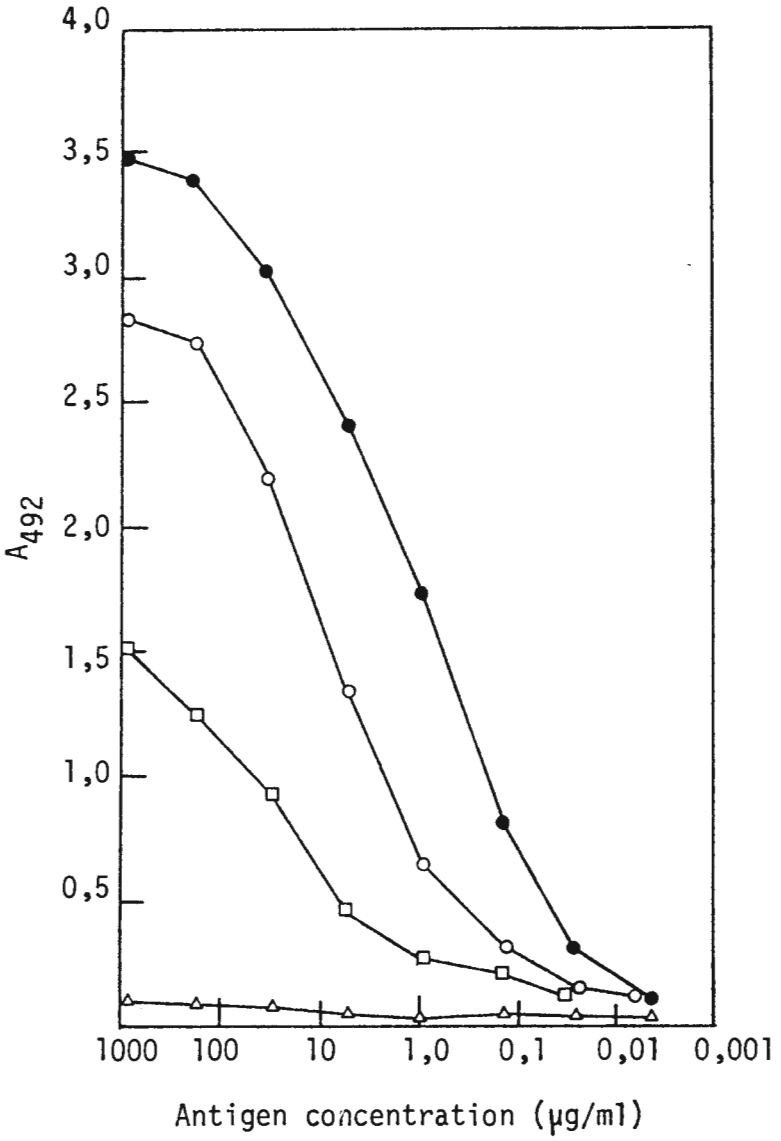


Fig. 4.2 MICRO-ELISA TITRATIONS

BCG-CF ●—●, BCG-CF neutralized with anti-BCG-CF serum △—△, BCG-CF100 □—□ and BCG-CF SDS ○—○ were titrated by the micro-ELISA procedure and A_{492} for each preparation is shown as a function of antigen concentration.

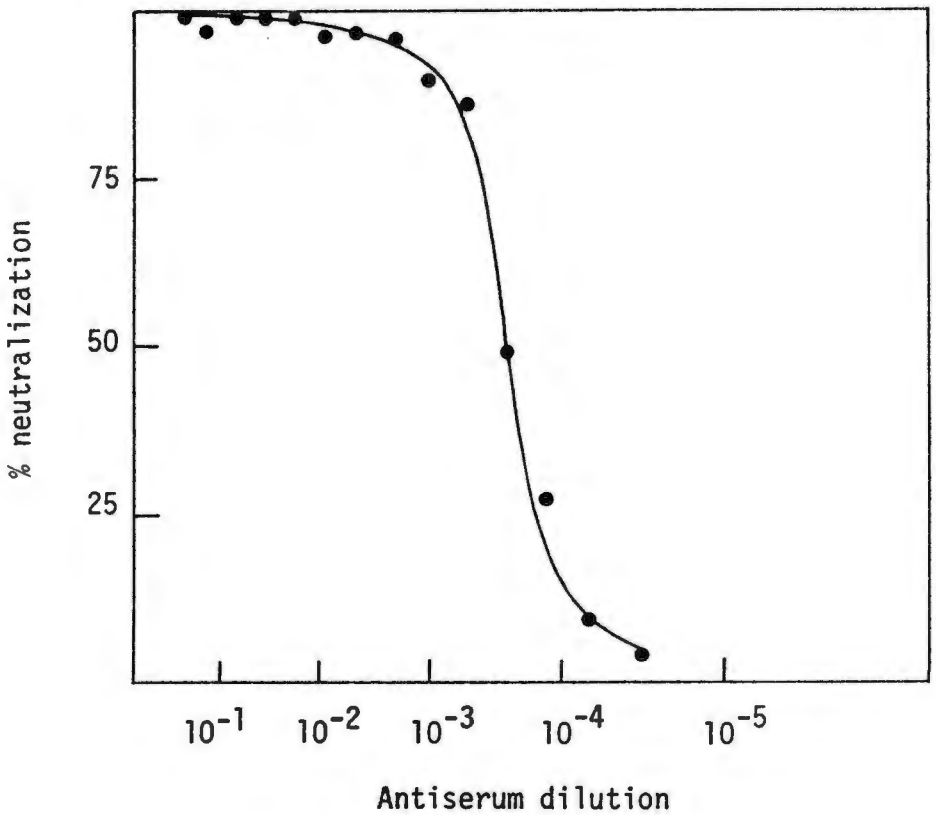


Fig. 4.3 DETERMINATION OF THE NEUTRALIZING CAPACITY OF ANTI-BCG-CF WITH THE MICRO-ELISA PROCEDURE

A series of doubling dilutions of anti-BCG-CF were mixed with equal volumes of BCG-CF polyvalent antigen (1 µg/ml). Following incubation at 37°C for 60 min the unbound antigen was determined by the micro-ELISA procedure. Percentage neutralization is plotted here as a function of antiserum dilution.

shown by the BCG-CF antigen when neutralized with specific antiserum over an antigen concentration range of 10 ng/ml to 1 mg/ml (Fig. 4.2). A neutralization curve in which the percentage neutralization of BCG-CF is plotted against anti-BCG-CF serum dilution at constant antigen concentration is shown in Fig. 4.3. The BCG-CF antigen (1 µg/ml) was effectively neutralized by anti-BCG-CF serum at high dilution and the 50% endpoint appeared at an antiserum dilution of 1/4096. The titration curve of BCG-CF(SDS) was very similar to that of BCG-CF except for lower peak absorbance (Fig. 4.2). The maximum absorbance produced by BCG-CF100 was similarly much less than that of the native preparation and did not attain peak absorbance even at an antigen concentration of 1 mg/ml.

4.3.3 TUBERCULIN SKIN TESTS

4.3.3.1 Potency of BCG-CF100 relative to BCG-CF

Results obtained in the assays of the potency of BCG-CF100 relative to BCG-CF in guinea pigs sensitized with live BCG and in those sensitized with heat killed BCG are shown in Table 4.1 and Fig. 4.4. BCG-CF and BCG-CF100 elicited comparable reactions at 0,1 µg and 1,0 µg dose levels in the guinea pigs sensitized with live BCG. The potency of BCG-CF100 relative to BCG-CF in this assay was 1,28 with fiducial limits of 0,98 and 1,67. The potency of BCG-CF100 was therefore not significantly different from BCG-CF in guinea pigs sensitized with live BCG.

When assayed in guinea pigs sensitized with heat killed BCG, the potency of BCG-CF100 relative to BCG-CF was 0,62 with fiducial limits of 0,38 and 0,99. Heating of BCG-CF therefore resulted in a 38% decrease in potency. However, reference to Fig. 4.4 shows that these differences are very small in terms of total reactivity. That is, a 38% decline in potency represents a reduction in reaction diameter of 0,6 mm or about 3%. It may be concluded that in guinea pigs sensitized with heat killed BCG, no significant differences in potency of BCG-CF and BCG-CF100 existed.

TABLE 4.1 COMPARISION OF SKIN TEST ACTIVITIES OF BCG-CF AND BCG-CF100 IN GUINEA PIGS SENSITIZED WITH LIVE BCG AND GUINEA PIGS SENSITIZED WITH HEAT KILLED BCG.

Relative potencies and fiducial limits were calculated as described in Appendix 2. BCG-CF100 was regarded as the test preparation and BCG-CF served as an assay standard for the calculations.

BCG sensitising antigen	Dose (µg of total solids)	Mean reaction diameter (mm)		Relative potency	Fiducial limits
		BCG-CF	BCG-CF100		
Live	1,0	14,3	15,0		
				1,28	0,98-1,67
	0,1	11,1	11,1		
Heat Killed	1,0	15,5	14,9		
				0,62	0,38-0,99
	0,1	12,7	12,2		

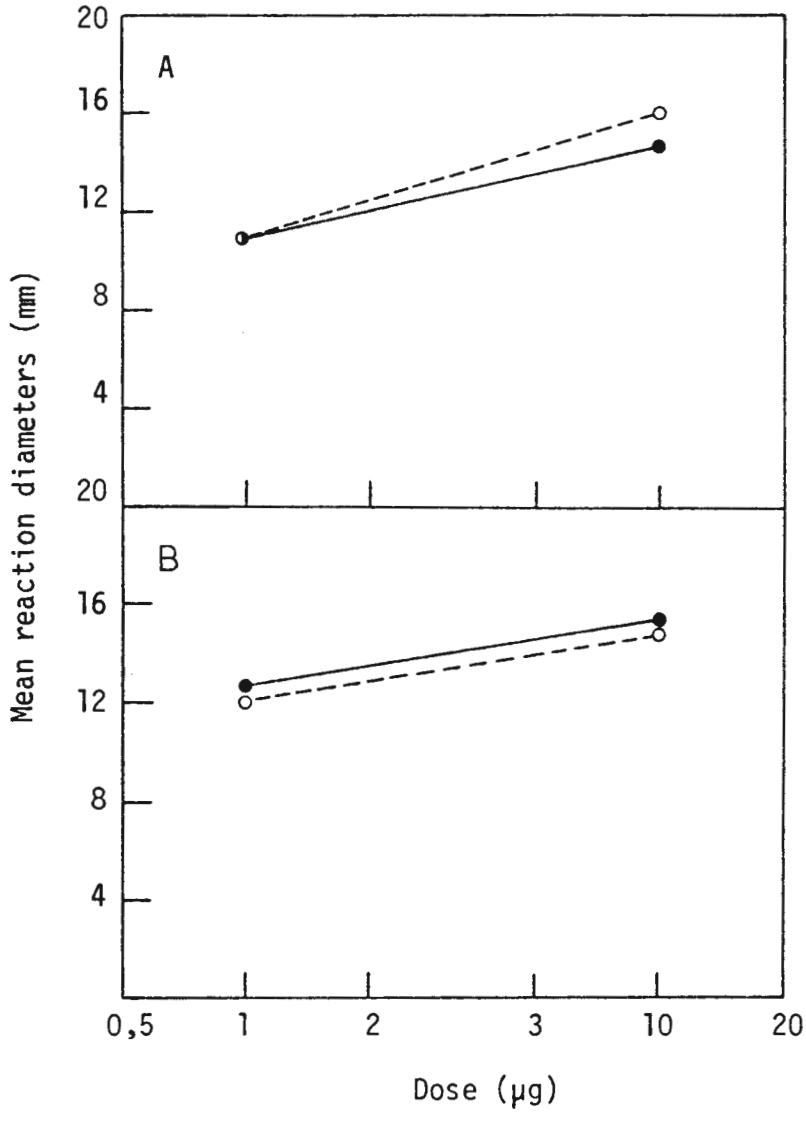


Fig. 4.4 TUBERCULIN SKIN TEST ACTIVITIES OF BCG-CF AND BCG-CF100

BCG-CF ●—● and BCG-CF100 o--o were assayed according to the parallel line protocol in guinea pigs sensitized with live BCG (Panel A) and guinea pigs sensitized with heat killed BCG (Panel B). Mean reaction diameters are plotted against antigen dose.

4.3.3.2 Effect of SDS-denaturation on elicitin activity

This investigation showed that reduction with 2-mercaptoethanol and denaturation with SDS did not affect skin test activities of BCG-CF and BCG-CF100. Mean reaction diameters for BCG-CF, BCG-CF100 and their SDS-denatured forms in Table 4.2 show no significant differences.

4.3.3.3 Elicitin properties of thermal precipitates of BCG-CF

Sensitized guinea pigs injected with the heat precipitated materials of BCG-CF and with control BCG-CF and BCG-CF100 solutions, showed no skin reactions at six hours. At 24 hours tuberculin lesions were present at all injection sites but the gross appearances of those elicited by precipitates differed from those caused by soluble BCG-CF and BCG-CF100. The area of reaction was smaller than that resulting from an equivalent amount of soluble tuberculin and induration was more prominent. Erythema which was marked at sites injected with soluble preparations, was almost absent in the case of heat coagulated protein. The reduced reaction area but greater degree of induration may reflect retention of the insoluble protein at the injection site as opposed to dispersion of the soluble antigen.

4.3.3.4 Effect of enzyme digestion on elicitin properties of BCG-CF and BCG-CF100

The skin sites of guinea pigs injected wtih enzyme digested and control samples of BCG-CF and BCG-CF100 were inspected at 4 hours, 24 hours and 48 hours. At 24 hours the enzyme blanks and enzyme digested preparations produced small, faintly erythematous zones with no induration, but at 48 hours these reactions were absent. The undigested control solutions of BCG-CF and BCG-CF100 produced large, characteristic tuberculin reactions with marked erythema and induration. No skin reactions were observed in unsensitized guinea pigs apart from faintly erythematous areas which were produced by the enzyme-containing preparations at 24 hours. The mean skin reaction diameters at 24 hours are given in Table 4.4.

TABLE 4.2 EFFECT OF DENATURATION WITH SDS AND 2-MERCAPTOETHANOL ON SKIN TEST ACTIVITIES OF BCG-CF AND BCG-CF100.

BCG-CF and BCG-CF100 proteins were reacted with SDS (0,1%, m/v) and 2-mercaptoethanol (0,1% v/v). These two preparations and equivalent samples from which SDS was removed by dialysis were assayed in eight guinea pigs. The resultant skin reactions are compared with those elicited by untreated BCG-CF and BCG-CF100.

Preparations	Treatments	Mean reaction diameter (mm)	
		24 hour reaction	48 hour reaction
BCG-CF	untreated	13,0	12,5
	SDS	12,9	12,1
	SDS (dialysed)	12,8	12,3
BCG-CF100	untreated	12,6	11,9
	SDS	12,7	12,3
	SDS (dialysed)	12,6	11,6
Buffer	SDS	1,3	0
	SDS (dialysed	2,9	0

TABLE 4.3 EFFECT OF ENZYME DIGESTION ON SKIN TEST ACTIVITIES OF BCG-CF AND BCG-CF100.

Comparision of the mean reaction daimeters of BCG-CF and BCG-CF100 following digestion with pronase and trypsin with the reactions of undigested samples of these preparations.

Preparation	Mean reaction diameters (mm)			
	Undigested preparations		Enzyme digested preparations	
	24 hours	48 hours	24 hours	48 hours
BCG-CF	13,7	13,3	7,2	0
BCG-CF100	13,3	12,7	5,8	0
BCG-CF standard	13,8	13,6	-	-
Enzyme blank	-	-	7,9	0

TABLE 4.4 EFFECT OF HEAT ON SKIN TEST ACTIVITIES OF GEL EXCLUSION CHROMATOGRAPHY FRACTIONS

Unheated and heated (100°C for 60 min) samples of the fractions EC1, EC2, EC3, EC4, EC5 and EC6 were assayed in guinea pigs sensitized with heat killed BCG and the 24 hour mean reaction diameters are compared below.

Fraction	Mean reaction diameter (mm)	
	Unheated	Heated
EC1	11,4	11,5
EC2	13,9	12,7
EC3	14,4	15,8
EC4	16,5	16,1
EC5	4,0	3,7
EC6	1,2	0,8
BCG-CF standard	14,4	-

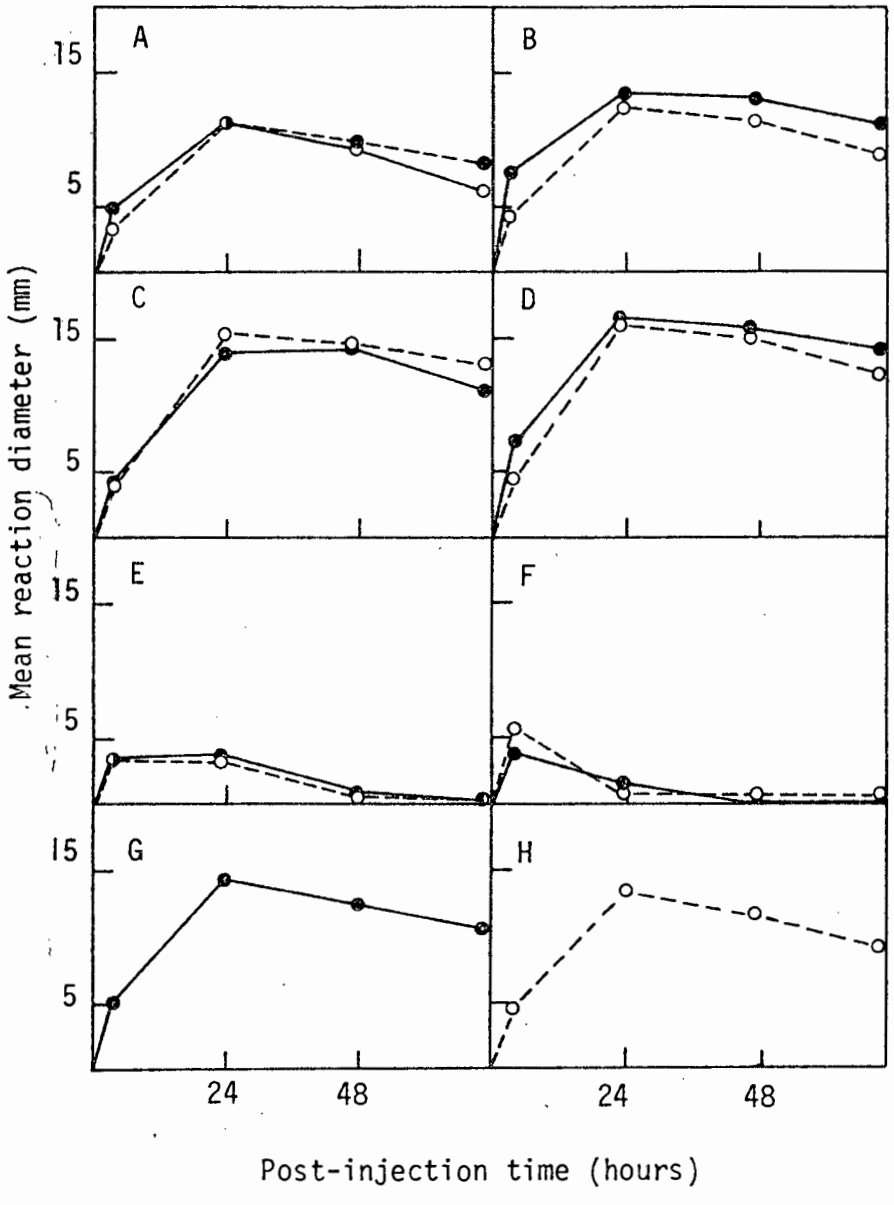


Fig. 4.5 ELICITIN ACTIVITIES OF NATIVE AND HEATED GEL EXCLUSION CHROMATOGRAPHY FRACTIONS OF BCG-CF.

A sample of each of the fractions EC1 (Panel A), EC2 (Panel B), EC3 (Panel C), EC4 (Panel D), EC5 (Panel E), EC6 (Panel F) and BCG-CF (Panels G & H) diluted to 10 µg/ml in skin test diluent was heated at 100°C for 60 min. Native, ●——● and heated, o-----o solutions were assayed in eight guinea pigs sensitized with heat killed BCG and mean reaction diameters were determined 4, 24, 48 and 72 hours post injection.

4.3.3.5 Elicitin properties of gel exclusion chromatography fractions

The elicitin activities of unheated and heated samples of the fractions EC1, EC2, EC3, EC4, EC5 and EC6 are compared in Table 4.4 and Fig. 4.5. No reactions were stimulated in unsensitized guinea pigs by any of the six fractions. In sensitized guinea pigs, considerable reactions were elicited by fractions EC1, EC2, EC3 and EC4. However, in comparison, the skin reactivities of fractions EC5 and EC6 were small. The reactions provoked by heated samples of the six fractions were not significantly different from those elicited by the native fractions (Table 4.5).

4.4 DISCUSSION

A summary of the antigen-antibody reactions and elicitin properties of BCG-CF, BCG-CF100 and BCG-CF(SDS) is given in Table 4.5. It can be seen that the heated antigens of BCG-CF100 had markedly reduced capacities to produce immunoprecipitates with antibody. By way of illustration it was calculated that a mass of BCG-CF100 approximately 50 times that of BCG-CF was required to produce detectable immunoprecipitation with anti-BCG-CF. The SDS denatured preparation, BCG-CF(SDS) showed a similar reduction in the capacity to form immunoprecipitates with anti-BCG-CF serum, and a mass of BCG-CF(SDS) five times the mass of BCG-CF was required to obtain equivalent immunoprecipitation.

The micro-ELISA system which was used was sensitive, reproducible and high absorbances were obtained at very low antigen concentrations. This was no doubt due to the use of polyvalent antiserum where the proportion of IgG molecules reacting with antigen is very much greater than in a system where monovalent IgG is used. The titration curves obtained for BCG-CF, BCG-CF100 and BCG-CF(SDS) were analogous with those described by Levine and Van Vunakis (1967) for native and denatured pepsin and pepsinogen in micro-complement fixation titrations. Since both the micro-ELISA and micro-complement fixation procedures measure primary binding of antigen by specific antibody, it is probable that the vertical

TABLE 4.5 COMPARISON OF ANTIGENIC REACTIVITIES OF BCG-CF, BCG-CF100 AND BCG-CF(SDS) IN IMMUNOPRECIPITIN, MICRO-ELISA AND TUBERCULIN SKIN TEST SYSTEMS

Immunoprecipitation is shown as the inverse mass of antigen required to produce a visible immunoprecipitate with anti-BCG-CF serum as a ratio of the activity of BCG-CF in the interfacial ring test. Figures quoted for the micro-ELISA assay represent the ratio of maximum absorbance of each preparation relative to the maximum absorbance of BCG-CF. Skin test results reflect the approximate potency relative to BCG-CF in guinea pigs sensitized with heat killed BCG. These estimates were calculated from data in Table 4.2.

Antigen	Immunoprecipitation (Interfacial ring test)	Micro-ELISA	Tuberculin skin test potency
BCG-CF	1,0	1,0	1,0
BCG-CF100	0,02	0,4	1,0
BCG-CF(SDS)	0,2	0,8	1,0

and lateral shifts in the two systems are indicative of the same conformational alterations. Although polyvalent antigens and a polyvalent antiserum were used in this study, a broad indication of the average conformational changes which accompanied SDS-denaturation and heat denaturation of BCG-CF proteins was obtained.

The heat denatured preparation BCG-CF100 produced a micro-ELISA titration curve which displayed a vertical and a lateral shift relative to the native preparation, BCG-CF. These shifts were comparable with the shifts displayed in micro-complement fixation curves of heat denatured pepsin and pepsinogen (Gerstein et al., 1963). This suggests that when BCG-CF proteins were heated a portion of the total antigenic determinants was irrevisibly inactivated and that a general decrease occurred in the affinity of those determinants which remained functional.

Sodium dodecyl sulphate is known to bind with proteins resulting in denaturation and the formation of ellipsoidal shaped molecules in which the secondary structure is largely α-helical (Fish et al., 1970). These conformational alterations accompanying SDS denaturation may largely be reversed when the detergent is removed (Weber and Osborn, 1975). The small downward vertical shift which was seen when the titration curve of BCG-CF (SDS) was compared with that of BCG-CF, showed that denaturation with SDS and its subsequent removal irreversibly denatured a limited number of antigenic determinants. The remainder, however, retained their affinity for the specific antibody.

The sensitivity with which heat denatured antigens were detected in the micro-ELISA system was much greater than with the immunoprecipitin reaction (Table 4.5). This difference might be due to the direct detection of antigen-antibody binding in the micro-ELISA system as opposed to the immunoprecipitin reaction where the detection system depends on processes secondary to antigen binding. It is probable that extensive binding of heat denatured antigen took place in immunoprecipitin assays but that the secondary formation of macroscopic aggregates did not occur. Since the proteins of the heated culture filtrate preparation were of low molecular weight and had fewer antigenic determinants per molecule, it is possible that the heat

denatured antigens had insufficient valency, or antibody binding sites for cross-linking to occur.

For the study of the effect of heat on the elicitin properties of BCG-CF proteins, two approaches could have been followed. Firstly samples of a BCG-CF solution could have been heated for various time intervals and the potencies of these solutions determined relative to BCG-CF. Since the proteolytic enzyme digestion studies confirmed that protein is responsible for virtually all the elicitin potency of unheated and heated BCG-CF solutions and since heat coagulated materials retain some activity, it would be expected that the potency might be reduced according to the amount precipitated from solution. In this way an inverse relationship between protein precipitation and elicitin potency might have been expected. Alternatively, the potency of the materials in heated and unheated culture filtrates could have been compared directly on the basis of mass. The latter course was adopted for this investigation because it would give information on any changes in the intrinsic properties of the heated materials. It was found in both qualitative and quantitative studies that denaturation caused no changes of any significance in the elicitin activity of BCG-CF proteins regardless of whether assayed in guinea pigs sensitized with live or heat killed BCG. The slopes of dose response lines for both native and heat denatured BCG-CF preparations were greater when assayed in guinea pigs sensitized with live BCG than in guinea pigs sensitized with heat killed bacilli. But, at the dose levels used in the assay (0,1 µg and 1 µg), the reactions elicited in those guinea pigs sensitized with heat killed BCG were greater than those sensitized with live BCG. This is probably attributable to the use of Freund's adjuvant for the injection of guinea pigs with the heat-killed sensitizing antigen.

The tuberculin skin reactions elicited by native and heated samples of the fractions EC1, EC2, EC3, EC4 and the BCG-CF standard were typical of a delayed hypersensitivity response with characteristic delayed onset, peak intensity at approximately 24 hours and slow regression. The reactions of fractions EC5 and EC6 were different in character with peak intensity at six hours and rapid regression. This is characteristic of non-specific irritation or of a very mild immediate type hypersensitivity

response (Schroff et al., 1980). The low molecular weight polysaccharide of fraction EC5 which accounted for a large proportion of the total polysaccharides of BCG-CF was therefore not active as a delayed hypersensitivity elicitin.

CHAPTER FIVE

ISOLATION AND CHARACTERIZATION OF PROTEINS FROM BCG-CF

5.1 INTRODUCTION

In this Chapter the isolation of heat stable and heat sensitive immunoprecipitin protein antigens from BCG-CF and the subsequent characterization of these components by electrophoresis, immunoelectrophoresis, gel immunoprecipitation, ultracentrifugation, amino acid analysis and tuberculin skin test assay procedures is described.

Fractions EC1 and EC4 obtained by gel exclusion chromatography of BCG-CF (Section 3.3.3.1) were used as starting materials. Fraction EC1 was selected because it contained a number of high molecular weight proteins representative of those which were irreversibly denatured on heating. Fraction EC4 was selected because it contained low molecular weight culture filtrate components, several of which displayed resistance to heating. Another feature of fraction EC4 was that although it contained the largest chromatographic protein peak, the number of electrophoretic and antigenic components was relatively small.

Fractionation techniques used included ion exchange chromatography and preparative polyacrylamide gel electrophoresis (preparative PAGE). DEAE-Sephadex was initially used but it was found unsuitable due to contraction of the gel bed with increasing buffer concentration. It was replaced by DEAE-cellulose which provided a satisfactory flow rate and did not display bed shrinkage. The use of isoelectric focussing was considered but rejected due to the strong likelihood of isoelectric precipitation of proteins (Moulton et al., 1971). Preparative PAGE was the most efficient separation technique and together with ion-exchange chromatography provided isolation of several purified fractions and purified polypeptides. Gel immunoprecipitin and analytical PAGE systems were utilized to monitor the isolation of heat stable and heat sensitive antigens from fractions EC1 and EC4.

Biophysical and immunological procedures were used to establish the degree of purity of the fractionated materials and to determine the number of proteins and subunit polypeptides which they contained. The molecular weights of the isolated polypeptide components were determined by the SDS-PAGE procedure described in Section 3.2.4 and by sedimentation equilibrium ultracentrifugation. The latter technique depends on centrifugation at relatively low rotor velocity over an extended period resulting in the formation of an equilibrium condition in which the rate of sedimentation of solute particles is balanced by the rate of back diffusion of these particles. The development of a mathematical expression relating molecular weight to the concentration of solute throughout the cell at sedimentation equilibrium is attributed to Svedberg, who derived the same expression using two independent approaches: a kinetic transport approach incorporating sedimentation and diffusion relationships and a thermodynamic approach (Haschemeyer and Haschemeyer, 1973). The basic mathematical equation derived from these considerations is

$$M = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{dc}{dr} \qquad \text{equation 5.1}$$

where M is the molecular weight of the solute component, $\bar{v}$, the partial specific volume (ml/g), ρ, the density of the solvent (g/ml), ω, the rotor velocity (radians/sec), R, the universal gas constant (ergs/ degree/ mole), T, absolute temperature, c, the concentration of the solute (g/100ml) and, r, the distance from the centre of rotation (cm).

In practice, the change in solute concentration across the centrifuge cell (dc/dr) is determined as an interference fringe shift and is expressed in interference fringe units, C(r). The initial concentration of the solute is determined in interference fringe units by an accompanying synthetic boundary run in which solvent is layered over the protein solution and the interference fringe shift, j_{sb}, at the interface of the two phases is determined. This provides initial solute concentration, j(a), from the relation

$$j(a) = \Delta j_{sb} - \frac{r_b^2(j_{eq} - r_b\int^{r_a} r^2 dj)}{r^2_b - r^2_a} \qquad \text{equation 5.2}$$

where $\triangle j_{sb}$ represents the total interference fringe shift, j, the integral number of interference fringes, and r_a, r_b, and r_n represent the distances from the centre of rotation to the meniscus, the bottom of the solution column and to each interference fringe respectively.

For reliable molecular weight determinations the term $(1 - \bar{\nu}\rho)$ must be accurately known since an error of 1% in estimation of this value may result in an error of at least 3% in molecular weight determinations (Charlwood, 1957). Partial specific volume, $\bar{\nu}$, is described as the volume increase obtained by addition of one gram of dry isoionic protein to a large excess of solvent (Haschemeyer and Haschemeyer, 1973). The partial specific volume is valid for non-interacting two component systems but for more complex systems it is replaced by apparent isopotential specific volume, ϕ which accounts for preferential binding of solvent and solutes such as salts and detergent molecules to the protein (Van Holde, 1975). This is determined from the equation

$$\phi = \frac{1}{\rho_o}\left(1 - \frac{\Delta\rho}{C_2}\right) \qquad \text{equation 5.3}$$

where ρ_o is the solvent density, $\triangle \rho$ is the difference between densities of the macromolecule solution and it's solvent at dialysis equilibrium and C_2 is the concentration of protein (g/100 ml).

For experimental purposes equation 5.1 may be rewritten in a form which substitutes apparent isopotential specific volume for partial specific volume and concentration is expressed in interference fringe units.

$$M = \frac{RT}{(1 - \phi\rho)\omega^2} \cdot \frac{d\ln C(r)}{dr^2} \qquad \text{equation 5.4}$$

Protein molecular weight is calculated from the slope of a plot of $d\ln C(r)/dr^2$ which is linear for pure non-interacting ideal proteins.

5.2 MATERIALS AND METHODS.

5.2.1 ION EXCHANGE CHROMATOGRAPHY

Lyophilized samples (100 mg) of fractions EC1 and EC4 were dissolved in 50 mM Tris-HCl, pH7 starting buffer and subjected to ion exchange chromatography on DEAE-cellulose as described in Section 2.10.2. Components were eluted with a Tris-HCl gradient from 50 mM to 0,5 M, samples were collected and A_{260} was read. Protein concentrations were determined by the Lowry method and carbohydrate concentrations of a number of samples were determined by the anthrone method. The elution of antigens was established by the gel immunoprecipitin reaction and skin test reactivity was qualitatively determined by injection of 0,1 ml volumes of eluate diluted 1/10 into single sites on the shaved flanks of one to several sensitized guinea pigs.

5.2.2 PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS.

Appropriate pooled materials from ion exchange chromatography were concentrated by pervaporation, dialysed against PAGE sample solution and subjected to preparative PAGE as described in Section 2.9.2. Isolated components were concentrated by pervaporation, dialysed against 50 mM Tris-HCl buffer and stored at -20°C.

5.2.3 ANALYSIS OF ISOLATES

5.2.3.1 Polyacrylamide gel electrophoresis

Purified fractions were analysed by the PAGE and SDS-PAGE procedures described in Section 2.9.1. Effects of heat on the electrophoretic properties of purified fractions of BCG-CF were investigated as follows. A sample of each at a concentration of 0,1-1 mg/ml in 50 mM Tris-HCl was drawn up and sealed in a capillary tube and heated at 100°C for 60 min. Any precipitates which appeared were removed by

centrifugation, and the supernatant materials were mixed with a drop of double strength electrophoresis sample solution and subjected to PAGE or SDS-PAGE. The molecular weights of polypeptide components of the BCG-CF isolates were determined by comparison of relative mobility data with those of protein standards as described in Section 3.2.3. A best fit regression coefficient was determined by the least squares method described in Appendix A3.

5.2.3.2 Gel immunoprecipitation

Native and heated (100°C for 60 min) samples of purified BCG-CF antigens were investigated by the gel immunoprecipitin technique with anti-BCG-CF serum. Heated samples were prepared in capillary tubes as described in Section 5.2.3.1.

5.2.3.3 Immunoelectrophoresis

Four isolates were investigated with the reference immunoelectrophoretic system described in Section 2.11.5. Solutions of these isolates at a concentration of 1 mg/ml were dialysed overnight against a buffer containing 3 mM barbital, 17 mM sodium barbital, and 1,7 mM calcium lactate. Volumes of 5 µl were applied to immunoelectrophoresis slides and precipitin lines were developed with either anti-BCG-CF, Ref-CF antiserum or Ref-CE antiserum.

5.2.3.4 Amino acid analysis

Samples of BCG-CF isolates were extensively dialysed against distilled water, lyophilized and submitted to Prof. C. van Holt of the Biochemistry Department, University of Cape Town for amino acid analysis. Hydrolysis in constant boiling (6M) HCl proceeded for 24 hours at 110°C and the digests were subjected to analysis on a Beckman 116 automatic amino acid analyser according to the procedure of Spackman, Stein and Moore (1958). Approximate tryptophan contents were estimated from UV absorbance spectra in 10 mM Tris-HCl, pH7 according to the equation

$$A_{288} = 4515\ M_{trp} + 385\ M_{tyr} \qquad \text{equation 5.5}$$

where M_{trp} and M_{tyr} represent the concentrations of tryptophan and tyrosine in moles per litre (Haschemeyer and Haschemeyer, 1973).

5.2.4. SEDIMENTATION EQUILIBRIUM ULTRACENTRIFUGATION

5.2.4.1 Centrifugation procedure

Procedures for sedimentation equilibrium and synthetic boundary centrifugation described by van Holde (1967) and Chervenka (1969) were followed. The sample for centrifugation was prepared by dialysis to equilibrium against several changes of 50 mM Tris-HCl containing 0,1% (m/v) SDS. Final washings at dialysis equilibrium were collected and used as a solvent control. A double sector cell with a 12 mm optical path, synthetic boundary centrepiece, and sapphire windows was used for centrifugation procedures. Levels of the solute and solvent columns were respectively raised from the centrepiece bottom by addition of 0,2 ml and 0,1 ml of FC-43 fluorocarbon oil. A 0,1 ml volume of the sample solution was pipetted into the right hand sector and 0,12 ml of solvent into the left hand sector of the cell. Centrifugation was performed for 24 hours in an An-D rotor at 22 000 rev/min and temperature equilibrated at 23,7°C. At sedimentation equilibrium, Rayleigh interference patterns were photographed and the run terminated.

A synthetic boundary run to determine the solute concentration as an interference fringe shift was performed with the same sample. The centrifuge cell was gently tilted several times to redistribute solute components, and the solvent sector was filled with the appropriate solvent. The cell was returned to the rotor and centrifuged at 8 000 rev/min. During acceleration the interference pattern was observed through the viewer and several photographs were taken as soon as the fluid menisci were equalized in the two sectors.

Synthetic boundary and sedimentation equilibrium plates were analysed with a Nikon comparator and the meniscus concentration, j(a) was calculated from equation 5.2. This result was used to calculate the value of C(r) for each interference fringe position, r, across the centrifuge cell and used for the calculation of molecular weight according to equation 5.4.

5.2.4.2 Determination of apparent isopotential specific volume

The apparent isopotential specific volume of the polypeptide under investigation was calculated from measurements of microdrop density according to Polson et al. (1980). With this procedure the density difference between the solute at unit concentration and it's equilibrium dialysed solvent is determined by the rate of microdrop sedimentation in a calibrated sedimentometer column filled with hydrophobic organic fluids.

The sedimentometer consisted of a vertical, cylindrical glass column with a sealed lower extremity (Fig. 5.1). Hydrophobic sedimentation fluid was prepared by mixing equal volumes of silicone fluid (Dow Corning, 200 fluid CS) and paraffin (Merck, DAB7, NFXIV). The density of this mixture was adjusted approximately to that of water by addition of 1,2-dichlorobenzene (Merck, density 1,3) until a drop of distilled water would settle slowly. Temperature was stabilized at 22,5°C by means of a water jacket within which the column was mounted. Two photoelectric sensors were fixed at sites within the waterjacket with an accurately measured interval of 15 cm between them. These sensors were connected to an automatic timer which was triggered and stopped by sedimentation of a microdrop across the sensor interval. Microdrop samples (10 µl) were formed and released below the surface of the sedimentation fluid by a micrometer controlled syringe.

Volumetrically prepared solutions of NaCl in distilled water were used to calibrate the sedimentometer column and densities of solute and solvent solutions were calculated. Protein concentration of the solute was determined from a calibration graph of protein microdrop sedimentation velocity versus protein concentration for a bovine serum albumin standard.

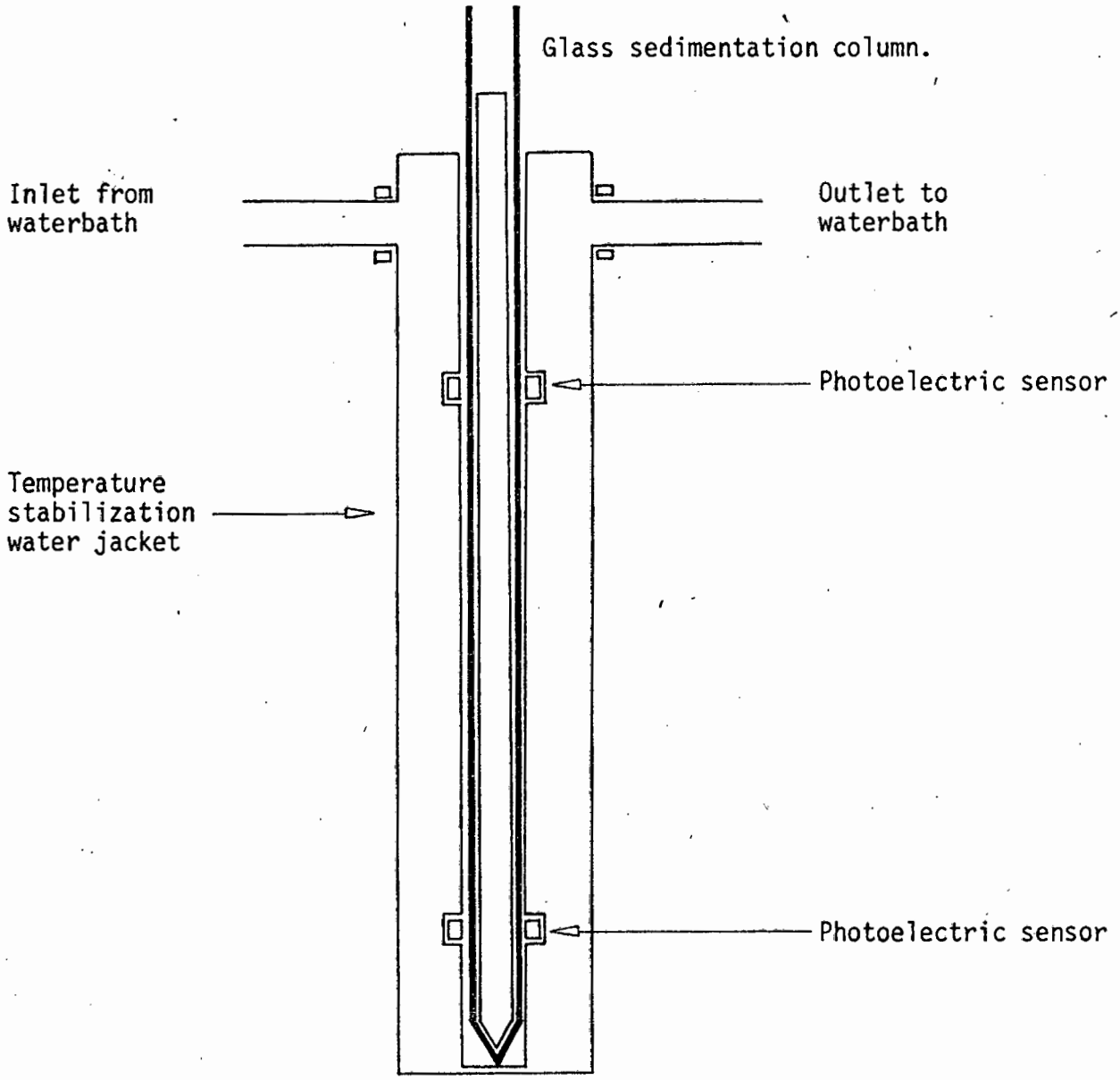


Fig. 5.1 SEDIMENTOMETER APPARATUS USED FOR DENSITY DETERMINATIONS BY THE MICRODROP TECHNIQUE ACCORDING TO POLSON et al. (1980).

The glass sedimentation column was filled with fluid silicone, paraffin and 1,2-dichlorobenzene.

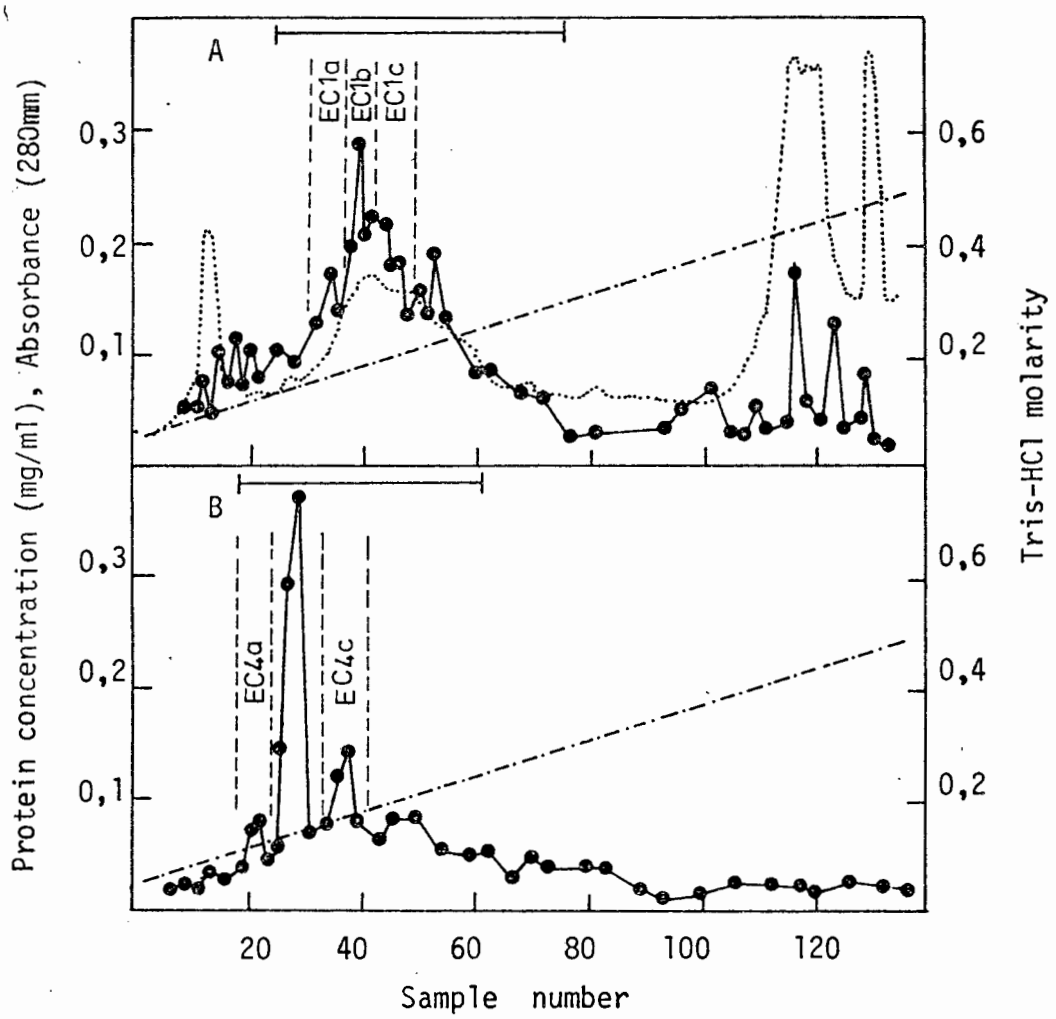
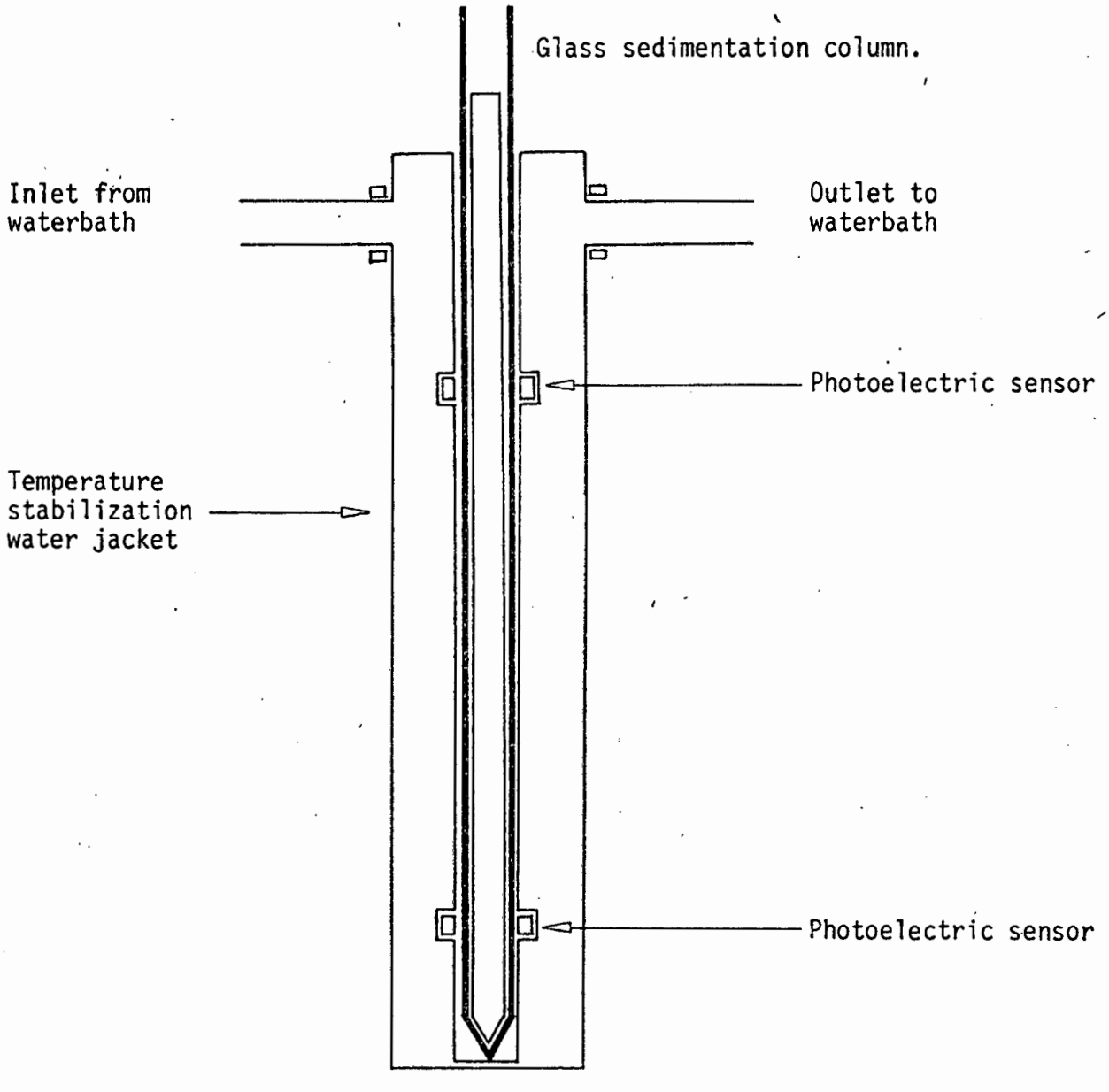


Fig. 5.2 ION EXCHANGE CHROMATOGRAPHY ON DEAE-CELLULOSE

Fractions EC1 (Panel A) and EC4 (Panel B) were eluted from DEAE-cellulose with a Tris-HCl buffer concentration gradient (-·-·-·-) from 50 mM to 0,5 M at pH7. Protein concentration, ●——●; A_{260}, ·····; and areas of elution profile containing tuberculin skin test elicitins, ⊢——⊣ are indicated. Fractions which were collected are indicated by broken lines.



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The glass sedimentation column was filled with fluid silicone, paraffin and 1,2-dichlorobenzene.

These results were substituted into equation 5.3 for the calculation of the apparent isopotential specific volume.

5.2.5 TUBERCULIN SKIN TESTS

Native and heated samples of purified BCG-CF fractions were assayed for tuberculin skin test activity according to the point assay design described in Section 2.11.7. Six guinea pigs sensitized with heat killed BCG and two unsensitized negative control animals were included in the assay. The protein concentrations of solutions of the isolates were determined by the Lowry method and these were accordingly diluted with skin test diluent to final concentrations of 10 µg/ml. A 2 ml sample of each diluted fraction was sealed in a glass vial and heated at 100°C for 60 min. Heated and unheated samples were coded, randomized and injected in 0,1 ml volumes. Skin reactions were measured 24 hours later.

5.3 RESULTS

5.3.1. ISOLATION OF COMPONENTS FROM FRACTION EC1

5.3.1.1 Fractionation of EC1

The elution of components of fraction EC1 from DEAE-cellulose is shown in Fig. 5.2 and in the flow diagram in Fig. 5.3. The A_{260} trace showed a sharp peak at the front of the elution profile, a broad central peak and two large peaks toward the tail. Polysaccharide eluted in the front peak and brown material characteristic of fraction EC6 eluted in the tail peaks. Proteins were largely associated with the central A_{260} peak and were not well resolved according to PAGE investigations. Tuberculin skin test activity and immunoprecipitin antigens were associated only with samples from the central protein peak. Preparative PAGE was used to purify protein components from this peak as shown in Fig. 5.3 and three fractions which were labelled EC1a, EC1b and EC1c were obtained.

These results were substituted into equation 5.3 for the calculation of the apparent isopotential specific volume.

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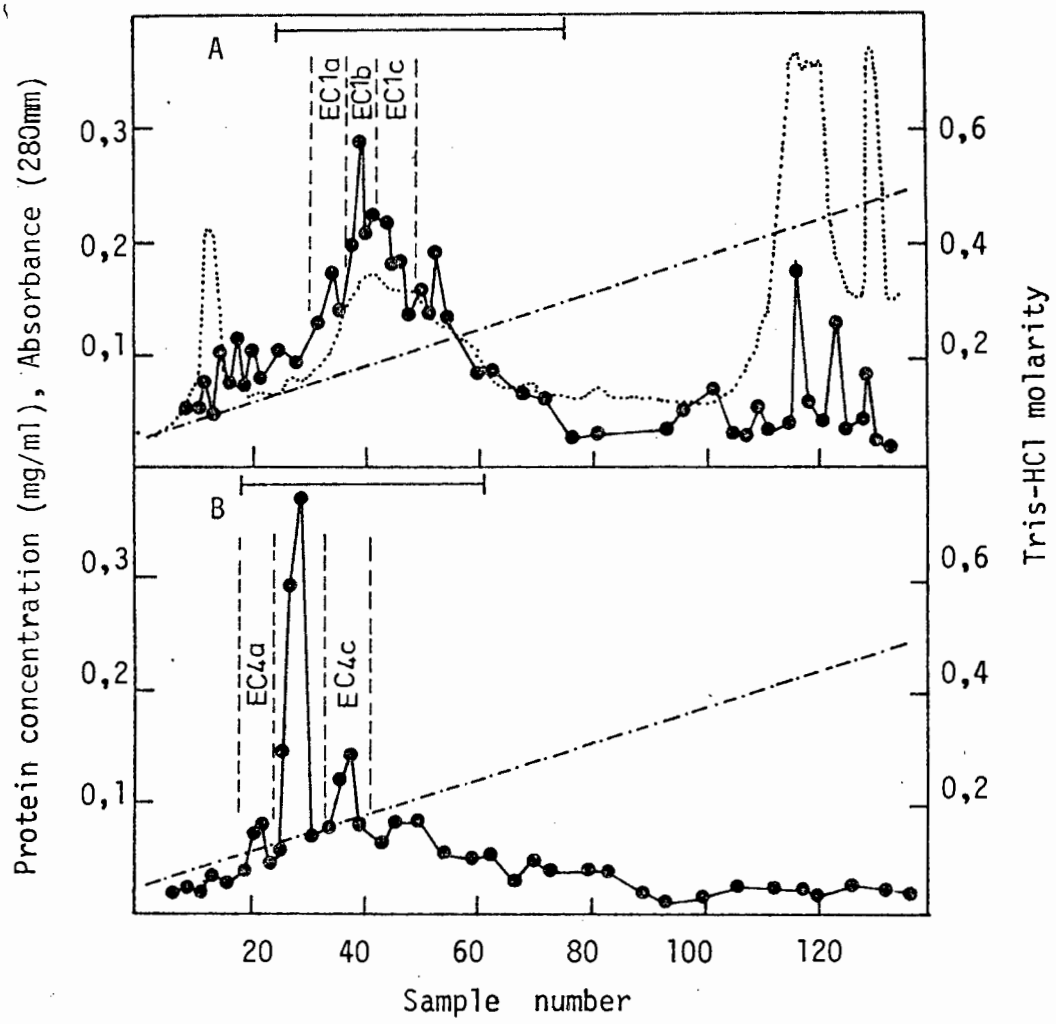
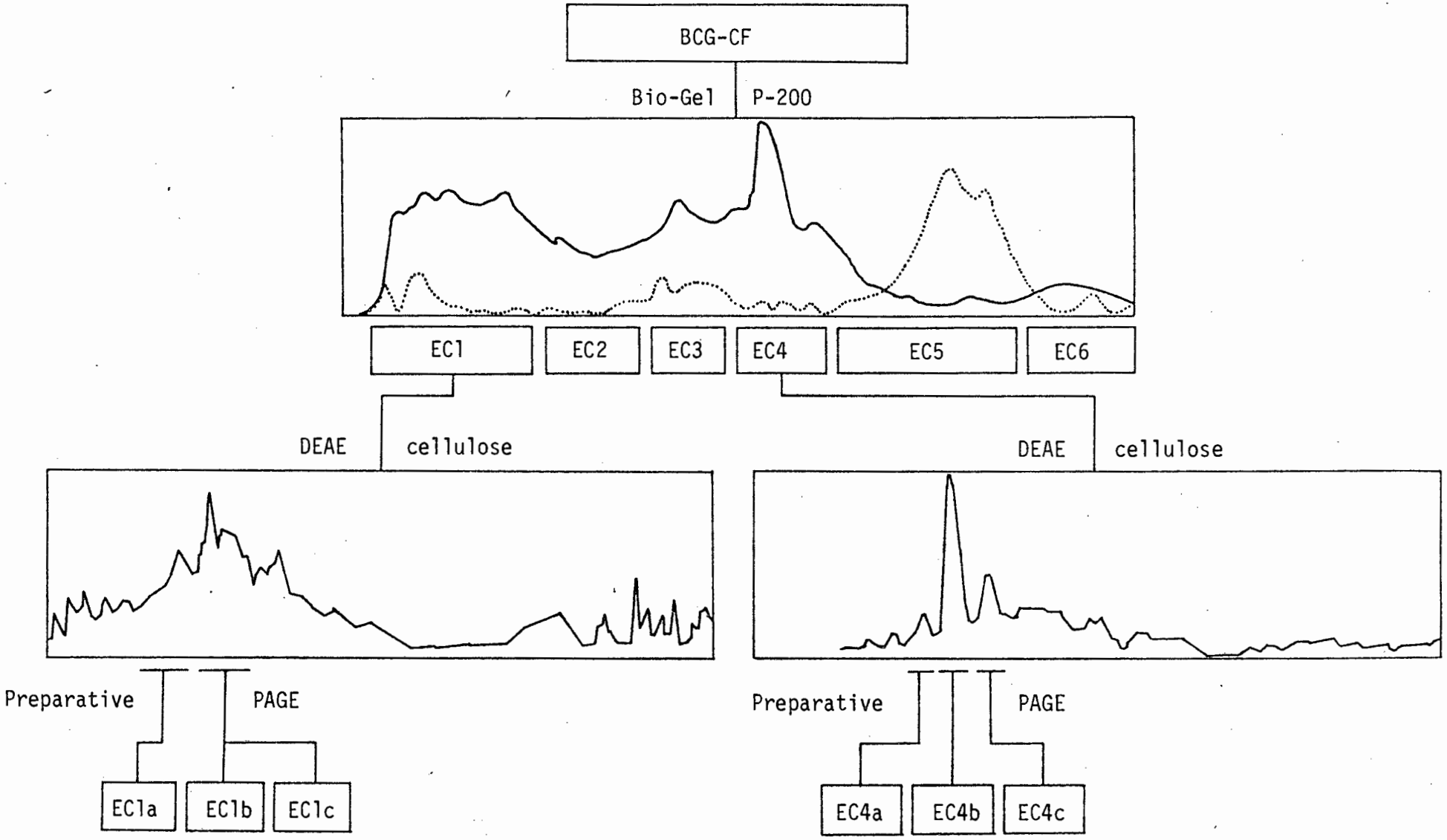


Fig. 5.2 ION EXCHANGE CHROMATOGRAPHY ON DEAE-CELLULOSE

Fractions EC1 (Panel A) and EC4 (Panel B) were eluted from DEAE-cellulose with a Tris-HCl buffer concentration gradient (-·-·-·-) from 50 mM to 0,5 M at pH7. Protein concentration, ●——●; A_{260}, ·····; and areas of elution profile containing tuberculin skin test elicitins, ⊢——⊣ are indicated. Fractions which were collected are indicated by broken lines.

Fig. 5.3. FRACTIONATION OF BCG-CF

Schematic flow chart showing fractionation of BCG-CF to yield purified protein isolates.
Refer also to Fig. 3.1 and Fig. 5.2



5.3.1.2 Biophysical properties of ECla

Fraction ECla contained two immunoprecipitin antigens (Fig. 5.4) and on PAGE two proteins which migrated close together in both 7,5% and 10% polyacrylamide gels were detected (Fig. 5.5A). Treatment with 1% SDS and subjection to PAGE resulted in a single major band representing a molecular weight of 54 000. When dissociated with 1% (m/v) SDS and reduced with 0,1% (v/v) 2-mercaptoethanol, the 54 000 molecular weight band was accompanied in addition by four bands representing polypeptides with calculated molecular weights of 19 000, 26 000, 28 000 and 35 000 (Fig. 5.5B, Table 5.1).

Heating of a 1 mg/ml solution of fraction ECla at 100°C for 60 min resulted in the appearance of a limited quantity of precipitate. The clarified supernatant fluid contained significant protein, but immunoprecipitin and electrophoretic properties were lost. Heating of a 0,1 mg/ml solution produced no precipitation but immunoprecipitin and electrophoretic properties were still destroyed.

5.3.1.3. Biophysical properties of ECIb

A single polypeptide with a molecular weight of 40 000 was found in fraction ECIb by SDS-PAGE (Fig. 5.5, Table 5.1). Homogeneity of the fraction was confirmed by gel immunoprecipitation where a single antigenic species was present (Fig. 5.4). The response of this fraction to heating was similar to that observed with fraction ECla.

5.3.1.4 Biophysical properties of ECIc

Immunoprecipitin (Fig. 5.4) and electrophoretic (Fig. 5.5) studies of this fraction indicated that a number of polypeptides were present. The production of a single strong immunoprecipitin line by the freshly isolated preparation suggested the presence of a single antigenic species. But on standing at 4°C for one week a number of immunoprecipitin lines were produced as may be seen in the example in Fig. 5.4. Similarly, when the fraction was subjected to PAGE, a broad band which was ill-

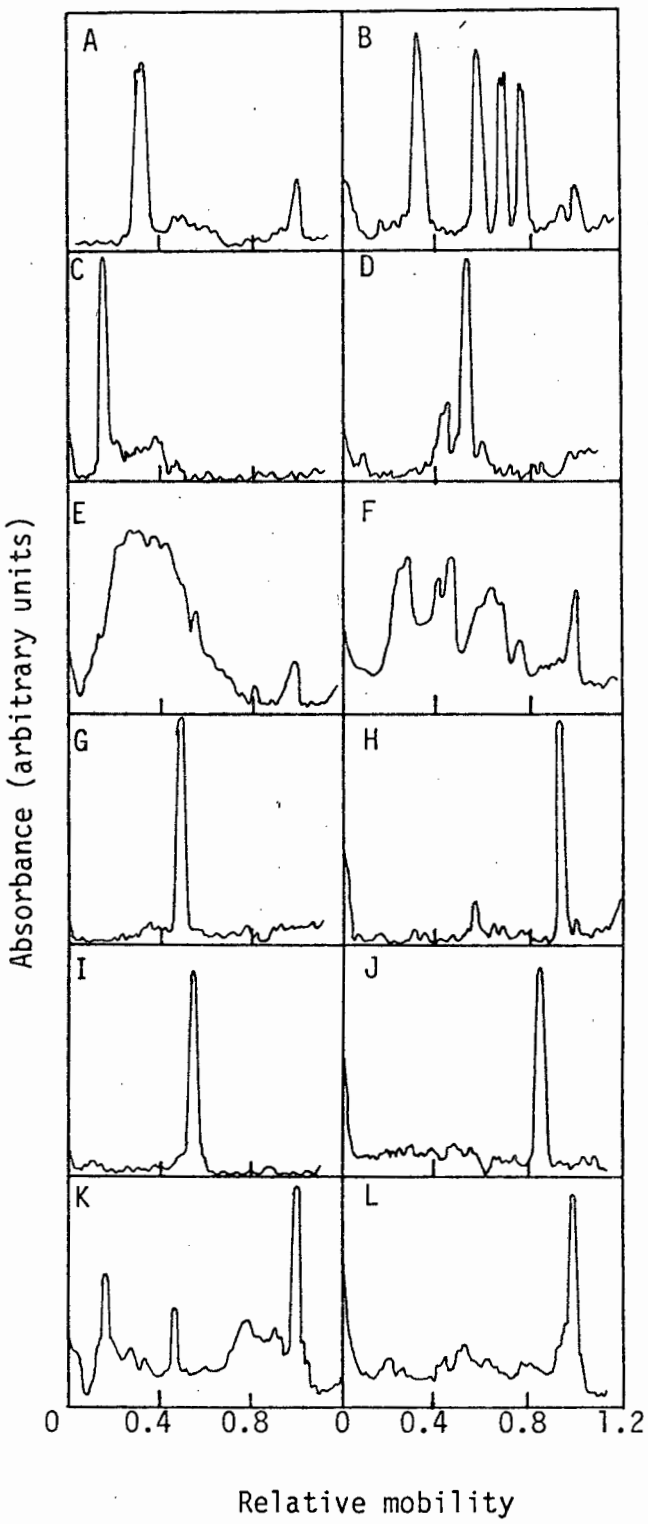


Fig. 5.5. POLYACRYLAMIDE GEL ELECTROPHORESIS OF ISOLATES AND FRACTIONS OF BCG-CF.

Fractions EC1a (Panels A & B), EC1b (Panels C & D), EC1c (Panels E & F), EC4a (Panels G & H), EC4b (Panels I & J) and EC4c (Panels K & L) were subjected to PAGE and SDS-PAGE in 10% gels. Densitometer traces of PAGE studies are shown in panels on the left and SDS-PAGE results on the right.

TABLE 5.1 SDS-PAGE DETERMINATION OF MOLECULAR WEIGHTS OF PURIFIED BCG-CF POLYPEPTIDES

Molecular weights of the polypeptides and proteins of fractions EC1a, EC1b, EC1c, EC4a, EC4b and EC4c were calculated by the least squares method (Appendix A3.1) from an SDS-PAGE calibration graph prepared with standard proteins. Values used for the regression coefficient (b_{y-x}) and the intercept(a) were 1,2 x 10^{-5} and 1,01 respectively (Appendix A3.2).

Fraction	Relative mobility (Y)	Y-a	Calculated molecular weight (Y-a)/b
EC1a	0,78	-0,23	19 000
	0,70	-0,31	26 000
	0,67	-0,34	28 000
	0,59	-0,42	35 000
	0,34	-0,67	54 000
EC1b	0,53	-0,48	40 000
EC1c	0,79	-0,22	18 000
	0,71	-0,30	25 000
	0,65	-0.36	30 000
	0,59	-0,42	35 000
	0,45	-0,56	46 000
	0,40	-0,61	51 000
	0,33	-0,68	56 000
	0,30	-0,71	59 000
	0,26	-0,75	62 000
EC4a	0,91	-0,11	8 700
EC4b	0,84	-0,17	14 000
EC4c	0,00	-0,01	less than 8 000

defined and rather diffuse appeared (Fig. 5.5E). It was not immediately obvious whether this was due to contamination of the desired fraction with material from adjacent protein bands during preparative PAGE, or whether the material within the fraction displayed erratic mobility. Therefore the fraction was repurified by preparative PAGE. Spreading of the protein fraction during electrophoresis was somewhat reduced by this procedure, but nevertheless still occurred. Several darker zones which might have represented discrete bands were apparent. This type of pattern is characteristic of that found where association and dissociation of complex protein subunits actively occurs during electrophoresis. Nine polypeptides were liberated when the fraction was denatured with SDS and 2-mercaptoethanol (Fig. 5.5F), the molecular weights of which are given in Table 5.1. It is probable that this fraction consisted of one to several proteins each of which contained polypeptide subunits.

5.3.2 ISOLATION OF COMPONENTS FROM EC4

5.3.2.1 Fractionation of EC4

Fraction EC4 contained far fewer components than fraction EC1 and consequently ion exchange chromatography provided a clearer separation than in the former case. Proteins eluted from DEAE-cellulose in one large and three small peaks (Fig. 5.2) all of which contained immunoprecipitin antigens and elicited tuberculin skin reactions in sensitized guinea pigs. The first three peaks were relatively homogeneous electrophoretically, with only minor quantities of contaminating polypeptides as opposed to the fourth peak which was electrophoretically heterogeneous. Preparative PAGE was used to remove the traces of contaminating materials from the major protein of each of the first three peaks and these isolates were labelled EC4a, EC4b and EC4c as shown in Fig. 5.3. Fractions EC4a and EC4b were subjected to a second fractionation with preparative PAGE in order to ensure greater purity.

5.3.2.2 Biophysical properties of EC4a

Fraction EC4a contained a single immunoprecipitin antigen (Fig. 5.4)

which produced a single corresponding electrophoretic band with a relative mobility (R_F) of 0,52 on PAGE in 10% gels (Fig. 5.5G). This band corresponded with the band with R_F of 0,52 produced by BCG-CF100 (Fig. 3.8C). Apparent molecular weight was estimated by SDS-PAGE to be approximately 8 700 (Table 5.1). Heating of a 1 mg/ml solution of this polypeptide at 100°C for 60 min did not induce any precipitation and the heated solution produced an electrophoretic band indistinguishable from that of the native protein. An immunoprecipitin line which was just marginally weaker than that of the native protein was produced by the heated solution (Fig. 5.4) and a reaction of identity with unheated material was found.

5.3.2.3. Biophysical properties of EC4b

A single immunoprecipitin antigen (Fig. 5.4) with an R_F of 0,56 on PAGE in 10% gels (Fig. 5.5I) was present in Fraction EC4b. This band corresponded very closely with the major heat stable band of BCG-CF100 which had a relative mobility of 0,57 (Fig. 3.8C). SDS-PAGE studies (Fig. 5.5J) showed that the protein of this fraction consisted of a single polypeptide with an approximate molecular weight of 14 000. Heating of a 1 mg/ml solution of the polypeptide at 100°C for 60 min resulted in some precipitation but the clarified supernatant retained immunoprecipitin activity and the electrophoretic mobility was unchanged.

Apparent isopotential specific volume of EC4b

Results obtained in the determination of the apparent isopotential specific volume of EC4b in 50 mM Tris-HCl containing 0,1 M NaCl and 0,1% (m/v) SDS are shown in Table 5.2 and Fig. 5.6. From microdrop sedimentation data the term $\Delta\rho$ was calculated as 0,00187 g/ml and protein concentration as 0,625% (m/v). These figures were substituted into equation 5.3 and the apparent isopotential specific volume was calculated as 0,7 ml/g.

TABLE 5.2 DENSITY DETERMINATION OF FRACTION EC4b BY THE MICRODROP SEDIMENTATION VELOCITY METHOD

Microdrop sedimentation velocities and accompanying density data are given for standard NaCl solutions of known density; bovine serum albumin (BSA) standards in distilled water; fraction EC4b dissolved in 50 mM Tris-HCl containing 0,1 m NaCl and 0,1% (m/v) SDS and its equilibrium dialysed buffer.

Sample	Mean sediment-ation time (sec)	Mean sediment-ation velocity (cm/sec)	Density (g/ml)
0,1 M NaCl	132,3	0,113	1,00185
0,15 M NaCl	102,0	0,147	1,00393
0,2 M NaCl	72,1	0,208	1,00600
0,85% BSA	290,7	0,052	1,00391
1,7% BSA	145,1	0,103	1,00604
EC4b	93,2	0,161	1,00395
Equilibrium dialysed solvent	122,1	0,123	1,00208

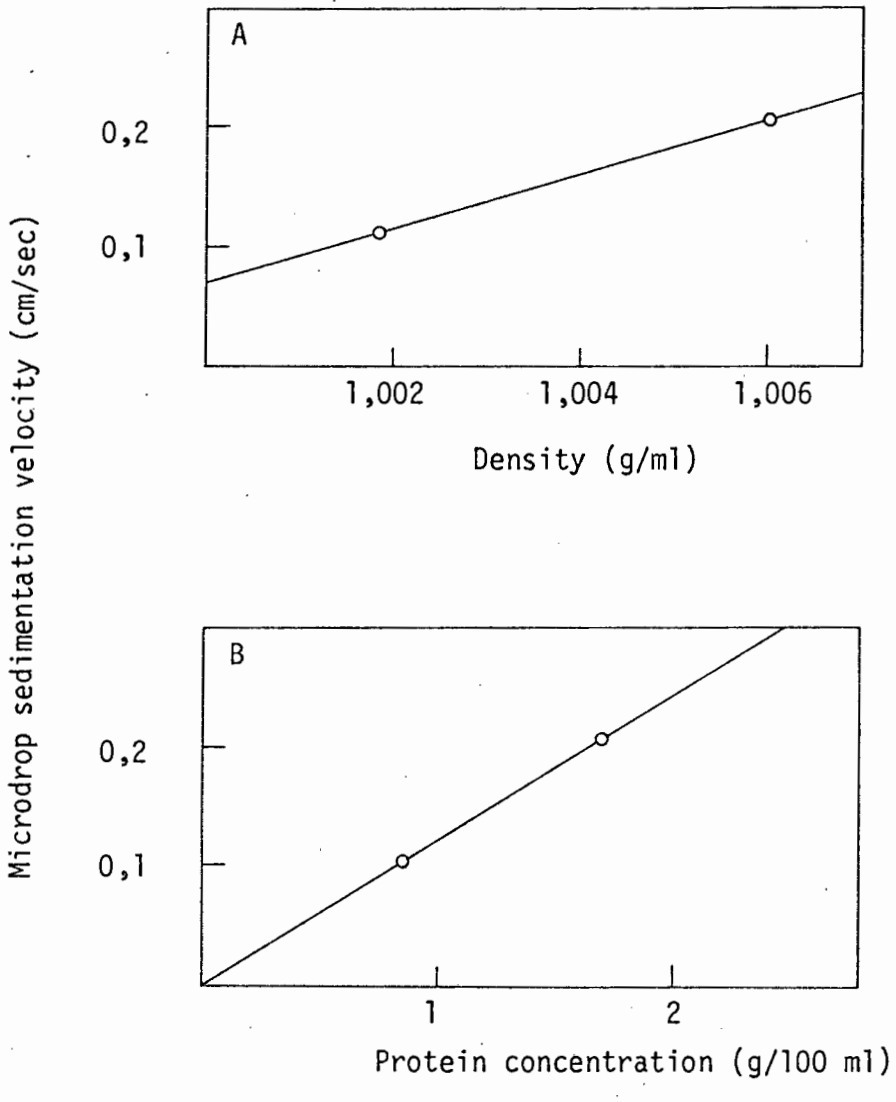
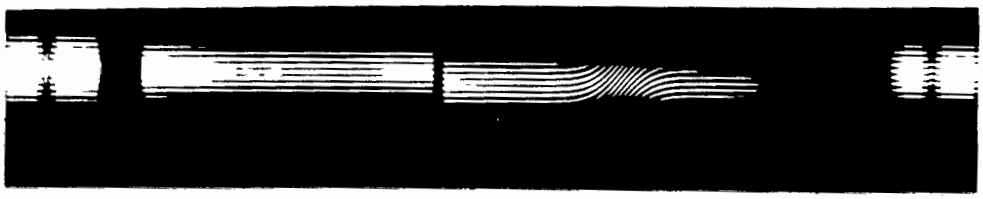
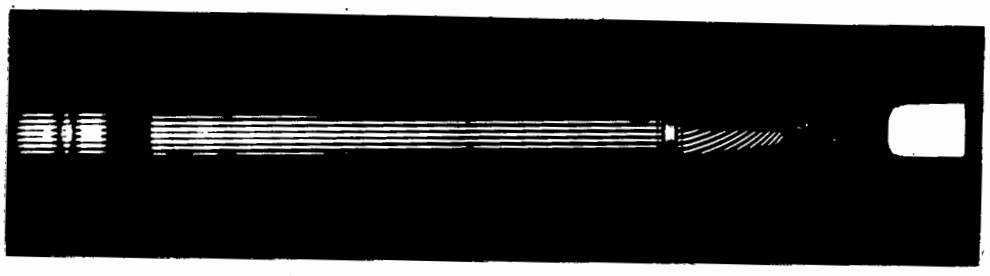


Fig. 5.6. DENSITY DETERMINATION BY THE MICRODROP SEDIMENTATION VELOCITY METHOD.

Microdrop sedimentation velocity is plotted against density for 0,1 M and 0,2 M solutions of NaCl in Panel A. Panel B shows the relationship between microdrop sedimentation velocity and the concentration of bovine serum albumin.



Synthetic boundary experiment



Sedimentation equilibrium experiment.

Fig 5.7 SEDIMENTATION EQUILIBRIUM ULTRACENTRIFUGATION

Rayleigh interference patterns for sedimentation equilibrium ultracentrifugation experiments with fraction EC4b.

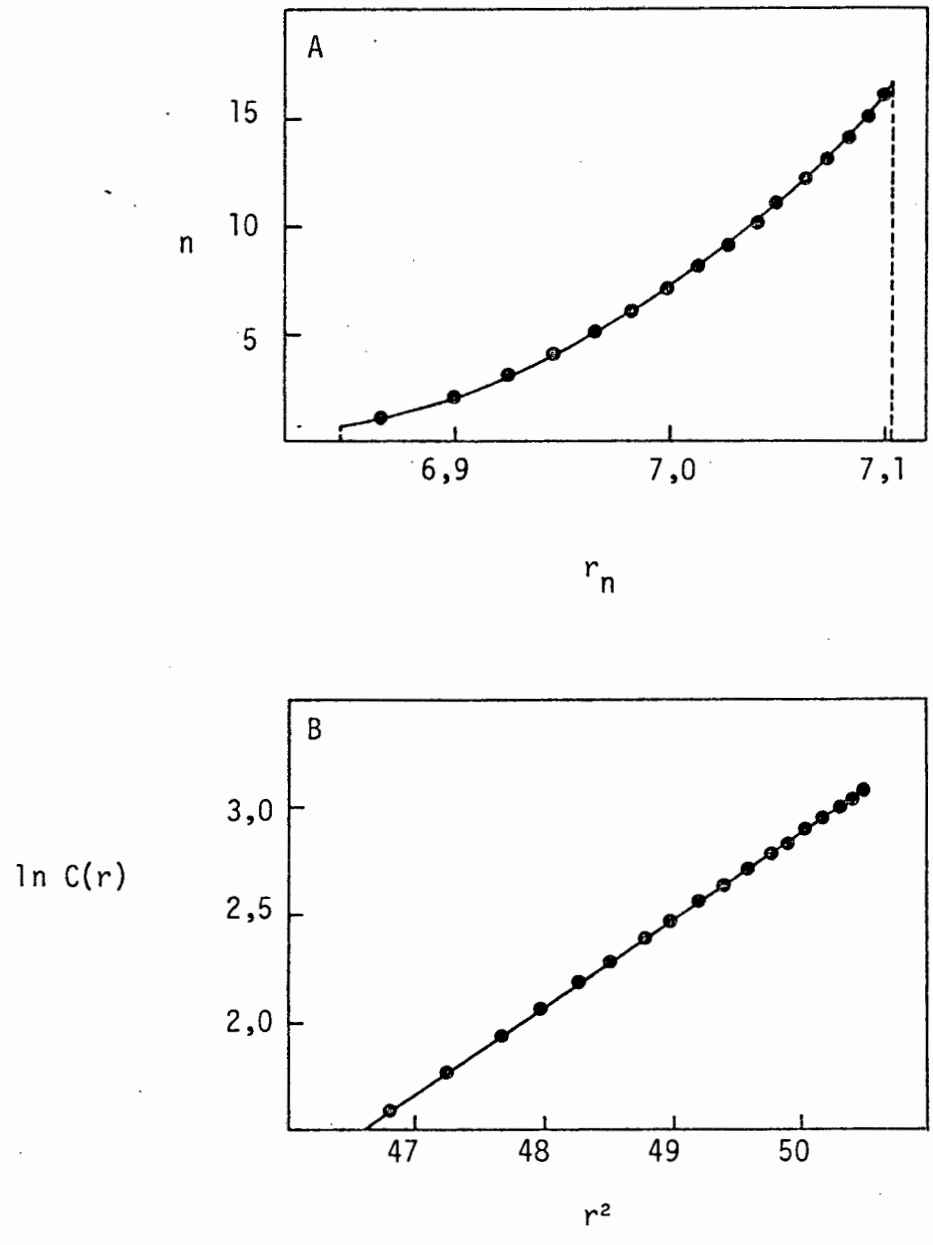


Fig. 5.8 DETERMINATION OF MOLECULAR WEIGHT BY SEDIMENTATION EQUILIBRIUM ULTRACENTRIFUGATION.

In panel A synthetic boundary centrifugation data for the calculation of the meniscus concentration of fraction EC4b as a fringe shift are shown. Integral interference fringes (n) are plotted versus distance from the centre of rotation (r_n). Panel B shows the evaluation of the relation $d\ln C(r)/d\ r^2$ for the sedimentation equilibrium ultracentrifugation determination of the molecular weight of EC4b.

TABLE 5.3 SEDIMENTATION EQUILIBRIUM ULTRACENTRIFUGATION DATA FOR EC4b

Calculation of lnC(r) and r^2 values for molecular weight determination (See Section 5.2.4.1)

Fringe number	Radial distance (r) cm	r^2	C(r) fringes	lnC(r)
r(a)	6,842	46,813	4,916	1,59331
1	6,847	47,252	5,916	1,77834
2	6,901	47,624	6,916	1,93442
3	6,926	47,969	7,916	2,06939
4	6,947	48,261	8,916	2,18830
5	6,966	48,525	9,916	2,29455
6	6,984	48,776	10,916	2,38876
7	6,999	48,986	11,916	2,47654
8	7,015	49,210	12,916	2,55723
9	7,029	49,407	13,916	2,63189
10	7,042	49,590	14,916	2,70136
11	7,055	49,773	15,916	2,76632
12	7,065	49,914	16,916	2,82731
13	7,075	50,056	17,916	2,88480
14	7,085	50,197	18,916	2,93916
15	7,094	50,325	19,916	2,99072
16	7,101	50,424	20,916	3,03975
r(b)	7,105	50,481	21,336	3,06339

Molecular weight of EC4b by sedimentation equilibrium ultracentrifugation.

Rayleigh interference patterns for synthetic boundary and sedimentation equilibrium experiments are shown in Fig. 5.7 and the evaluation of these data according to methods described in Section 5.2.4.2. are shown in Table 5.3 and Fig. 5.8. A linear relationship was obtained for the plot of $\ln C(r)$ versus r^2 which indicated ideal behaviour of the solute and confirmed its homogeneity. The slope of this graph and the apparent isopotential specific volume which was calculated above were substituted into equation 5.4 and the molecular weight of EC4b was calculated as 12 200.

5.3.2.4 Properties of EC4c

This fraction contained the polypeptides of fraction EC4 which migrated with the tracking dye in SDS-PAGE studies (Fig.5.5L). The molecular weights of these components were approximately 8 000 or less and gel immunoprecipitin studies confirmed the heterogeneity of the fraction which contained at least five antigens or more (Fig. 5.4).
During preparation of fraction EC4c the well separated front band which migrated with the tracking dye on preparative PAGE was excised and eluted. On subsequent PAGE this band was accompanied by a second strong band of low mobility and traces of several other weak bands of various mobilities were also detected (Fig. 5.5K).
Immunoprecipitin and electrophoretic properties of this fraction were unaffected by heating at 100°C for 60 min at a concentration of 1 mg/ml.

5.3.3 AMINO ACID COMPOSITIONS

The results obtained in the analysis of amino acid compositions of the fractions EC1a, EC1b, EC1c, EC4a and EC4b are given in Table 5.4. The content of each amino acid has been expressed as a percentage (nanomoles %) of the total protein (nanomoles) to enable a comparison of the relative proportions of the various amino acids of the five fractions.

5.3.4 ANTIGENIC RELATIONSHIPS OF ISOLATES

Antigenic relationships between the fractions EC1a, EC1b, EC1c, EC4a, EC4b and EC4c were deduced from the gel immunoprecipitin reactions shown in Fig 5.4 and from other studies not shown here. It was observed that no antigenic identity was shared by the three high molecular weight fractions EC1a, EC1b and EC1c either with each other or with the three low molecular weight isolates. The polypeptides of EC4a and EC4b showed reactions of non-identity with each other but each produced a reaction of identity with a different antigenic component of the low molecular weight fraction, EC4c.

5.3.5 IMMUNOELECTROPHORETIC STANDARDIZATION OF FRACTIONS

Line drawings and photographs of immunoelectrophoresis patterns of the fractions EC1a, EC1c, EC4a and EC4b when developed with Ref-CF antiserum, Ref-CE antiserum and anti-BCG-CF serum are shown in Fig. 5.9. The number of antigens detected in each fraction by the various antisera are given in Table 5.5.

5.3.6 TUBERCULIN SKIN TEST ACTIVITY.

Native and heated samples of the fractions EC1a, EC1b, EC1c, EC4a and EC4b elicited significant tuberculin skin reactions in sensitized guinea pigs (Table 5.6). In all cases the differences between the mean reactions of the heated and unheated preparations were not statistically significant at the $P = 0,05$ level. No skin reactions were elicited in unsensitized control guinea pigs by any of the isolates tested.

5.4 DISCUSSION

In this Chapter the isolation of two polypeptides, EC4a and EC4b and an heterogeneous fraction, EC4c, which displayed resistance of their immunoprecipitin and electrophoretic properties to heating at 100°C

TABLE 5.4 AMINO ACID COMPOSITIONS OF BCG-CF PROTEINS

Lyophilized samples of fractions EC1a, EC1b, EC1c, EC4a and EC4b were hydrolysed in constant boiling (6 M) HCl at 110°C for 24 hours and the digests were subjected to analysis on a Beckman 116 automatic amino acid analyser. Tryptophan was estimated from UV absorbance spectra. The content of each amino acid is expressed in nanomoles per 100 nanomoles of total amino acids (nmoles %).

Amino acid	Amino acid contents (nmoles %)				
	EC1a	EC1b	EC1c	EC4a	EC4b
Lysine	6,6	5,7	4,1	11,5	12,5
Histidine	0,9	1,5	0,6	1,8	2,3
Arginine	0,5	2,8	0	0	0
Aspartic acid	9,2	9,7	12,8	6,8	8,1
Threonine	0	4,5	4,9	5,2	6,0
Serine	23,9	13,8	12,4	33,1	29,2
Glutamic acid	14,8	13,0	14,6	0,3	2,9
Proline	0	0,4	0,5	0	0
Glycine	23,3	15,6	16,0	21,5	19,0
Alanine	9,8	11,9	10,7	11,6	9,3
Half cystine	1,3	0,6	1,3	0	0
Valine	3,0	5,2	6,2	0,4	1,0
Methionine	0	1,2	1,4	0,4	0,9
Isoleucine	1,7	3,0	3,7	2,5	2,9
Leucine	2,8	6,1	6,1	3,1	3,7
Tyrosine	0,5	1,9	1,0	0,4	0,7
Phenylalanine	0,6	2,3	1,2	0,5	0,6
Tryptophan	1,1	1,0	2,6	0,9	1,0

TABLE 5.5 IMMUNOELECTROPHORETIC STANDARDIZATION OF BCG-CF ISOLATES

Immunoelectrophoretic patterns of the fractions EC1a, EC1c, EC4a and EC4b were developed with anti-BCG-CF serum and with the Ref-CF and Ref-CE antisera. The number of antigens detected in each fraction by each of the three antisera are compared in this Table.

Fraction	Number of antigens detected		
	Ref-CF antiserum	Ref-CE antiserum	Anti-BCG-CF serum
EC1a	0	2	2
EC1c	1	1	5
EC4a	0	0	1
EC4b	0	0	1

TABLE 5.6 TUBERCULIN SKIN TEST ACTIVITY OF NATIVE AND HEATED BCG-CF ISOLATES

Samples of fractions EC1a, EC1b, EC1c, EC4a and EC4b at concentrations of 10 µg/ml were heated at 100°C for 60 min. Heated and native samples of these fractions were assayed according to the point assay protocol in six guinea pigs sensitized with heat killed BCG.

Preparation	Mean reaction diameter at 24 hours (mm)	
	Native preparation	Heated preparation
EC1a	11,9	11,4
EC1b	12,1	10,7
EC1c	10,7	9,7
EC4a	11,2	11,6
EC4b	11,5	11,9
BCG-CF	13,3	13,3

TABLE 5.7 SUMMARY OF PROPERTIES OF PURIFIED BCG-CF FRACTIONS.

The compositions, biophysical properties and immunological properties of the purified fractions EC1a, EC1b, EC1c EC4a, EC4b and EC4c are summarized below. This includes the number of proteins (from PAGE data), the number of polypeptides (from SDS-PAGE), molecular weights of polypeptide components (from SDS-PAGE and sedimentation equilibrium ultracentrifugation data), partial specific volume, the number of immunoprecipitin antigens detected by anti-BCG-CF serum, the number of antigens detected with the Ref-CF and Ref-CE immunoelectrophoretic antisera and the heat stability of the immunoprecipitin and electrophoretic properties.

Fraction	Proteins	Polypeptides.	Polypeptide molecular weight		Partial Specific Volume ($\bar{v}$)	Immunoprecipitin Antigens.	Reference antigens		Heat Stability
			SDS-PAGE	Ultracentrifugation			Ref-CF	Ref-CE	
EC1a	2	4	19 000 26 000 28 000 35 000			2	0	2	Unstable
EC1b	Variable	9	18 000 25 000 30 000 35 000 46 000 51 000 56 000 59 000 62 000			5	1	1	Unstable
EC1c	1	1	40 000			1			Unstable
EC4a	1	1	8 700			1	0	0	Stable
EC4b	1	1	14 000	12 200	0,72	1	0	0	Stable
EC4c			8 000			5			Stable

has been described. These fractions contained the few protein antigens of BCG-CF which were resistant to very high temperatures. From the summary of the properties of purified BCG-CF components in Table 5.7 it may be seen that the two antigens, EC4a and EC4b had molecular weights of 8 700 and 12 200 respectively. The apparent isopotential specific volume of EC4b was calculated as 0,7 ml/g. Since the apparent isopotential specific volumes of proteins in detergent solutions are usually less than their partial specific volumes by approximately 0,02 ml/g (Sakura and Reithel, 1972), the partial specific volume of EC4b may be estimated at about 0,72 ml/g. This value falls midway within the range of 0,7 to 0,75 ml/g found for most proteins (Van Holde, 1975). The molecular weights of the several polypeptides detected in fraction EC4c were approximately 8 000 or smaller, but were nevertheless large enough not to pass through dialysis membranes with specified molecular weight retentions of approximately 8 000.

For comparative purposes a polypeptide (EC1b), a fraction containing 2 dimeric proteins (EC1a) and an heterogeneous fraction (EC1c) which were heat sensitive were prepared. Immunoprecipitin and electrophoretic properties of these fractions were inactivated by heating to 100°C although tuberculin skin test activity was not measurably affected by this treatment.

During the fractionation of BCG-CF it became apparent that the total number of polypeptides and proteins of BCG-CF was far greater than was indicated by the 34 bands which were distinguished in SDS-PAGE studies of whole BCG-CF. This rendered the isolation of discrete proteins extremely difficult and the combination of gel exclusion chromatography, ion exchange chromatography and preparative polyacrylamide gel electrophoresis became essential. These procedures, although somewhat lengthy, were satisfactory for the isolation of the heat stable low molecular weight antigens, but were less efficacious with the higher molecular weight proteins where the yields of purified materials were very low. The preparation obtained in greatest quantity was fraction EC4b where about 8 mg was obtained from approximately 4,5g of BCG-CF powder.

Antibody immobilized affinity chromatography was considered for the

isolation of EC4a and EC4b in larger quantity but problems encountered with antiserum preparation prevented its use. Affinity chromatography has until very recently (Daniel and Anderson, 1978; Payne and Daniel, 1980) not been widely used in mycobacterial antigen fractionations, due largely to the difficulty of isolating sufficient purified antigen for the initial preparation of the necessary reagents. In this study the inability to raise antibody to EC4a and EC4b was no doubt due to insufficient antigen (two injections of 1 mg of antigen emulsified in Freund's incomplete adjuvant), the low molecular weight of the antigens and the necessary use of incomplete adjuvant.

It was noted in PAGE and SDS-PAGE investigations of semipurified and purified fractions that a polypeptide band often appeared at the position of the tracking dye (see Fig. 5.5). In freshly isolated fractions this band was weak or absent but became noticable if the solution was stored at 4°C for several days, frozen and thawed, or electrophoresed in the presence of SDS. It could be removed from fractions by preparative SDS-PAGE. This apparently spontaneous appearance of low molecular weight polypeptides could possibly be due to adsorption of low molecular weight polypeptides to proteins of BCG-CF by weak intermolecular links such as hydrogen bonds or by association of hydrophobic regions of polypeptide chains. This view is supported by the solubilization and elimination of these components by treatment with SDS. Low molecular weight polypeptides are likely to originate during the long incubation of cultures, when proteolytic enzymes are liberated into the medium following bacterial autolysis and the digestion of culture filtrate proteins might result. Adsorption of digested polypeptides to proteins could result in the formation of protein complexes which appear to liberate lower molecular weight components spontaneously following fractionation.

Unusual electrophoretic behaviour was also observed with the low molecular weight fraction EC4c where certain materials excised at the position of the tracking dye migrated with low mobilities (0,19, 0,42) on re-electrophoresis under identical conditions (see Fig. 5.5 K). These changes in mobility were too large to be explained by simple aggregation therefore it must be presumed that components of EC1c were able to exist in states of different net charge. This may be explained by the presence of

polypeptides which assume more than one conformation. In one form highly charged groups could be protected within the molecule and in the other exposure of such groups might result in changed electrophoretic mobility.

A qualitative indication of the amino acid compositions of the fractions EC1a, EC1b, EC1c, EC4a and EC4b was obtained from the results shown in Table 5.4. Since only a single 24 hour acid digestion procedure was employed due to the limited availability of materials, the reported values for several of the amino acids are not true values. These include asparagine and glutamine which are converted to aspartic and glutamic acid; cysteine which is converted to cystine; serine, theonine, cystine and tyrosine which are partially destroyed and tryptophan which is totally destroyed during acid hydrolysis (Haschemeyer and Haschemeyer, 1973). Tryptophan was determined from UV absorbance spectra but the results shown in Table 5.4 for this amino acid are likely to be lower than the real values as the spectra should ideally be determined in the presence of 6 M guanidine hydrochloride.

The major amino acids of fractions EC1a, EC1b and EC1c were serine, glycine, glutamic acid and alanine. The amino acid compositions of the heat stable antigens EC4a and EC4b were very similar to each other with very high contents of serine and relatively high proportions of glycine, lysine and alanine. The similarity in composition of these two antigens suggests that they may have had a common source. They were, however, shown to be antigenically distinct. Therefore it is unlikely that the low molecular weight component EC4a could have been a degradation product of EC4b, although both could well have been fragments of a larger protein.

In immunoelectrophoresis studies two anodic antigens were detected in the heat labile fraction EC1a by anti-BCG-CF and by the Ref-CE antiserum. One of the two antigens detected in this fraction with the Ref-CE antiserum corresponded with antigen CE-10 of the Ref-CE preparation. The second antigen migrated in a position which indicated that it might represent one of the unlabelled antigens described by Daniel *et al.* (1975). No reactivity towards fraction EC1a was obtained with the Ref-CF antiserum. This is in agreement with the reported prominence of the immunoprecipitin arc 10 in cell extract antigens of batch 002 as opposed to

limited distribution in the 002 culture filtrate antigen preparation (Daniel et al., 1975).

Of five antigens detected in fraction EC1c by anti-BCG-CF, only one reacted with the Ref-CF and Ref-CE antisera. This antigen was tentatively identified as representing antigen 6 of the reference system. The two heat stable antigens of EC4a and EC4b were not reactive with the reference antisera although they reacted with anti-BCG-CF. This demonstrates that a greater degree of species specificity exists between the antigens of M. tuberculosis and BCG than might have been apparent from previous studies of crude culture filtrates of these species (Chaparas and Hedrick, 1973; Chaparas, 1975).

CHAPTER SIX

THERMALLY INDUCED CONFORMATIONAL CHANGES IN BCG-CF PROTEINS

6.1 INTRODUCTION

The conformational status of the proteins of fractions EC1a, EC1c, EC4a and EC4b and the subsequent conformational alterations which occur when these fractions are heated to 98°C and cooled to 20°C are described in this Chapter. Theoretical and practical aspects of protein conformation and protein denaturation reactions have recently been comprehensively reviewed (Urnes and Doty, 1961; Schellman and Schellman, 1964; Hermans, 1965; Tanford, 1968; 1970; Jirgensens, 1973; Mathews, 1977; Privalov, 1979; Schulz and Schirmer, 1979).

The denaturation reactions of proteins may be monitored by a number of techniques which include assays of reactivity of certain groups, enzymatic digestion, optical rotatory dispersion, circular dichroism, various spectroscopy procedures and investigations of hydrodynamic, charge and immunological properties. The choice of technique depends on the homogeneity of the protein to be investigated, the degree of sensitivity required and the availability of suitable instrumentation. Data for this study were obtained from optical rotatory dispersion, UV difference spectroscopy and micro-ELISA studies. These procedures were the most sensitive which were available for the investigation of the small quantities of materials isolated from BCG-CF.

The spectrum of optical rotations, or optical rotatory dispersions, of proteins provide a reliable indication of their conformational complexity. In practice observed optical rotations are corrected for protein concentration and are expressed either as specific rotations, $[\alpha]_{\lambda}^{T}$, or reduced mean residue rotation, $[m']_{\lambda}^{T}$ (Jirgensens, 1973). This specific rotation is calculated according to the formula

$$[\alpha]_{\lambda}^{T} = \frac{100\alpha}{cxl} \qquad \text{equation 6.1}$$

where α represents the observed rotation(degrees), l the length of the sample tube (dm) and c, the concentration of the sample (g/100ml). Mean reduced residue rotation, $[m']_{\lambda}^{T}$, is obtained from the relation

$$[m']_{\lambda}^{T} = \frac{3}{n^2 + 2} \cdot \frac{\bar{M}\alpha}{100} \qquad \text{equation 6.2}$$

where $3/(n^2 + 2)$ represents the Lorentz correction factor, n the refractive index of the solvent and $\bar{M}$ the mean molecular weight of the amino acid residues.

Optical rotatory dispersion measurements are taken over a broad wavelength range of 185-600nm. In the far ultraviolet region of the spectrum strong absorbance bands accompanied by large optical rotations occur. These are associated with the polypeptide backbone and are very much dependent on conformation (Jirgensens, 1973). At longer wavelengths the conformations of proteins and polypeptides have been related to their optical rotatory dispersions by the Drude and the Moffitt relationships (Moffitt and Yang, 1956).

The simplified one-term Drude equation is written in the form

$$[\alpha]_{\lambda}^{T} = \frac{A}{(\lambda^2 - \lambda^2_c)} \qquad \text{equation 6.3}$$

where λ represents the wavelength of incident radiation, λ_c is the dispersion constant and A is a constant proportional to the rotatory strength of the major optically active transition in the far ultraviolet region of the spectrum. The dispersion constant is determined from the slope of a plot of $\lambda^2[\alpha]_{\lambda}$ versus $[\alpha]_{\lambda}$. This relationship is linear for synthetic polypeptides and denatured proteins in the random coil form and λc values cluster in the region of 220 nm (Urnes and Doty, 1961). Synthetic polypeptides in α-helical or β-pleated sheet conformations do not produce linear functions although most native proteins do. The λ_c values of native proteins are higher than those

of denatured proteins and the variation in λ_c between approximately 220-280nm has been used as a measure of the content of α-helical secondary structure in proteins (Jirgensens, 1961).

The complex dispersions of synthetic polypeptides in α-helical and β-pleated sheet conformations and of certain proteins may be accounted for by the use of the two-term Moffitt equation

$$[m']^T_\lambda = a_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + b_0\lambda_0^4/(\lambda^2 - \lambda_0^2)^2 \qquad \text{equation 6.4}$$

where λ represents the wavelength of the incident light, λ_0 the wavelength of the major optically active transition and a_0 and b_0 are dispersion coefficients. The value of λ_0 for synthetic polypeptides has been experimentally determined as 212 ± 5 nm (Moffitt and Yang, 1956). The dispersion coefficients a_0 and b_0 are calculated from intercept and slope values of plots of $[m']^T_\lambda(\lambda^2 - \lambda_0^2)$ versus $1/(\lambda^2-\lambda_0^2)$. Over a wavelength range of approximately 350-600nm the relationship is linear but beyond these limits marked deviations from linearity occur.

The parameter a_0 represents both intrinsic residue rotations and interactions with helical structures and varies with environment. In contrast b_0 is principally a function of the helical skeleton and is relatively insensitive to environmental factors such as solvent variation and side chain interactions. The value of b_0 for helically coiled synthetic polypeptides is about -630 and for random coils approaches zero. The extent to which portions of the polypeptide chain are involved in α-helical structures is given by the partical helical content, f_H, which is calculated from observed b_0 values according to the relation

$$f_H = (b_0/630) \times 100 \qquad \text{equation 6.5}$$
(Urnes and Doty, 1961)

Positive b_0 values indicate parallel and anti-parallel β-pleated sheet conformations and have also been attributed to α-helical synthetic polypeptides with reverse screw sense (Jirgensens, 1973).

Ultraviolet absorbance spectra are frequently utilized for studies of the conformational changes which occur in denaturation reactions. In the far ultraviolet spectrum polypeptides in the random coil form and polypeptides in the β-pleated sheet conformation display large absorbance peaks at wavelengths of 192 nm and 194 nm respectively. Polypeptides in α-helical conformations absorb much less strongly in this region (Rosenheck and Doty, 1961). At wavelengths shorter than 220 nm ultraviolet absorbance measurements are complicated by absorptions resulting from the ionization of oxygen and also to strong absorption phenomena in buffer components of protein solutions. Consequently longer wavelengths are more commonly used for denaturation studies.

Within the 220-240 nm wavelength range a prominent peak is invariably associated with difference spectra of native and denatured proteins (Glazer and Smith, 1960). This peak results from changes in the environment of the polypeptide backbone and from altered distributions of aromatic side chains. The wavelength at which maximum difference occurs depends chiefly on the protein studied and to a minor degree on the method of denaturation employed. At longer wavelengths (260-300 nm) absorption phenomona due to tyrosine and tryptophan side chains occur. Difference spectra in these regions provide an indication of changes in the conformation of the polypeptide chain in regions adjacent to these residues (Hermans, 1965).

6.2 MATERIALS AND METHODS

6.2.1 OPTICAL ROTATION STUDIES

Samples of the fractions EC1a, EC1c, EC4a and EC4b at concentrations of approximately 1 mg/ml were dialysed extensively against several changes of 10 mM Tris-HCl buffer, pH7 and filtered through 0,2 μm membranes to remove large light scattering particles. Protein concentrations were determined by the Lowry technique and samples were transferred to a 1 dm pathlength polarimeter cuvette fitted wtih a waterjacket. Temperature

of the cuvette was regulated by a thermostatically controlled waterbath. Optical rotation readings were made with a Perkin-Elmer 141 polarimeter at wavelengths of 365 nm, 436 nm, 546 nm 578 nm and 589 nm heating was commenced at a rate of 1°C per min. Optical rotation was monitored during heating over the temperature range of 20-98°C and during cooling which was controlled at a rate of 1°C per min. After returning to 20°C samples were filtered to remove any precipitate, protein concentrations were determined and optical rotatory dispersions were read. Optical rotation of the Tris-HCl buffer was measured over the spectral and temperature ranges used and readings for the protein solutions were corrected for interference effects by subtraction of the optical rotation of the solvents. For the calculation of $[m']^T_\lambda$ an approximate value of 1,37 was assumed for n and a mean residue weight of 115 was used (Jirgensens, 1973).

6.2.2 ULTRAVIOLET DIFFERENCE SPECTROSCOPY

Ultraviolet difference spectra for heated and native solutions of the fractions EC1a, EC4a and EC4b were prepared as follows. Solutions containing 0,1 mg/ml of each fraction in 10 mM Tris-HCl, pH7 were prepared and a portion of each was heated at 100°C for 60 min. Where necessary, clarification by centrifugation was performed. Heated and unheated reference solutions were dialysed to equilibrium against 10 mM Tris-HCl, pH7 and filtered through 0,2µm membranes to remove large light scattering particles. Samples of equilibrium dialysed buffer were similarly filtered for use as blanks. A matched pair of Suprasil quartz cuvettes which displayed uniform light absorbance in the UV spectrum over the wavelength range of 220-325 nm were used in the measurement of absorbance spectra. Protein concentrations of all solutions were determined by the Lowry method and absorbance data were standardized to a concentration of 1% (m/v). Absorbance difference, $\triangle A^{1\%}_{cm}$, was calculated according to the relationship

$$\triangle A^{1\%}_{cm} = A^{1\%}_{D} - A^{1\%}_{N} \qquad \text{Equation 6.6}$$

where $A_D^{1\%}$ represents the absorbance of a 1% (m/v) solution of the denatured protein and $A_N^{1\%}$ the absorbance of the native protein at the same concentration.

6.2.3 MICRO-ELISA STUDIES

Native and heated solutions of fractions EC1a, EC1c, EC4a and EC4b were diluted to yield concentration ranges of 10^0-10^5 ng/ml and were assayed by the micro-ELISA double-antibody sandwich method described in Chapter 2.11.6.

6.3 RESULTS

6.3.1 OPTICAL PROPERTIES OF NATIVE ISOLATES

Optical rotatory dispersions of native solutions of the fractions EC1a, EC1c, EC4a and EC4b are shown in Fig. 6.1. All four preparations produced laevorotatory dispersions over the 365-589 nm wavelength range with specific rotations at the sodium-D line, $[\alpha]_{589}$, of -32°, -67°, -30° and -91° respectively (Table 6.1). The smooth dispersion curves which best fitted the optical rotation readings were used to prepare modified Drude plots of $\lambda^2[\alpha]_\lambda$ versus $[\alpha]$ according to the method described by Yang and Doty (1957). The Drude relationships were linear over the 400-500 nm wavelength range but exhibited relatively complex deviations from linearity at wavelengths beyond this range (Figs. 6.2, 6.3, 6.4). Dispersion constants (λ_c) were calculated from the slopes of the linear portions of these plots and are recorded in Table 6.1.

Moffitt plots of $[m']_\lambda$ $(\lambda^2-\lambda_0^2)$ versus $1/(\lambda^2-\lambda_0^2)$ for the four isolates yielded linear relationships. But, as with the Drude plots, deviations from linearity were noted beyond the 400-500 nm wavelength range (Figs. 6.5, 6.6). The dispersion coefficient b_0 was calculated from the slopes

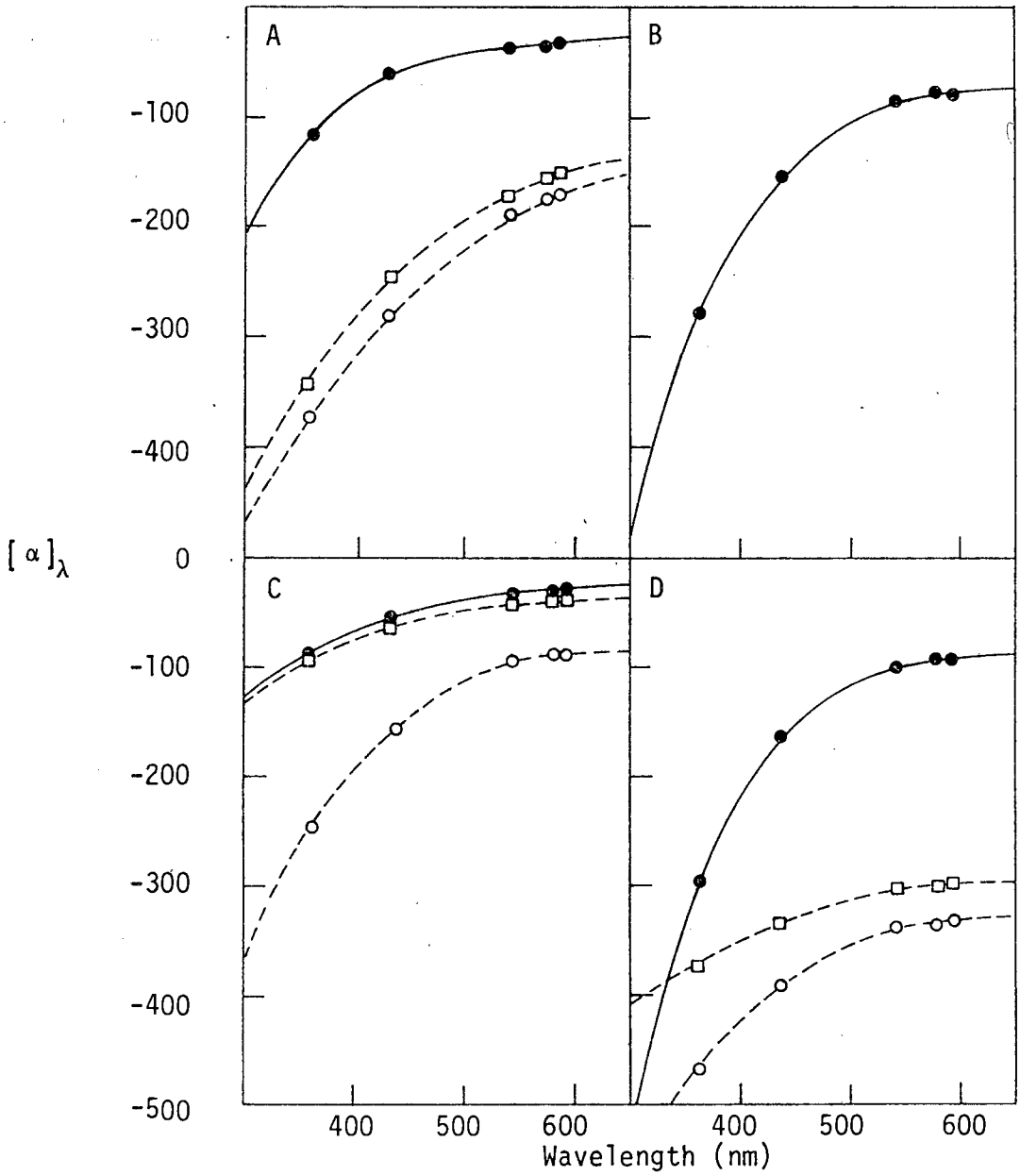


Fig. 6.1. OPTICAL ROTATORY DISPERSIONS OF NATIVE AND HEATED BCG-CF ISOLATES.

Optical rotations of fractions EC1a (Panel A), EC1c (Panel B), Ec4a (Panel C) and EC4b (Panel D) at 20°C, ●——●; at 98°C, o----o; and following cooling to 20°C, □----□ , were read at wavelengths of 365 nm, 436 nm, 546 nm, 578 nm and 589 nm. Dispersion curves of specific rotation, $[\alpha]_\lambda$, versus wavelength for these preparations are shown. Fraction EC1c was examined at 20°C only due to precipitation which occurred on heating.

of the linear portions of the Moffitt plots and the values obtained have been recorded in Table 6.1. Partial helical contents (f_H) were calculated from observed b_o values according to equation 6.5 and have been recorded in Table 6.1.

6.3.2 OPTICAL PROPERTIES OF HEATED SOLUTIONS

Extensive protein preicpitation occurred when fraction EC1c was heated to 76°C despite the dilute nature of the preparation (0,1 mg/ml). A small quantity of precipitate was similarly produced when fraction EC4b was heated to 82°C. No precipitates appeared in 0,1 mg/ml solutions of fractions EC1a and EC4a when heated to 98°C.

6.3.2.1 Optical rotation

Heating of the four fractions was attended by significant laevorotatory shifts in optical rotation over the wavelength range investigated (Fig. 6.1). Values of $[\alpha]_{589}$ for isolates heated to 98°C are given in Table 6.1. At this wavelength shifts from -32° to -170° and -30° to -88° were recorded for fractions EC1a and EC4a. At shorter wavelength (365 nm) specific rotation changes from -95° to -250° and -60° to -250° were observed for these fractions.

Heating curves in which variation in $[\alpha]_{365}$ is plotted versus temperature for each of the isolates during heating to 98°C are shown in Fig. 6.7. In all instances the laevorotatory shifts which accompanied heating to 98°C, or to the temperature at which precipitation occurred, could be divided into two smooth and distinct sigmoidal transitions. The first of these occurred at relatively low temperatures (20-40°C) in fractions EC1a, EC1c and EC4b and the magnitude varied from very small in fraction EC1c to relatively large in the other two fractions. The second transition observed for each of these fractions occurred at temperatures above 50°C. Fraction EC1a differed from those discussed previously in that no significant changes in optical rotation occurred at temperatures below 58°C. However two shifts at 58 - 75°C and 80 - 95°C could be distinguished.

TABLE 6.1 SUMMARY OF OPTICAL PROPERTIES OF NATIVE SOLUTIONS, HEATED SOLUTIONS AND COOLED SOLUTIONS OF THE ISOLATES EC1a, EC1c, EC4a and EC4b

Data represented for the fractions include their specific rotations at the sodium D line, $[\alpha]_D$, dispersion coefficients, λ_c, calculated from slopes of modified Drude plots according to Yang and Doty (1957), Moffitt parameter, b_o, based on λ_o = 212 nm, and partial helical contents, f_H, calculated from $f_H = b_o / -630 \times 100$ (Urnes and Doty, 1961). Certain values could not be calculated and have been left blank.

Preparation	Temperature (°C)	$[\alpha]_D$ (°)	λ_c (nm)	b_o	f_H (%)
EC1a, native	20°C	- 32	281	-242	38
EC1a, heated	98°C	-170		+470	
EC1a, cooled	20°C	-148		+470	
EC1c, native	20°C	- 67	261	-325	52
EC4a, native	20°C	- 30	208	0	
EC4a, heated	98°C	- 88	214	+ 5	
EC4a, cooled	20°C	- 38		+ 79	
EC4b, native	20°C	- 91	258	-263	42
EC4b, heated	98°C	-270			
EC4b, cooled	20°C	-225		+400	

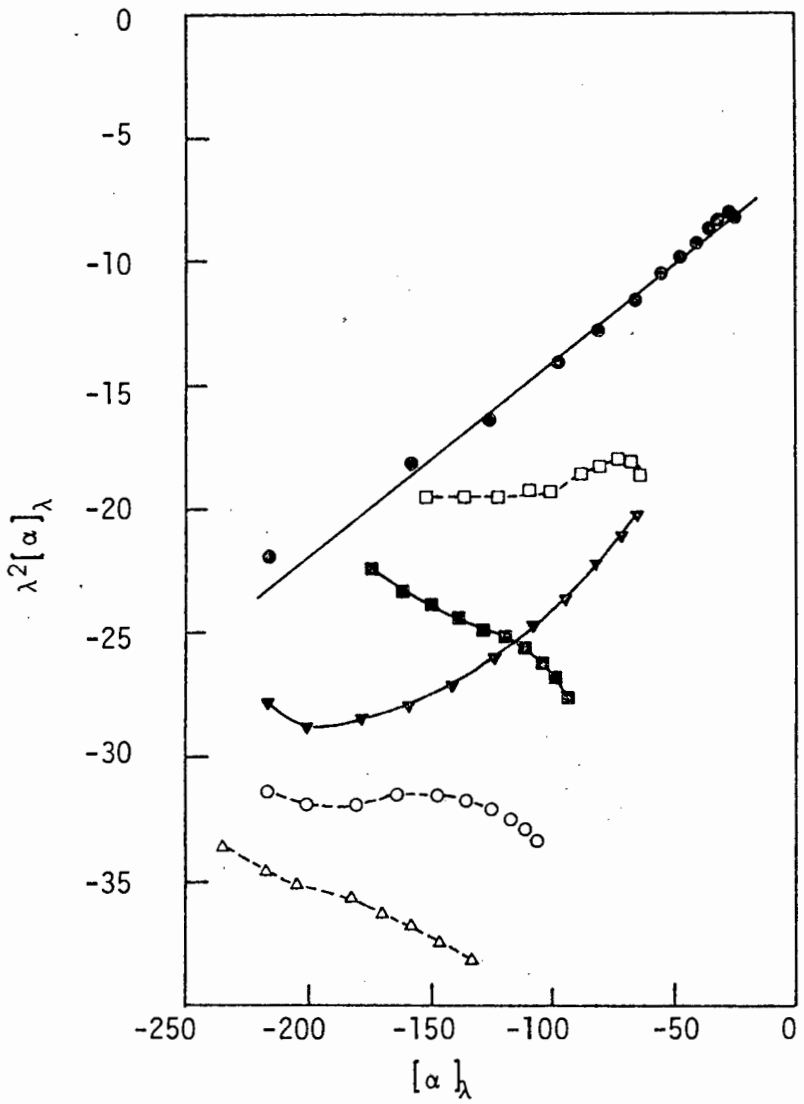


Fig. 6.2. DRUDE PLOTS FOR FRACTION EC1a

Drude plots for fraction EC1a at 20°C, ●——● ; 50°C, ▼——▼ 58°C, □----□ ; 65°C, ■——■; 98°C, △----△ and following cooling to 20°C, o----o. The dispersion constant (λ_c) for EC1a at 20°C is given in Table 6.1.

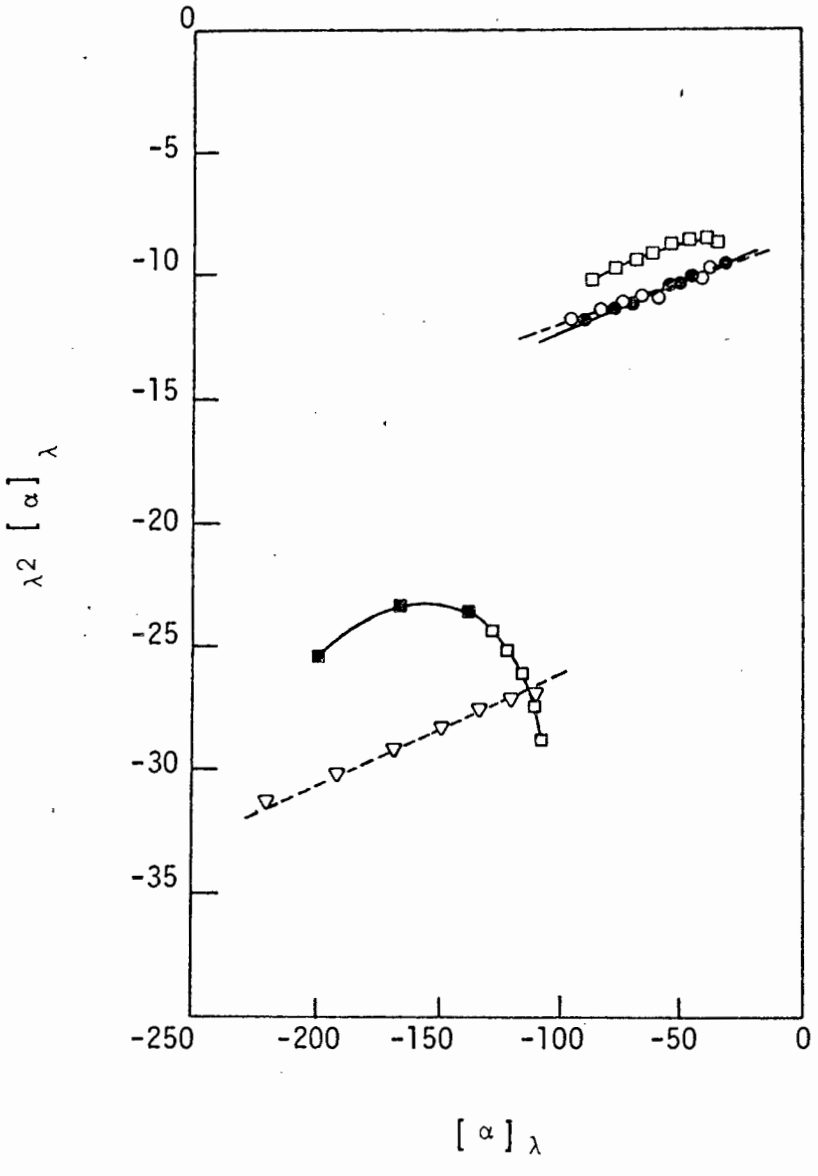


Fig. 6.3 DRUDE PLOTS FOR FRACTION EC4a

Drude plots for fraction Ec4a at 20°C, ●—● ; 56°C, □—□; 65°C, ■—■ ; 98°C, ▽---▽ ; and following cooling at 20°C, ○--○. Values for dispersion coefficients (λ_c) are given in Table 6.1.

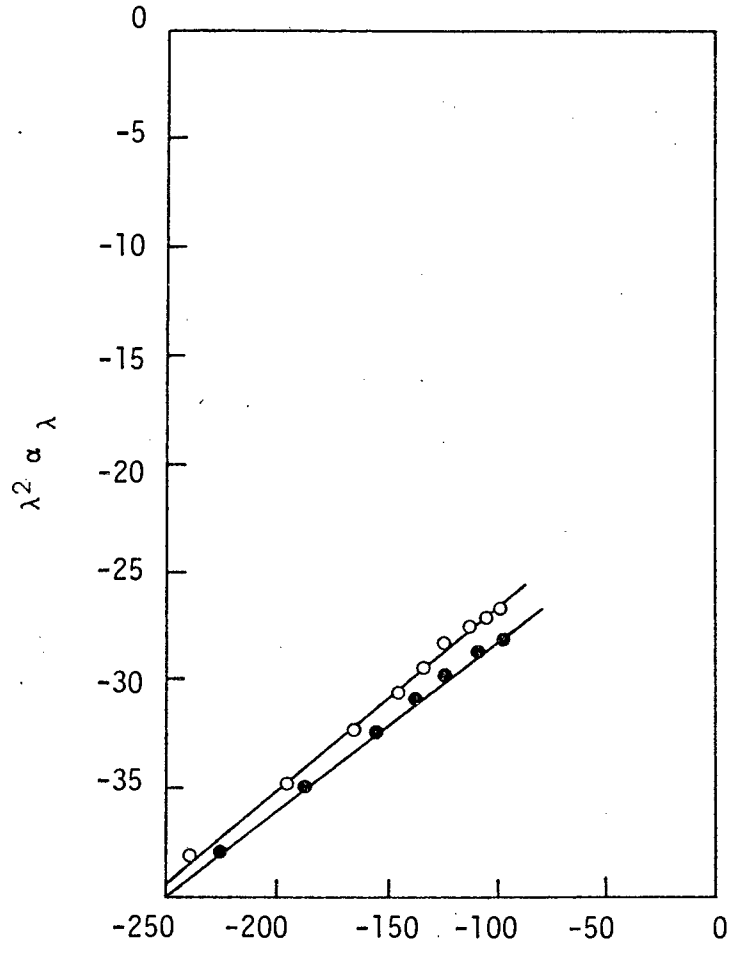


Fig. 6.4 DRUDE PLOTS

Drude plots for fractions EC1c, o——o and EC4b, ●——● at 20°C. Values calculated for the dispersion coefficient, λ_c, are given in Table 6.1.

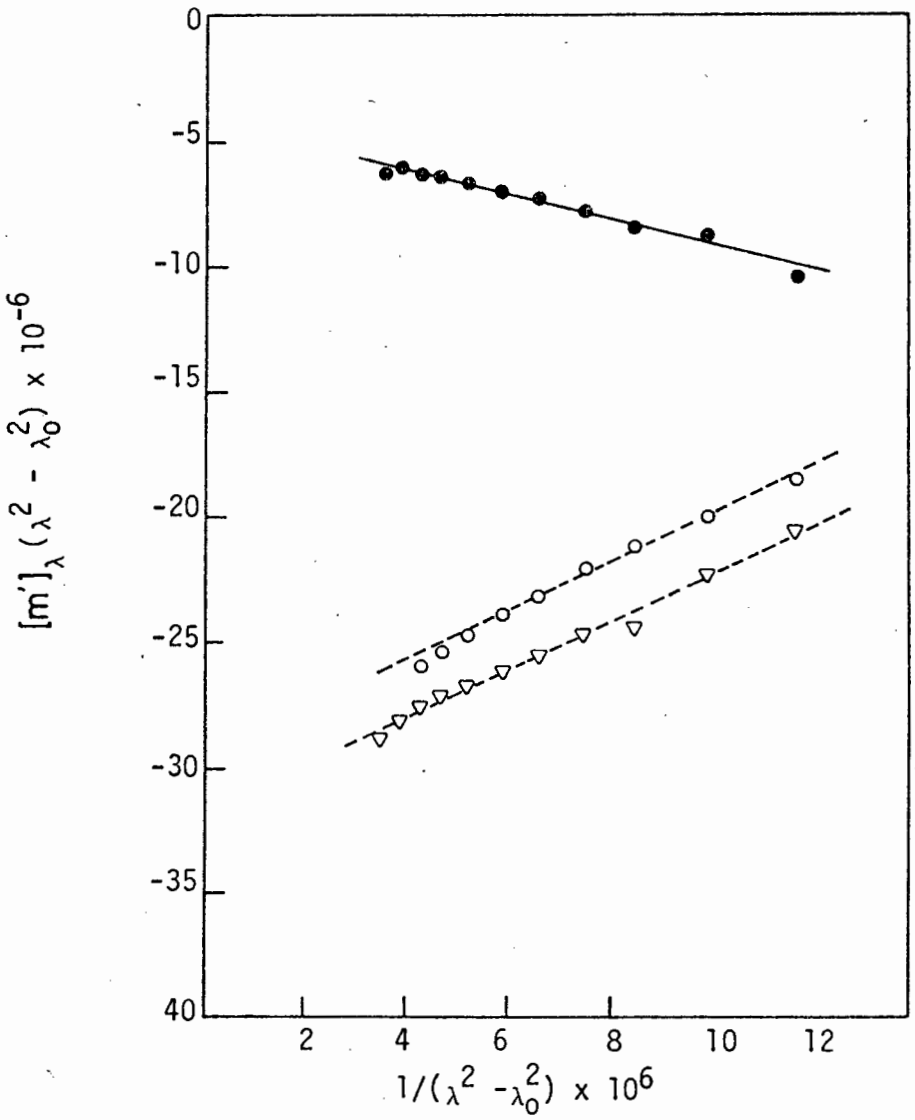


Fig. 6.5 MOFFITT PLOTS, FRACTION ECla

Moffitt plots for fraction ECla at 20°C, ●——●; 98°C, ▽--▽; and following cooling to 20°C, o--o. Values calculated for the parameter b_o are shown in Table 6.1.

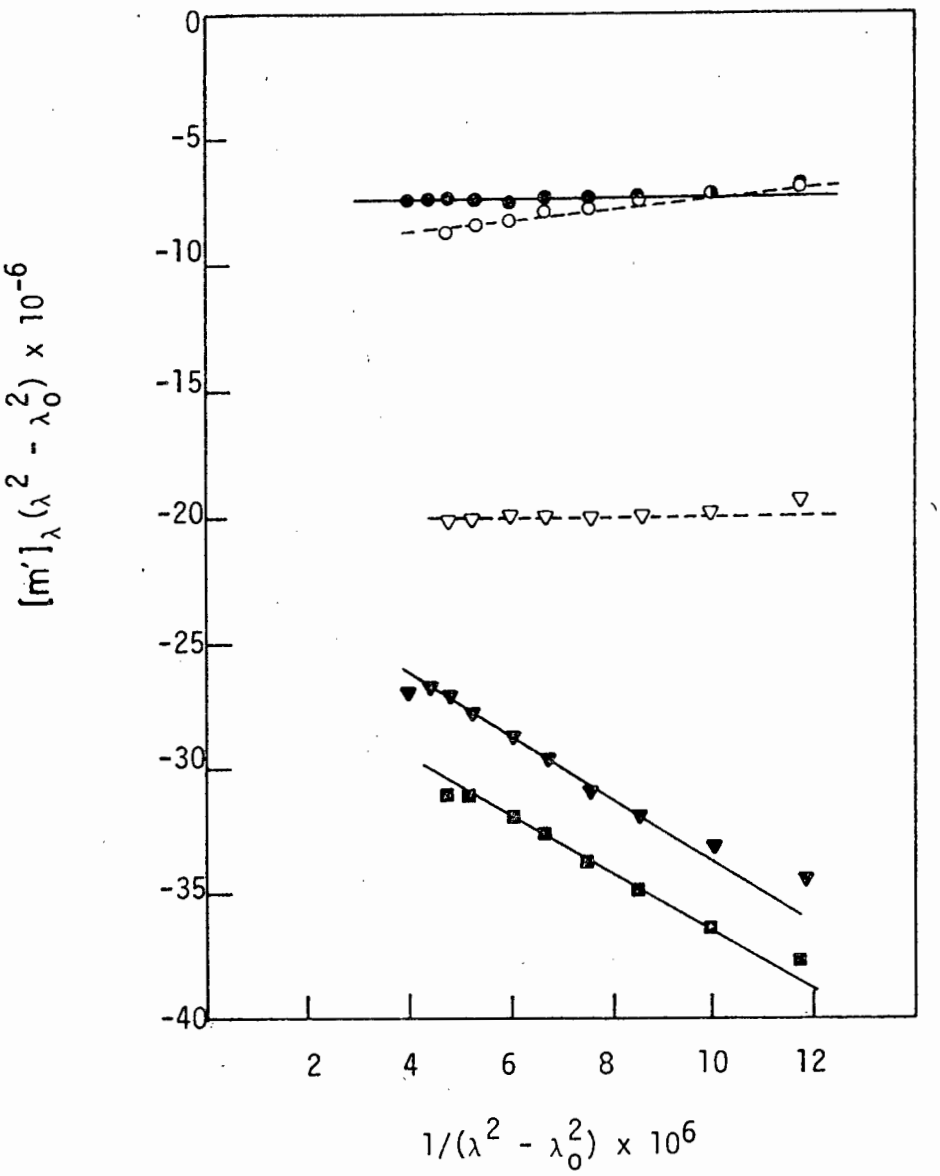


Fig. 6.6 MOFFITT PLOTS

Moffitt plots for fraction EC4a at 20°C, ●—●; 98°C, ▽--▽; following cooling to 20°C, ○--○; fraction EC1c at 20°C, ▼—▼; and fraction EC4b at 20°C, ■—■. Values calculated for the parameter b_o are shown in Table 6.1.

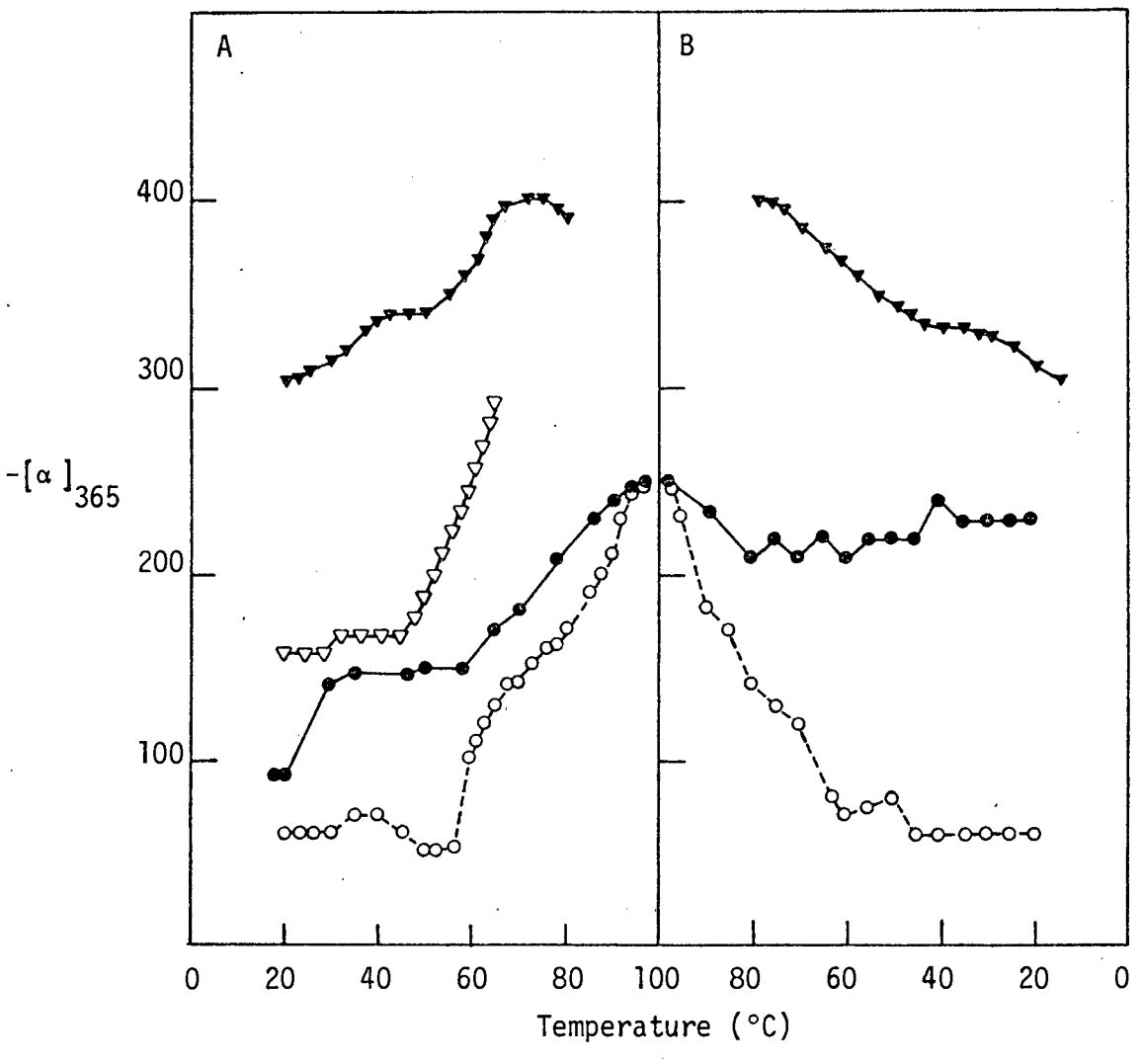


Fig 6.7 OPTICAL ROTATION HEATING AND COOLING CURVES

Fractions EC1a, ●—●; EC1c, ▽—▽; EC4a, ○---○; and EC4b, ▼—▼; were heated from 20°C to 98°C (Panel A) and cooled to 20°C (Panel B). Optical properties, $[\alpha]_{365}$, are shown as functions of temperature. Note that EC1c precipitated above 60°C and results beyond this temperature were not obtained.

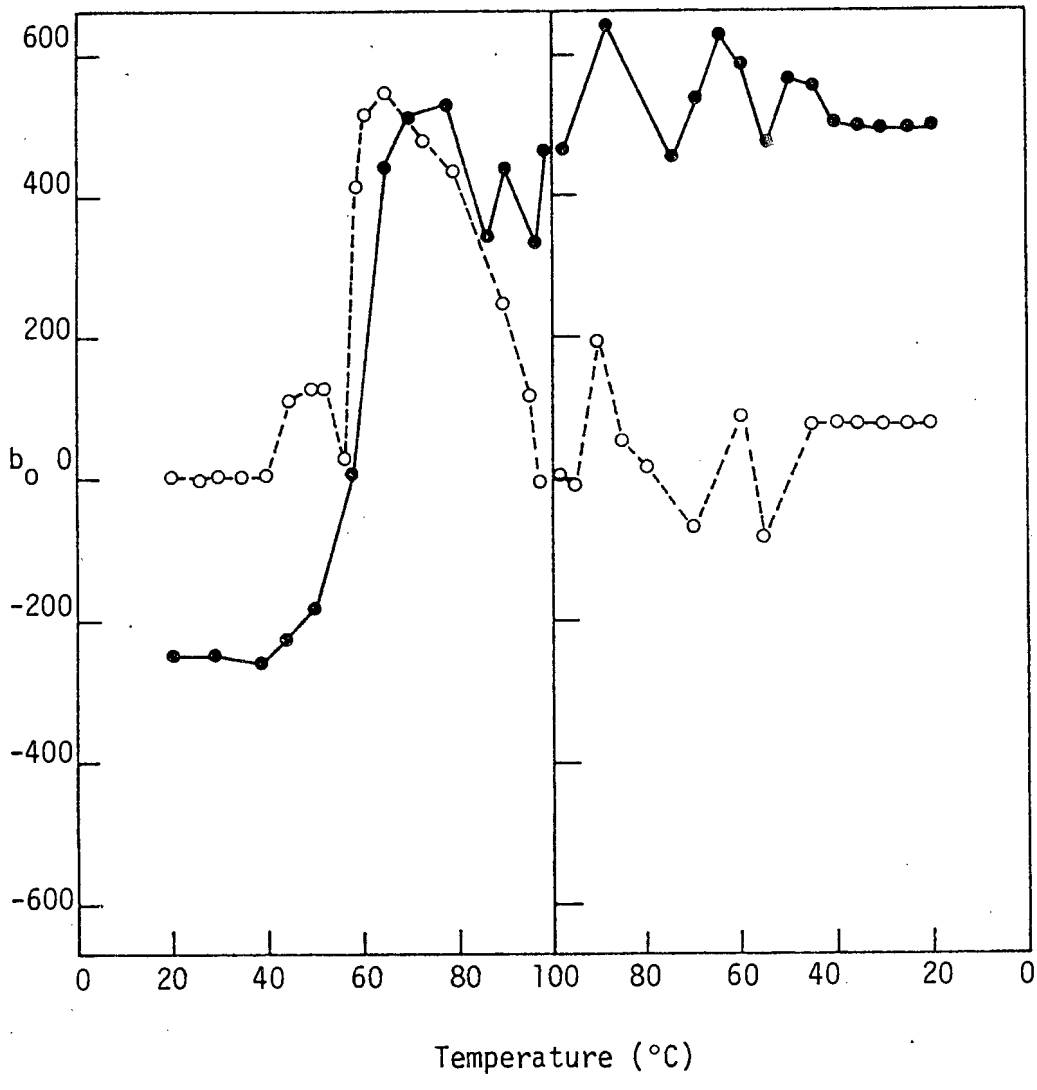


Fig. 6.8 HEATING AND COOLING CURVES ACCORDING TO THE MOFFITT PARAMETER, b_o.

The Moffitt parameter b_o was calculated for solutions of fraction EC1a, ●——● and EC4a, o---o which were heated from 20°C to 98°C (Panel A) and cooled to 20°C (Panel B).

6.3.2.2 Drude plots

Drude plots for solutions of the fractions EC1a and EC4a heated at various temperatures are shown in Fig. 6.2 and Fig. 6.3 respectively. At 48°C the Drude relationship for fraction EC1a was linear with a slope unchanged from that of the unheated solution. Deviation from linearity occurred at 50°C and the general slope of the plot changed from positive to negative with further heating to 65°C and to 98°C. The response of fraction EC4a to heating was similar but at 98°C the Drude relationship became linear with a λ_c value of 214 nm.

6.3.2.3 Dispersion coefficient, b_o

Variation in the dispersion coefficient b_o with temperature for each of the isolates EC1a and EC4a is shown in Fig. 6.8. The linearity of plots of $[m']_\lambda$ $(\lambda^2 - \lambda_o^2)$versus $1/(\lambda^2-\lambda_o^2)$ was good over most of the temperature range investigated. It was, however, observed that, at temperatures close to major transitions, linearity was often reduced to a very limited wavelength range. No noteworthy changes in b_o occurred when the two fractions were heated to 50°C. But heating of fraction EC1a from 50-70°C resulted in an exponential positive shift in b_o. The same effect was noted when fraction EC4a was heated from 58°C to 64°C.

6.3.3 OPTICAL PROPERTIES OF COOLED SOLUTIONS

The optical rotatory dispersions of fractions EC1a, EC4a and EC4b following heating and cooling to 20°C are shown in Fig. 6.1. Cooling curves of $[\alpha]_{365}$and b_o versus temperature are shown in Figs. 6.7B and 6.8B.

No significant reversal of thermally induced changes in the optical properties of fraction EC1a occurred when cooled from 98°C to 20°C. In contrast, the optical properties of fraction EC4a when cooled to 20°C were virtually indistinguishable from those of the native polypeptide. The $[\alpha]_{365}$ cooling curve for this isolate was an

approximate reversal of the heating curve (Fig. 6.7.B). The transient positive shift in b_o which occurred in the heating curve was not, however, found in the cooling curve (Fig. 6.8B).

The thermally induced laevorotatory shift in $[\alpha]_{365}$ which accompanied heating of fraction EC4b was reversed on cooling (Fig. 6.7B). But due to the altered shape of the dispersion curve (Fig. 6.1), $[\alpha]_{589}$ showed very little return to the initial value of the native protein.

6.3.4 ULTRAVIOLET DIFFERENCE SPECTRA

Difference spectra of heated solutions of the isolates EC1a, EC1b and EC4b relative to native solutions are shown in Fig. 6.9. Large negative difference peaks appeared in the spectra of EC1a and EC4b within the 220-240 nm wavelength range with $\Delta A^{1\%}_{1\ cm}$ max values at 226 nm in both instances. Small difference peaks due to side chain chromophores were also produced at longer wavelengths. A broad difference zone from 260-285 nm with maxima at wavelengths 262, 274 and 285 nm characterized the spectrum of EC1a. These could have represented altered environments of tryptophan, tyrosine and possibly phenylalanine residues (Hermans, 1965). The difference spectrum of fraction EC4b over the 240-300 nm wavelength range was negligible apart from a small positive shift at 258-265 nm. Since phenylalanine was not detected in EC4b, it is probable that this peak might have been due to perturbed tryptophan residues (Nicola and Leach, 1976).

The difference spectrum of fraction EC4a displayed a very small negative shift over the 220-250 nm wavelength range and a positive shift at longer wavelengths (270-300 nm). This suggests that the polypeptide backbones in native and heated solutions were very similar in conformation but that the spatial organization of side chain chromophores in the heated preparation differed to some extent from that of the native polypeptide.

6.3.5 MICRO-ELISA STUDIES

Micro-ELISA titrations of native and heated solutions of the isolates EC1a, EC4a and EC4b may be seen in Fig. 6.10. Exponential regions of the titration curves occurred within antigen concentration ranges of 1-1000

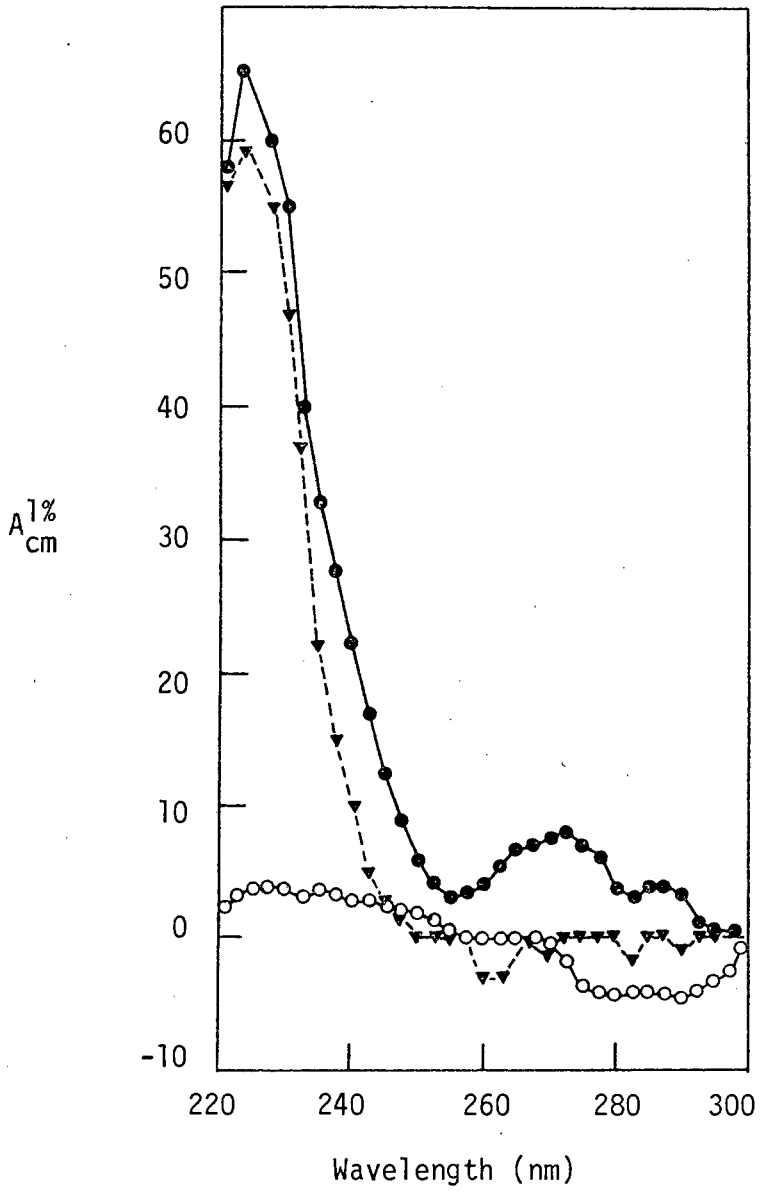


Fig. 6.9. UV DIFFERENCE SPECTRA

UV absorption differences between heated (100°C for 60 min) and native samples of the fractions EC1a, ●——● ; EC4a, o——o; and EC4b, ▼- - - -▼ were standardized to values for 1% (m/v) solutions and were plotted against the wavelength of incident-light.

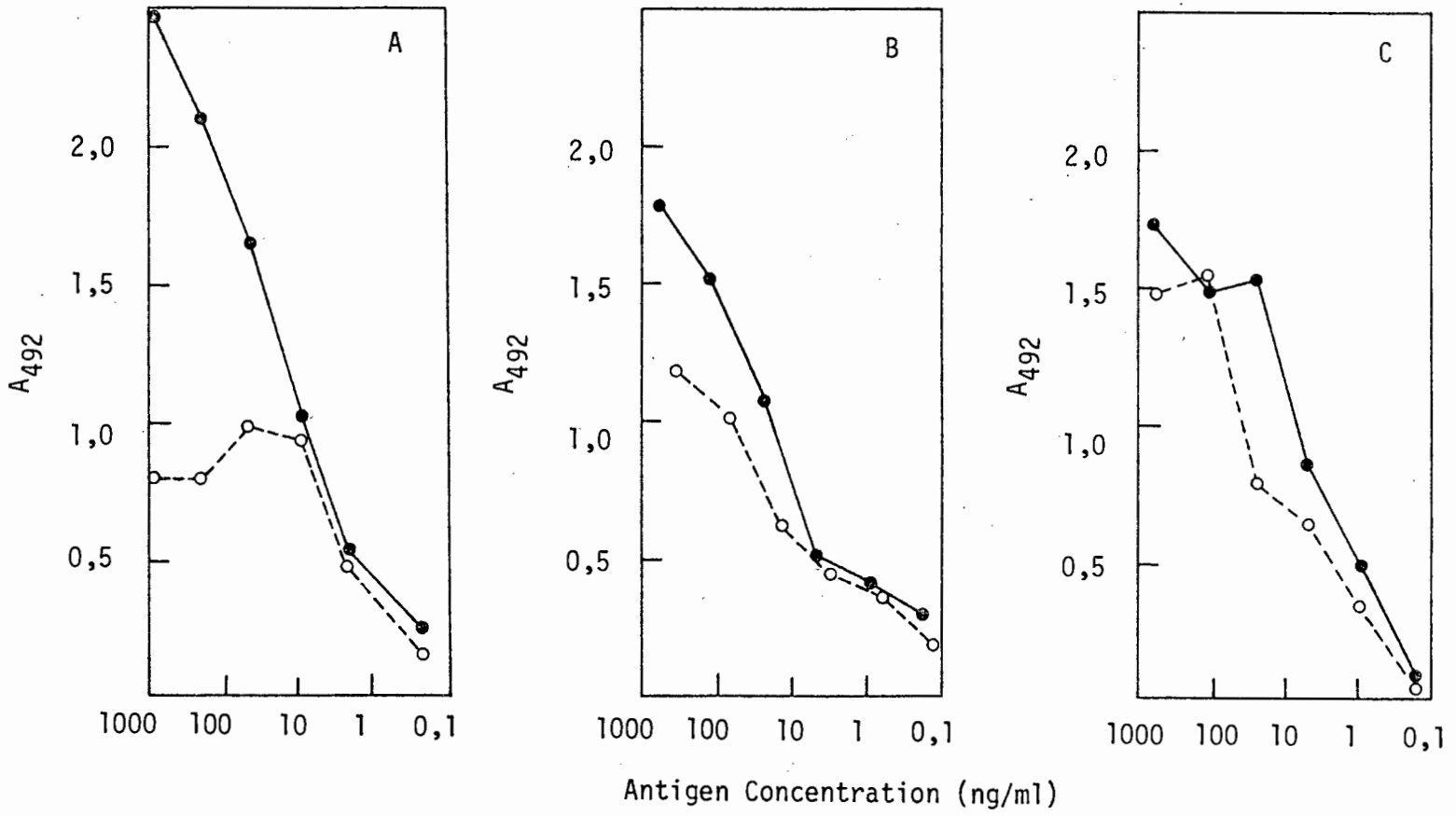


Fig. 6.10. MICRO-ELISA TITRATION CURVES

Comparison of titration curves of native ●——● and heated (100°C for 60 min) o----o samples of fractions EC1a (Panel A), EC4a (Panel B) and EC4b (Panel C).

ng/ml. Maximum absorbances obtained with the isolates EC1a, EC4a and EC4b were less than that obtained with the BCG-CF polyvalent antigen used in Section 4.3.3.2. It was therefore necessary to increase the concentrations of coating IgG and antibody-enzyme conjugate to contain 20 µg/ml of IgG for optimum antigen binding.

Comparison of titration curves of native and heated isolates indicated that antigenic determinants of the isolates were affected differently by heating. The titration curve of fraction EC1a showed a significant downward shift when heated. A similar but smaller shift was displayed by fraction EC4a. In contrast, the heated sample of fraction EC4b attained the same absorbance as the native preparation. However, a lateral shift occurred such that a six-fold increase in antigen concentration was required to obtain the same degree of binding.

The titration curve for fraction EC1c, which has not been shown here, was anomalous in comparison with those of the other three fractions. Very high absorbances (as high as BCG-CF polyvalent antigen) were obtained at very low antigen concentrations. It was concluded from the shape of the titration curve that fraction EC1c contained either a catalase or peroxidase enzyme which caused interference effects by interaction with the micro-ELISA substrate.

6.4 DISCUSSION

It has become apparent only relatively recently that β-sheets are essential elements of the spatial orientation of proteins (Chou and Fasman, 1978). In this respect the Moffitt treatment of optical rotatory dispersion which depends on an α-helix-random coil model is somewhat limited. Negative b_0 values are regarded as indicative of α-helical structure but in fact reflect the sum of negative contributions of α-helical regions and positive contributions by β-sheet structures (Urnes and Doty, 1961). Nevertheless, the Moffitt and Drude relationships provided the best available indication of overall configuration. This was particularly the case with the two isolates EC1a and EC1c each of which contained more than one protein.

6.5 NATIVE CONFORMATIONS OF EC1a, EC1c AND EC4b

Optical properties of native solutions of EC1a, EC1c and EC4b showed that the proteins of these fractions were compactly folded with complex secondary structure. The dispersion constants (λ_c) of 281 nm, 261 nm and 258 nm calculated for the three fractions fall within the range of proteins classified by Jirgensens (1961) as Class 1 proteins. These are characterized by high dispersion constants and include compact ordered proteins such as ovalbumin, serum albumin, amylase and lysozyme. Calculations of the dispersion coefficient b_0 for EC1a, EC1c and EC4b yielded large negative values which characterize proteins with α-helical secondary structure (Imahori, 1961). The extent to which amino acid residues of the isolates were engaged in α-helical structures is given

by the calculated partial helical contents. These indicate 38%, 52% and 42% α-helical involvement for EC1a, EC1b and EC4b. Partial helical contents of 35-46% and 45-50% have been reported for bovine serum albumin and ovalbumin from optical rotatory dispersion and hydrogen-deuterium exchange data (Urnes and Doty, 1961).

6.6. THERMALLY INDUCED CONFORMATIONAL CHANGES IN EC1a

The heating curves for fraction EC1a show that conformations of the two proteins of this fraction were readily affected by mild heating. An increase in temperature from 20°C to 30°C resulted in a laevorotatory shift indicative of a denaturation transition. But, since λ_c and b_o parameters were not altered by this temperature increase, the observed changes reflected only tertiary and/or quaternary structure re-arrangements.

The second optical transition which occurred on heating from 50-70°C was related to denaturation as shown by the major transition in b_o from negative to positive. This change indicated disruption of organized α-helical secondary structure and the formation of β-pleated sheet structures. The transition from α-helical to β-structure was sharply defined. Therefore, it may be concluded that subunits of the two proteins comprising fraction EC1a underwent simultaneous α-helix to β-structure transformations. In their mathematical treatment of the β-form, Wada *et al.*, (1961) assumed that in synthetic polypeptides α-helices are converted to β-forms via a random coil intermediate. This has been demonstrated by Gill *et al.*, (1972) for poly-L-lysine which undergoes a transition from α-helical conformation to the random coil form when heated from 20°-40°C. With further heating to 50°C β-structure is assumed and at higher temperature aggregation ensues.

Since a b_o parameter of zero characterizes the random coil form (Imahori, 1961) it is possible that random coils might have formed as intermediates in the denaturation of EC1a at 56-58°C. But, Drude plots for EC1a at 55°C and 58°C were complex and non-linear (Fig. 6.2). Therefore when b_o was zero both α-helical and β-structures were represented in this fraction.

Further heating of EC1a to 98°C did not result in any further conformational changes of note. On cooling, some rearrangements occurred but optical rotation data showed that thermal denaturation was irreversible. This was confirmed by the large negative peak at 224 nm in the UV-difference spectrum.

The irreversible thermal denaturation of EC1a was previously shown (Section 5.3.1.2) to result in total loss of immunoprecipitin properties. In the micro-ELISA system a downward shift in peak absorbance followed heating of EC1a although a significant degree of primary binding by antibody nevertheless occurred. Since B lymphocyte and hence antibody specificity is preferentially directed against conformational determinants of protein antigens (Singh et al., 1980), it is probable that certain regions of conformation were either preserved during heating or renatured during cooling. The first of these alternatives would agree with Aune et al. (1967) and Matthews and Westmoreland (1975) that protein unfolding in thermal denaturation reactions is incomplete as judged by further changes in optical and viscocity properties upon the addition of chemical denaturants. This view has recently been contested by Privalov (1979). But, since the denaturation of EC1a was not a simple α-helix-random coil transformation only, but included β-structure conversion and the possible formation of small aggregated structures, it is likely that a number of antigenic determinants might have been preserved during heating.

6.7 CONFORMATION OF NATIVE AND HEATED EC4a

The optical properties of the polypeptide of EC4a differed from those of the other three fractions. The λ_c value of 208 nm and a b_o parameter of zero suggested a very randomized conformation. However, the various changes in optical properties which occurred during heating indicated that this was not the case. At temperatures below 56°C no significant conformational alterations were apparent. When heated from 56-60°C optical transitions including a laevorotatory shift in $[\alpha]_{365}$, a positive shift in b_o and departure of the Drude relationship from linearity occurred. These indicated extensive assumption of β-structure.

On further heating to 98°C the λ_c value of 214 nm and b_o value of + 5 indicated disruption of β-structure at very high temperature and the appearance of a random coil form.

The development of β-structure in proteins during heat denaturation is generally considered to be irreversible. However, reversal of this process, as in the case of EC4a, has been observed with Bence Jones proteins (Urnes and Doty, 1961) and with the synthetic polypeptide poly-L-lysine (Gill et al., 1972). The optical properties of the polypeptide of EC4a in the native and denatured state (98°C) were remarkably similar to those reported for Bence Jones proteins (IgG L-chains), γ-globulins and macroglobulins. These proteins have complex native conformations containing an unusually high proportion of β-sheets. In the native state they are characterized by low Drude constants (λ_c = 190-210 nm) and b_o parameters close to zero. When denatured by pH alteration or by chemical denaturing agents their λ_c parameters generally rise to within the region of approximately 220 nm. (Jirgensens, 1961; 1973; Urnes and Doty, 1961). It may therefore be concluded that secondary structure of the native polypeptide of EC4a included disorganized regions, some α-helical regions and a significant proportion of β-pleated sheet structure.

When EC4a was cooled from 98-20°C the change in $[\alpha]_{365}$ indicated that renaturation occurred, although a small positive shift in b_o to + 79 showed a greater proportion of β-structure than was present in the native molecule. The UV difference spectrum agreed with optical rotatory dispersion data by showing that the backbone structure of the heated polypeptide differed very little from that of the native form, but that some differences in the exposure of side chain chromophores to solvent were apparent. This was confirmed by micro-ELISA studies where the downward vertical shift in peak absorbance indicated that a fraction of the antigenic determinants remained disrupted after heating. Therefore, it may be concluded that the renaturation of the polypeptide which occurs on cooling produces a structure very similar to the original but with some altered regions.

6.8 EFFECT OF HEAT ON THE CONFORMATIONS OF EC1c AND EC4b

When these preparations were heated the initial denaturation reactions were followed by β-aggregate formation and precipitation from solution. In the case of EC1c virtually all the protein precipitated from solution. Although a small proportion of EC4b precipitated from solution when heated to 98°C, the reversion of $[\alpha]_{365}$ to its original value suggested that renaturation occurred to some extent when cooled. The b_o parameter of the cooled solution was positive suggesting a greater proportion of β-structure than in the native molecule. The negative peak at 226 nm in the UV difference spectrum provided additional evidence that the polypeptide backbone conformation following heating differed from that of the native molecule. These differences were accompanied by a lateral shift in peak absorbance in micro-ELISA studies. This could be interpreted as a generalized decrease in affinity of the antigenic determinants indicating that partial renaturation occurred when EC4b was cooled. Fujita and Imahori (1974) have shown that protein populations which have been heated and which appear to be partially denatured do not contain any appreciable numbers of partially denatured proteins, but in fact consist of a mixture of fully native and fully denatured molecules. Therefore, it is probable that the lateral shift seen in the micro-ELISA system indicated that the cooled solution of EC4b consisted of a population of irreversibly denatured molecules, and a population of molecules which renatured to a conformation very similar to that of the native form. This would explain the unchanged immunoprecipitin and electrophoretic properties of heated solutions of EC4b.

6.9. AMINO ACID COMPOSITION, CONFORMATION AND THERMOSTABILITY

The enzyme proteins of thermophilic bacteria are able to function efficiently at temperatures as high as 80°C (Fujita and Imahori, 1974). There has consequently been much interest in determining how these enzymes differ from their heat sensitive counterparts of mesophilic bacteria. The comparison of amino acid compositions of thermostable and thermolabile forms of a number of specific enzymes has not revealed any significant differences (Singleton et al., 1977). There is evidence,

however, that the relative thermostability of thermophilic enzymes is not dependent on any uncommon features such as covalent cross linkages, unusual amino acids, lipids and sugar moeities or the absence of higher structure, but rather depends on subtle differences such as efficient hydrophobic packing and the favourable distribution of charges and hydrogen bonds in the molecule (Fujita and Imahori, 1974).

It may be seen from the amino acid compositions of the BCG-CF proteins presented in Table 5.4 that the heat stable antigens EC4a and EC4b had higher contents of serine and lysine and lower contents of glutamic acid than the heat sensitive fractions EC1a, EC1b and EC1c. The data base for this comparison was rather limited and it is therefore not possible to assess the significance of these differences without further investigation.

The amino acid analyses and other investigations of BCG-CF proteins, as with other reports (Fujita and Imahori, 1974), revealed no lipid, carbohydrate or unusual amino acid residues in the heat stable antigens. Furthermore both the heat sensitive and the thermostable BCG-CF proteins had compact ordered structures in the native state. The heat stable antigen of EC4a was shown to contain predominantly β-structure and the second thermostable antigen, EC4b to have predominantly α-helical structure. It may therefore be concluded that the predominant form of secondary structure of a protein molecule does not determine its thermostability properties.

CHAPTER SEVEN

CONCLUSION

The object of this thesis was to investigate the conformational changes which occur in so called "heat stable" and "heat labile" protein components of a mycobacterial culture filtrate during heating and relate these to the more obvious indications of denaturation studied by other workers. This required the initial preparation of a suitable culture filtrate (M. bovis, strain BCG 172 culture filtrate) and an investigation of the effects of heat which has involved : (a), a study of the changes which occur in the composition and immunological properties of BCG culture filtrate as a result of heating, including factors which influence the precipitation of BCG culture filtrate proteins during heating, (b), the isolation and characterization of proteins representing the "heat stable" and "heat sensitive" fractions in the culture filtrate, (c), an investigation of the conformational status, electrophoretic and immunological properties of these proteins before and after heating and (d), the structural changes which occur during heating and cooling of thse antigens.

The non-dialysable components of the BCG culture filtrate (BCG-CF) consisted mainly of proteins and polysaccharides which accounted for 41% and 38% respectively of the total solids. The protein fraction was heterogeneous containing many more than 34 individual polypeptides with molecular weights which ranged from less than 10 000 to much greater than 70 000. The majority of native proteins were found within molecular weight ranges of 8 000 - 30 000 and 70 000 - 150 000, the latter of which contained many multisubunit proteins and nonspecifically aggregated proteins. There was evidence that many of the proteins of BCG culture filtrate had peptides or low molecular weight polypeptides adsorbed to them. It is possible that these components may have arisen as a result of proteolysis during the incubation of the cultures. Certain low molecular weight polypeptides displayed variable electro-

phoretic mobility which could not be ascribed to changes in molecular size, such as aggregation. It was proposed that alteration in conformation resulted in net charge alteration giving rise to the observed variable mobility. Polysaccharides of BCG-CF existed in a high molecular weight fraction, an intermediate molecular weight fraction and a low molecular weight fraction.

Heating of the BCG culture filtrate at 100°C resulted in the precipitation of some protein, the extent of which was dependent on the conditions under which heating occurred. The higher molecular weight proteins were found to aggregate and precipitate more readily than low molecular weight proteins. Those proteins which remained in solution were mainly of low molecular weight and most were denatured as demonstrated by the loss of ability to function as immunoprecipitin antigens and to migrate in discrete bands in polyacrylamide gel electrophoresis. Factors which contributed to the degree of denaturation and precipitation included the temperature, heating time, the concentration of culture filtrate materials, the composition of the medium and the pH of the medium. The affect of pH was particularly marked, with a small reduction in pH below neutral leading to a large increase in the quantity of protein precipitated. This provided quantitative confirmation that the increased yields of mycobacterin protein reported by others following adjustment of the medium pH to greater than neutral prior to heat sterilization (Kim and Magnusson, 1964; Lesslie et al., 1975), is due more to protection against protein precipitation than to improved extraction of tuberculin proteins. These studies showed that the number of proteins which undergo denaturation during heating, and the degree of denaturation will vary with the conditions and therefore account for much of the variability of the compositions of PPD preparations.

Two antigens which displayed resistance to heating at 100°C were isolated from BCG culture filtrate in homogeneous form by a combination of gel exclusion chromatography, ion exchange chromatography and preparative polyacrylamide gel electrophoresis. These isolates labelled EC4a and EC4b were immunologically distinct, tuberculin skin test active and comprised single polypeptide chains with molecular weights of 8 700 and 12 200 respectively. Their amino acid compositions were

similar, the major components being serine, glycine and alanine. Three heat sensitive high molecular weight, antigenic fractions designated EC1a, EC1b and EC1c were prepared by the same procedures used for the heat stable antigens. Fraction EC1a contained two 54 000 molecular weight proteins each of which comprised two polypeptide subunits, the molecular weights of which were 19 000, 26 000, 28 000 and 35 000. Fraction EC1b contained a single polypeptide with a molecular weight of 40 000 and fraction EC1c was somewhat heterogeneous containing as many as nine polypeptides.

Immunoelectrophoretic characterization of BCG-CF according to the United States-Japan reference system which uses antisera prepared from cell extracts and culture filtrates of M. tuberculosis H37Rv, confirmed earlier reports of the similarity of the immunoelectrophoresis patterns of M. tuberculosis and a number of BCG strains of M. bovis (Chaparas and Hedrick, 1973; Chaparas, 1975). The presence of antigen 5 which has recently been shown to be a protein with a molecular weight of 35 000 (Daniel and Anderson, 1978) with a species distribution limited to strains of M. tuberculosis and M. bovis (Daniel et al., 1979), was also demonstrated in the BCG 172 strain of M. bovis used in this investigation.

Immunoelectrophoretic studies of the protein fractions isolated from BCG culture filtrate showed that the two heat sensitive antigens of fraction EC1a reacted with the reference cell extract antiserum but not with the reference culture filtrate antiserum. This suggested that the two proteins of the fraction were possibly membrane bound and only released into the medium on autolysis. The difference in reactivity of the reference cell extract and culture filtrate antisera of batch 002A underlines the importance of using both of these antisera when studying the antigenic specificity of mycobacterial culture filtrates. One of the two antigens of fraction EC1a was identified as representing the reference immunoprecipitin band 10, which has been reported to have a wide distribution among mycobacteria (Chaparas, 1975). In the BCG strain studied here, this antigen was represented by a protein which was membrane bound, possibly an enzyme, having a molecular weight of 54 000 and comprising two polypeptide subunits.

Only one of the five antigens of fraction EC1c was detected with the reference cell extract and culture filtrate antisera. This antigen appeared to be identical with antigen 6 of the reference system. Antigen 6 has been shown (Daniel et al., 1975) to correspond with the a_2 protein (Molecular weight of 45 000 - 48 000) isolated by Daniel and Ferguson (1970) from culture filtrates of M. tuberculosis H37Ra. The molecular weight of one of the polypeptides of fraction EC1c was estimated at 46 000. It is possible that this component was the antigenic element which represented antigen 6 in this study.

The heat stable antigens EC4a and EC4b were unreactive with the reference antisera. This showed that despite the obvious similarities between the antigenic compositions of M. tuberculosis and BCG strains of M. bovis, specificity differences between these two species nevertheless do exist.

Analysis of the native conformations of the heat sensitive antigens of fractions EC1a and EC1c by the Drude relationship and by the empirical Moffitt treatment indicated that the polypeptides of these fractions exist as organized folded structures with α-helical regions of secondary structure.

The conformations of antigens of fraction EC1a were readily susceptible to heating and tertiary or quaternary structural rearrangements occurred over the temperature range of 20-30°C. At 40-60°C irreversible denaturation with alteration of α-helical secondary structure to the extended β-sheet form occurred. This denaturation reaction resulted in the loss of immunoprecipitin properties although micro-ELISA studies have shown that primary binding with antibody still occurred. Therefore, although fraction EC1a was irreversibly denatured according to electrophoretic, optical rotatory dispersion and UV difference spectroscopy data, antigenic determinants and hence some portions of conformation were preserved during heating.

The heat stable antigen of fraction EC4a contrasted from the heat sensitive antigens in having a λ_c of 208 nm and a b_o value of zero, showing it to exist mainly in β-structures but with some α-helical

regions and some regions which may be disorganized. When heated to 56-60°C the α-helical structure was converted to the β-form but with further heating to 98°C the β-structure melted out and was replaced by a random coil form. On cooling the molecule renatured and the resulting conformation was very similar to that of the native molecule, and although some altered regions existed, the electrophoretic mobility and the formation of immunoprecipitates was not materially affected.

The conformational parameters (λ_c, b_o) of the polypeptide of the other heat stable fraction, EC4b, indicated that in the native state it existed as an organized folded structure with a partial helical content (α-helical) of 42%. Denaturation occurred when heated from 25-40°C and the irreversible formation of precipitates when heated to 70-98°C. On cooling the optical rotatory dispersion, UV difference spectra and micro-ELISA studies suggested that a population of irreversibly denatured molecules and a population of molecules with native properties existed. This latter population could be implicated as the fraction responsible for the unchanged electrophoretic and immunoprecipitin properties of EC4b following heating.

From this it is clear that apparently heat stable mycobacterial protein antigens which retain immunoprecipitin and electrophoretic properties following heating do in fact denature at elevated temperatures. Furthermore, on cooling these proteins renature to give molecules which to a large extent are correctly folded, but contain some regions which differ from the original. Similarly, heat labile proteins may retain certain antigenic determinants in spite of obvious irreversible denaturation. The ability of the heat resistant antigens to renature was not dependent on the initial degree of resistance of the molecule to denaturation, since the heat sensitive fraction EC1a and the heat stable antigen EC4b underwent temperature dependent conformational alterations between the incubation temperature of 37°C and the normal room temperature of about 20°C. Renaturation was also independent of the predominant form of secondary structure of the molecule, since both EC4a which contained largely β-structure and the polypeptide EC4b which had a high proportion of α-helical conformation were able to resume ordered conformations on cooling. It may therefore be concluded that

the ability of heat resistant mycobacterial antigens to renature on cooling depended on how readily the β-structure which formed at high temperature melted out on cooling.

The distinction between heat stable and heat labile mycobacterial antigens is thus one of degree and depends largely on the method of assay. The skin test activities of all the isolates were unaltered by any change in the conformation of the molecule, and specificity for this activity must therefore rest in the primary structure of the molecule. This is in agreement with the mounting evidence from other sources that the antigenic signal to the T lymphocytes in mycobacterin delayed hypersensitivity skin reactions is dependent on amino acid sequence (Arnon, 1974; Singh et al., 1980). The delayed hypersensitivity reaction has therefore great limitations in respect of denaturation studies, although the value of in vitro delayed hypersensitivity reactions in determining evolutionary relationships among antigens of closely related or unrelated species has been realised (Arnon, 1974).

The development of a micro-ELISA procedure, which measures primary binding of antigen and antibody, was of great value in this work. It provided a sensitive indication of the changes in conformation which accompanied heating, and good correlation was obtained between the micro-ELISA results, UV absorbance spectroscopy and optical rotatory dispersion data. There has recently been interest in the application of highly sensitive immunological procedures to the study of mycobacterial antigens and radio-immunoassays have been used in specificity studies of mycobacteria (Axelsen et al., 1974; Ferguson et al., 1978). The micro-ELISA system described in this investigation should have wide application in this field particularly with respect to the ease of preparation of micro-ELISA reagents, the simplicity of the assay and the minimal amounts of antigen and antibody required. It would provide an extremely sensitive measure of the specificity of partially purified fractions and isolated antigens and could be applied to the long term goal of characterization of specific mycobacterial antigenic determinants, and ultimately, the synthetic production of species specific diagnostic mycobacterial antigens.

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APPENDIX 1

A1 COMPOSITION OF LIQUID BACTERIOLOGICAL CULTURE MEDIA.

Medium components	BAI medium	Sauton medium
L-Asparagine	14,0 g	4,0 g
Citric acid	-	2,0 g
Sodium citrate	0,9 g	-
K_2HPO_4	1,4 g	0,5 g
$MgSO_4.7H_2O$	1,5 g	0,5 g
Glycerol	100 ml	60 ml
Glucose	10,0 g	-
Ferric ammonium citrate	0,3 g	0,05 g
Trace element stock	10 ml	-
$ZnSO_4.7H_2O$ (2g/l)		
$MnCl.4H_2O$ (0,5g/l)		
$CaCl_2$ (0,35g/l)		
Distilled Water	890 ml	950 ml
pH adjusted with concentrated ammonia to	6,8 - 7,1	6,8 - 6,9
Media were sterilized by filtration through 0,2 µm membranes		

APPENDIX 2

A2 EVALUATION OF TUBERCULIN POTENCY BY PARALLEL LINE ASSAY

The calculation of the potency of the test preparation relative to the standard preparation and the determination of confidence limits was performed according to the methods outlined in the European Pharmacopoeia.

A2.1 Calculation of relative potency

Total responses for the standard (S) and test preparations (U) in guinea pig potency assays were obtained by addition of the mean reaction diameters for the high and low doses of the standard ($S = S_1 + S_2$) and test ($U = U_1 + U_2$) preparations respectively. Mean responses for standard (Y_s) and test (Y_u) preparations were calculated from

$$Y_s = \frac{S}{N_s} \quad \text{and} \quad Y_u = \frac{U}{N_u}$$

where N_s is the total number of responses of high and low doses of the standard preparation and N_u the total number of responses of the test preparation. Linear contrast values for standard (L_s) and test (L_u) preparations were calculated according to the formulae

$$L_s = S_2 - S_1 \qquad L_u = U_2 - U_1$$

The slope (b) of the regression line of response versus log dose based on all preparations in the assay was determined according to the relation

$$b = \frac{L_S + L_u}{Inh}$$

where I is the interval between adjacent log doses, n is the number of replicates of each treatment and h is the number of preparations including the standard in the assay. The log potency ratio (M_u') of the test preparation relative to the standard preparation was calculated from the mean responses of the standard and test preparations and the slope of the regression line by the formula

$$M_u' = \frac{Y_u - Y_S}{b}$$

The estimate of potency ratio R_u' was obtained as the antilog of M_u'.

A2.2 Calculation of fiducial limits

Fiducial (confidence) limits which have a 95% probability of including the true potency were calculated as the antilog of the expression

$$\text{Fiducial limits} = CM_u \pm \sqrt{(C - 1)(CM_u'^2 + c'I^2)}$$

where c' is a constant (in this case 1) and C which is a measure of the significance of the regression was calculated from

$$C = E/(E - s^2t^2)$$

and approaches unity in an assay with a well defined slope. The regression sum of squares E was calculated from the equation

$$E = \frac{(L_s + L_u)^2}{2nh}$$

The value of s^2 was obtained by division of the residual error estimated as in Section A3.3 divided by its degrees of freedom. A value of t was obtained according to the degrees of freedom of s^2 from Student's statistic tables.

A2.3 The calculation of residual error

Designation	Expression	Degrees of freedom (f)
Correction term, K, for analysis of variance	$K = \frac{(\Sigma Y)^2}{N}$	-
Variation from treatments	$\frac{(S_1^2 + S_2^2 + U_1^2\ U_2^2) - K}{h}$	K - 1
Variation from rows ($R_1 \ldots\ldots\ldots R_n$ is the total response in each of rows 1 to n)	$\frac{(R_1^2 + R_2^2 + \ldots\ldots R_n^2) - K}{dh}$	n - 1
Reduced sum of squares	$Y^2 - K$	N - 1
Residual error	Residual error = reduced sum of squares - (variation from treatments + variation from rows)	-

APPENDIX 3

A3 MOLECULAR WEIGHTS OF BCG-CF PROTEINS FROM SDS-PAGE DATA

A3.1 Computation procedure

The least squares method described by Sokol and Rohlf (1969) was used for the calculation of a best-fit calibration graph of the relative mobilities of protein standards versus their molecular weights in SDS-PAGE studies. A mean value $\overline{Y}$ was calculated from the observed relative mobilities of the protein standards, and a mean value $\overline{X}$ was calculated from the sum of their molecular weights. The regression coefficient (slope of best fit line) was calculated from the relation

$$b_{Y.X} = \frac{\Sigma xy}{\Sigma x^2}$$

where x represents the deviation of the relative mobility of each protein standard from the mean value ($\overline{Y}$), and y similarly represents the deviation of the molecular weight of each standard from the mean ($\overline{X}$).

The intercept on the relative mobility axis, a, was calculated from the equation.

$$a = \overline{Y} - b_{X.Y}$$

The molecular weight, X, of each unknown protein was then calculated from relative mobility data by

$$X = \frac{Y - a}{b_{Y.X}}$$

A3.2 Preparation of calibration curve

Data from SDS-PAGE studies were used to prepare a calibration graph of relative mobility versus log molecular weight according to the procedure described in Section A3.1.

Protein Standard	Molecular weight * (X)	Relative mobility ** (Y)	x $(X - \bar{X})$	y $(Y - \bar{Y})$	xy	x^2 $(X\ 10^{-7})$
Ribonuclease	13 700	0,891	-15 967	0,239	-3 816	2,55
Lysozyme	14 700	0,873	-15 367	0,221	-3 396	2,36
Haemoglobin	15 500	0,857	-14 167	0,205	-2 904	2,01
Trypsin	23 300	0,694	- 6 367	0,042	- 267	0,41
IgG (L chain)	23 500	0,689	- 6 167	0,037	- 228	0,38
Carbonic anhydrase	29 000	0,549	- 667	-0,103	69	0,04
Ovalbumin	43 000	0,445	13 333	-0,207	-2 760	1,78
IgG (H chain)	50 000	0,422	20 333	-0,230	-4 677	4,13
Leucine amino peptidase	53 000	0,337	23 333	-0,315	-7 350	5,44
Bovine serum albumin	68 000	0,275	38 333	-0,3	-14 452	14,69
SUM OF COLUMN	356 000	7,822			-48 684	40,47
MEAN VALUES $(\bar{X}, \bar{Y})$	29 667	0,652				

* Molecular weight data were taken from Weber and Osborn (1969).

** The relative mobility represents the distance migrated by proteins relative to the bromophenol blue tracking dye.

APPENDIX 4

A2 CALCULATION OF STANDARD DEVIATION.

Standard deviation, δ, was calculated according to the formula

$$\delta = \sqrt{\frac{\Sigma d^2}{n-1}}$$

where d represents the deviation of each reading from the mean and n the total of readings.

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