ELECTROPHORETIC AND IMMUNOELECTROPHORETIC ANALYSES OF SERUMS FROM NORMAL COWS AND COWS EXPERIMENTALLY AND NATURALLY INFECTED WITH MYCOBACTERIA

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
George Leonard Wright, Jr.
1966

This is to certify that the

thesis entitled

Electrophoretic and Immunoelectrophoretic Analyses of Serums from Normal Cows and Cows Experimentally and Naturally Infected with Mycobacteria

presented by

George Leonard Wright, Jr.

has been accepted towards fulfillment of the requirements for

Ph.D degree in Microbiology

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Date May 10, 1966

ABSTRACT

ELECTROPHORETIC AND IMMUNOELECTROPHORETIC ANALYSES OF SERUMS FROM NORMAL COWS AND COWS EXPERIMENTALLY AND NATURALLY INFECTED WITH MYCOBACTERIA

by George Leonard Wright, Jr.

Apparatus and techniques for starch-gel electrophoresis and for the rapid staining and destaining of both starch and disc gels were developed. This facilitated the mechanical manipulation of starch-gels during the preparation and slicing procedures and eliminated handling of gels during the staining and destaining processes.

Serums from normal and tuberculous cattle were analyzed by various electrophoretic procedures. A modified polyacrylamide gel electrophoretic procedure, tentatively named differential disc electrophoresis, was the most sensitive single procedure. Thirty to thirty-eight fractions were detected in normal bovine serum by this technique. A nomenclature was proposed for the fractions separated by cellulose acetate electrophoresis, starch-gel electrophoresis, and immunoelectrophoresis.

A hypergammaglobulinemia and a decrease in albumin were detected by all electrophoretic procedures in the serums from calves inoculated with M. bovis, virulent

Group III's and serums collected from naturally infected cows prior to tuberculin testing. No changes were detected in the electrophorograms of serums from calves inoculated with M. avium or avirulent Group III, or from tuberculin negative cows.

Changes in the albumin/gamma globulin (A/G) ratio calculated from cellulose acetate electrophorograms were differential for cattle inoculated with virulent mycobacteria (except one virulent Group III, strain 51C).

Starch-gel, differential disc electrophoresis, and immunoelectrophoresis detected an increase in an $alpha_2$ globulin fraction in serums 7 to 28 days after injection of virulent mycobacteria (including strain 51C) and in serums collected prior to tuberculin tests from tuberculin-positive gross-lesion and no-gross-lesion cows. This fraction was unchanged in serums from calves inoculated with M. avium and avirulent Group III mycobacteria. The fraction has been tentatively named the $alpha_2$-V (virulence) fraction and was most reliably detected by starch-gel electrophoresis.

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By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1966

PREFACE

> Tuberculosis occupies a peculiar place in the history of medicine. By the end of the nineteenth century, more was known of its clinical manifestations and pathological characteristics than was the case for any other important infectious disease. Yet, the genesis of its lesions and the factors which influence their course constitute today one of the most obscure chapters of pathology. Similarly, while the tubercle bacillus was one of the first pathogenic microorganisms to be isolated and extensively studied both *in vivo* and *in vitro*, knowledge of its pathogenic determinants has hardly advanced during the past two decades. Factual information concerning its metabolism and composition, its immunological behavior, and the properties responsible for its virulence is so deficient that it contributes but little to the understanding of the disease. In other words, the study of tuberculosis, which was in the forefront of medical sciences fifty years ago, now lags several decades behind that of many other human infections.
>
> Rene J. Dubos (52)

The paradox of tuberculosis is also apparent from the attempt to eradicate bovine tuberculosis. Enough was known about the mode of transmission, and methods of detection such as the tuberculin test that the incidence of the disease was reduced in the United States from 4.5 percent in 1917 to 0.2 percent in 1940. Since 1940 the incidence has decreased only 0.1 percent. The disease continues to exist at a surprisingly high incidence in geographic pockets in spite of the eradication program. Why does the organism

persist in animals in these areas where the eradication program is as vigorously pursued as in areas where the disease has essentially been eradicated? A logical answer to this provocative question has been made by Dr. W. L. Mallmann (119), Department of Microbiology, Michigan State University:

> It is apparent that our knowledge of bovine tuberculosis is inadequate. Otherwise the procedures in use to detect and eliminate bovine tuberculosis would have continued to reduce the incidence and the disease should have disappeared. Certainly the uses we have made of the procedures for eradication were good. Thus, the faults lie in lack of knowledge.

Because a large number of tuberculin positive cattle failed to show any gross lesions indicative of tuberculosis upon slaughter, the United States Department of Agriculture decided that a rigorous investigation of this problem was needed. Research was initiated at the College of Veterinary Medicine, in the Department of Microbiology and Public Health and the Department of Pathology at Michigan State University in 1959 by means of a contract with Animal Disease and Parasite Research Division and a Cooperative Agreement with the Animal Disease Eradication Division (now the Animal Health Division), United States Department of Agriculture.

The research at M.S.U. was designed primarily to investigate the so-called no-gross-lesion (NGL) cattle. The initial investigations were concerned primarily with methods

of isolation and identification of acid-fast organisms from tissues of gross-lesion and no-gross-lesion tuberculin positive cattle. Mycobacterium bovis was isolated from some of these NGL cattle. A large number of the isolants from NGL cattle and some from gross lesion cattle were "atypical" acid-fast organisms. They were identical or very closely related to those isolated from tuberculosis-like disease in man.

The isolation of the "atypical" acid-fast organisms from tuberculin positive cattle presented new problems: Do they cause tuberculosis or tuberculosis-like disease? Do they cause tuberculin sensitivity without disease? Their relative virulence in cattle, swine, guinea pigs, rabbits, and chickens, and the routes by which calves were susceptible to these "atypical" organisms were investigated. Many of the atypical organisms were Runyon Group III (slow growing, nonpigmented and nonphotochromogenic) and varied markedly in infectivity in guinea pigs and calves. Some produced a generalized progressive disease similar to the disease caused by M. bovis, some were of low virulence and produced only a primary complex and some produced no disease in experimental calves. Those which were infectious usually induced a tuberculin reaction of both mammalian and avian tuberculins. The response varied depending largely upon the extent of the disease, whether the disease was progressive

or nonprogressive, and whether it was generalized or localized.

Investigations were initiated to evaluate specific sensitins and serologic tests as aids in differential diagnosis of mycobacterial infections. Serologic tests such as the hemagglutination (HA), complement fixation, and immunodiffusion tests were found to yield false negative and false positive reactions. In some cases, a false positive response was attributed to an anamnestic-like reaction elicited by tuberculin. A recent epidemiologic study indicates that on a herd basis, the cervical skin test and 640 HA titer are supportive tests for M. bovis infection.

The ambitious effort at M.S.U. has contributed greatly to our knowledge of bovine tuberculosis but continued study is required before the eradication program can be effectively modified. Time is a critical factor as indicated in the field of human tuberculosis by Dubos (53):

> All biologic experience indicates that we are working against time in the campaign to eradicate tuberculosis. The period of time may be ten years or more, but it has very definite limits. Chemotherapy has a chance of fulfilling its full potential effect in tuberculosis control, only if the public health attack is made before the current irregular and disorganized treatments have swelled the number of drug-resistant bacilli to the danger point. It is for this reason that the time for a campaign of eradication of tuberculosis is now . . . lest it be never.

The need still exists to differentiate between tuberculous infection and disease, progressive or nonprogressive, that is to detect the animals which are of potential hazard to man and other animals. A single specific serodiagnostic test and/or specific sensitins for each species of mycobacteria is needed to accurately detect bovine tuberculosis and tuberculosis-like disease in the herd. Because something is now known about the kinds of mycobacteria which exist, studies of antigen-antibody reactions can make direct or indirect meaningful contributions toward the ultimate goal of tuberculosis eradication. Such studies are under investigation at M.S.U.

ACKNOWLEDGMENTS

Because of the scope and team effort of the bovine tuberculosis project, many individuals have contributed in various ways to this study. The author wishes to express his sincere appreciation and thanks to Drs. W. L. and V. H. Mallmann for their continued interest and guidance throughout this study. The author wishes to thank all others on the project who participated in the research which provided the supportive pathologic, bacteriologic, and serologic information. Appreciation is also extended to the U.S.D.A. personnel who worked closely with the project and provided meaningful field interpretations. To those who have participated directly or indirectly, the author is indebted.

A special appreciation goes to the author's wife, Yolonda for encouragement and her faithful help in many particulars, both large and small.

Preparation of this thesis has been greatly facilitated by financial assistance given to the author by the Michigan Tuberculosis and Respiratory Disease Association. Gratitude is extended to the United States Department of Agriculture who provided necessary equipment used in this study.

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INTRODUCTION

Many attempts have been made to use serologic tests to diagnose tuberculosis in man and animals. Various antigen preparations and serologic techniques such as agglutination, precipitation, complement fixation, and hemagglutination with its various modifications, have been investigated. No test has reliably differentiated tuberculosis, tuberculosis-like disease caused by "atypical" acid-fast organisms, or nontuberculosis.

Electrophoretic studies of the serum proteins in tuberculous and nontuberculous humans and animals have been made to find specific changes that might be of diagnostic or prognostic significance. Although some success has been achieved, most of the changes detected reflected the nonspecific physiologic change in the body rather than a change specific for tuberculosis. Moving boundary or paper electrophoretic methods were generally used. They lack the sensitivity of gellified media tests, such as starch-gel electrophoresis, acrylamide electrophoresis, and immunoelectrophoresis. It is possible that specific fractions, antibody or non-antibody proteins, or both, could be detected and isolated with improved fractionation precedures.

Considerable emphasis has been placed on the need to isolate specific mycobacterial antigens. To utilize specific antigens in a serodiagnostic test, antiserums must be examined to demonstrate the fractions that contain any diagnostic specific antibodies or antigen-antibody complexes. The failure to demonstrate specific antibodies from some tuberculous individuals may not be the failure of the animal to produce antibody, but failure in the design of the test procedure. Also the mechanisms of antibody production against mycobacterial antigens and natural resistance mechanisms in the animal must be investigated. According to Seibert (167), there may be little progress in the development of serologic tests for tuberculosis because of the interplay between antigens, antibodies, and lysozyme in vivo. More understanding of the interactions is needed before diagnostic procedures for the detection of tuberculosis and tuberculosis-like disease can be devised for the practicing physician and veterinarian.

This is a report of the studies made of serums from normal cattle and from infected cattle. Various separatory procedures were used for the fractionation of normal bovine serum proteins: cellulose acetate electrophoresis, starch-gel electrophoresis, acrylamide electrophoresis, and immunoelectrophoresis. A standard nomenclature is proposed for the electrophoretic and immunoelectrophoretic patterns of

normal bovine serum components. Results are presented of the electrophoretic and immunoelectrophoretic evaluations of serial changes in serum proteins from naturally infected cattle and from cattle experimentally infected with various strains and species of mycobacteria.

HISTORICAL REVIEW

There are numerous methods available for analyses of serum proteins. Electrophoresis, the separation of ionic mixtures in an electrical field, is one of the most important analytical techniques presently available to the research and clinical scientist.

Zone Electrophoresis

Zone electrophoresis refers to a number of techniques in which the electrophoretic separation is carried out in a solid or semisolid support medium which is a modification of the liquid system employed in the classical moving boundary or free solution electrophoretic method (190). It offers the advantage that particular zones may be stabilized and stained or isolated for further investigation.

Various stabilizers as solid supporting media have been employed such as: glass beads, sands, resins, powdered cellulose, asbestos, sponges, filter paper, cellulose acetate membranes, and various gels (103). The supporting medium is ideally inert and the separation of components results from their electrophoretic mobility. Gel filtration can be an additional separatory technique in some types of zone electrophoresis such as starch-gel and acrylamide gel.

The basic design of all zone electrophoretic systems consists of two electrode vessels with the electrolytic bridge (supporting medium) between them. A potential difference applied across the two vessels of electrolyte will cause a current to flow across the electrolytic bridge. The flow of electrons is toward the anode. An ionizable substance separates into its different electrophoretic components because of variation in charge, molecular weight, or both (46, 54, 103). A number of factors influence the electrophoretic separation: the concentration, constituents, consistency, and pH of the buffer; isoelectric points of the sample; temperature; and electroosmotic flow.

The main functions of the buffer solution are to conduct current and maintain pH of the sample. Since the majority of serum proteins have their isoelectric points between pH 4 and pH 8 the electrolyte solution is usually buffered between pH 8.2 and pH 8.6 (46). Above pH 8.0, most, if not all, serum proteins react as anions in an electrical field (33). The ionicity of the electrolyte solution must be sufficient to provide adequate buffering capacity. The maximum permissible ionicity is determined by the amount of heat due to resistance that can be tolerated. Because of the heating effect of currents the ionicity, temperature, voltage and pH must be carefully regulated, particularly when gellified media are used. Excessive current can cause drying of the supporting medium,

pH changes as a result of electrolysis of buffer salts, and increased ionic mobilities (54, 196). To avoid depleting the buffer capacity of the electrolyte solution due to electrolysis of buffer salts during electrophoresis, a buffer solution with low ionicites between 0.075 and 0.1 is used (106).

The constituents of the buffer solution may also influence the separation of an ionic mixture. A buffer consisting of tris (hydroxymethyl) aminomethane (tris), ethylenediaminetetracetic acid (EDTA), and boric acid was described by Aronson and Gronwal (1). Human serum proteins were separated into 9 fractions which was superior to the separation in the barbital buffer commonly used in paper electrophoresis.

A discontinuous buffer system in which tris-EDTA-borate buffer is applied to paper, and barbital buffer in the buffer vessels was described by Goldberg (63). Resolution of abnormal hemoglobulins was superior to that in barbital or tris-EDTA-borate single buffers. The addition of either calcium or magnesium salts to barbital buffer facilitates the separation of the beta globulins (107, 108).

Water tends to move toward the cathode during electrophoresis in alkaline solutions because of negatively charged carboxyl groups on many of the supporting materials (97, 103, 180). The movement of the water is called electro-osmotic flow. It is greater in buffers which are less

concentrated or at a pH which increases the ionization of the carboxyl group of the supporting material. Little electroosmosis occurs in filter paper and cellulose acetate relative to that which occurs in agar gel (46). These and other factors regulating the separation obtained by zone electrophoresis have been discussed (15, 16, 43, 109, 125, 157, 196, 211).

The greatest impetus to zone electrophoresis was the introduction of filter paper as a supporting medium. It afforded many advantages: complete separation of individual components; storage for a permanent record; required only a small sample volume; facile; many analyses performed simultaneously; no prior dialysis of sample required; and analyses made at room temperature (157). Despite its great usefulness and many applications, interest in paper electrophoresis as a clinical tool has diminished in the last several years. Because of albumin tailing, the added background color causes the percentage of albumin to always be low while the globulin values are correspondingly increased (166).

Cellulose acetate was introduced as a supporting medium for zone electrophoresis by Kohn (94). The strips are used the same as paper strips but it offers some notable advantages. The background is transparent and colorless, and the definition of the protein bands are much sharper (42, 94, 97). An investigation of the effect of buffer composition, ionicity, and pH, electrolyte volume, strip

length, voltage, sample size and position, and migration time, on the sample separation obtained with this medium was reported by Brackenridge (24). A microadaptation using cellulose acetate has been developed by Graham and Grunboom (66) which permits separation of serum proteins within 15 minutes. This medium may also be used for immunoelectrophoresis (95, 97, 165).

Agar gel electrophoresis is a widely used technique, particularly the method of Wieme (207) utilizing agar coated microscopic slides. A recent modification employs movie film leader strip (Cronar strips) which is firm yet flexible enough to fit into some of the commercial equipment originally made to accommodate paper strips (38). In agar, as with cellulose acetate, there is not tailing of the albumin and the bands are sharply delineated from the background (43, 45, 64). The chief disadvantage is that considerable electroosmosis occurs (101, 103).

Starch-gel (179, 180) and acrylamide gels (132, 153) are finely porous and act as sieves as the protein molecules migrate through them. Thus, the proteins are separated not only by differences in electrophoretic mobility but also by their molecular size (179). The substantial increase in resolving power of sieving gel matrices over that of conventional filter paper electrophoresis is effectively demonstrated with a complex protein mixture such as human serum.

In a single electrophoretic analysis, a filter paper technique commonly resolves 5 to 7 protein fractions; starch and acrylamide gels resolves 20 to 30 fractions. The separating power of these gels has been thoroughly discussed by Smithies (181, 182); Raymond (152, 154); and Ornstein (132).

Starch-gels can be used in horizontal (179) or vertical systems (180). In either method, starch-gel is made into a block and the sample inserted into a slot in the gel. Electrophoresis proceeds for 18 hours. Several layers are cut from the block. One layer is usually stained for protein and the others are stained for specific components of serum. The vertical method permits the sample to be inserted into the gel without any supporting substance, reduces electrodecantation, improved the resolution of serum proteins considerably, and reduced the difficulties of obtaining reproducible results. A discontinuous system of buffers in starch-gel medium permitted higher voltage gradients, reduced the time required for electrophoresis and increased resolution of toxins, enzymes, and human serums (141).

Starch-gel electrophoresis permitted the separation of certain serum proteins, such as the haptoglobins, which had not been possible with the standard electrophoretic procedures (183, 185). As a result, starch-gel has been employed extensively in investigation of serum proteins, enzymes, hemoglobins, transferrins, and haptoglobins.

It has also been employed to demonstrate the paraproteins found in cases of myeloma (14), and for separation and characterization of LDH isoenzymes (87, 212).

Starch-gel electrophoresis has not become popular in the routine diagnostic laboratory because it is technically quite difficult. However, Marsh et al. (121) have described a modification of the conventional technique which makes it simple to perform, flexible, more rapid, and more adaptable to routine clinical usage.

Difficulty in correlating findings of paper and starch-gel electrophoresis stimulated development of a two-dimensional electrophoretic analysis combining migration first in filter paper and second at right angles in starch gel (143). The protein components common to both the whole serum and the serum fraction obtained from filter paper electrophoresis can be identified as uninterrupted bands across the entire width of the gel. Ashton (6) found bands not detected by agar or starch-gel singly by using agar in the first direction and starch-gel in the second direction. The chief advantage of this method is to correlate bands detected in starch with those separated in a classical system (paper, cellulose acetate or agar).

The utilization of polyacrylamide gels for zone electrophoresis was reported independently by Raymond and Weintraub (155) and Davis and Ornstein (50). The vertical

slab procedure separates and resolves serum proteins and hemoglobin qualitatively similar to that seen in starch-gels (152, 155). The properties and preparation of acrylamide gels and applications was reviewed by Raymond (150). There is no retrograde migration of gamma globulin due to electroendosmotic flow as occurs in starch-gels (149, 153). Heremans et al. (76) described a two-dimensional technique employing paper in the first direction and cyanogum gel in the second direction. Raymond and Aurell (151) employed a low concentration acrylamide gel in the first direction followed by a high-concentration gel in the second direction to produce separations analogous to those obtained in two-dimensional paper chromatography.

Disc electrophoresis (50, 132) employs polyacrylamide gel in narrow glass cylinders. The salient characteristics of this technique reside in (1) the controlled variation of the pore size for the purpose of increasing the resolution of ions based on dimensional differences and (2) an electrophoretic step for concentrating the sample ions into a narrow starting zone prior to electrophoretic separation. Human serum proteins, in particular, have been extensively examined by this method with over 20 protein zones demonstrated (49, 122, 214). However, the number of protein bands are so numerous that the system is of no benefit to the routine diagnostic laboratory because satisfactory interpretation of the complex patterns is not yet

possible. Furthermore, the gamma globulins are not adequately separated. They migrate as a broad zone from point of origin to the transferrin band which makes the background gel hazy (49). The greatest use of this technique presently is with special histochemical stains particularly for the study of enzymes. Because a very small quantity of protein is required it is particularly effective for separation of proteins in body fluids with low protein content (49).

Electrophoretic separation of serum proteins in starch and acrylamide gels is technically far more advanced than our knowledge of the significance and characteristics of these substances. Until these are understood, the separating power of starch and acrylamide gels will not be fully utilized.

Measurement of Proteins Separated in Solid Media

After proteins are separated in solid media, the methods for measuring are generally grouped as chemical or densitometrical. For clinical purposes, scanning is a more convenient and simple procedure. The amount of dye that binds to a protein will vary with the dye and the protein. Errors in measuring protein by bound dyes are inherent since the binding capacity of many proteins is unknown.

If given systems are standardized, and automatic scanners used the errors can be negligible (4, 25, 31, 35, 91, 97, 136).

The densitometric analyses of proteins separated in starch-gels are more difficult. Before making transmission measurements of the starch-gel, the translucent destained gel must be made transparent by heating the gel slowly in 5 to 10% acetic acid or glycerol (67, 136, 160). Glycerol is preferable because no dimensional changes occur during the prolonged period of the gel manipulation. Heating in acetic acid makes the gels more fragile, and due to drying shrinkage may reduce reproducibility. Slicing the starch-gel introduces another error because the gel is too elastic and soft to cut at a uniform thickness (123). The use of acrylamide gels eliminates the need of treatment before scanning because of their transparency. Densometric analyses of proteins separated in gels are discussed in the following papers (73, 136, 160, 195, 213).

The elution of proteins from starch-gels is difficult but can be done by: (1) digesting the starch with amylase (2) freezing and thawing the gel which breaks the structure and leaves a sponge-like substance followed by centrifugation and (3) by electrodialysis (17). The latter is the most satisfactory method and is detailed by Moretti et al. (126).

Elution of stained bands from cellulose acetate strips is very satisfactory and rapid, more so than filter paper. Ponceau S stained bands can be eluted with sodium hydroxide followed by the addition of acetic acid (58, 97).

Immunoelectrophoresis

Immunoelectrophoresis (IE) is a sequential combination of electrophoretic separation and double diffusion in the same agar (65). The resolution is markedly increased. It resolved more than twenty distinct antigenic components in human serum. Scheidegger (164) used microscope slides which reduced the amount of reagents required and shortened the periods of electrophoresis and immunodiffusion.

Supporting materials other than agar have been used for IE: paper (59, 140, 205); starch-gel (3, 99, 142); cellulose acetate (94, 96, 98); and polyacrylamide gels (86). Agar was the most useful because it is negatively charged, has a high gel strength, is nearly transparent, is soluble in an aqueous medium, has few ionized groups, and undergoes little nonspecific reactions with proteins (46, 208). Starch-gels were opaque so that relatively high concentrations were required which increased the possibility of nonspecific interactions with proteins (99, 142) and reduced the rate of diffusion.

Highly purified agar is used to decrease electro-osmosis and extraneous salts (46, 80, 101). To insure reproducibility of results, agar should be prepared in a single batch (46).

Procedure for IE essentially consists of covering glass plates or slides with 1-2% agar dissolved in an appropriate buffer. After solidification, a well is cut in the

agar and filled with sample. It is connected to the two buffer vessels with filter paper, and electrophoresed as for zone electrophoresis (p. 5). Following electrophoresis, a trough is cut in the agar parallel to the axis of migration. Antibodies specific for the antigen constituents of the sample are added. The plates or slides are incubated in a moist chamber at a predetermined temperature. The antigens and antibodies diffuse radially from their respective centers and form visible bands of precipitates which can be stained (46). Other identification procedures can be used such as special staining techniques, enzymatic reactions, or radioimmunoelectrophoresis (45, 64, 209).

The same variables influencing the separation of ionic mixtures in zone electrophoresis (described on page 5) also influence IE. In addition, the electrophoresis and immunodiffusion must be carried out in the same environment. The required buffer system for electrophoresis may not be the optimum conditions for antigen-antibody precipitation. The low ionic strength used in agar electrophoresis will not support specific precipitation by some antiserums (46). The ionicity, pH, and type of salts used in the buffer have to be determined empirically, and frequently it is a compromise.

Low ionicity increases the rate of migration of fractions but broadens their zones and lowers resolution.

Increasing the ionicity causes overheating and decreases the diffusion rate of macromolecules. A pH should be selected which maintains the negative charge on the antigenic constituents to prevent their absorption to the gelling medium. Barbital buffers at pH 8.2 to 8.6 with low ionicities of 0.025 to 0.1 are generally used (46). It is not without its limitations. The barbital precipitates some non-antibody components of human serum (100), probably the alpha lipoprotein (46).

Optimal conditions for immunoprecipitation following electrophoresis are the same as those for gel double diffusion tests. At least three other problems may occur. First, electrophoresis can separate antigens from each other, and one may depend upon the other for their solubility in agar gels. When the protective substance, probably an $alpha_2$-liproprotein (194) is removed, the unprotected molecules may form irregular precipitin arcs in the agar which are not due to specific antigen-antibody reactions (46). The addition of gelatin or glycine to the agar may act as protective agents (46). Second, the slopes of precipitin arcs in immunoelectrophoresis are governed by more factors than those in double diffusion plates primarily: mobility, electrophoretic heterogeneity, slope of the antigen diffusion fronts, the diffusibility of the antigens, and the relative preparations of the reactants (46, 64, 80, 92).

Third, and probably most important, a molecule or complex with two different determinant groups may be split by electrophoresis or denatured during electrophoresis.

The probability of two or more antigens forming superimposed precipitates is decreased by IE because the electrophoretic mobility as well as the diffusibility and reactant ratios would have to be identical or very closely similar. The probability of the simultaneous occurrence of all those factors, during IE, however, is very slight (81).

One of the more important reagents and chief limiting factor of IE is the antiserum. Variations occur due to the differences in antigenicity of the various constituents in the test sample and the qualitative and quantitative differences in the antibodies elicited (22, 46, 147, 206). The quantity, quality, and kind of precipitating antibodies can be influenced by route of injection, physical state of antigens, and variability in the immunologic responsiveness of individual animals of the same and different species.

Crowle (46, p. 93) states:

> The dynamics of single and double diffusion tests and of immunoelectrophoresis are sufficiently complex to call for a great deal of caution in interpreting results obtained from those tests, but this very complexity contributes to their ability to yield remarkably varied information involving antigen-antibody precipitations consequently, efforts made by users of these tests to understand their intricacies will be rewarded with a generous return of knowledge.

Electrophoresis as a Diagnostic Tool

One of the most significant contributions of electrophoresis has been the identification of different hemoglobulins (88) and haptoblobulins (180). However, the analyses of serum and plasma requires that normal patterns be established. Patterns have been established for several animal species but the changes in pattern due to disease and nutritional deficiency have rarely been of direct diagnostic value. They are more frequently a general index to the physiologic condition of the patient (111).

To interpret changes in electrophoretic patterns it is essential to know the site of production, half life, and site of destruction of serum proteins in vivo. It is generally believed that albumins, fibrinogen and alpha globulins are produced in the liver. Gamma globulins and possibly some beta globulins are probably produced by plasma cells in splanchnic bed, bone marrow and lymph nodes (55). Some protein, primarily albumin, is filtered and reabsorbed in the kidney which maintains normal colloidal osmotic pressure (72, 137). Under certain pathological conditions, large amounts are lost. Significant changes in protein pattern may be encountered in liver disease, in disorders of the reticuloendothelial system, or when abnormal amounts are lost through the kidney. Non-specific, stress patterns and bacterial infections (13, 137) can cause a hypoalbuminemia, decreased beta globulins and increased alpha and gamma

globulins. Alpha globulins, particularly $alpha_2$, increase in high fever and tissue destruction. $Alpha_2$ increases markedly in the nephrotic syndrome. Beta globulins are often increased in conditions in which increased serum phospholipid and cholesterol are associated. Hypergammaglobulinemia occurs in chronic infection, in liver disease and collagenous disorders. Hypergammaglobulinemia was usually indicative of increased antibody production (11, 69, 190) although some gamma globulins may be serologically "inert" (12). Heremans (75) found that the gamma A, gamma M and the gamma G globulins of the human system possessed antibody activity. In most bacterial diseases, the gamma globulin concentrations did not parallel resistance (13).

Specific patterns have been described for relatively few diseases. Many of the disease-induced alterations that have been detected in the plasma constituents of men and animals, are noted in the following reviews (13, 32, 36, 37, 55, 69, 71, 89, 100, 105, 110, 111, 114, 115, 134, 137, 146, 156, 166, 186, 187).

Electrophoresis of Bovine Serum

With the moving boundary electrophoretic technique of Tiselius, bovine plasma were separated into five major fractions--albumin, alpha, beta, and gamma globulin and fibrinogen (84, 162). Alpha globulin was separated into two and gamma globulin into three subfractions (26, 27, 138).

Some differences are reported in the number of components separated in bovine serum by paper electrophoresis but, in general, the same major fractions were obtained with the moving boundary technique. Rooney (159, personal communication) could not separate the components in bovine serum as satisfactorily as those in human serum. Individual components diffused more and the alpha globulin did not separate with any of the phosphate or veronal buffer systems utilized. With paper electrophoresis bovine serum separated into more than six components (18, 19, 47, 93, 138). The beta fraction was difficult to separate by paper electrophoresis in veronal buffer (47). Tris-buffer increased the resolution of more globulin fractions (Czernicki, personal communication).

The various amounts of protein found in bovine serum by electrophoretic techniques was reviewed by Nilssen (130). The results obtained by various authors are not necessarily comparable. It is essential to consider the different apparatus, buffer solutions, etc. which were used (198).

Cellulose acetate has not been employed as a medium for the separation of bovine serum, and agar gel has been used only in a few cases (Cornelius, personal communication) with similar problems in separating the globulin fractions.

Bovine serums have been studied with starch-gel electrophoresis to detect serum protein polymorphism. The polymorphism is associated in the beta-globulin region of

the electrophoretic pattern. Cattle serums on the basis of the variation in the beta-region, can be divided into five groups (6, 184); a sixth group was subsequently recognized (8).

Whole bovine serum was separated up to 25 components (7, 104, 184) by one directional starch-gel electrophoresis and into 24 components by two-dimensional agar or starch-gel electrophoresis (7). Brummerstedt-Hansen (30) studied transferrin types in cattle with starch-gel electrophoresis and immunoelectrophoresis. Disc electrophoresis produced more discrete and more easily analyzed transferrin bands than starch-gel electrophoresis (148).

The more sophisticated gel electrophoretic procedures have had limited use in evaluating various diseases of cattle. Most reports have been on the use of moving boundary or paper electrophoresis. San Clemente and Huddleson (162) analyzed serums from cows infected with brucella by the moving boundary method. Moving boundary method detected hyper-beta- and hypergammaglobulinemia one and three weeks, respectively, after cattle were experimentally infected with the virus of foot-and-mouth disease (26, 27). Rooney (159) studied seven bovine disorders by paper electrophoresis: Ramsey-type mucosal disease (hyperalpha-globulinemia); lymphomatosis (double alpha peak, hypoalbuminemia and hypergammaglobulinemia); vegetative endocarditis (hypoalbuminemia and hyperalpha- and hypergammaglobulinemia); traumatic

reticulitis and hydronephrosis, amyloidosis (hypoalbuminemia and hypergammaglobulinemia); foot rot (hypoalbuminemia and hypergammaglobulinemia). A hyperalpha-, hyperbeta- and hypergammaglobulinemia was found by paper electrophoretic analyses of cows experimentally infected with anaplasmosis (51, 158).

Brown (28, 29) used agar electrophoresis, immunoelectrophoresis and paper electrophoresis of normal serums and serums from cows and guinea pigs experimentally infected with the virus of foot-and-mouth disease. The mobility of the precipitating antibodies altered considerably during convalescence was of prognostic value.

Electrophoresis in Human Tuberculosis

Many attempts have been made to find specific antigens, antibodies or other components in the blood which could be of diagnostic or prognostic value for tuberculosis. Measurement of erythrocyte sedimentation rate, blood volume, pH, non-nitrogenous protein content and inorganic ion content have been examined (113, 127). No single test or combination of tests is conclusively specific for tuberculosis. Some of the changes aid in determining the extent and activity of the disease and the prognosis.

The electrophoresis of the serum proteins from tuberculosis individuals has been studied extensively and with similar results. Early studies of serum by moving boundary electrophoresis revealed changes that roughly

parallel the extent and activity of the disease. Hypoalbuminemia and a reduction in the normal albumin/globulin ratio (A/G) were common (168, 169, 172). Increase in the $alpha_1$, $alpha_2$, and gamma globulins and decrease in albumin occurred in the serum as the disease progressed (12, 102). Similar changes have been shown by paper electrophoresis (20, 39, 41, 62, 74, 117, 124, 139, 197). Starch-gel electrophoresis detected no specific quantitative change in serums from 25 patients with active tuberculosis (90).

These electrophoretic results clearly demonstrate that there are definite departures from normal values in serum proteins in active human tuberculosis. An increase in $alpha_2$ globulin occurs in all forms of disease. Nymen (131) and Hever and Kalnai-Hever (77) reported that haptoglobins contributed most to the rise in the $alpha_2$ globulins. Bovornkitti (20) believed that the increase in $alpha_2$ globulin occurs as a result of hypersensitivity reactions and is not due to tissue destruction as previously suggested by others. However, changes in other components are related to liver impairment. Because of the relationship between hyperalpha 2-globulenemia and disease activity, some workers believe that the albumin $alpha_2$ globulin ratio is more valuable and specific than the A/G ratio (139, 174). McCuiston and Hudgins (124) found that the $alpha_2$ globulins were not elevated in the serums of patients with tuberculosis caused by nonphotochromogenic atypical mycobacteria. They

emphasized (1) the diagnostic value of the pattern rather than the quantitative values and (2) that the results obtained are important only when evaluated with the total clinical picture.

Grigorieva and Livshitz (68) interpreted the change in the beta globulins as being the most significant change during tuberculosis. The rise in beta globulin was proportional to severity and extent of disease and to the degree of amyloidosis. There was no definite pattern of relationship between the character of the disease and the gamma globulins.

The relationship between clinical progress and hypergammaglobulins often observed in advanced and far advanced tuberculosis is controversial. Some workers have found a rising or high gamma globulin level to be a good prognostic sign (163, 172, 215, 216, 217). Others have failed to find consistent relationships (135, 178). Freigang et al. (57) found as improvement occurred the albumin alpha$_2$ goes up and gamma decreases.

Significant changes in serum glycoprotein levels in human tuberculosis (171, 172, 193) suggest that glycoprotein determinations might be useful in diagnosing tuberculosis. However, electrophoretic patterns of carbohydrate distribution in serum from tuberculosis patients were of no diagnostic value (79).

Price (144) used disc electrophoresis to examine the seromucoid patterns in patients with a variety of different diseases, including tuberculosis. Intensive work on normal individuals and tuberculous patients indicated a typical pattern for normal and another for active tuberculosis. However, the significance of the seromucoid pattern and the nature of their association with different disease states is not known.

Value of Electrophoresis in Experimental Tuberculosis

Paper has been the principal medium used in the electrophoretic analysis of the serum protein changes that occurs during experimental tuberculosis.

Serum from tuberculous guinea pigs examined by paper electrophoresis showed a reaction very similar to those that occurred in man (203): moderate hypoalbuminemia and hyperglobulinemia. However, in contrast to man, a moderate hyperproteinemia accompanied the latter stages of the disease due to an increase in the concentration of all the globulin fractions (85, 175, 176).

An increase in the alpha globulins and alpha$_2$ glycoproteins was present within eight days following subcutaneous inoculation of guinea pigs with 0.1 mg of M. tuberculosis (200, 201, 202, 204). Hypoalbuminemia was common after the 15th day and hyperglobulinemia after the 19th day. Maximum alteration had occurred in all of the

fractions except the gamma globulins of the 15th day. Sher et al. (176) using paper elctrophoresis showed that the principal mucoprotein alterations occurred in the alpha$_2$ globulin region and that the serum lipids and lipoproteins remained unaltered during tuberculosis in guinea pigs. In spite of this intensive study plasma protein or glycoprotein patterns of diagnostic significance for tuberculosis have not been found.

Besides paper electrophoresis, immunoelectrophoresis has been used to study serum protein changes in tuberculous mice and guinea pigs. Williams and Wemysis (210) found an increase in the alpha$_2$ globulin which was haptoglobin. After 3 weeks post-inoculation all infected mice showed a marked increase in gamma globulin levels and the presence of a low mobility gamma globulin.

Hyperalpha$_2$-globulinemia was detected by cellulose acetate electrophoresis in serums from tuberculous guinea pigs 14 days after inoculation (48). Coincident with the hyperalphaglobulinemia was the detection by immunoelectrophoresis of an additional alpha$_2$ globulin which increased from sub-detection amounts in normal serums to detectable amounts in serums from tuberculous guinea pigs sometime between the eighth and fourteenth day after inoculation and remained increased until death. The hyperalphaglobulinemia did not occur in guinea pigs which were tuberculin hypersensitive but not infected.

Starch-gel electrophoresis and disc electrophoresis have not been used to study the serum protein fluctuations during experimental tuberculosis.

Serums from cows experimentally infected with tuberculosis have not been analyzed by electrophoretic procedures. However, there was one investigation which employed paper electrophoresis to study serum protein alterations in naturally infected tuberculin positive cows (199). A mild infection produced only small changes in the fractions, but animals with generalized disease showed a hyperalbumenia and a hypogammaglobulinemia. The same fractions were also altered, but to a lesser degree, after the animal was tuberculin tested.

MATERIALS AND METHODS

Cultures for Inoculation

The cultures used to inoculate experimental calves were isolated from cattle, swine and chickens in this laboratory. Isolants 50B, 51C, and 68C were selected for study because they were isolated from bovine tissues, had Group III culture and biochemical characteristics, but differed in their virulence for laboratory animals.

Isolant 50B was from thoracic lymph nodes of a no-gross-lesion, tuberculin positive cow. It caused no lesions in guinea pigs, rabbits or chickens except at the site of injection if injected intradermally. The virulence did not increase with indirect animal passage and it appeared to be identical with P39, a well-studied Group III of human origin. Subsequent studies indicated that all antigens of 50B and P39 were not identical (Flavin, unpublished data).

Isolant 51C was from lesions in mesenteric lymph nodes from a tuberculin positive cow. It caused no gross lesions in laboratory animals except at the site of injection and the regional lymph node when 0.1 mg was injected intradermally. This isolant increased in virulence for guinea pigs and rabbits by indirect passage in guinea pigs but was not equal to that of *M. bovis* or *M. tuberculosis*.

Isolant 68C was obtained from mesenteric lymph nodes from a tuberculin positive cow and its virulence was similar to that of 51C.

Besides the Group III organisms, calves were inoculated with four strains of M. bovis and three strains of M. avium (Table 1).

The inoculums were prepared from cultures seeded in 1.0 ml quantities of Dubos broth (Difco) containing 0.5% dextrose (no tween 80 or enrichment) and incubated at 35 C for 14-21 days. The supernatant fluid was removed and the cells resuspended in Dubos medium to 10 mg wet weight per ml. Each calf was inoculated intradermally with 0.1 ml unless indicated otherwise.

Experimental Animals

Serum was obtained from animals that were being used in other experiments. Inasmuch as serum was collected routinely from these animals, the selection of the animals serum to be used were made on the basis of the degree of pathogensity in each animal as judged by the gross and microscopic pathology at slaughter.

Serums collected at autopsy from two cows experimentally infected with Brucella abortus were also examined (Pathology Department, College of Veterinary Medicine, Michigan State University).

Table 1. List of animals inoculated with viable mycobacteria

Culture No.	Source	Mycobacterium	Route[3]	Animal No.
130-0[1]	Cattle	M. bovis[2]	ID	6
81-0	Swine	M. bovis	ID	20
			SC	81
310-2	Cattle	M. bovis	ID	113,116,128
278-3	Cattle	M. bovis	ID	135
Lab strain	Chicken	M. avium	ID	21
131-4	Chicken	M. avium	ID	114,115,118, 138
51C-0	Cattle	III	ID	43,59,60,104 105,106,107, 108,109,127, 136
			IU	75,77
			A	84,91
68C-0	Cattle	III	ID	64,137
			IU	70,71
			A	86
50B-0	Cattle	III	ID	66,67,126
			IU	80
			A	88,89

[1]The letters following the culture number refer to the tissues from which the strain was isolated: B=thoracic lymph nodes, C=mesenteric lymph nodes. The number following the letters refers to the year in which the culture was isolated: 0=1960, 1=1961, 2=1962, 3=1963, and 4=1964.

[2]Where species is not indicated, organisms are atypical mycobacteria. The Roman numberal indicates the Runyon Group.

[3]ID=intradermal: 1.0 mg; IU=intrauterine: 1.0 mg; SC=subcutanious: 1.0 mg; and AE=aerosol: 15 ml of culture medium containing approximately 1 x 10^8 organisms per ml were sprayed into the aerosol chamber during a one hr exposure for each calf.

Naturally Infected Animals

Serum samples were obtained from 38 cattle in 17 infected herds. Cattle in these herds included gross- and no-gross-lesion reactors, as well as 4 tuberculin-negative cows. In most cases, a sample was obtained before and after tuberculin testing.

Single serum samples were collected at autopsy from a cow with actinomycosis, one with brucellosis, one with leptospirosis, and one case of Johnes disease.

Normal Animal and Bird Serum

A number of normal animal and bird serums (sheep, pig, guinea pig, human, dog, horse, mouse, rabbit, duck, and chicken) were obtained from various projects under investigation in the Department of Microbiology and Public Health, Michigan State University. These serums were used primarily for evaluating the differential disc electrophoresis procedure.

Collection of Serum from Experimental Calves

Blood was collected by venipuncture prior to inoculation and at various post-inoculation times. After clotting, serum was removed, distributed in 1 ml amounts, and used immediately or stored at -20 C. Fresh frozen normal serum was examined periodically by cellulose acetate and starch-gel electrophoresis.

Rabbit Antiserum Specific for Normal Bovine Serum

Portions of the pre-inoculation serums from each of the calves were pooled. The mixture was precipitated with alum as described by Proom (145). The inoculums were prepared by mixing 12.5 ml of the pooled normal serum, 40 ml of distilled water and 45 ml of 10% aluminum potassium sulfate. The mixture was adjusted to pH 6.5 with 5 N sodium hydroxide and centrifuged at 4,000 Xg for five minutes. The sediment was washed twice with isotonic saline solution containing 0.01% merthiolate and resuspended to 50 ml in saline solution. It was stored at 4 C for no longer than 14 days.

Eighteen Dutch Belted rabbits approximately 6 months of age were inoculated with alum precipitated normal bovine serum and bled according to the following schedule:

Day	Operation	Amount	Route
1	Bled	10.0	Cardiocentesis
14	Injected	6.0	Intramuscular
24	Injected	1.0	Intramuscular
34	Bled	10.0	Cardiocentesis
48	Injected	6.0	Intramuscular
62	Injected	6.0	Intramuscular
72	Injected	1.0	Intramuscular
77	Bled	10.0	Cardiocentesis
91	Injected	6.0	Intramuscular
105	Injected	6.0	Intramuscular
115	Injected	1.0	Intramuscular
121	Bled	30.0	Cardiocentesis

Blood was obtained from the rabbits by cardiocentesis with a 2½ inch 22 gauge needle on a 12 ml syringe. The blood was allowed to clot in slanted tubes overnight. The serums were decanted, centrifuged at approximately 4,000 X g

for 5 min and 3 ml amounts transferred to sterile brucella tubes and stored at -20 C. Each antiserum was tested by immunoelectrophoresis (IE) with normal bovine serum before being pooled. Equal portions of each of the antiserums were pooled.

Cellulose Acetate Electrophoresis

Preliminary analyses with a range of buffer systems were used to establish the conditions for subsequent work. The buffer systems employed were:

1. Phosphate buffer pH 7.4, M = 0.15, 12.8 gm Na_2HPO_4 and 2.62 gm $NaH_2PO_4 \cdot H_20$ per liter.
2. Tris-EDTA-boric acid buffer (Aronsson and Gronwall, 1).
3. Tris-EDTA-boric acid discontinuous buffer (Goldberg, 63).
4. Barbital-barbituric acid buffer (Laurell et al., 107).
5. Barbital-acetate buffer (Owen, 133).

The latter was used subsequently. The Owen buffer solution pH 8.6, ionicity 0.07, has the following formula:

Sodium diethylbarbiturate	5.00 gm
Sodium acetate (anhydrous)	3.25 gm
Hydrochloric acid (0.IN)	34.20 ml
Calcium lactate	0.38 gm
Distilled water q. s. ad.	1,000.00 ml

Electrophoresis was done in a Shandon migration chamber (173) with variable voltage DC power supply (Heath-kit, model PS-3). Cellulose acetate strips (OXOlD, 2.5 X

12 cm) was the supporting material. Five ul of serum were applied directly over the cathode on each buffer impregnated strip. It was electrophoresed with a current of 1 ma per strip for 2 hours at 4 C. Following electrophoresis, the strips were stained, dried under weighted absorbent paper, and scanned.

Agar gel Electrophoresis

Glass microscope slides, 1 x 3 inch, were covered with 2.5 ml of a melted one percent agar solution prepared as described under IE. Each agar-covered slide was "aged" at least three hours in a humidified diffusion chamber prior to use. Just before the samples were applied, a circular well, 7 mm in diameter, was cut in the center of the gel 3.5 mm from the cathode end of each slide. The agar plugs were removed by vacuum with a Pasteur pipette. Approximately 5 ul of serum was placed in the well and electrophoresed at 1.25 ma per slide at 4 C for 2½ hours. Following electrophoresis the slides were immersed in a 5% solution of glacial acetic acid for 30 minutes, dried under filter paper at 37 C and stained.

Vertical Starch-gel Electrophoresis

Preliminary analyses were with a wide range of buffer systems and gel concentrations were used to establish the conditions for subsequent work. The buffer systems examined were:

1. Phosphate buffer pH 7.6 (Ashton, 8).
2. Boric buffer (Smithies, 180).
3. Tris buffer (Raymond, 150).
4. Tris-EDTA-boric acid buffer (Aronsson and Gronwall, 1).
5. Discontinuous buffer (Poulik, 141).
6. Discontinuous buffer (Ashton and Braden, 10).

The discontinuous buffer system (10) with a 15% gel concentration was used throughout the investigation.

The electrolyte in the buffer compartments was a solution containing 1.2 gm of lithium hydroxide and 11.8 gm of boric acid per liter. The gels were prepared with a buffer made by adding 90 volumes of a solution containing 1.6 gm citric acid and 6.2 gm tris per liter to 10 volumes of electrolyte.

The gels were prepared by adding 75 gm of hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada) to 500 ml of gel buffer in a 5 liter vacuum flask. The mixture was heated over a flame with constant and vigorous swirling and became a viscous or semisolid opaque mass. Heating was continued just short of boiling until the mixture changed consistency and was a homogenous, viscous, translucent liquid. After the heating a negative pressure was applied to the flask until the entire contents boiled vigorously for 30 seconds. The vacuum was turned off and the gel slabs prepared by pouring the hot gel solution into

the mold (Fig. 1) and covering the surface with the plastic cover containing the slot formers. The cover was weighted to obtain a gel of uniform thickness. After 30 minutes the gel was equilibrated with the well buffer by placing the gel at 4 C for 2 hours.

Following equilibration, the cover was removed and approximately 0.1 ml sample inserted into the slot. The exposed surface was covered with warm petroleum jelly (45 C) which sealed the slots in position and prevented evaporation. The retainer plate (Fig. 2) was placed over the surface of mold. (The bottom edges of the mold rest on a platform protruding from the retainer plate, the bottom and front open to allow direct contact with the buffer.)

The plate and mold were carefully lifted and placed on the bolts (a) projecting from the front of the electrophoretor (Fig. 3) the two parts were clamped by wing-nuts on the bolts. The mold holder and buffer chambers (electrophoretor) were one unit for rigidity and to facilitate leveling the gel. A spirit level was mounted at the top center of the retainer plate (Fig. 2). Leveling in all planes was made by adjusting the 3 leveling screws (LS) mounted in the base of the electrophoretor (Fig. 3). The electrodes were 5-mill platinum wire stretched across the back of the buffer chambers. Electrical contact with the electrodes was made by connecting the wire directly to the pole of a co-axial

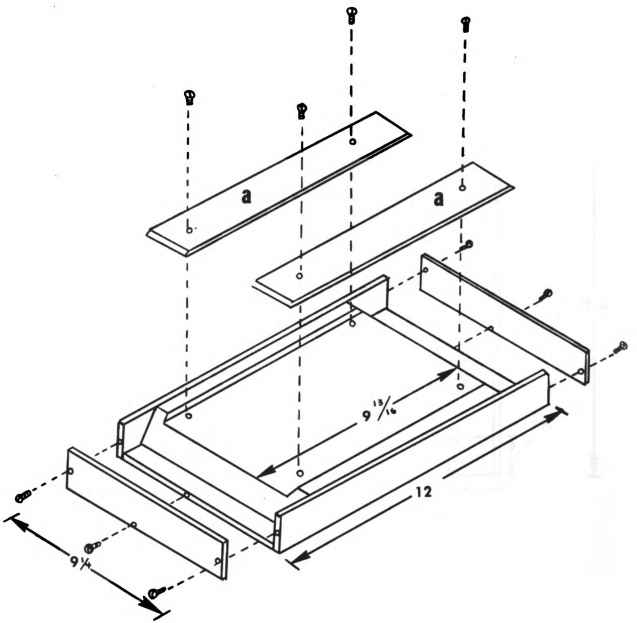


Fig. 1. Exploded view of a modified mold for starch gel electrophoresis. Removable spacers (a). (Dimensions are in inches and only critical dimensions illustrated.)

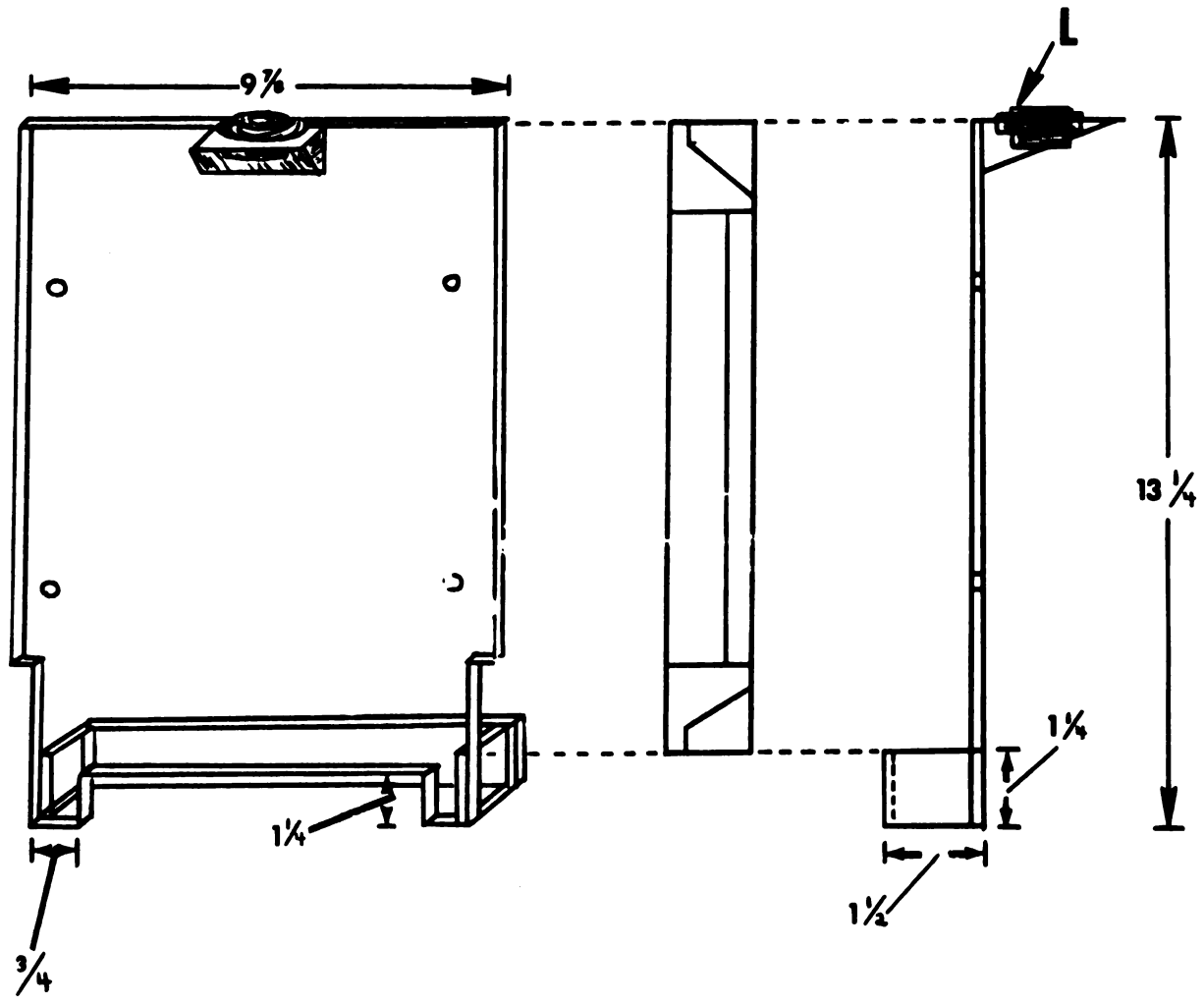
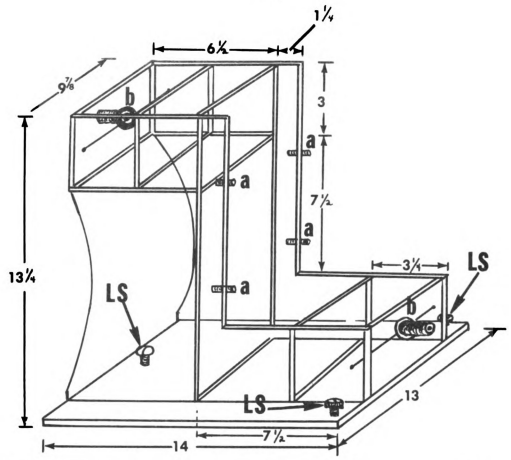


Fig. 2. Retainer plate. A: front view. B: cross section of gel mold. C: cross section of retainer plate. Spirit level (L). (Dimensions in inches and only critical dimensions illustrated.)



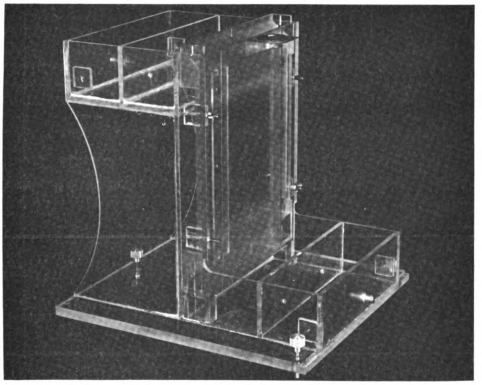


Fig. 3. Modified gel-mold holder and buffer compartments (electrophoretor) for starch gel electrophoresis. Schematic representation showing only the important dimensions. Bolts (a) for clamping the retainer plate in place. Co-axial terminals (b). (Dimensions in inches.)

connector (b). The upper buffer chamber has a capacity of 2,500 ml. The lower chamber was slightly larger holding approximately 2,750 ml. Current was provided by a variable voltage DC power supply (Heathkit model PS-3).

After completion of electrophoresis, the gel mold was removed from the holder and the petroleum jelly scraped from the gel surface. Saran wrap (Dow Chemical, Midland, Michigan) was placed on the gel surface to give support to the gel as it is lifted from the mold and placed in the slicer-holder (Fig. 4b).

The base of the gel cutter (Fig. 4) consists of a heavy metal plate with two metal bars (a) accurately spaced to permit the gel holder (b) to slide between them. A fine piano wire (approx. 0.008 inch in dia.) was drawn taut across notches cut in the metal bars. The width of the gel holder (b) is adjusted by two 1.5 mm thick spacers to correspond to the gel width. The base was clamped to the work bench for additional support.

A ¼ inch thick glass plate was placed on top of the Saran wrap. The holder (b) guided by the metal bars and plexiglass runners (c), was pushed with one quick movement into the wire (W). By moving the wire to a different notch (4.5 mm, 3 mm, and 1.5 mm), four 1.5 mm slices could be obtained from a 6 mm gel. Each slice was easily removed by aid of the Saran wrap. If the wire was clean and taut and if the gel was cut with one quick movement, all slices were smooth and of uniform thickness.

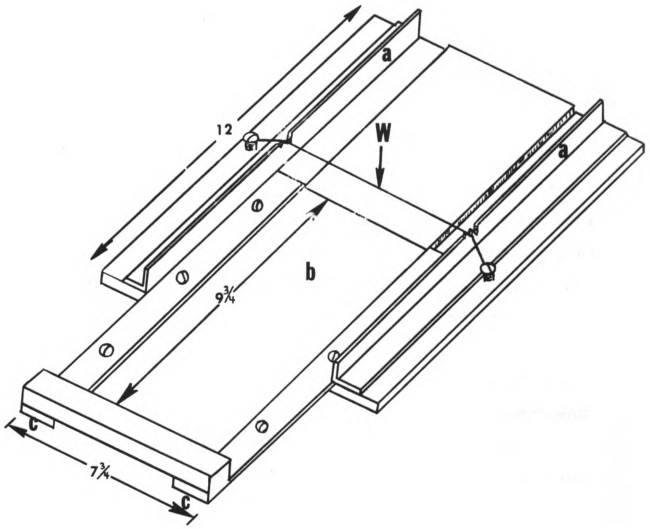
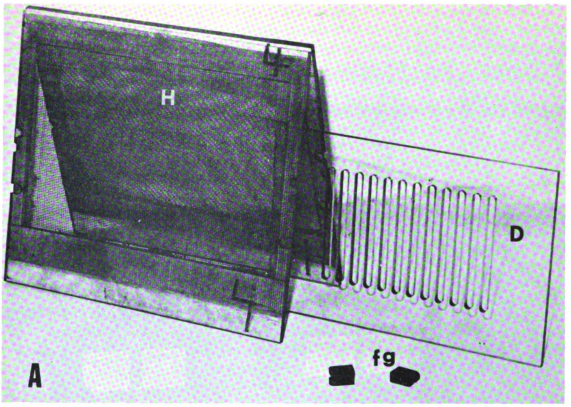


Fig. 4. Slicing device. Metal bars (a). The notches in the middle of the two metal bars are for varying the thickness of the slice. Plexiglass gel holder (b). The gel holder is guided by (a) and by the plexiglass runners (c). 0.008 inch piano wire (W). (Dimensions are in inches and only critical dimensions illustrated.)

The sliced gel was lifted with the Saran wrap as support and placed into the bottom half of a framed-plastic-screen-holder (Fig. 5). Each frame was made of ¼ inch plexiglass and plastic screen mounted on one side. The internal dimensions were 9 11/16 inches in length and 6 1/4 inches in width; the outer dimensions were 10 3/4 inches x 9 1/4 inches. Both halves were placed screen side down so that the space between screens was approximately ¼ inch, and the halves were held together with fiberglass U-clamps placed in grooves at each side. The screen gel was lifted by aid of a spring clamp and placed into the compartment of the staining tank (Fig. 6) containing the protein stain, (1% Amido Black 10 B in 5% acetic acid). Each individual compartment was slightly longer and wider, in order to hold four screens. The illustrated tank with 10 compartments measures 27 3/4 inches x 12 1/2 inches x 10 1/2 inches and each compartment had a drainage valve, so that when a stain or solution became exhausted it could be removed without disturbing the other compartments.

The gel holder was removed from the stain, washed with running tap water to remove excess stain and placed into the destainer (Fig. 7) that was especially designed to be coupled with the convenient procedure for staining gel matrices. It has 2 compartments so that two gel slabs could be destained at one time. Destaining was done at 4 amps. for 30-45 min with continuous circulation of the destaining solution (10% glycerol in 5% acetic acid).



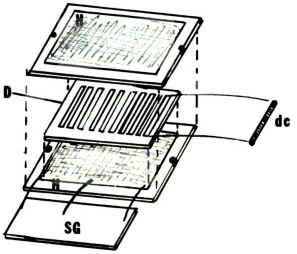


Fig. 5. Plastic-screen-gel-holder. A: gel holder (H), disc gel adaptor (D) and fiber glass clips (fg). B: diagramatic illustration showing how gel holder (H) is assembled for starch-gel slabs (SG) and disc gel columns (dc). Note that the disc gel adaptor is not used for starch-gel slabs.

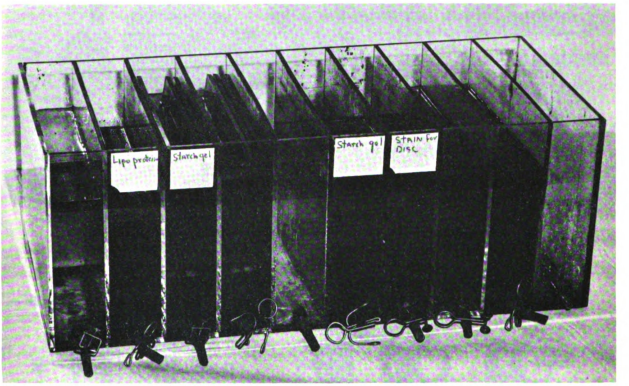


Fig. 6. Multiple staining tank.

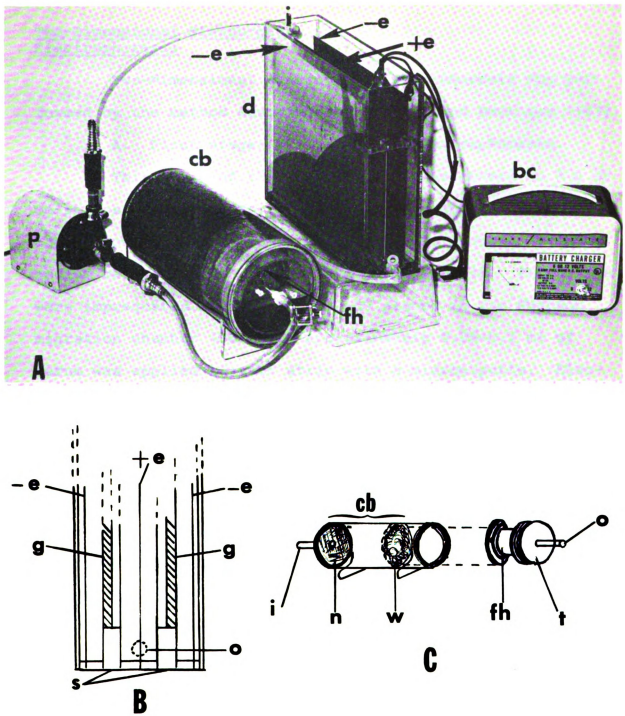


Fig. 7. A: destaining assembly. Destainer (d), 11½ x 10½ x 2½ in. i.d. (bc) 6-12 volt battery charger. The destaining solution circulates through charcoal bed (cb) by pump (p). B: Saggital section through destainer. Gel holder (H) placed in slot (s) so cathode is ⅛ and anode ½ in. from gel (g). C: Filter unit (13 x 5½ x ½ in. o.d.). 8 in. charcoal bed (cb) holds 2 lbs granular charcoal, held in place by two ½ in. thick glass wool filter pads (W) held in place by filter holder (fh) mounted on back of screw-on cover (t). Electrodes (e), inlet (i), plastic screen (n), and outlet (o).

Two-Dimensional Starch-gel Electrophoresis

Two-dimensional starch-gel electrophoresis was performed by the method described by Poulick and Smithies (143).

A. First stage: Filter paper electrophoresis.

This stage of the separation was performed on 3 x 15 cm strips of Whatman No. 3 filter paper using a buffer of the following composition: 0.048 M sodium acetate, 0.048 M sodium barbital, and 0.0073 M hydrochloric acid. The strips were impregnated in this buffer and placed in the Shandon migration chamber (Colab). Approximately 0.05-0.1 ml of serum was applied to each strip with a micropipette. Electrophoresis was done at 120 volts for 12 hours. After electrophoresis one of the strips was stained as a guide to the positions of the protein zones on the unstained strips.

In a few cases, 5 cm x 15 cm cellulose acetate strips were used in place of filter paper. Owen buffer, as described for cellulose acetate electrophoresis was substituted for the above buffer.

B. Second stage: Starch-gel electrophoresis.

A starch slab was prepared as described above, except that a cover without the slot former was used. The gel mold was adjusted to give the following dimensions: 16 cm long, 12 cm wide and 0.6 cm thick.

A cut was made across the full width of the gel about 5 cm from the cathode end. A second cut was made

above, parallel to and 3 cm from the first and a section 1 cm wide was removed. The sample slot was formed by displacing the gel into the space left by the removed section.

Using the stained filter paper strip as a guide a 12 cm x 6 mm section was cut from the middle of an unstained strip. This strip was inserted into the slot in the gel, the displaced gel pushed back against the strip and the removed section replaced. The gel was covered with melted petroleum jelly and electrophoresis performed under the same conditions as for one dimensional starch-gel electrophoresis.

After electrophoresis, the gel was not sliced but stained directly, and destained as described for starch-gel electrophoresis.

Immunoelectrophoresis

Immunoelectrophoresis was employed according to the technique of Hirschfeld (80). The agar was prepared as follows. A two percent solution of Difco Bacto agar was poured into a flat dish to a depth of 1 cm and allowed to solidify. The gel was cut into 1 cm cubes and washed in running tap water for 72 hours followed by frequent changes of distilled water for an additional 72 hours. The agar cubes were stored in distilled water at room temperature. All of the agar used in this investigation was prepared in a single batch.

A barbital buffer system was employed (80):

	Well buffer (pH 8.6, I=0.06)	Agar buffer (pH 8.6, I=0.093)
Diethylbarbituric acid	1.38 gm	1.66 gm
Sodium diethylbarbiturate	8.76 gm	10.51 gm
Calcium lactate	0.38 gm	1.54 gm
Distilled water q. s. ab.	1000 ml	1000 ml

Two parts of the agar buffer was mixed with one part distilled water and the mixture heated in a steamer. An equal volume of freshly melted agar was added with merthiolate in a final concentration of 1:10,000. While the solution was warm, 2½ ml were spread evenly over the surface of thoroughly washed microscope slides and allowed to solidify. Each agar-covered slide was "aged" at least 3 hours in a humidified diffusion chamber prior to use.

A 1 x 66 mm antiserum trought equidistant between 2 antigen wells, 2 mm in diameter, 35 mm from the cathode end and 5 mm from each edge were cut in the agar with a LKB punch, type 6808A (112). The agar plugs in the antigen wells were removed by vacuum with a Pasteur pipette. The plug in the antibody trough was removed after electrophoresis was completed.

The slides were supported in a Shandon migration chamber as modified for IE by Dardas and Mallman (48). A 6 x 8½ inch plexiglass plate was placed across the bridge supports. Sixteen slides were used at one time; eight on each side. Electrical contact between the slides and buffer chamber, and between slides were made with filter paper impregnated

with well buffer. Current was supplied by a variable voltage DC power supply (Heathkit, model PS-3). Electrophoresis was carried out at 4 C for 2½ hours at 1.25 ma per slide. Following electrophoretic separation the agar plugs in the antiserum trough were removed and approximately 0.25 ml of antiserum added. The patterns were allowed to develop for 18 to 24 hours at 28 C in a humidified diffusion chamber.

The slides were washed for 48 hours in repeated changes of saline solution and in repeated changes of distilled water for 24 hours. The slides were dried at 37 C under a single moistened piece of bibulous paper and stained.

Starch-gel Immunoelectrophoresis

Twenty-five milliliters of buffered agar (same as for agar and IE) was spread evenly over the surface of an 8 x 18 cm glass plate forming a layer 3 mm thick. The serum specimen was separated in starch-gel as above. The gel was then sliced longitudinally into 2 parts; one was stained for protein and the other placed cut side down on the agar layer. The agar plate was then placed into a humidified diffusion chamber to preincubate for 12 hours. Then two troughs, 2 mm wide, were cut in the agar 1 cm from each edge of the starch-gel slice. The troughs were filled with the antiserum and the pattern allowed to develop for 3-5 days at 28 C in a humidified diffusion chamber.

The starch-gel slice was removed and the plate washed and dried. The immunoprecipitates were stained and recorded.

Continuous Flow Electrophoresis

Serum was diluted 1:4 with 0.02 M barbital buffer and poured into the feed-device of the Spinco Continuous-Flow Paper Electrophoresis Cell. The apparatus was prepared according to the directions of the manufacturer (16, 120) and the flow rate of the sample was fixed at 4 ml per hour. A constant current of 50 ma requiring approximately 550 v was maintained by a Spinco Constat. The apparatus was cooled in a walk-in refrigerator. The buffer (barbital, pH 8.6, 0.02 ionic strength) was recirculated continuously and only the condensation buffer was discarded during the run. The collection of the fractions into 32 tubes was begun with the appearance of the yellowish-stained albumin fraction at the serrated edges of the lower curtain. At the end of each run, the lower curtain was removed immediately from the cell and, after drying in an oven at 120-130 C for 30 min, stained with Ponteau S or bromphenol blue to reveal the position of the different serum fractions.

The tubes containing the same fractions were pooled, concentrated by prevaporization, and each fraction adjusted to twice its amount in the original serum. The separated fractions were examined for purity by cellulose-acetate electrophoresis.

Polyacrylamide Electrophoresis

Disc electrophoresis. The apparatus and technique were employed according to the earlier version of the disc electrophoresis method as reported in detail by Ornstein and Davis (132). The latest modification of the stock and working solutions (49) were employed (Tables 2 and 3).

Six ul of serum were added to 0.14 ml of sample gel, the mixture layered on top of the spacer gel, photopolymerized for 30 minutes and the cylinders placed in the upper buffer compartment. Electrophoresis was done at 4 ma/tube at room temperature until the tracking dye reached the bottom end of the tube (approximately 1 hour). Current was supplied by a 5,000 volt, 200 ma DC power supply (Canadian Research Institute Model EP-5K/200).

Differential disc electrophoresis. The stock and working solutions for the spacer-sample gel and electrophoretic procedure. The only difference in reagents was the following two acrylamide solutions:

1. Acrylamide stock solution No. I for the 10% working solution:
   40 gm acrylamide
   0.4 gm BIS
   Dissolve and bring to 100 ml with distilled water.

2. Acrylamide stock solution No. II for the 4.75% working solution:
   19 gm acrylamide
   0.4 gm BIS
   Dissolve and bring to 100 ml with distilled water.

Table 2. Stock solutions

(A)		(B)	
1 N HCL	48 ml	1 N HCL	approximately 48 ml[1]
TRIS	36.6 gm	TRIS	5.98 gm
TEMED	0.23 ml	TEMED	0.46 ml
DHOH	to 100 ml	DHOH	to 100 ml
(pH 8.9)		(pH 6.7)	
(C)		(D)	
Acrylamide	28.0 gm	Acrylamide	10.0 gm
BIS	0.735 gm	BIS	2.5 gm
DHOH	to 100 ml	DHOH	to 100 ml
(E)		(F)	
Riboflavin	4 mg	Sucrose	40 gm
DHOH	to 100 ml	DHOH	to 100 ml

[1]pH adjusted by titrating with 1 N HCl.
TRIS=tris(hydroxymethyl)aminomethane
TEMED=N,N,N',N',-tetramethylethylenediamine
BIS=N,N'-methylenebisacrylamide
DHOH=distilled water.

Table 3. Working solutions

Small-pore solution #1	Small-pore solution #2	Large-pore solution	Stock buffer solution for reservoirs[1]
1 part A 2 parts C 1 part DHOH pH 8.9 (8.8-9.0)	Ammonium persulfate 0.14 gm	1 part B 2 parts D 1 part E 4 parts F pH 6.7 (6.6-6.8)	TRIS 6.0 gm Glycine 28.8 gm DHOH to 1 liter pH 8.3

[1]Diluted 1:10 before use.

Pyrex tubes (6 mm i. d. x 12 cm) were marked at 11 cm, 7.5 cm, 4 cm, and 2 cm starting from the base end (Fig. 8). Prior to use, the tubes were cleaned in a detergent solution. The inner walls thoroughly cleaned with a cotton tipped applicator stick, rinsed thoroughly in tap water, then in distilled water and stored in a solution consisting 1-200 parts Kodak Photo Flo solution in distilled water. The tubes were drained and allowed to dry. Then they were inserted vertically into vaccine-bottle stoppers (49), and filled to the 7.5 cm mark with the 10% acrylamide solution. The 4.75% acrylamide solution was carefully added to the 4 cm mark. A thin layer of water was carefully added by means of a 1 ml tuberculin syringe with a 25 gauge needle, and allowed to polymerize for 40 min. The water layer was shaken off and rinsed with spacer gel. Spacer gel was added to the 2 cm mark, water layered and photo-polymerized for 15 min. Sample gel, prepared by mixing 8 ul of fresh serum with 0.4 ml of spacer gel was added and photo-polymerized for 30 min.

Electrophoresis was started at 2 ma/tube until the marker dye reached the separating gel (4 cm mark), approximately 20 min. The current was adjusted to 4 ma/tube until the albumin reached the 11 cm mark, approximately 1½ hours.

Following electrophoresis, the gel columns were removed from the glass tubes after placing in ice water for 5 min by rimming with a finely tapered steel needle.

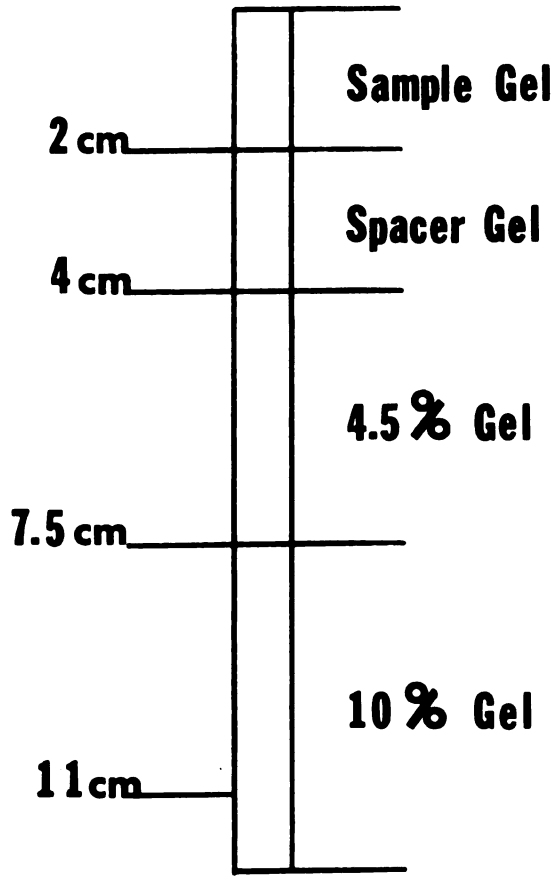


Fig. 8. Diagramatic representation of the position of the gels in the differential disc electrophoretic column.

The gel columns were stained and destained with a modified apparatus designed for starch and acrylamide gel slabs (Figs. 5, 6, 7). The disc gel adapter (H), which has 12 slots (Fig. 5), was placed into the bottom half of the gel holder (D) and the gels inserted into the slots. The top half of the gel holder was then connected to the bottom half by two fiberglass clips.

The gel holder was placed in the compartment of the staining tank (Fig. 6) containing 0.5% Amido Black 10 B in 5% acetic acid. The gels were stained for 30 min at room temperature.

The gel holder was removed from the stain, washed with running tap water to remove excess stain and placed in the destainer (Fig. 7). Sixteen to 24 gels were destained in 30-45 min at 4 amps. Following destaining the gels were photographed and scanned.

Protein, Lipoprotein, and Glycoprotein Stains

Cellulose acetate. Pontceau S, Nigrosin, Light green SF, Coomassie brilliant blue, Lissamine green and Procion Brilliant Blue M-RS were tested comparatively for protein staining on cellulose acetate. Pontceau S was selected for routine serum protein staining. Cellulose acetate strips were immersed for 10 min in a 0.2% solution of Pontceau S in 3% trichleroacetic and differentiated in 2% glacial acetic acid.

Lipoproteins were separated on cellulose acetate electrophoretically according to Gelman (58). Five parts of serum were mixed with 1 part of the following Sudan Black B staining solution: 0.1 gm of Sudan Black B was dissolved in 1 ml of ethyl acetate and 9 ml of propane glycol added, mixed thoroughly and centrifuged at 4,000 X g for 5 min. The serum-staining solution mixture was allowed to stand 3 hours and centrifuged at 4,000 X g for 5 min. Twenty-five microliters of the mixture was applied to the cellulose acetate strip directly over the cathode. Electrophoresis was done as described on pages 33 and 34.

Glycoproteins and/or mucoproteins were stained by the periodic acid Schiff (PAS) method as outlined by Gelman (58). After electrophoresing a sample in the usual way, the strips were immersed in 0.5% periodic acid for 5 min, washed twice with distilled water and placed in Schiff reagent at room temperature for 10 min. The strips were washed 3 times in 0.5% sodium metabisulfite, washed in tap water 5-10 minutes and dried at room temperature under weighted absorbent filter paper.

Agar gel and immunoelectrophoresis. Proteins on dried agar following electrophoresis and immunoelectrophoresis in agar gel were stained with Crowle's (46) Triple Stain:

Thiazine red R	0.1 gm
Amidoswarz 10 B	0.1 gm
Light green SF	0.1 gm
Acetic acid	2.0 gm
Mercuric chloride	0.1 gm
Distilled water	100.0 ml

The slides were immersed solution for 5 min, differentiated in 2% acetic acid, washed in tap water, and dried.

Following electrophoresis and IE in agar gel, lipoprotein and glycoproteins were stained according to the methods described by Gabar (64). Lipoproteins were stained by immersing the dried agar slide in a saturated solution of Oil Red O (37C) in 60% ethanol. Slides were differentiated in 50% ethanol, washed in tap water, and dried. Glycoproteins were stained by the PAS method of Uriel and Grabar (194), according to Crowle (46):

A. Solutions

a. Schiff reagent
1.5 gm basic fuchsin
500 ml boiling distilled water
Filter at 55C and cool to 40C. Add
25 ml 2 N hydrochloric acid
3.75 gm $Na_2S_2O_5$
Agitate to ensure rapid solution. Allow to stand stoppered in a refrigerator for 6 hours. Add
1.2 gm animal charcoal

Mix vigorously for 50 seconds and filter rapidly, filtration time not exceeding 2-3 min. Store stoppered in refrigerator.

b. 1.0 gm periodic acid
0.82 gm anhydrous sodium acetate
100 ml distilled water

c. 0.54 gm acetic acid
0.89 gm anhydrous sodium acetate
10 gm hydroxylamine hydrochloride
100 ml distilled water

d. 5 ml 10% $Na_2S_2O_5$
5 ml 2 N hydrochloric acid
90 ml distilled water

Solution should be made just before it is used. This also serves as the final wash bath when it contains 20% glycerol.

B. Procedure

The pre-dried agar slides were placed in c for 15 min, washed in running water for 15 min, placed in b for 10 min, washed in running water for 10 min, and placed in Shiff reagent, diluted immediately before use with an equal volume of distilled water, for 3 min. The slides were then washed 3 times for 2 min each in d, 3 times for 1 hour each in glycerinated d, dried and recorded.

Starch-gel. Nigrosin, Amidoschwarz, and Wool Fast Blue BL (Natural Aniline) were tested comparatively for staining proteins. A 0.5% solution of amidoschwarz in 5% acetic acid was selected for routine protein staining. The gel slabs were stained for 10 min at room temperature.

Polyacrylamide gel. A 0.5% solution of amidoschwarz in 5% acetic acid and filtered twice, was used routinely for protein staining. The gel columns were stained for 30 min at room temperature.

The detection of lipoproteins was investigated by two methods: prestaining of the serum lipoproteins as described for cellulose acetate or by immersing the gel column, following electrophoresis in a saturated solution

of Oil Red O (37 C) in 30% ethanol and differentiating in 20% alcohol. The former method gave better results and was used routinely.

Glycoproteins were stained according to the PSA method described by Canalco (35). After electrophoresing the sample in the usual way the gel column was immersed in 7.5% acetic acid at room temperature for 1 hour. The column was transferred to 0.5% solution of periodic acid (PA) solution at 4 C for 1 hour and the PA moved electrophoretically in 7.5% acetic acid for 1 hour. Then the columns were placed in Schiff reagent at 4 C until bands appeared. The columns were stored indefinitely in fresh Schiff reagent.

Continuous flow filter paper curtain. Immediately following electrophoresis the curtain was dried in 120-130 C oven for 30 min. The dried curtain was stained by immersing in 0.2% Pontceau S in 3% TCA, differentiated in 5% acetic acid and dried at 37 C.

Densitometric Evaluation of Electrophorograms

All scanning was done with the N. I. L.-Joyce Chromascan, a double-beam recording and integrating microdensitometer, incorporating facilities for scanning by transmission and reflectance. The four variables: (a) the kind of light filter used, (b) the slit width of the aperature, (c) the slope of the grey wedge or cam, and (d) the gear ratio of sample movement to recording pen were used to

obtain maximum sensitivity as follows: (1) cellulose acetate: no filter, 1:1 gear ratio, 40° cam, a slit width of 1/20 mm, and scanned by reflectance; (2) starch-gel: (reflectance) no filter, 1:1 gear ratio, 90° cam, and a slit width of 1/24 mm; (transmission) no filter, 1:1 gear ratio, 90° cam, a 1 mm slit width in front of light source and a 1/24 mm slit width in front of transmission photomultiplier; (3) polyacrylamide gel columns: no filter, 1:1 gear ratio, 90° cam, a 1 mm slit width in front of light source, a 1/24 mm slit width in front of the transmission photomultiplier and scanned by transmission.

Photography

A number of 35 mm films were tested for photography of starch-gel slabs and polyacrylamide gel columns: Kodak High Contrast Copy, Kodak Plus X, Kodak Infrared, Kodak Panatomic X, Ansco Versapan and Adox KB-14. Kodak Plus X and Ansco Versapan were used during the entire investigation.

A Lieca 35 mm camera (model M3) with a 90 mm Elmarit lens was mounted on a specially constructed camera stand. The specimen to be photographed was placed on the surface of the plate glass top. A flashed Opal glass plate was mounted 10 inches below the upper clear plate glass and twenty-four 50 watt daylight type light bulbs were mounted underneath so that the gels could be photographed by transmitted light.

Starch-gel slabs were placed on the glass top, all air bubbles removed, and photographed with Ansco Versapan for 0.1 and 0.2 seconds at f/11 with a Lieca red filter. The film was developed in Ansco Hyfinol for 4 min.

Polyacrylamide gel columns were placed in unetched test tubes, filled with 5% glacial acetic acid and stoppered. The tubes were arranged on the surface of the upper glass plate and photographed with Kodak Plus X for 0.125 sec at f/4. The film was developed with Kodak D-76 developer, full strength, for 10 min. Immunoelectrophoretic slides were placed in the negative carrier of the enlarger and medalist single weight F-4 enlargement paper was exposed and developed in Kodak Dektol.

RESULTS

Effect of Freezing on Normal Bovine Serum Proteins

Freezing and thawing a serum twice did not produce any changes detectable by cellulose acetate or starch-gel electrophoresis.

Examination of Rabbit Anti-Normal Bovine Serums

No detectable anti-albumin antibodies were found in the antiserums from 3 of 18 rabbits and 2 failed to produce anti-gamma globulin after 3 immunization schedules. Antiserums collected 10 days after the last intraperitoneal inoculation of normal serum were unsuitable for use because a large number of anti-globulins were no longer detectable. Only those antiserums collected between 5-7 days after the last intraperitoneal inoculation and which contained all the anti-normal bovine serum components were pooled.

Fractionation of Normal Bovine Serum by Continuous Flow Electrophoresis

Normal bovine serum proteins were separated into four main components by continuous flow electrophoresis (Fig. 9). Fractions 9 and 10 contained gamma globulin (Fig. 10) by cellulose acetate electrophoresis of the

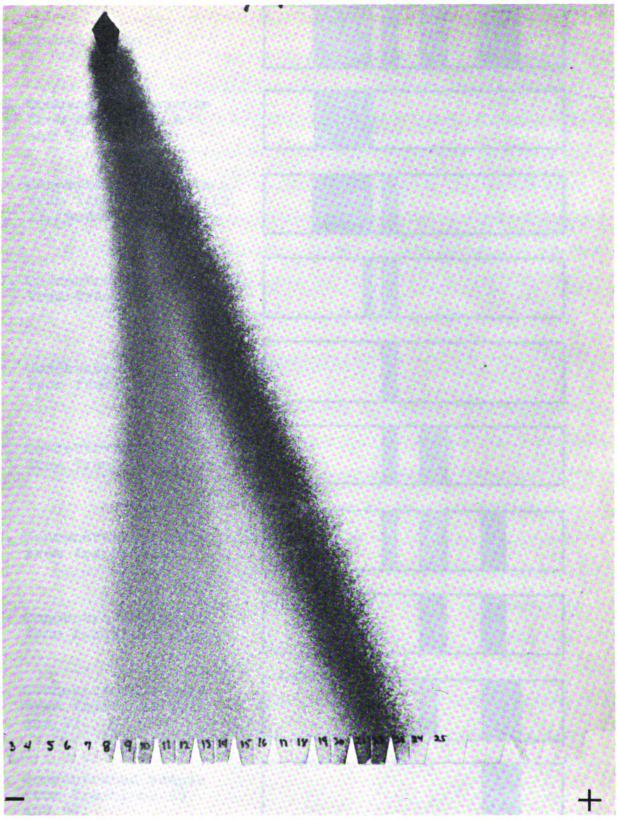


Fig. 9. Paper curtain electrophorogram of normal bovine serum after separation by continuous flow electrophoresis. The numbers at the bottom of the curtain represent fraction numbers.

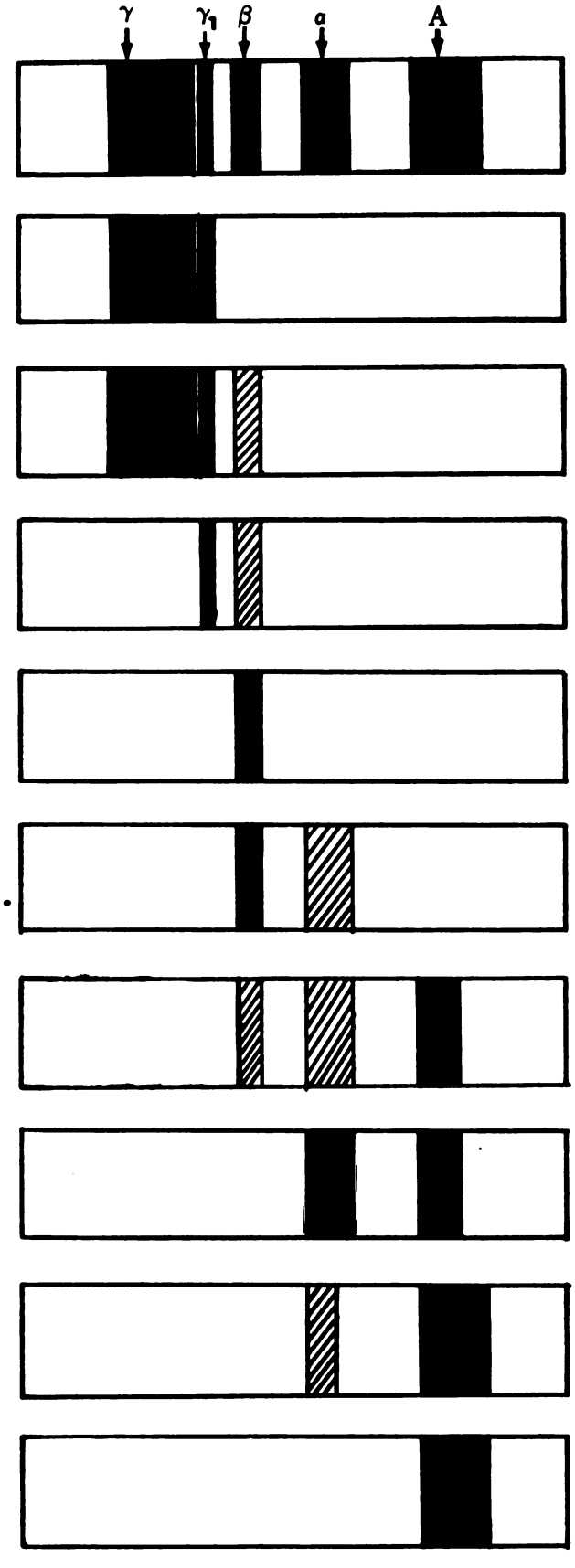


Fig. 10. Cellulose acetate electrophorograms of continuous flow electrophoretic effluent serum fractions.

concentrated effluent. Fractions 11, 12, 13, 14, and 15 contained both gamma and beta globulins. Fractions 16 contained only beta globulin. Beta and alpha globulins were in fractions 17 and 18. Albumin, and alpha and beta globulins were in fraction 19. Fraction 20 contained alpha globulin in high concentration and a low concentration of albumin. A trace of alpha globulin and an increasing concentration of albumin were in fractions 21 and 22; and fractions 23 and 24 contained only albumin.

Cellulose Acetate Electrophoresis of Normal Bovine Serum

Normal bovine serum was separated into 6 fractions with the tris-EDTA-boric acid buffer system (1): prealbumin, albumin, 2 alpha globulins, beta globulin and gamma globulin but the pattern of any given serum was not reproducible.

The only buffer system with which reproducible patterns were obtained was the continuous barbital-acetate buffer system (133). Normal bovine serum was separated into 4 major major fractions by electrophoresis for 2 hours with this system. These included albumin, and alpha, beta, and gamma globulins. An additional gamma component ($gamma_1$) was separated in many serums electrophoresed under the same conditions. Five ul of human serum separated into 5 fractions by the system: albumin, 2 alpha globulins, beta globulin and gamma globulin (Fig. 11).

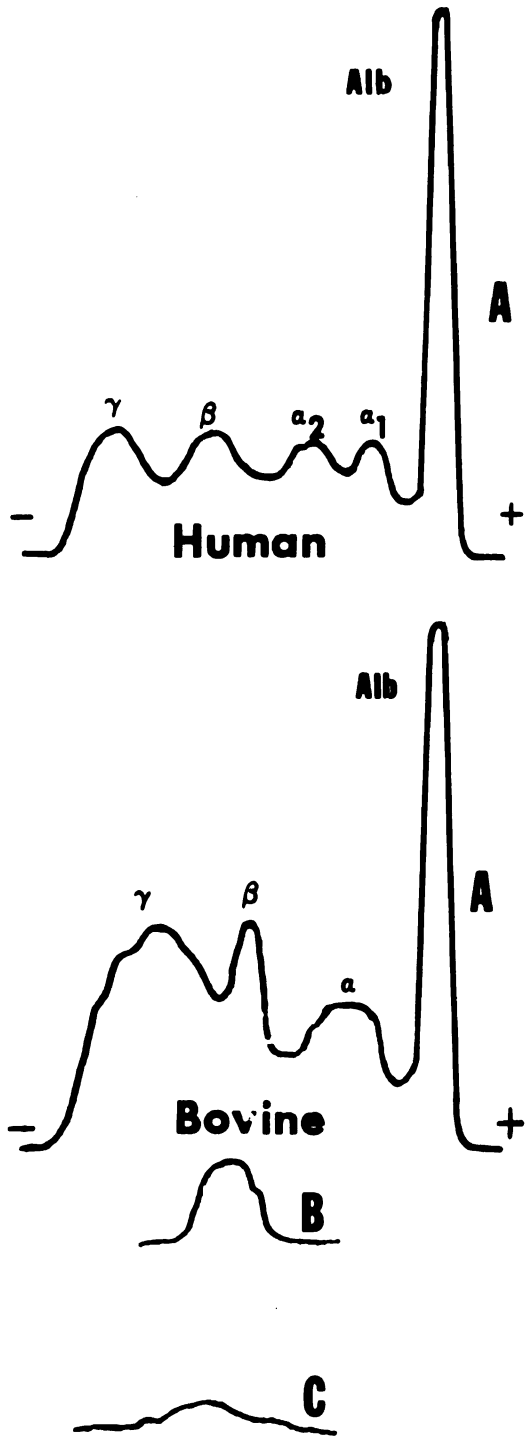


Fig. 11. Typical densitometric recordings of cellulose acetate electrophorograms after electrophoresis with the Owen buffer system. A: protein. B: protein-bound carbohydrate. C: protein-bound lipid.

Stained normal bovine serum after cellulose acetate electrophorograms contained one lipoprotein and 1 glycoprotein fraction. Protein-bound lipids were in low concentration with the major component migrating in the beta region (Fig. 11). One great glycoprotein fraction was in the beta region.

Agar Gel Electrophoresis of Normal Bovine Serums

Four major fractions in normal bovine serum were detected in agar gel electrophoresis (Fig. 12) using either the continuous barbital-acetate system (133) or the discontinuous veronal buffer system (80). Neither separation nor resolution was better than that obtained by cellulose acetate electrophoresis.

Starch-gel Electrophoresis of Normal Bovine Serum

Sixteen to 20 fractions were routinely separated with a 15% gel concentration using the Ashton discontinuous buffer system (10). This system yielded the clearest and most consistent separation of the normal serum proteins. Separation of serum proteins with the other buffer systems were unsatisfactory.

Figure 13 is a composite, schematic representation of the relationships between protein zones following single dimensional cellulose acetate, starch-gel, and two-dimensional electrophoresis. The zones in starch-gel were assigned

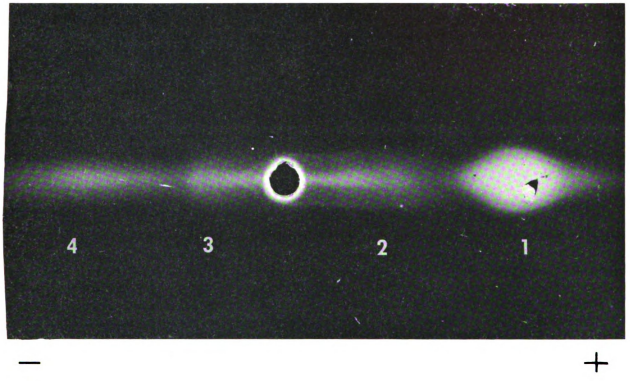


Fig. 12. Electrophorogram of normal bovine serum in agar gel with the Owen buffer system. (1) albumin, (2) alpha globulin, (3) beta globulin, (4) gamma globulin.

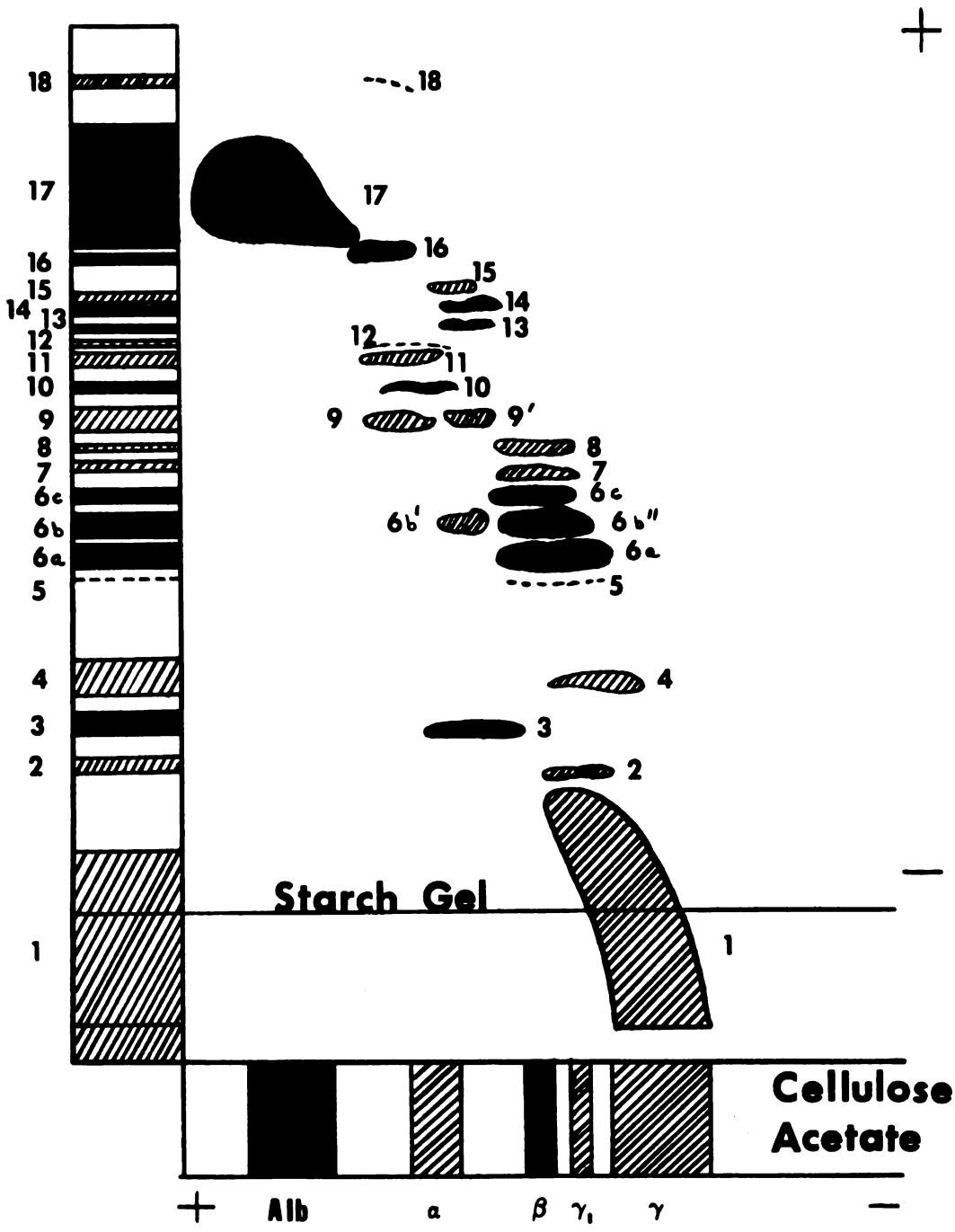


Fig. 13. Single dimensional starch gel, cellulose acetate, and two dimensional electrophoretic patterns of normal bovine serum. (The cross hatched areas represent lightly stained bands.)

numbers according to the mobility in the gel (with number 1 for the slowest moving). Two additional zones were detected in the two dimensional analyses in which the serum proteins were electrophoresed on cellulose acetate and then placed in a starch-gel for electrophoresis at a right angle to the original direction of migration. The zones resolved by this procedure were classified according to their electrophoretic mobility in cellulose acetate. Further definition was dependent upon the isolation and characterization of the individual zones.

Band 1 was a light staining zone of gamma-globulin. Some serum samples contained 2 proteins which migrated only slightly toward the anode as discrete bands.

Band 2 was not consistently detected and was a part of the gamma globulin.

Band 3 stained intensely and corresponded to the slow moving alpha$_2$ globulin described for human serum (180).

Band 4 migrated as a gamma globulin.

Bands 5, 6a, b, and c, 7, and 8 migrated as beta globulins. Three to six beta globulins were in this zone. The number of bands varied with breed of the animal (7, 8, 9). Band 5 was present in varying concentrations in all serum samples except serums from Guernsey cattle. Bands 6a, b, and c were always present, always intensely stained and were the major beta bands in serum from Guernsey cattle.

Holstein or Holstein-Angus cattle serums contained band 7 in addition to 6a, b, and c. Band 8 was lightly stained and variable in serum from Jersey cattle.

Bands 9 to 16 were classified as postalbumin bands. Band 9 contained $alpha_1$ and $alpha_2$ globulin. Bands 10, 11, 12, and 16 had only $alpha_1$ globulins while bands 13, 14, and 15 migrated as $alpha_2$ globulins. Bands 10, 13, 14, and 16 stained intensely. Bands 11, 12, and 15 were not always present. Band 16 was frequently marked by the albumin band.

Band 17 corresponded to the albumin zone on cellulose acetate.

Band 18 was the only prealbumin demonstrable and migrated as an $alpha_1$ globulin.

Immunoelectrophoresis of Normal Bovine Serum

Thirty-two antigenic components were detected in normal bovine serum by IE: one pre-albumin, one albumin, 6 $alpha_1$ globulins, 13 $alpha_2$ globulins, 4 $beta_1$ globulins, 5 beta globulins, 1 $beta_3$ globulin and 1 gamma globulin (Fig. 14). The 32 immunoprecipitates were demonstrated by the use of several antiserums. The maximum number of antigenic components which could be observed by the use of a single antiserum was 28. These precipitin arcs have been labeled according to their mobility in the whole serum pattern and in the individual fractions obtained by

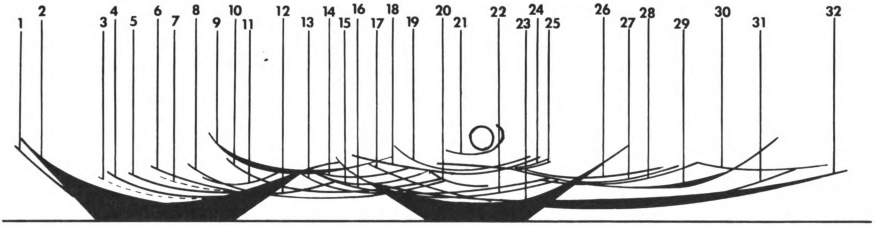


Fig. 14. A composite drawing of agar immunoelectrophoretic patterns of normal bovine serum proteins.

1. Pre-Albumin
2. Albumin
3. $Alpha_1$-1
4. $Alpha_1$-2
5. $Alpha_1$-3
6. $Alpha_1$-4
7. $Alpha_1$-5
8. $Alpha_1$-6
9. $Alpha_2$-1
10. $Alpha_2$-2
11. $Alpha_2$-3
12. $Alpha_2$-4
13. $Alpha_2$-5
14. $Alpha_2$-6
15. $Alpha_2$-7
16. $Alpha_2$-8
17. $Alpha_2$-9
18. $Alpha_2$-10
19. $Alpha_2$-11
20. $Alpha_2$-12
21. $Alpha_2$-13
22. $Beta_1$-1
23. $Beta_1$-2
24. $Beta_1$-3
25. $Beta_1$-4
26. $Beta_2$-1
27. $Beta_2$-2
28. $Beta_2$-3
29. $Beta_2$-4
30. $Beta_3$
31. $Beta_2$-5
32. Gamma

continuous flow electrophoresis (page 50). The $alpha_1$ globulin with the greatest mobility was designated $alpha_1$-1 (a_1-1) and so forth.

The prealbumin and a_1-1 and a_1-5 areas were often difficult to observe with the protein stains whereas the albumin and the other four a_1-globulins were readily and consistently seen. The a_1-1 arc stained poorly for lipid. The use of the concentrated effluent fractions obtained from continuous flow electrophoresis, and antiserum from rabbits which produced no detectable anti-albumin antibodies facilitated the identification of the $alpha_1$ and $alpha_2$ regions.

The $alpha_2$ zone had 5 precipitin arcs. $Alpha_2$-13 stained for lipid. The intensity of the lipoprotein stain of this arc was markedly greater than the a_1-1 arc. In addition, there was a strong Oil Red O stained area lying behind the a_2-13 lipoprotein and immediately adjacent to the antigen well. This lipid spot, which has none of the characteristics of a precipitation band, corresponds to a similar fraction in human and sheep serums (40).

One strong glycoprotein (a_2-1) and 6 weak glycoproteins (albumin, a_2-2, a_2-9, a_2-11, $beta_1$-2 (b_1-2), and b_2-4) were stained with the Schiff reagent.

None of the beta globulins except b_1-2 and b_2-4 have been identified. If serum contained hemoglobin the hemoglobin would migrate in the $beta_1$ zone. Except at the

cathodic end, the $beta_2$-5 precipitate was often obscured by the broad gamma globulin precipitate and resembled a biphasic precipitin curve. The $beta_3$ arc was not consistently detectable.

The gamma globulin precipitate formed a continuous arc from the $alpha_2$ zone to the most cathodic end of the slide.

Starch-gel Immunoelectrophoresis of Normal Bovine Serum

Many of the bands, especially in the post albumin region, obtained by electrophoresis in starch-gel consisted of multiple antigenic components when analyzed by immunodiffusion in agar after electrophoretic separation in starch-gel (Fig. 15). Many of these bands could not be identified with specific precipitin arcs. Individual bands cut from the gel and placed on agar did not resolve the exact antigenic moeity because of trailing in the highly concentrated bands, especially gamma, beta, and albumin bands.

Not as many antigenic components were detected as by agar immunoelectrophoresis. The number of immunoprecipitates was generally the same as or less than the number of bands separated in starch-gel. It was necessary to preincubate the starch-gel 12-18 hours before adding the antiserum to assure maximum diffusion of the antigens into the agar gel. This did not improve on the number of arcs but preincubation less than 12 hours resulted in a further decrease in precipitin arcs.

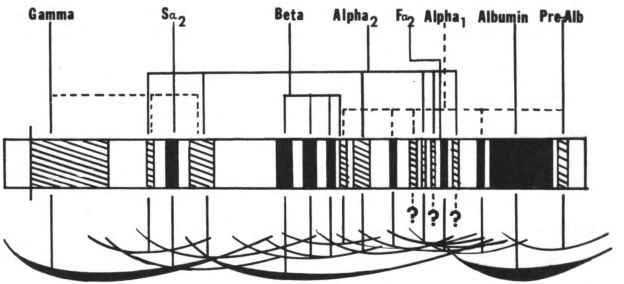


Fig. 15. Starch gel immunoelectrophoretic pattern of normal bovine serum. (The cross hatched areas represent lightly stained bands.)

Polyacrylamide Gel Electrophoresis of Normal Bovine Serum Proteins

The conventional disc electrophoretic procedure (132) yielded poor separation and resolution, and was unsatisfactory for the fractionation of bovine serum proteins.

The differential disc electrophoretic procedure increased the separation and resolution of human and bovine serum proteins (Fig. 16, 17, 18). Human serum separated into 44-50 components with this procedure; into only 22-28 by the conventional procedure (Fig. 16). With the conventional system, bovine serum separated into 14-22 bands with poor resolution; into over 30 bands by the differential procedure (Fig. 17). Because of the better resolution, the corresponding densitometric tracings (Fig. 18) were markedly improved, particularly the post albumin region of bovine serum.

Ten glycoprotein fractions in normal bovine serum were stained with Schiff reagent. The more deeply stained bands were in the gamma, slow $alpha_2$ and beta globulins and in the cathodic end of the albumin band. Only 4 glycoproteins were detected by the conventional procedure. The prestaining procedure for protein-bound lipids was unsatisfactory. Two weak lipoproteins and 3 major lipoproteins were stained with Oil Red O. Two of the major lipoproteins were in the gamma zone and one corresponded to the slow $alpha_2$ band. The carbohydrate- and lipid-bound protein bands are

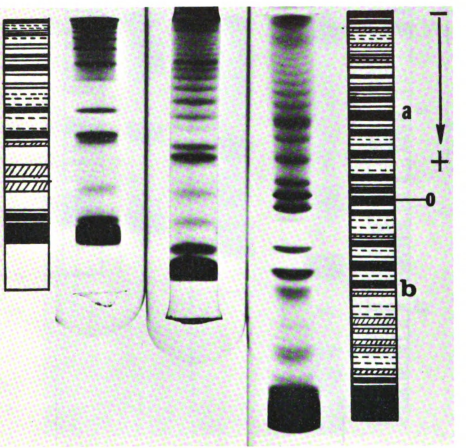


Fig. 16. Comparison of the separation of human serum in standard 7% gel (left), 4.75% gel (middle), and differential gel (right). Cross hatched areas and dotted lines indicate lightly stained and fine bands, respectively. (o) origin of the 10% separation gel, (a) 4.75% gel, (b) 10% gel. Spacer gel not shown.

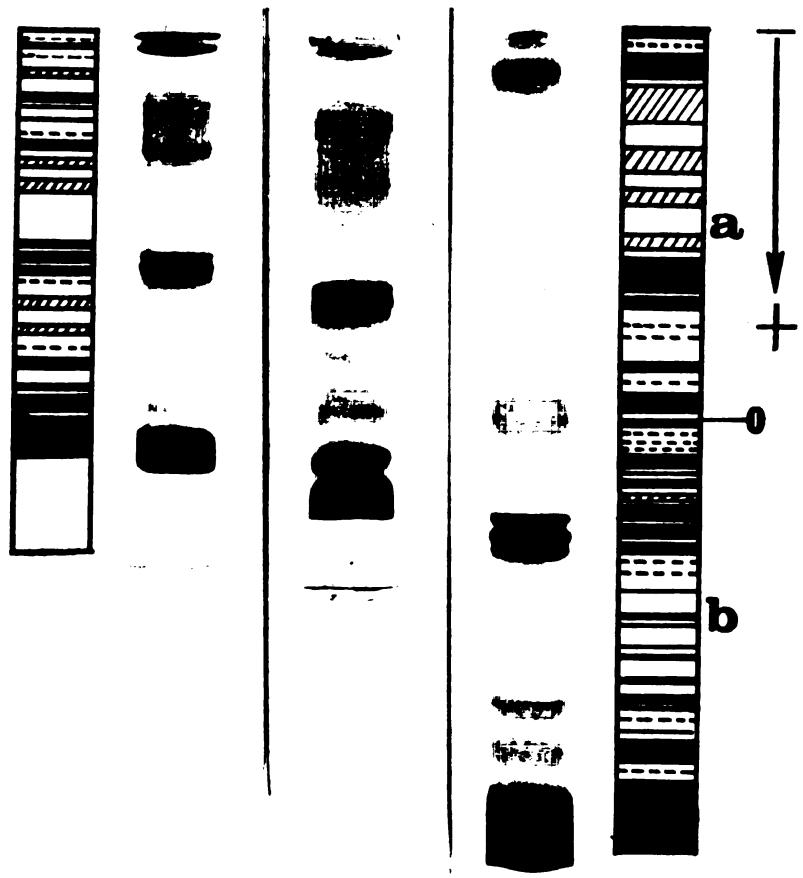


Fig. 17. Comparison of the separation of normal bovine serum in standard 7% gel (left), 4.75% gel (middle), and differential gel (right). Cross hatched areas and dotted lines indicate lightly stained and fine bands, respectively. (o) origin of the 10% separation gel, (a) 4.75% gel, (b) 10% gel. Spacer gel not shown.

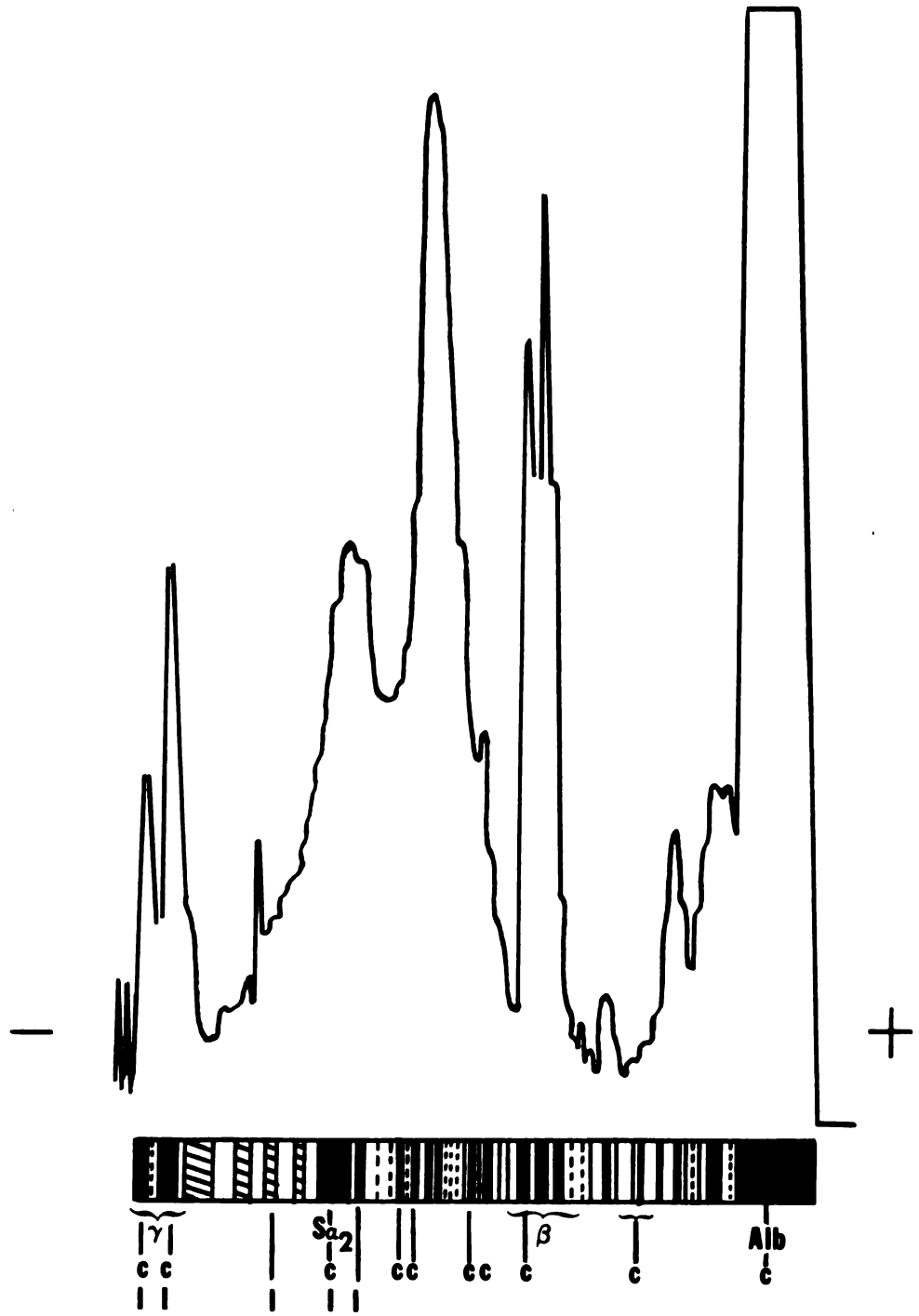


Fig. 18. Densitometric tracing of the normal bovine serum separated by the differential disc electrophoretic method illustrated in Figure 17. The glycoprotein fractions (c) and lipoprotein fractions (l) are also indicated. (Sa_2) slow alpha$_2$ lipoprotein.

presented in Fig. 18. Densitometric analyses of these specific stained bands was unsatisfactory.

A 4.75% gel in a standard tube (6.5 cm long) afforded better separation than the standard procedure (7% gel), especially of human serum, which increased by 5-8 more bands. Figure 16 shows 22 bands separated by the standard gel (7%), 33 by the 4.75% gel and 50 by the differential gel. The number of bands, however, did not increase if the differential tubes (12 cm long) were filled only with 7%, 4.75%, or 10% separating gels and electrophoresed under the same conditions described for the differential system. Because of diffusion the post albumin bands were extremely fuzzy (Fig. 19).

Reproducible results were obtained when 16 tubes containing the same sample were run simultaneously (Fig. 20). The tubes in Fig. 20 were picked at random from a 16 column run. Figure 21 illustrates variation in protein fractions found in 8 individual bovine serums, and also demonstrates the over-all reproducibility of the distance migrated by the albumin band in a single run.

The superiority of this system was clearly demonstrated with serums collected before inoculation (initial) and prior to autopsy (post-inoculation) from a cow experimentally infected with Mycobacterium bovis (Fig. 22).

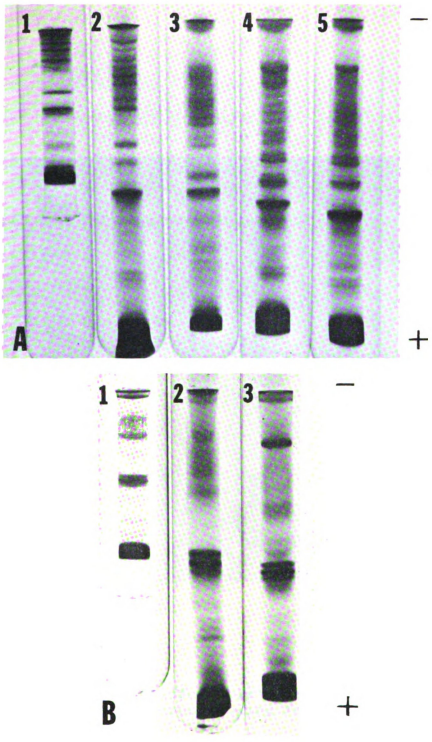


Fig. 19. Comparative electrophorograms of normal serum proteins. A: human serum: (1) conventional procedure, (2) 7% gel-12 cm column, (3) 4.75% gel-12 cm columns, (4) and (5) differential procedure. B: bovine serum: (1) conventional procedure, (2) 4.75% gel-12 cm column, (3) differential procedure.

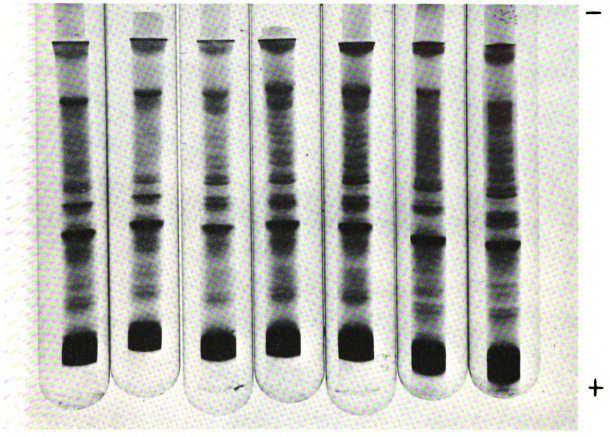
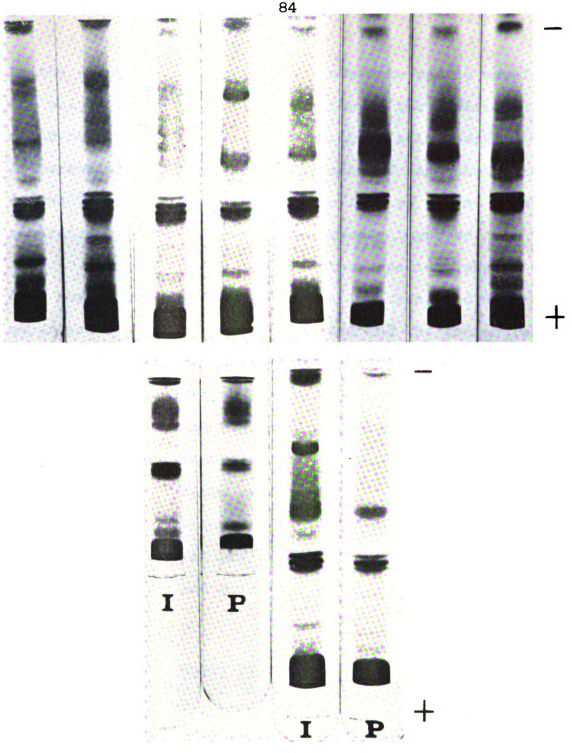


Fig. 20. Differential disc electrophorograms of normal serum showing reproducibility of the method.

Fig. 21. Eight individual bovine serums from a 16 column run by the differential system, illustrating variation among serums and reproducibility as measured by the distance migrated by the albumin in a single run.

Fig. 22. Comparison of the separation by the conventional (left) and differential (right) systems of the initial serum (I) and post-inoculation serum (P) from a cow experimentally infected with Mycobacterium bovis.


−
+
−
I
P
I
P
+

By the standard procedure, the bands were poorly resolved and only a decrease in concentration of the individual bands could be demonstrated in the post-inoculation serum. The differential system revealed a number of bands not detected in the 7% gel, particularly in the initial serum. Both the separation and resolution were markedly improved.

Serums from a number of other species (sheep, rabbit, dog, horse, guinea pig, pig, mouse, chicken, and duck) were separated by the differential system and the conventional procedure. The separation of sheep serum was increased from 19 to 31 bands. Five to 8 more bands were separated in rabbit, horse, guinea pig, and dog serums. This method did not increase the separation of mouse, dog, chicken, or pig serum. The resolutions were as good as, or better than, that found in the standard gel. With all serums tested, a 4.75% gel by the standard procedure gave better separation, better resolution and more reproducible results than the 7% gel.

Comparison of the Major Electrophoretic Procedures Used for the Analyses of Normal Bovine Serum

The relationships of the patterns obtained in cellulose acetate, starch-gel, differential disc and agar immunoelectrophoresis are presented in Figure 23. Other than the 4 zones that have been identified (gamma, Sa_2, beta and albumin zones), no nomenclature can be given for the numerous

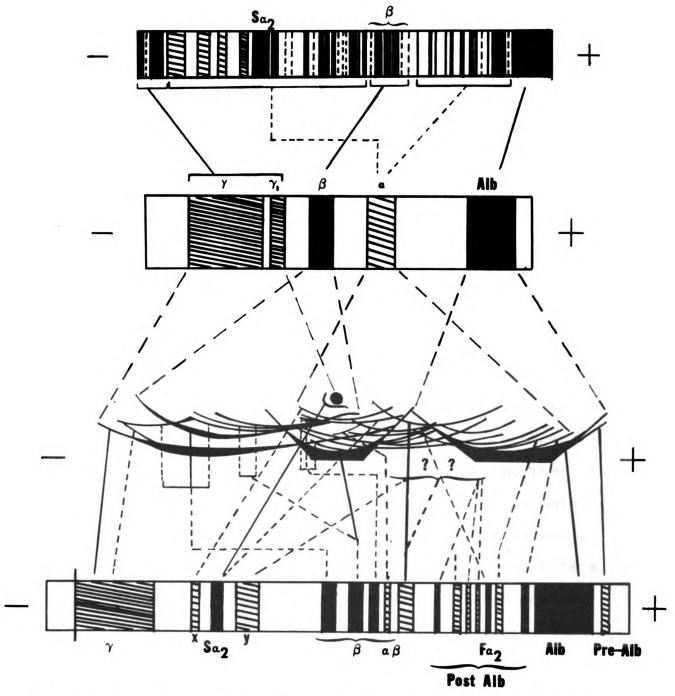


Fig. 23. Diagramatic representation showing the relationships among differential disc electrophoresis, cellulose acetate electrophoresis, agar immunoelectrophoresis, and starch gel electrophoresis of normal bovine serum (top to bottom).

protein bands separated by the differential disc electrophoretic procedure. Further definition is dependent upon the isolation and characterizations of each individual zone.

Most of the relationship between starch-gel and agar immunoelectrophoresis is based only on presumptive evidence.

Cellulose Acetate Electrophoresis of Serum from Tuberculous Cows

Experimentally infected animals. Representative densitometric tracings of cellulose acetate electrophorograms of serum collected from the experimental animals are shown in Figures 24, 25, and 26. From this type of tracing it was possible to calculate the relative percent protein values for each fraction.[1] A plot of these values from electrophorograms from representative animals showing the fluctuations and alterations in serum protein and protein bound carbohydrate patterns at various stages during the disease and the effect of tuberculin testing on these patterns are presented in Figures 27 through 31. The pathologic changes, the pre and post hemogglutination (HA) titers, and serum protein changes determined from cellulose acetate electrophorograms of the experimental animals are presented in Table 4.

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[1]Relative percent protein was calculated by dividing the area of each fraction by the total area of the electrophorogram and multiplying by 100.

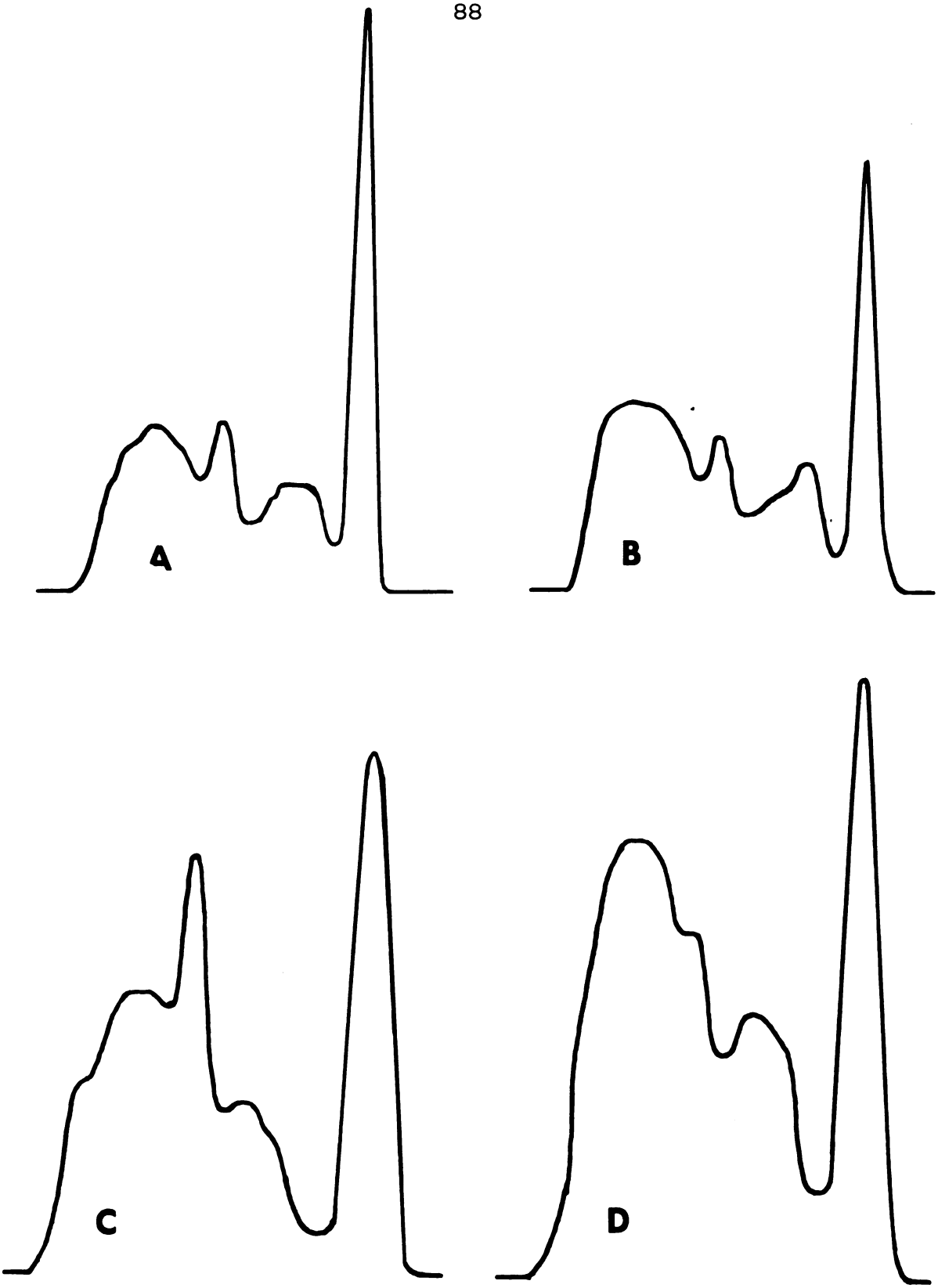

Fig. 24. Typical densitometric recordings of cellulose acetate electrophorograms of bovine serums. (Top) M. bovis. A: pre-inoculation, B: 132 days post-inoculation. (Bottom) 68C (virulent Group III). C: pre-inoculation, D: 122 days post-inoculation.

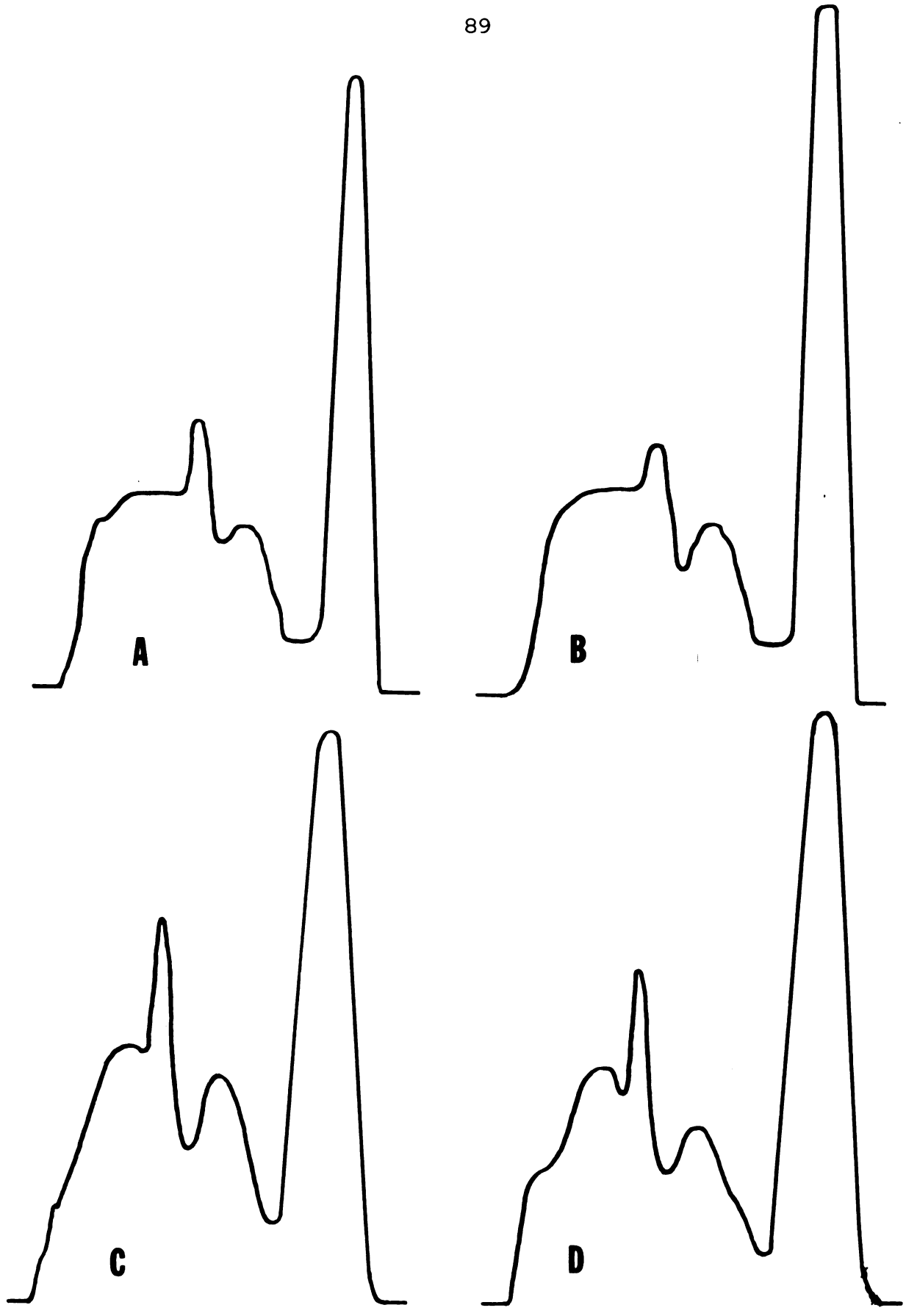

Fig. 25. Typical densitometric recordings of cellulose acetate electrophorograms of bovine serums. (Top) M. avium. A: pre-inoculation, B: 132 days post-inoculation. (Bottom) 50B (avirulent Group III). C: pre-inoculation, D: 172 days post-inoculation.

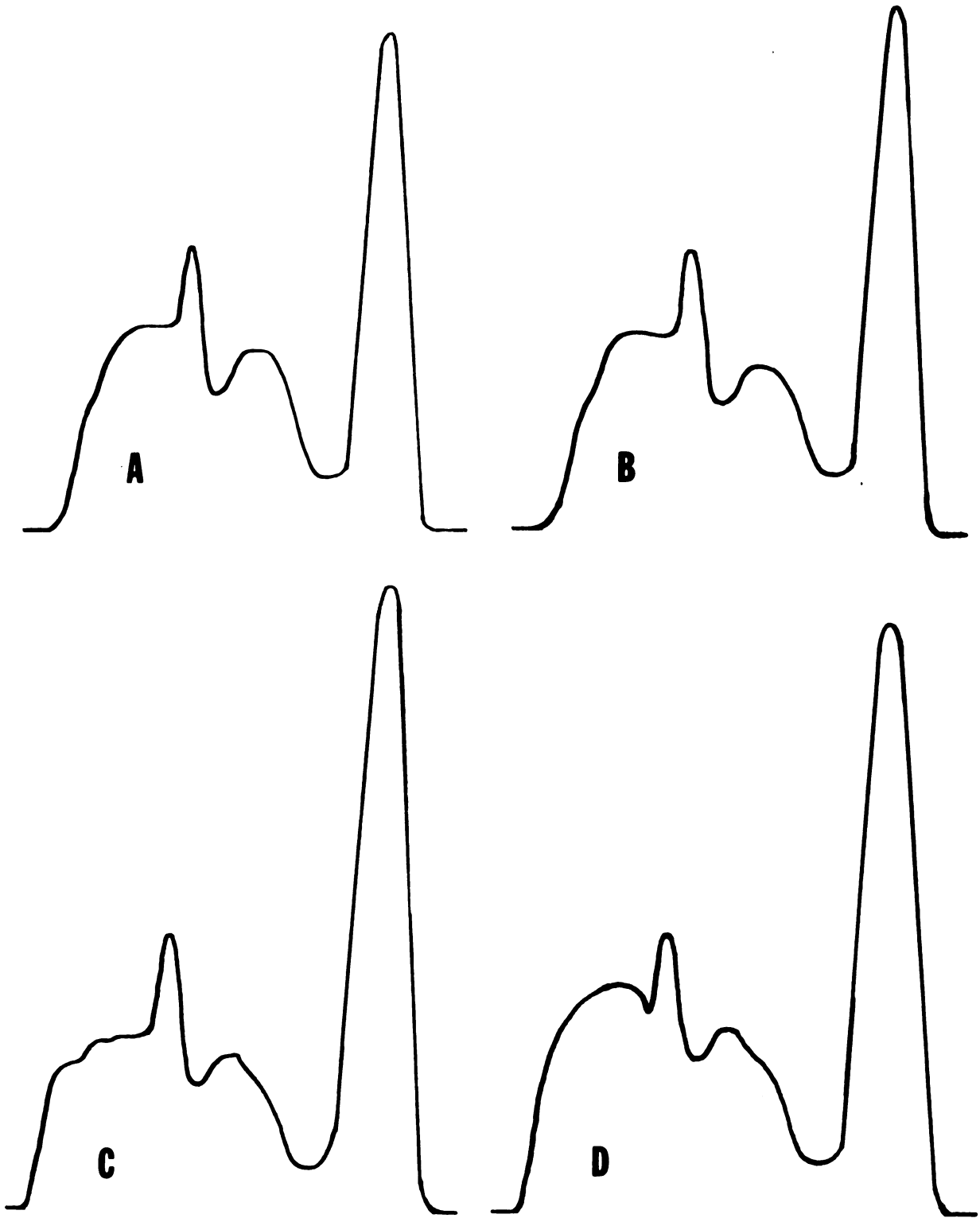

Fig. 26. Typical densitometric recordings of cellulose acetate electrophorograms of the two types of patterns from cattle infected with 51C (virulent Group III). (Top) Type I. (Below) Type II. A: and C: pre-inoculation, B: and D: 179 days post-inoculation.

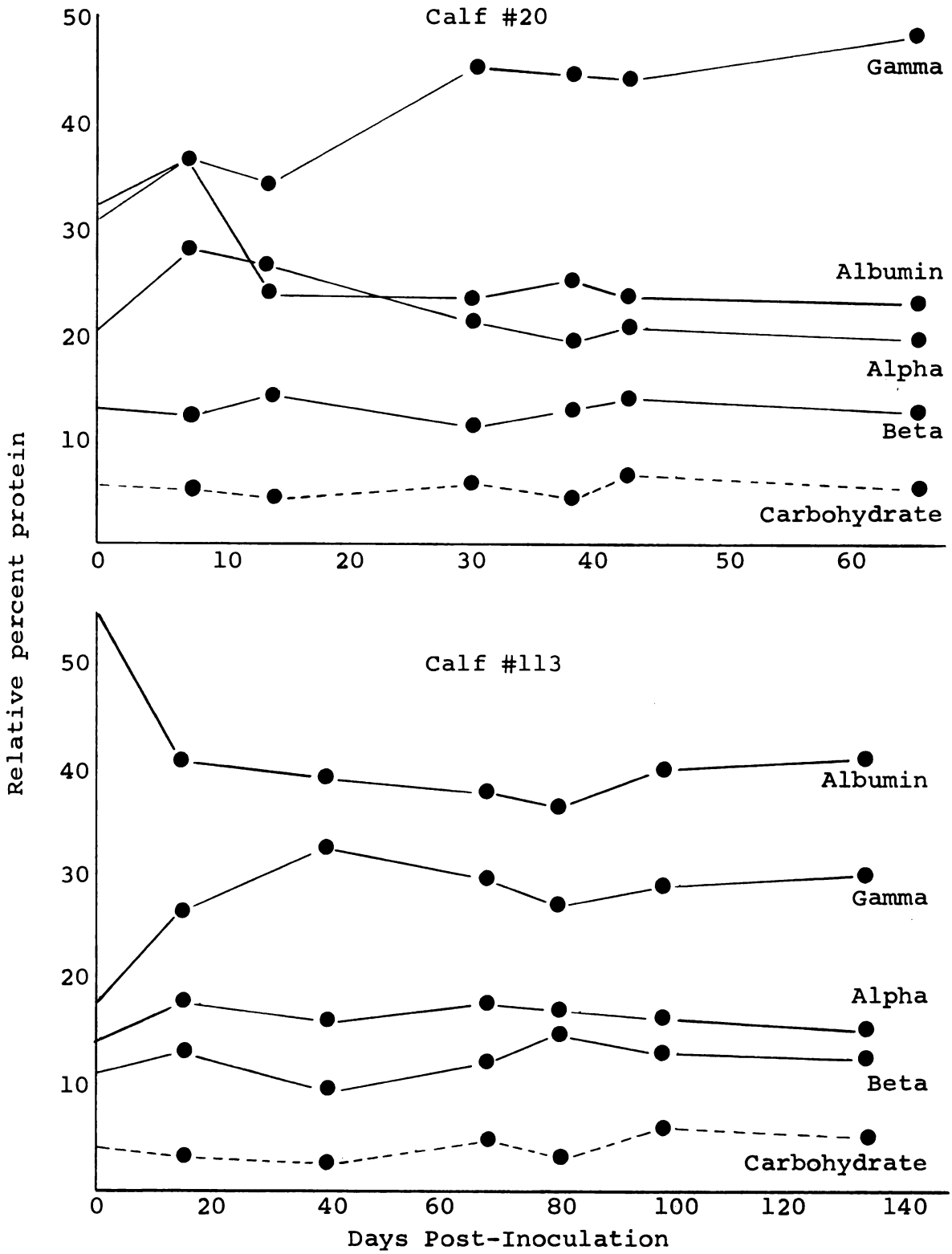

Fig. 27. Alterations in serum protein and protein-bound carbohydrate patterns in calves experimentally infected with M. bovis.

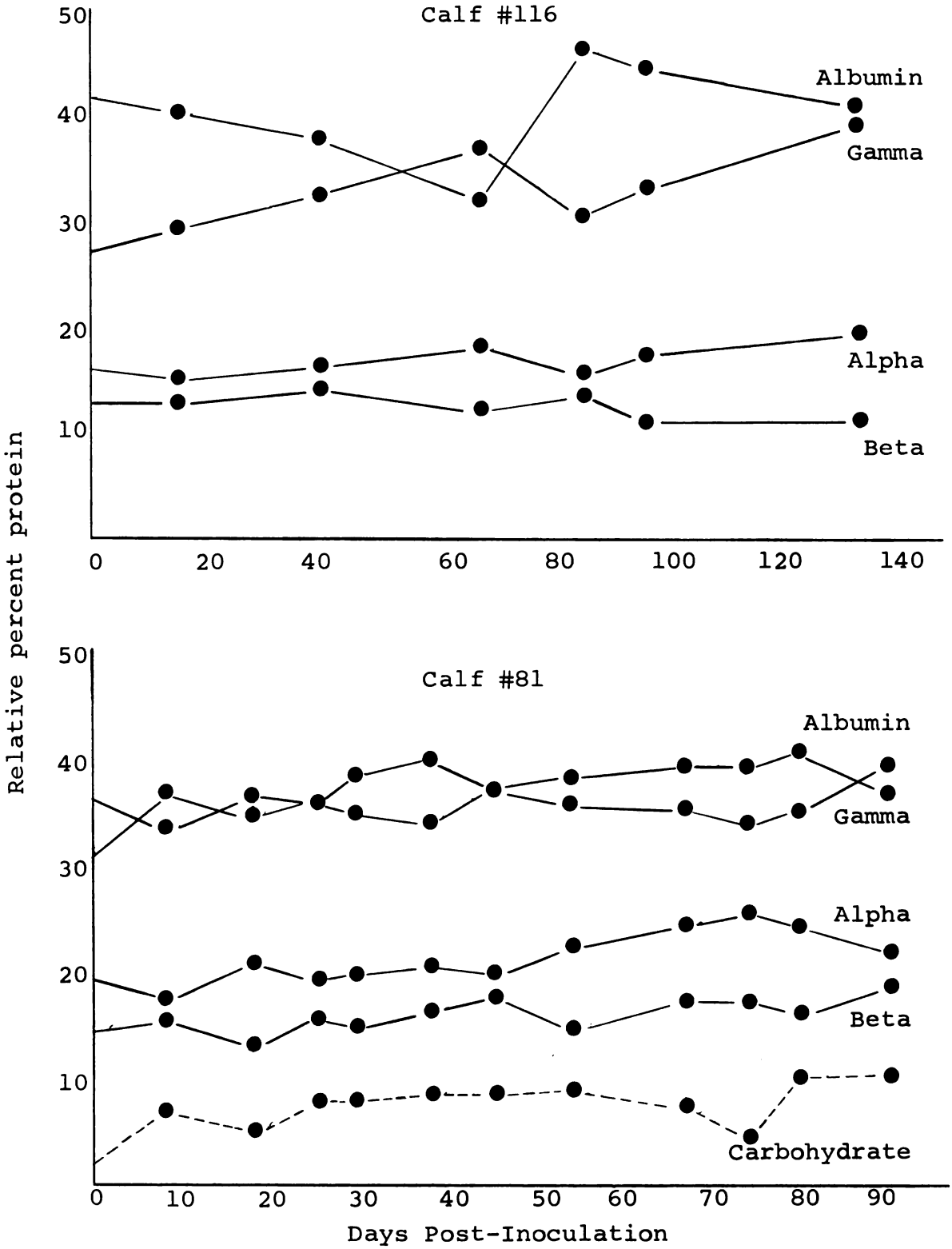

Fig. 27. Continued.

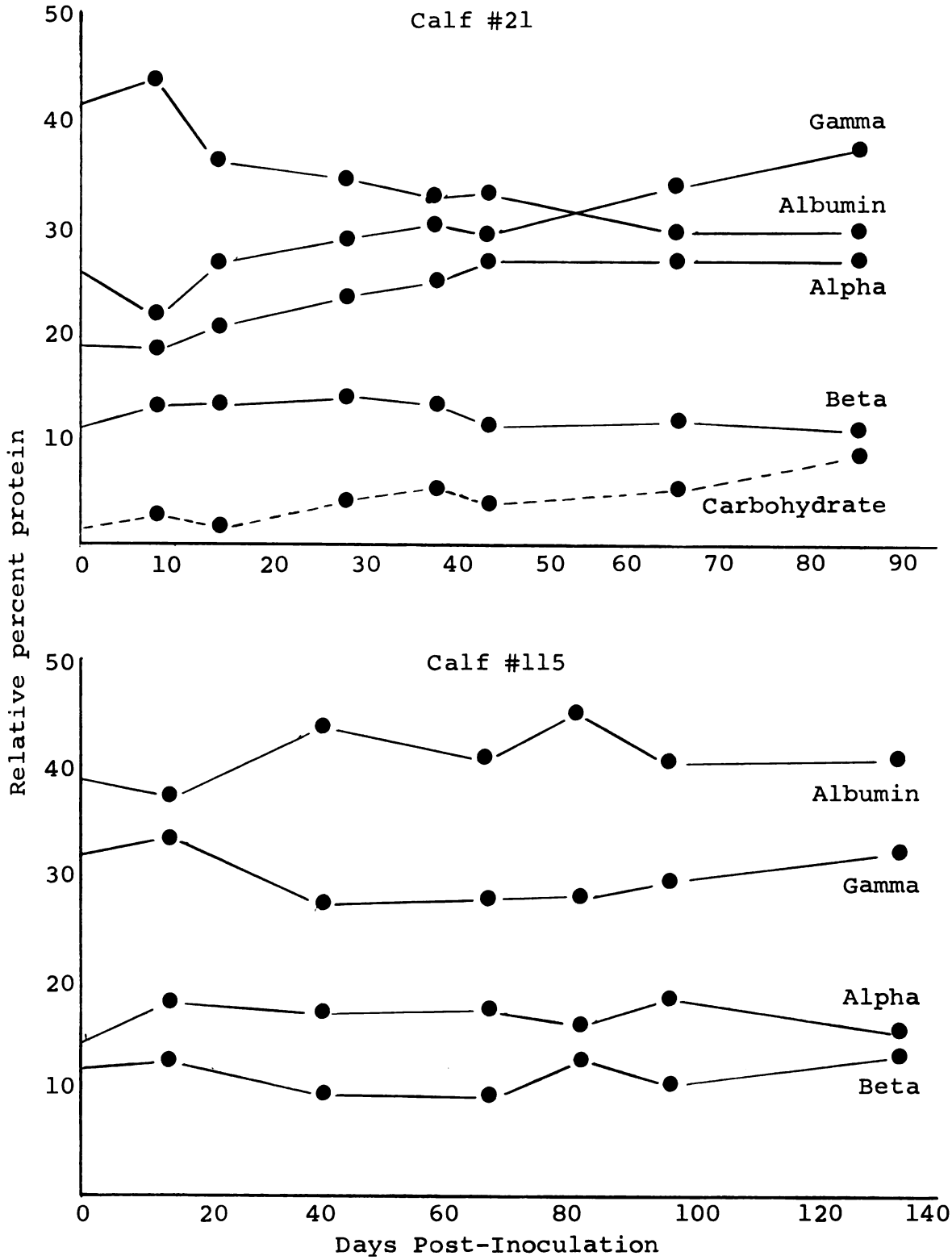

Fig. 28. Alterations in serum protein and protein bound carbohydrate patterns in calves experimentally infected with M. avium.

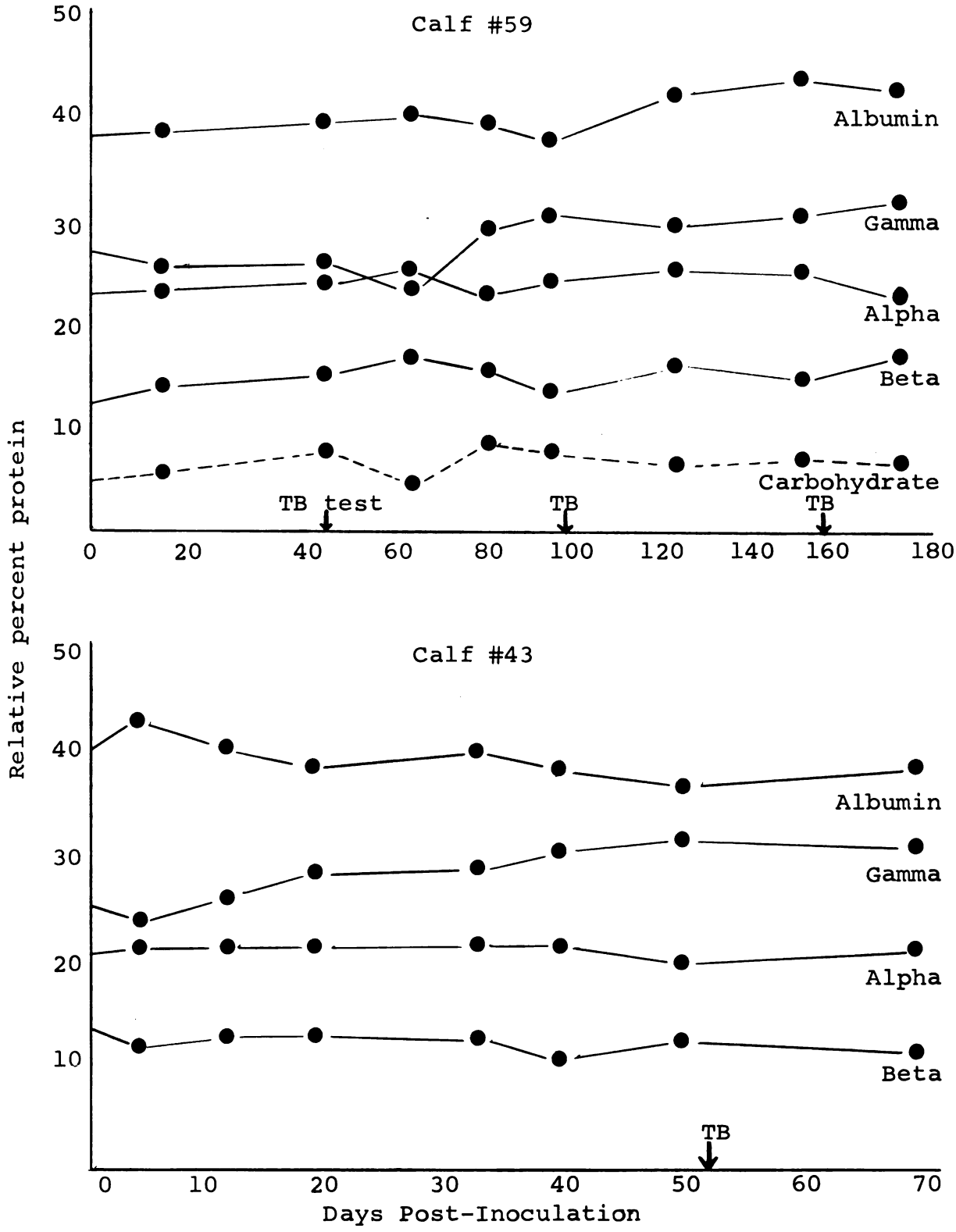

Fig. 29. Alterations in serum protein and protein-bound carbohydrate patterns in calves experimentally infected with strain 51C (virulent Group III).

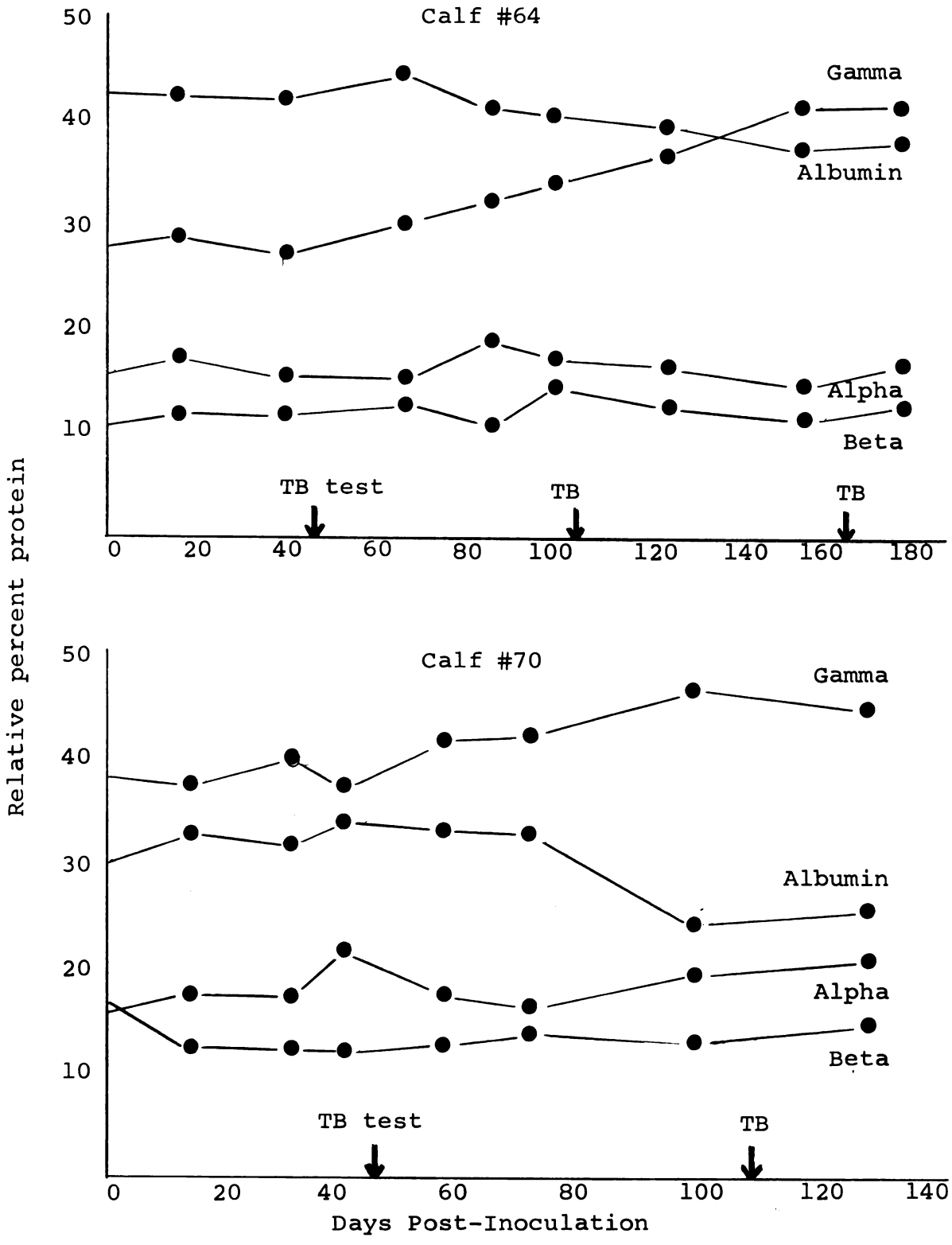

Fig. 30. Alterations in serum protein patterns in calves experimentally infected with strain 68C (virulent Group III).

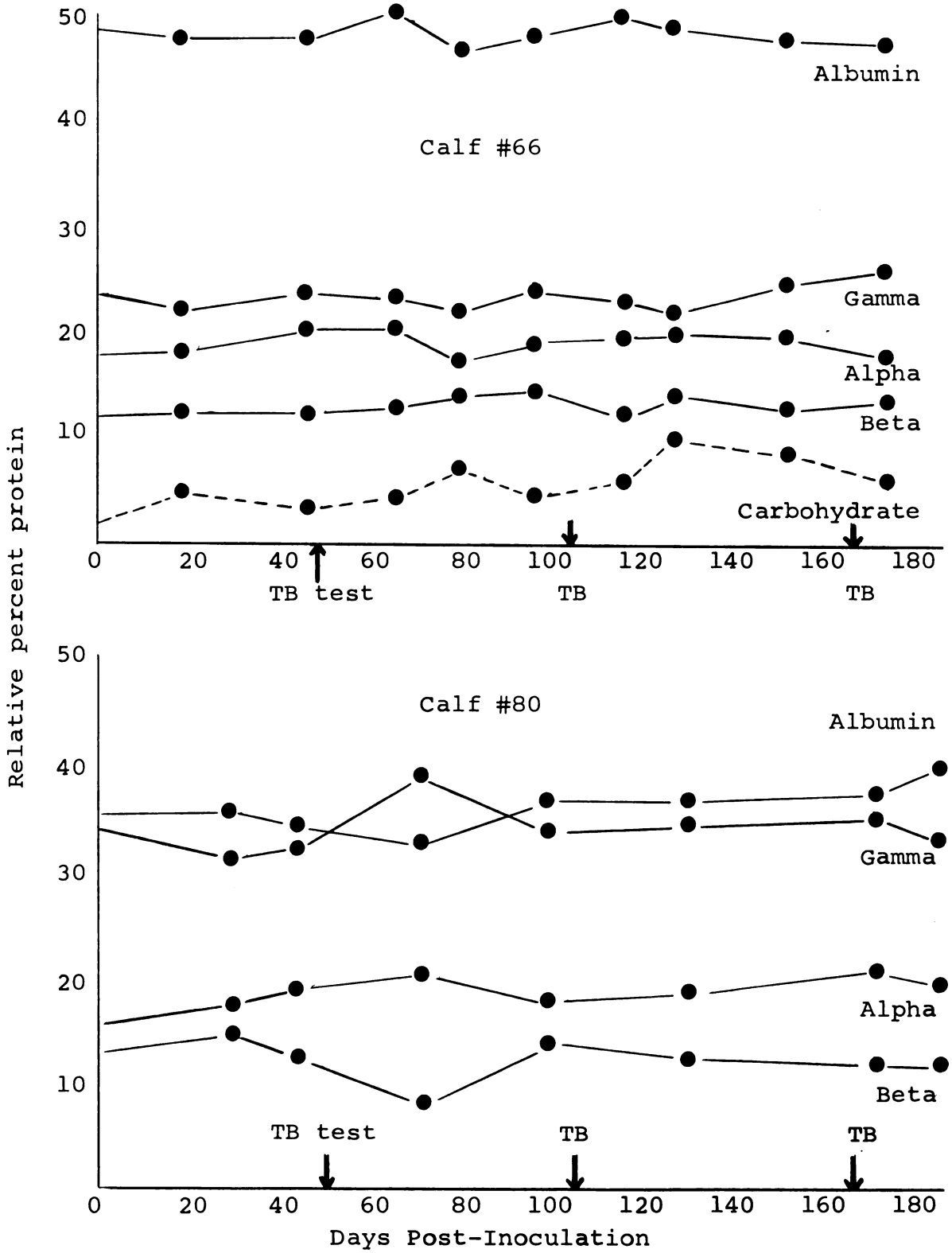

Fig. 31. Alterations in serum protein and protein-bound carbohydrate patterns in calves experimentally infected with 50B (avirulent Group III).

Table 4. Serum protein changes, hemaagglutination titers (HA) and pathology of calves inoculated with mycobacteria

Calf	Pathology[1]			HA[2]		Pre and Post Inoculations[3]				
						A/G	Albumin	Globulins		
No.	Days	G	P	Pr	Po			Alpha	Beta	Gamma
M. bovis										
6	37	+	+	-	40	1.1/0.8	35/33	18.1/16	15/10.3	32/41.3
20	65	+	+	16	64	1.0/0.6	33.3/25	20/19.3	13/12.4	34/43.5
113	132	PC		40	320	3.2/1.3	56.3/40.4	15.3/16.3	11.3/13	17.1/30.1
116	132	+		80	640	1.5/1.0	42/36	16.3/17.5	14/12	28/35.3
135	64	PC	-	20	40	1.7/1.3	39/38	22.4/18.3	16/15.1	23.3/29
128	75	PC	+	-	20	1.3/1.4	39/39.5	20/20	11.5/13	30/29
81	88	-	-	ND	ND	1.0/1.1	33/37	21/19	14/12	33.2/33

[1]Days=days post inoculation final serum sample collected prior to autopsy. G=generalized lesions anywhere beyond the primary complex; PC: primary complex= lesions in the lymph node draining inoculation site; -=no gross or microscope lesions or only lesion at site of inoculation. P=progressive disease: +=generalized disease; -=encapsulation of lesion if present.

[2]Pre and post tuberculin hemagglutination titers of samples collected prior to autopsy: Pr=serum collected before injection of tuberculin; Po=serum collected 7-14 days post tuberculin testing. ND=not done.

[3]Pre-inoculation sample (normal)=numerator; post-inoculation sample collected prior to autopsy=denominator.

*Adult cows.

Table 4--Continued

Calf No.	Pathology[1] Days	G	P	HA[2] Pr	HA[2] Po	Pre and Post Inoculations[3] A/G	Albumin	Globulins Alpha	Globulins Beta	Globulins Gamma
68C (III)										
64	173	PC	-	40	160	1.5/0.9	43.2/37	16/14	12.3/10	29/40
70	122	+	+	80	160	0.8/0.6	30/25	16/21.1	17.3/11.1	37/43.2
71	176	+	+	640	1280	1.0/0.7	32.4/27.5	20.3/17	16/14	32/42
86	76	+	+	40	160	0.9/0.9	33/31.4	17.7/19.6	12.2/14.5	37.1/34.5
51C (III)										
59	173	+	-	40	320	1.8/1.4	39.1/41	21/19	19/12	21.3/28.4
60	112	+	-	40	320	1.4/1.6	37.2/40	18.3/18.4	17.1/17	27.4/25.3
43	68	+	-	160	160	1.7/1.3	41/39	20.2/21.1	14.8/11.4	24.2/29
77	176	+	+	160	160	0.8/1.0	30.3/35.1	20/18	14.5/11.5	36/36
84	45	+	+	20	-	1.4/0.9	39/32.4	18.7/21.6	13.6/11.5	28.8/34.5
91	44	+	+	20	20	1.6/1.0	42.4/32.1	17/22	14/15	27.2/31
2		+	+	-	80	1.6/1.2	40/37	22/19.2	13.3/13	25/32
108*	96	PC	-	20	40	1.4/1.4	38/38	19/20	17/15	26.4/28

Table 4--Continued

Calf No.	Pathology[1] Days	G	P	Pre and Post Inoculations[3] HA[2] Pr	Pò	A/G	Albumin	Globulins Alpha	Beta	Gamma
107*	201	+	+	40	160	1.5/3.0	40/48	16.4/16	17.2/13	27/24
109*	279	+	+	40	80	1.5/1.2	42.4/36	17.4/21	11.1/13	29.2/31
104	96	PC	+	40	320	1.1/0.8	36/30.5	20/20	11.5/11	33.1/39
106	202	PC	-	40	320	0.7/1.0	30/36	15/16	13/12.4	42/36
105	279	PC	-	40	80	1.7/0.9	31.4/33	17.1/16	15/13.3	19/38.2
M.avium 21	84	+	-	-	80	1.7/0.9	43.5/29.3	19.4/26.8	11.8/10.4	25.3/34
118	88	-	-	-	-	1.1/1.2	34/35	20.2/22	15/15	31.5/29
138	64	-	-	-	-	1.4/1.6	38/42	21.3/13.3	15/11.2	26.5/27
114	132	-	-	-	80	1.0/1.0	35.4/34.5	17/19	12.3/13	35.8/33.5
115	132	-	-	-	80	1.2/1.3	37.5/39.2	18/19	12.4/12.4	32.2/30
50B (III) 66	172	-	-	160	320	2.1/1.7	48/45.1	17.2/17	12.3/12	23/26.1
67	84	-	-	160	640	1.2/1.4	33.3/37	22/21	16.4/15.4	28.3/27.2
80	181	-	-	40	160	1.1/1.2	36/38.4	16.3/17.2	14/12	34/32.5
88	188	-	-	-	40	1.2/2.5	36/48	19/19.4	14.3/13.8	31/19.4
89	64	-	-	-	40	1.5/1.4	41.1/36	17.1/25.3	14.9/13.4	26.9/25.3

In calves inoculated with M. bovis, serum albumin was moderately reduced and gamma globulin significantly elevated resulting in a decreasing albumin/gamma globulin (A/G) ratio (Table 4). The alpha and beta globulin remained constant except for minor fluctuations. The hypergammaglobulinemia was detected 7-14 days post-inoculation and remained until death.

When culture 81 was injected intradermally into calf 20, it caused death. The same culture did not produce any lesions in calf 81 when injected subcutaneously and the albumin and globulin fractions remained constant (Fig. 27). No significant differences were found between the normal and post-inoculation serum protein patterns of calf 128 which was injected with culture 310. Only a primary complex developed. However, other calves (113 and 135 inoculated with culture 310 and 278 respectively) developed a primary complex and also had hypergammaglobulinemia.

No consistent differences were detected in the protein distribution between normal serums and serums collected from 4 calves inoculated with M. avium. The A/G ratios were constant or slightly increased.

Calf 21 (inoculated with M. avium) had protein changes similar to calves infected with M. bovis. There was a lesion at the site of inoculation and in the head lymph nodes. In addition to a rise in gamma globulin and decrease in albumin, there was a rise in the alpha globulin fraction

similar to the changes observed in human tuberculosis and experimental tuberculosis in guinea pigs. Protein-bound carbohydrate also increased.

Responses of animals inoculated with Group III mycobacteria varied. Culture 50B did not produce any demonstrable lesions and no consistent changes in serum protein fractions could be detected. The A/G ratio remained constant or slightly increased which was similar to calves inoculated with M. avium.

Culture 68C produced the same pathology and serum protein changes as described for M. bovis injected intradermally.

Culture 51C produced the most inconsistent changes in serum proteins. Two different patterns could be detected: The first type was similar to the serum protein changes from animals inoculated with M. avium and 50B (Fig. 26 and 29: Calf #59). The second type mimicked the serum protein changes demonstrated with animals infected with M. bovis and 68C (Fig. 26 and 29: Calf #43). This organism was pathogenic producing gross lesions but no consistent A/G ratio resulted.

In general, carbohydrate and lipid staining revealed no consistent differences between serums collected from tuberculous calves before and during infection.

In a few cases, the gamma globulin fraction increased after tuberculin testing (Figs. 29, 30, 31). It was not consistent as previously reported by Mallmann et al. (118) (Table 4).

Naturally infected animals. The serum protein changes, pathologic changes, pre-and post-tuberculin HA titers, and bacteriology of naturally infected cows are presented in Table 5.

Generally, serum samples collected from both GL and NGL animals before and after tuberculin testing had higher A/G ratios than tuberculin negative cows. The total serum protein level dropped considerably following the tuberculin test in all GL and NGL animals. Tuberculin testing had little or no effect on the A/G ratios in tuberculin negative animals.

A comparison of the mean relative percent protein values from normal and experimentally and naturally infected cattle are presented in Table 6.

The mean A/G ratio from both GL and NGL (naturally infected group) was slightly higher than calves experimentally infected with M. bovis or 68C but sufficiently lower than the normal mean A/G ratio. This and the fact that the mean A/G ratio from tuberculin negative cows was approximately a normal A/G ratio was indicative that these animals were infected with a virulent mycobacteria.

Table 5. Serum protein changes, hemagglutination titers (HA), pathology, the bacteriology of naturally infected cows

Animal No.	Pathology[1]	Bacty[2]	Pre and Post Tuberculin Samples[3]					
			HA	A/G	Albumin	Globulin		
						Alpha	Beta	Gamma
2495	GL	M. bovis	20/80	0.7/0.8	30.1/39	19.3/12	10/19	41/31
9775	GL	M. bovis	20/80	1.0/0.8	34.4/36	18/17.1	15/17.1	33/30.2
4102	GL	M. bovis	20/40	0.9/0.8	32/27	20/24.4	14.2/13.4	34.2/36
4215	NGL	M. bovis	20/1280	1.3/1.3	39.3/36	18/22	13/15	30.1/28.1
0586	GL	M. bovis	80/640	1.2/1.0	37/33	20.3/24	13.3/10	30/34.2
5049	NGL	M. bovis	40	0.8	30	20.1	11.2	39
9825	GL*	NS	40/80	1.0/0.8	35/36	22/12	9.3/23.2	34.2/29
5220	NGL	M. bovis or III	80/640	0.8/0.7	32.4/28	18.4/16.3	11/14	38.2/42
0018	NGL	NS	40/80	0.9/0.9	33.2/34	17/18.3	11.4/10	39/38

[1]GL=gross lesion; NCL=no gross lesion; SL=skin lesion only; TB neg.=tuberculin negative; GL*=tuberculin negative and gross lesions.

[2]Bacteriology: NS=no sample; NI=no isolation.

[3]Pre tuberculin sample before injection of tuberculin=numerator; Post tuberculin sample=denominator.

Table 5--Continued

Animal No.	Pathology[1]	Bacty[2]	Pre and Post Tuberculin Samples[3]					
						Globulin		
			HA	A/G	Albumin	Alpha	Beta	Gamma
4586	NGL	NS	80/160	0.9/1.0	31/33.3	18/19.5	16/13	36/25
2422	NGL	NI	40/40	0.7/0.7	29/29	19/23	14/10	39/39
6175	NGL	NS	80/80	0.9/0.7	33.5/28.4	17.1/19	13.4/14	36.1/39
4706	NGL	NS	40/160	0.9/1.0	33/33	20/25	12.4/12	35.1/31
1386	NGL	NI	40/320	0.8/0.8	30/30.2	21/17.4	13/14	37/39
8000	NGL	NS	640/320	0.8/1.2	30/37	17.4/17.3	14/16	36.30
4706	SL	NS	40/160	0.9/1.1	33/33	20/25	12.4/12	35.1/30.5
2940	SL	NS	20/160	0.9/0.9	35/33	18/20.1	10/10.4	38/36.5
2365	NGL	NS	160/160	0.9/0.8	36/31.2	12/28	13/10.3	40/39
0062	NGL	NS	160	1.0	35	17.2	13	35.2
2249	NGL	NS	40/160	0.9/0.9	34/32.1	19.4/22	11.5/11	38/36
9926	TB neg.	NS	20/40	1.1/1.2	37/37	16.4/19.4	12.2/12	35/32
2696	TB neg.	NS	40/160	1.1/1.1	34/38	19.3/19	15.2/16.3	32/33.1
0040	TB neg.	NS	80/640	1.4/1.0	40.1/35.2	17.3/18.1	14.3/13	28.3/34
2234	TB neg.	NS	20/80	1.2/0.9	40/33	16/19	11/11.1	34/37.4

Table 6. Mean values of serum protein fractions and variations due to disease activity in bovine tuberculosis

	No. of Cases	A/G	Albumin	Globulins		
				Alpha	Beta	Gamma
Normal calves	38	1.4±0.1	37.3±8.2	19.1±1.8	14.4±1.0	29.7±6.6
Experimentally infected calves						
M. bovis	7	0.8±0.07	30.2±2.5	18.9±1.6	12.2±1.2	38.3±1.0
M. avium	5	1.3	38.8±5.0	16.8±2.4	13.4±1.9	29.3±1.3
Unclassified						
68C	9	0.7	27.5±1.3	19.7±1.0	13.7±2.7	39.7±2.4
51C	14	1.3±0.4	38.2±3.7	20.1±2.0	13.3±1.3	28.8±2.8
50B	9	1.6±0.3	40.9±1.9	19.9±1.2	13.3±0.8	26.1±1.6
Naturally infected calves						
M. bovis[1]	5	1.0±0.2	34.1±3.0	17.4±1.3	12.7±1.0	34.8±2.7
TB neg.[2]	4	1.2±0.2	37.0±1.6	17.6±2.4	13.9±1.9	31.7±1.4
NGL[3]	13	0.9±0.02	32.1±2.6	18.5±1.4	12.8±0.3	37.1±0.9

[1]Tuberculin positive animals which had gross lesions and *M. bovis* isolated.

[2]Tuberculin negative animals.

[3]Tuberculin positive but no gross lesions (NGL) found. *M. bovis* isolated from 3 cases (see Table 5).

Starch-gel Electrophoresis of Serum from Tuberculous Cows

Starch-gel electrophoresis of serums collected from calves inoculated with M. bovis or 68C revealed, in addition to an increase in gamma globulin and decrease in albumin, an increase in post albumin fraction 9 (Fig. 13) 7-14 days post-inoculation and remained elevated during the disease period (Figs. 32 and 33). This band was always elevated 7-28 days post-inoculation in serum from calves inoculated with 51C regardless of whether an increase in gamma and decrease in albumin occurred (Fig. 34). It was not elevated in serums from calves inoculated with M. avium or 50B (Fig. 35).

The fraction has been tentatively designated as the a_2-V (virulence) fraction. The increase in the a_2-V fraction is clearly illustrated in Fig. 36. It was also elevated in all serums from GL and most serums from NGL cows selected from naturally infected herds and not evident in serum from tuberculin negative cows.

No other consistent changes could be observed in other bands separated by starch-gel electrophoresis.

Differential Disc Electrophoresis of Serum from Tuberculous Cows

It was impossible to obtain reproducible densitometric analysis of the differential columns with the chromoscan. Therefore, only visual qualitative evaluation of the numerous fractions could be made.

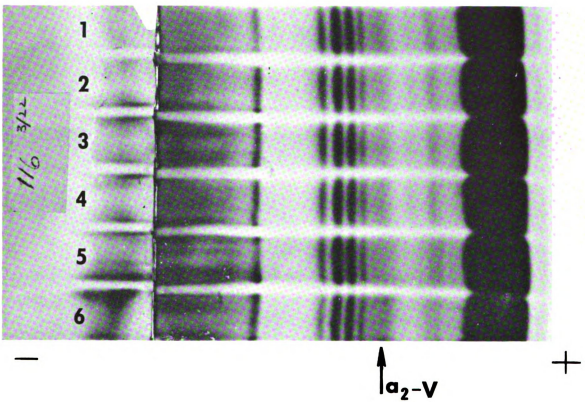

Fig. 32. Typical starch-gel electrophorograms of serums from experimental calves inoculated with M. bovis.

(1) pre-inoculation serum (normal)
(2) 12 days post-inoculation serum
(3) 39 days post-inoculation serum
(4) 82 days post-inoculation serum
(5) 98 days post-inoculation serum
(6) 132 days post-inoculation serum.

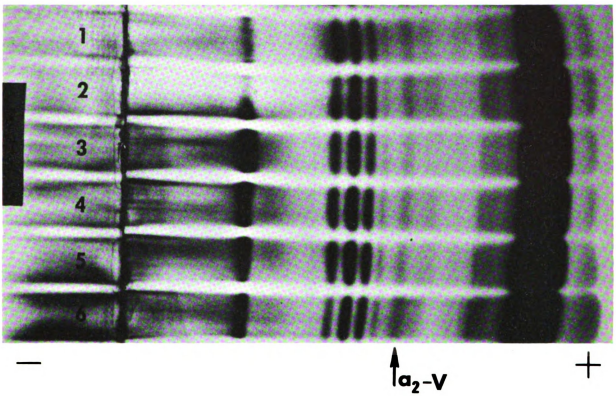

Fig. 33. Typical starch-gel electrophorograms of serums from experimental calves inoculated with 68C (virulent Group III).

(1) pre-inoculation serum (normal)
(2) 14 days post-inoculation serum
(3) 39 days post-inoculation serum
(4) 82 days post-inoculation serum
(5) 124 days post-inoculation serum
(6) 173 days post-inoculation serum.

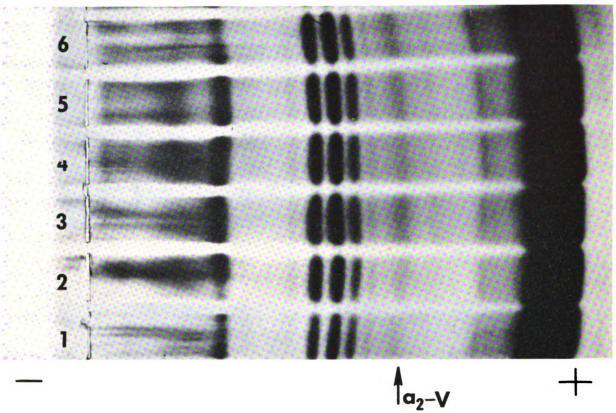

Fig. 34. Typical starch-gel electrophorograms of serums from experimental calves inoculated with 51C (virulent Group III).

(1) pre-inoculation serum (normal)
(2) 14 days post-inoculation serum
(3) 45 days post-inoculation serum
(4) 82 days post-inoculation serum
(5) 124 days post-inoculation serum
(6) 173 days post-inoculation serum.

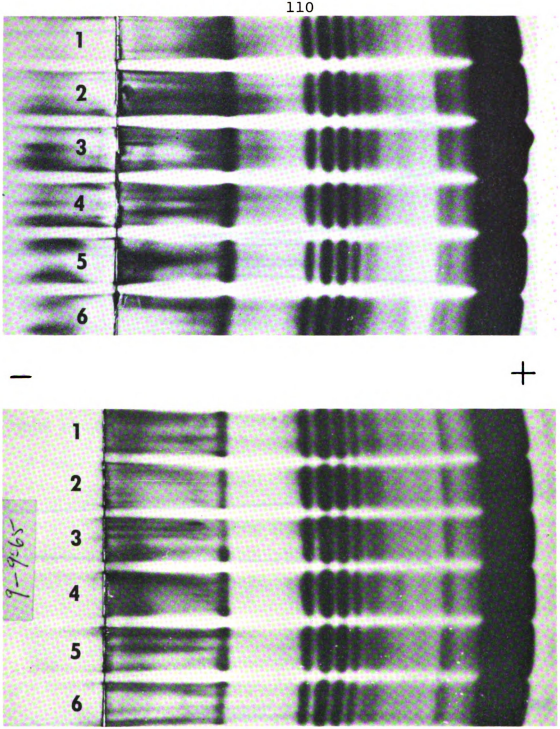

Fig. 35. Typical starch-gel electrophorograms of serum from experimental calves inoculated with M. avium (top) or (below) 50B (avirulent Group III).

(1) pre-inoculation serum (normal)
(2) 14 days post-inoculation serum
(3) 45 days post-inoculation serum
(4) 82 days post-inoculation serum
(5) 98 days post-inoculation serum
(6) 132 days post-inoculation serum.

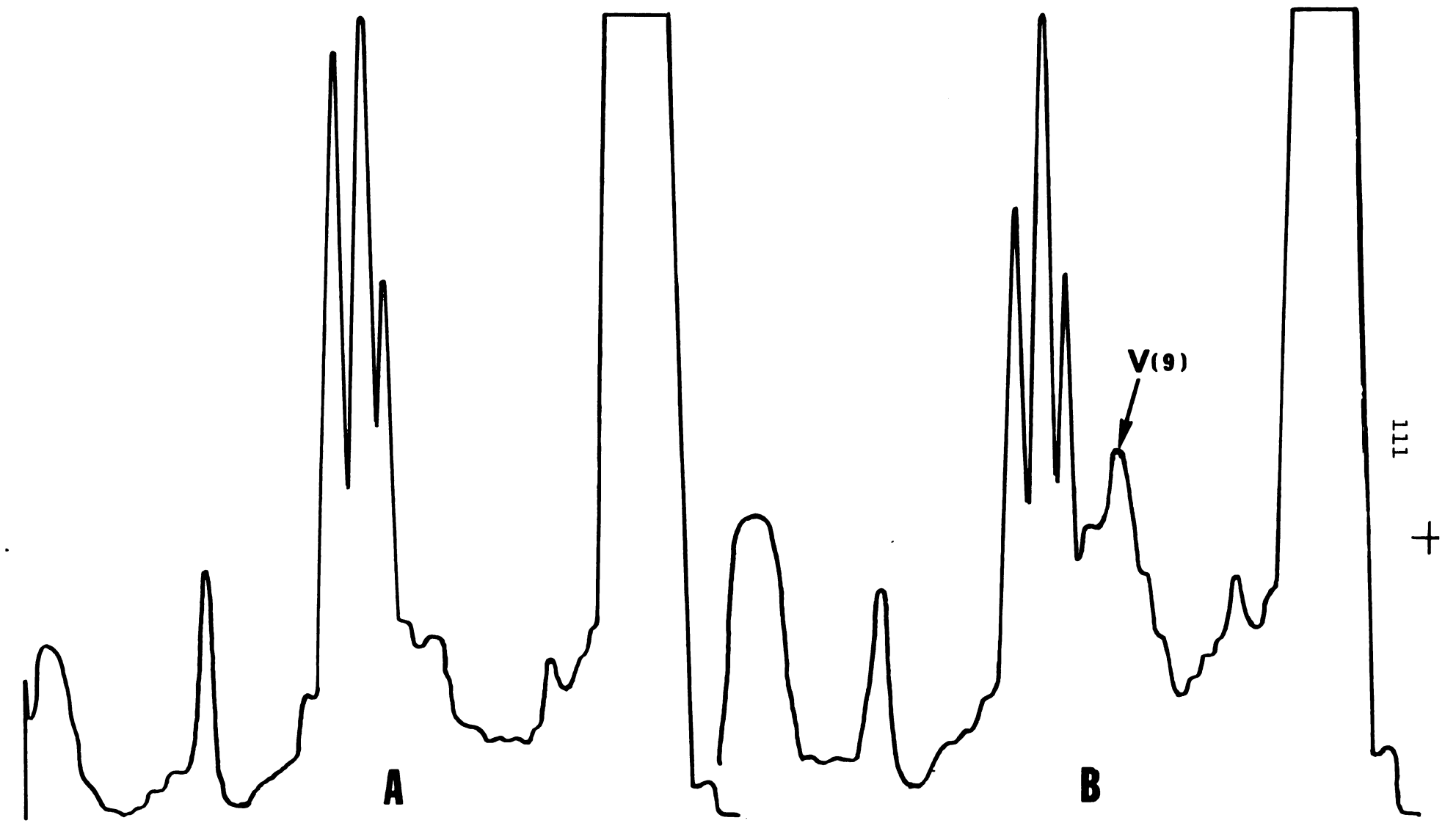

Fig. 36. Typical densitometeric recordings of starch gel electrophorograms of (A) normal bovine serum and (B) post-inoculation serum from experimental cattle inoculated with M. bovis, 68C or 51C, and gross-lesion and no gross lesion cows from naturally infected herds.

The hypergammaglobulinemia and decrease in albumin demonstrated in serums from calves infected with M. bovis or 68C by cellulose acetate and starch-gel electrophoresis was also detected by this method. There were in addition to the increase in gamma and decrease in albumin, increase in the Sa_2 band (see Fig. 21), increase in the first post albumin band and a marked increase in a thick zone just posterior to the beta zone (Figs. 37 and 38). The latter may correspond to the a_2-V fraction detected in starch-gel electrophoresis. These bands also increased in post-inoculation serum from calves experimentally infected with 51C but did not increase in serum from calves inoculated with M. avium or 50B (Fig. 38).

These bands also increased in pre-tuberculin serum samples from GL and NGL (naturally infected) animals but not in pre or post-tuberculin samples from tuberculin negative cows. Post-tuberculin samples from GL and NGL animals could not be evaluated qualitatively due to a marked decrease in total protein (Fig. 39). A decrease in total protein did not occur in post-tuberculin serum samples from tuberculin negative cows.

Of the ten bands which stained for carbohydrate, only the Sa_2 and the a_2-V band were increased in serums from the M. bovis, 51C and 68C infected calves. These bands were not increased in serum from calves inoculated with 50B and M. avium.

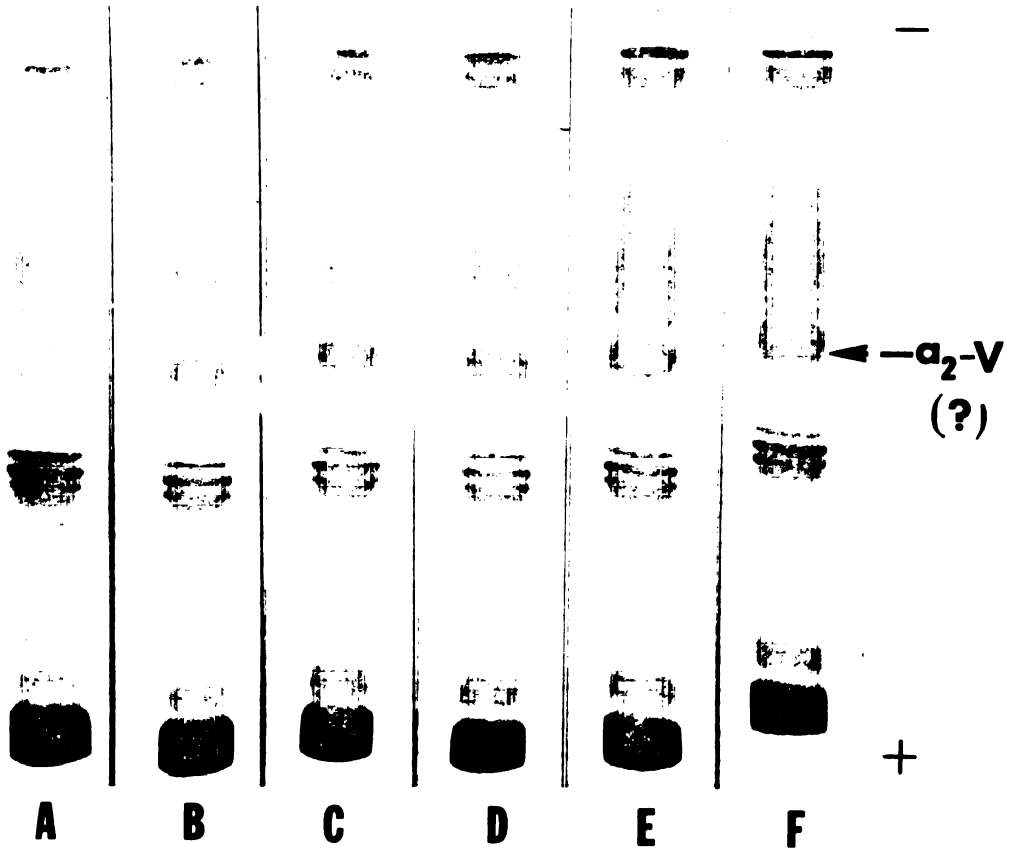

Fig. 37. Differential disc electrophorograms of pre- and post-inoculation serums from experimental calves inoculated with M. bovis, 68C or 51C (virulent Group III).

(A) pre-inoculation serum (normal)
(B) 14 days post-inoculation serum
(C) 39 days post-inoculation serum
(D) 82 days post-inoculation serum
(E) 98 days post-inoculation serum
(F) 132 days post-inoculation serum.

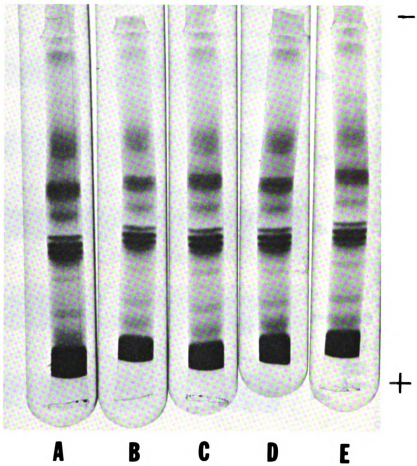

Fig. 38. Typical differential disc electrophorograms of pre- and post-inoculation serums from experimental calves inoculated with M. avium or 50B (avirulent Group III).

(A) pre-inoculation serum (normal)
(B) 14 days post-inoculation serum
(C) 45 days post-inoculation serum
(D) 82 days post-inoculation serum
(E) 132 days post-inoculation serum.

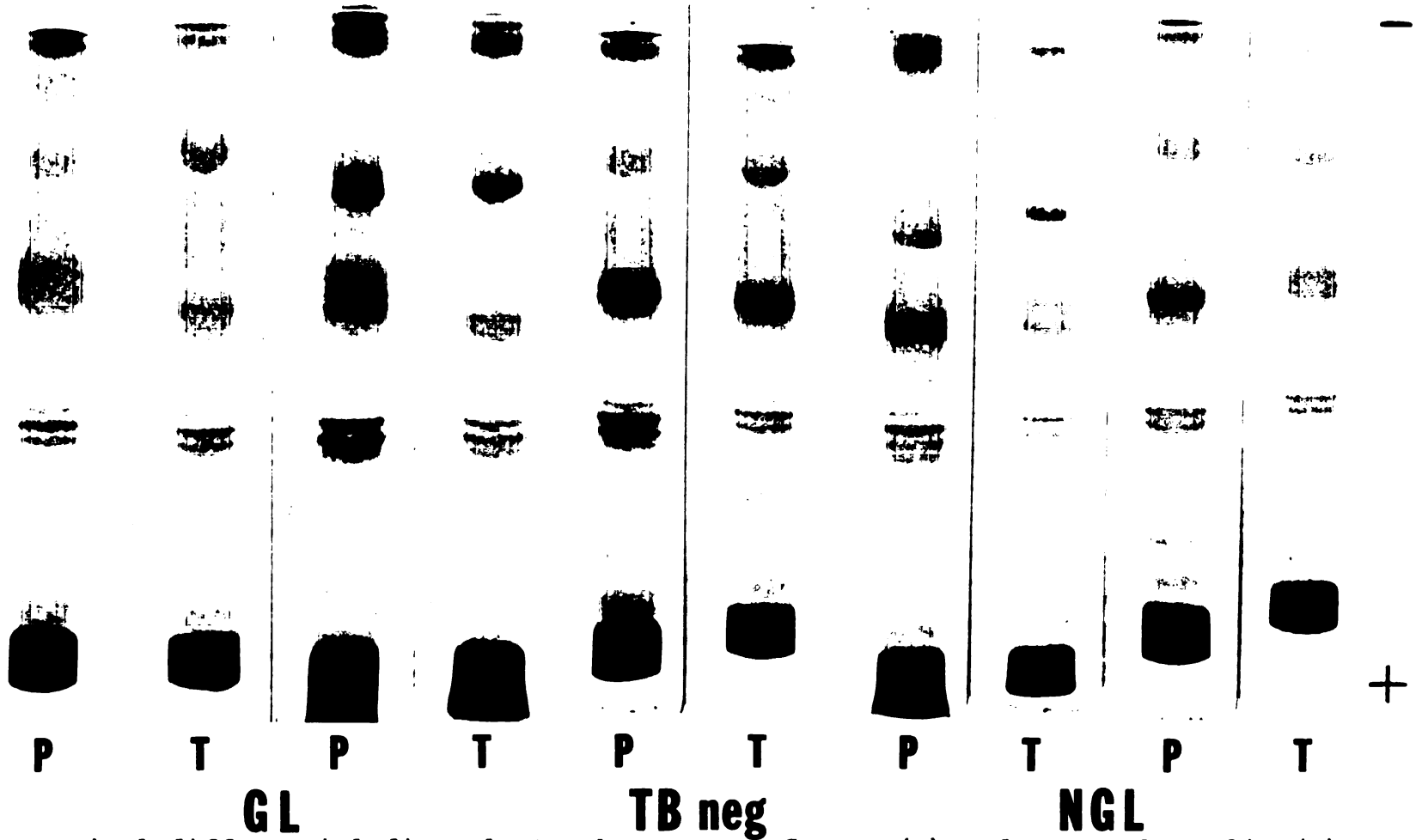

Fig. 39. Typical differential disc electrophorograms of pre- (P) and post-tuberculin (T) serums from tuberculin negative (TB neg.) cattle, and from tuberculin positive gross-lesion (GL) and no-gross-lesion (NGL) cattle from naturally infected herds. Note the decrease in all protein bands in the (T) samples from both GL and NGL animals but not in the (T) serum from TB neg. animals.

The analyses for similar changes in serums from cows natually infected with other chronic disorders are presented in Figure 40. Paratuberculosis, brucellosis, and leptospirosis caused serum protein patterns similar to both the terminal serum protein patterns of a calf experimentally infected with M. bovis. Serums from cattle with paratuberculosis had denser gamma globulin zones than the serums from tuberculous animals. A band just prior to the major beta zone (see Fig. 40) was markedly greater from a cow with brucellosis than in serums from normal and tuberculous cattle. The serum from a cow with antinomycosis was similar to a normal serum.

Immunoelectrophoresis of Serums from Tuberculous Cows

Generally a_2-2, a_2-3, a_2-4, a_2-5, a_2-6, a_2-7, and a_2-8 precipitates were slightly increased 7-14 days post-inoculation and remained constant until death. In serums from animals inoculated with M. bovis, 68C or 51C the a_2-1 arc became markedly increased 28 days post-inoculation and increased in density until death (Figs. 41, 42, and 43). The immunoprecipitate was stained with protein and carbohydrate specific stains. All evidence indicated that this immunoprecipitate is identical with the a_2-V fraction separated by starch-gel electrophoresis. An increase in the gamma globulin precipitate also developed simultaneously

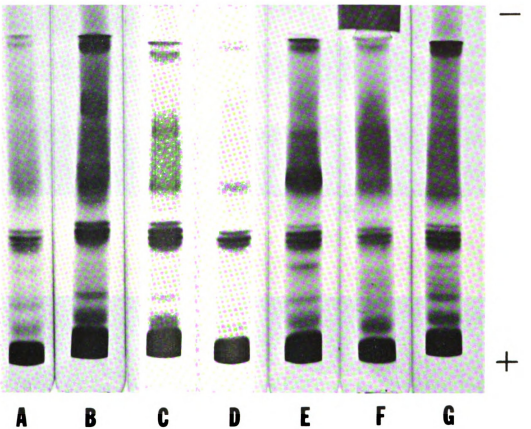

Fig. 40. Comparison of differential disc electrophorograms of normal bovine serum and serum from tuberculous cattle with serums from other chronic inflamatory diseases of cattle.

(A) actinomycosis pattern
(B) paratuberculosis pattern (Johne's disease)
(C) naturally infected tuberculosis pattern (M. bovis)
(D) normal bovine serum pattern
(E) brucellosis pattern
(F) experimental tuberculosis pattern (M. bovis)
(G) leptospirosis pattern.

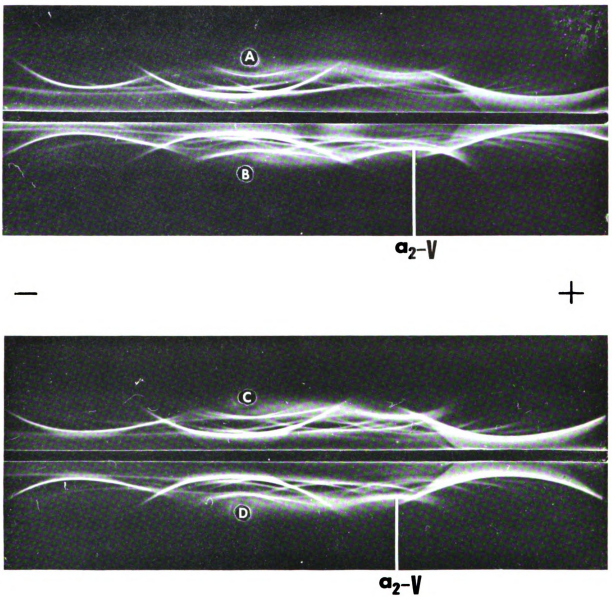

Fig. 41. Immunoelectrophorograms of pre- and post-inoculation serums from calves with M. bovis. (A) pre-inoculation serum (normal), (B) 14 days post-inoculation serum, (C) 82 days post-inoculation serum, (D) 132 days post-inoculation serum.

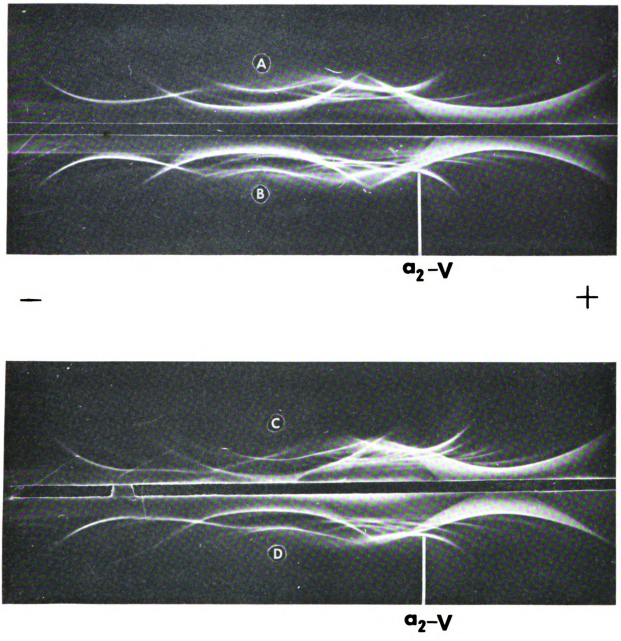

Fig. 42. Immunoelectrophorograms of pre- and post-inoculation serums from calves with 68C (virulent Group III). (A) pre-inoculation serum (normal), (B) 14 days post-inoculation serum, (C) 82 days post-inoculation serum, (D) 173 days post-inoculation serum.

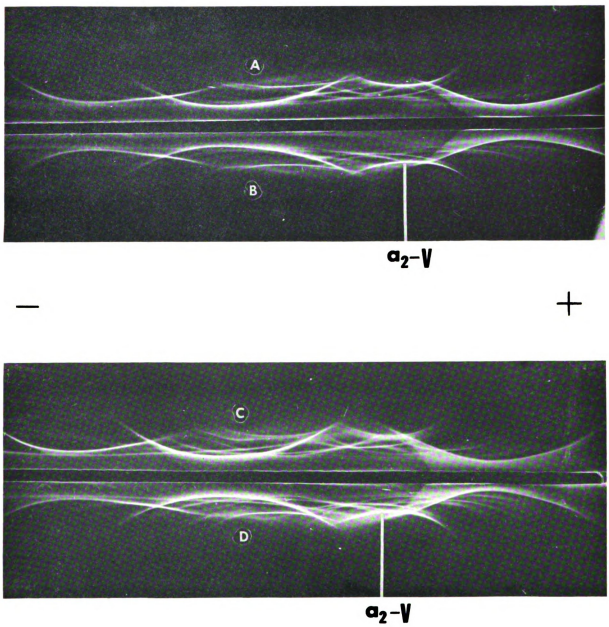

Fig. 43. Immunoelectrophorograms of pre- and post-inoculation serums from calves with 51C (virulent Group III). (A) pre-inoculation serum (normal), (B) 14 days post-inoculation serum, (C) 82 days post-inoculation serum, (D) 173 days post-inoculation serum.

with the increase in the a_2-1 arc. An increase in the gamma or decrease in albumin was often difficult to determine quantitatively.

The a_2-13 lipoprotein precipitate decreased slightly during the disease process in serums from the above group of animals. No changes were detected in any of the carbohydrate staining arcs other than the increase in the a_2-1 precipitate.

None of these changes were detected in serums from calves inoculated with M. avium or 50B (Figs. 44 and 45) or tuberculin negative cows.

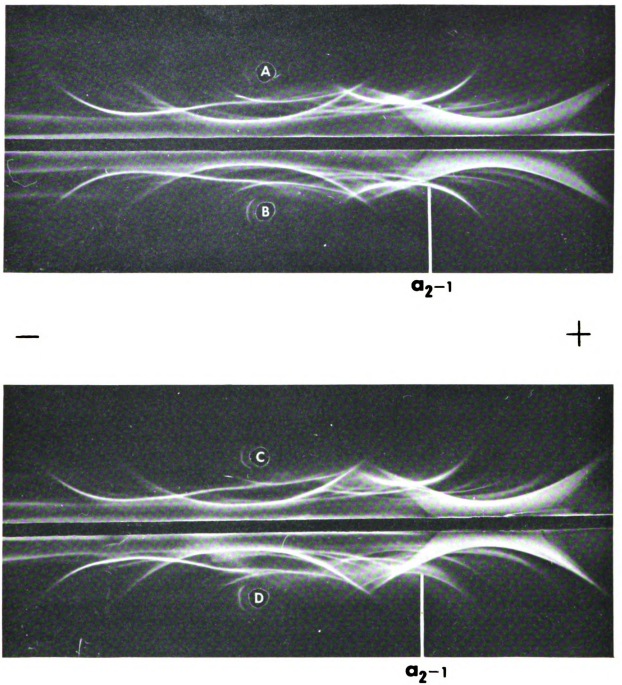

Fig. 44. Immunoelectrophorograms of pre- and post-inoculation serums from calves with M. avium. (A) pre-inoculation serum (normal), (B) 14 days post-inoculation serum, (C) 82 days post-inoculation serum, (D) 132 days post inoculation serum.

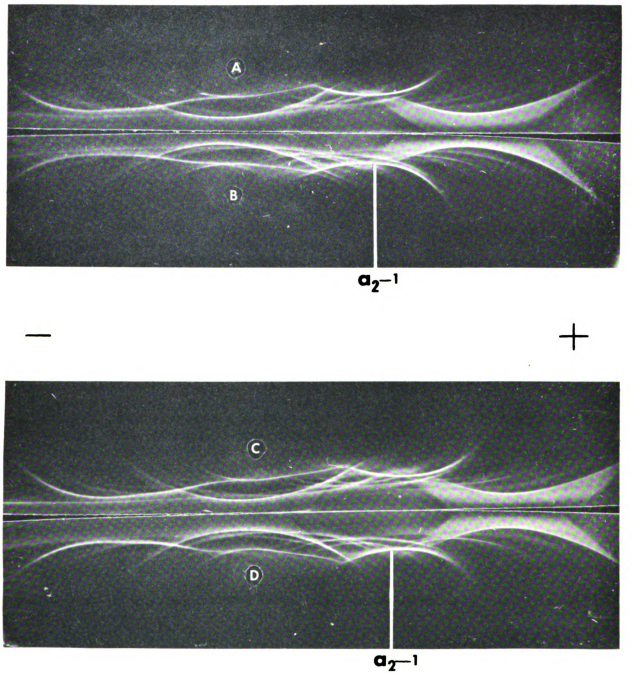

Fig. 45. Immunoelectrophorograms of pre- and post-inoculation serums from calves with 50B (avirulent Group III). (A) pre-inoculation serum (normal), (B) 14 days post-inoculation serum, (C) 82 days post-inoculation serum, (D) 132 days post-inoculation serum.

DISCUSSION

Development of Techniques and Analyses of Normal Bovine Serum Proteins

Relatively few studies have been concerned with the characterization of bovine serum proteins. A more complete knowledge of the chemical, physiochemical, and immunochemical properties of the serum proteins is a prerequisite to an understanding of their normal physiologic functions and changes induced during pathologic processes.

The electrophoretic composition of bovine serum characterized by cellulose acetate electrophoresis was essentially the same as reported from studies employing a single procedure (18, 19, 26, 27, 47, 93, 138, 159). The resolutions of the protein bands were markedly better than that obtained with paper electrophoresis.

The selection of the appropriate buffer system to be used with blood serum from any animal species is empirical because of the differences in composition and electrophoretic properties of the protein constituents. For example, the alpha component in the serums from human, swine, horse, dog, and mouse separated into two components ($alpha_1$ and $alpha_2$) after cellulose acetate electrophoresis with Owen buffer

system. The alpha component in bovine serum did not separate under the same conditions but was separated into two fractions with a tris-EDTA-borate system. The barbital-acetate system was employed because it gave consistent results.

A buffer system that provides ideal separation of the serum constituents from one species is not necessarily satisfactory for serums from other species. Although a system may appear to give ideal separation and resolution, the results may not be consistently reproducible as with the electrophoretic separation of bovine serum. Therefore, a complete analyses of buffer systems (pH, species of salts, ionicity) for both separation and reproducibility is required. It is also of paramount importance to consider the different fractions of proteins which occur when different apparatuses, buffer solutions, etc. are used. Establishing normal conditions for each investigation is essential.

Because of the simplicity of separation, lack of protein trailing, and clear background; cellulose acetate electrophorograms were the only medium from which reliable relative percent protein values could be calculated. However, errors in measuring protein by measuring bound dye are inevitable especially since the binding capacity of many proteins is not known. There is a wide variation among proteins in lipid, carbohydrate, and nitrogen content, within these limitations, dye-binding is a convenient parameter to measure.

Simple agar gel electrophoresis did not improve the separation or resolution of bovine serums that was obtained by cellulose acetate electrophoresis. Agar electrophorograms were unsuitable for densitometric analysis due to considerable protein trailing and electroosmosis. Deionized agar might decrease one or both of these difficulties. The large pore size of agar may contribute to a poorer separation and resolution of serum proteins than that in starch or acrylamide gels. The use of a more highly purified agar would permit a longer period of electrophoresis by reducing the amount of electroosmosis. A better separation of the globulin zones could be accomplished. This agar can also be prepared directly in the buffer and used immediately, eliminating the necessity of further purification.

The apparatus for starch-gel electrophoresis, and the electrophoretic destaining of starch and polyacrylamide gels was developed during this study. Most of the commercial apparatus and techniques for starch-gel electrophoresis had several inconvenient features: (a) excessive handling and exposure to harsh solvents cause an injury to the gels; (b) a separate gel mold was required to change dimensions of the gel; (c) the gel slicing operation was tedious when using either a dermatome knife (179) or fine wire (23); (d) the methods for staining and destaining the gels were cumbersome and time consuming; and (e) destaining by electrophoresis often resulted in soft and friable gels.

The apparatus and techniques for starch-gel electrophoresis was modified and improved.

The gel mold, which was similar to Smithies (180) incorporates two spacers (Fig. 1) to facilitate changing the depth and width of the gel. In this manner, only one gel frame was required, all changes being made with the appropriate spacers. The cover and removable slot formers were identical to Smithies (180) but the slot formers were made to correspond with the width and depth of the spacers. The volume of sample could be adjusted by varying the length and thickness of the slot formers. The gel mold eliminated the necessity of having a separate mold for each size gel, making it usable for a variety of experiments such as two-dimensional electrophoresis.

Using acrylamide gel with this apparatus was moderately successful. The synthetic gels are flexible and were supported by a sponge in the lower buffer compartment and saran wrap on the surface of the gel prior to placing the retainer plate in place. Preliminary studies indicated little advantage in using acrylamide gel slabs for analyses of bovine serum.

The slicing operation was improved by slicing with a fine wire. This provided smooth and nearly uniform sections. Excellent results were obtained because the gel was firmly supported during the slicing operation. Furthermore, less dexterity was required on the part of the operator.

The improved procedure for staining and destaining starch and acrylamide gels saved considerable time and lessened the chance of breakage. In previous procedures the sliced gel was placed directly into a staining tray or supported by a glass plate or heavy filter paper and the stain poured over the gel. After staining was completed, the stain was removed and the solvent which usually consisted of a mixture of methanol and acetic acid was added. Prolonged contact with methanol caused the gel to become very brittle and handling usually resulted in a broken gel. Destaining by electrophoresis (56, 78) has eliminated the use of methanol and decreased the time required to destain a gel.

In the procedure developed, the gel is supported between two framed screens (gel holder), which is placed into the staining tank (Figs. 5, 6). The destainer (Fig. 7) was designed and coupled conveniently with the staining tank which eliminated any handling of the gel during staining and destaining. The addition of 10% glycerol to the acetic acid solution for destaining starch slabs, causes a slight dehydration of the gel which results in a more resilient, easily handled flexible gel. The gel becomes semi-transparent from which densitometric recordings can be made.

The major difference between the results of one dimensional starch-gel and those reported by Ashton (6), was in the number of components in the post albumin fractions.

Nine bands were detected. Only one post albumin band was reported by Ashton (6). This difference in number of post albumin bands is largely due to different buffer systems employed in the two investigations. Ashton used a phosphate buffer system (pH 7.8). A discontinuous buffer system (10) was employed in this study. Ashton (10) had used a discontinuous buffer system for starch-gel electrophoresis of mouse serum proteins, not bovine serum.

By two-dimensional analyses, Ashton detected 26 fractions in normal bovine serum: 13 alpha globulins, 8 beta globulins, one gamma globulin zone, one albumin band and two pre albumin bands. This represents two more alpha globulins and one additional pre albumin band than was detected in the present investigation. The buffer systems differed and the breed of cow was not indicated.

Calculation of relative percent protein values from starch-gel electrophorograms was unsatisfactory. If the baseline were adjusted too high for some gels (10 counts per inch), the sensitivity was too low and an incomplete scan resulted. If the baseline were too low, the sensitivity was too great. Surface defects and unevenness in the sample were represented as protein fractions. Once the baseline was established for a given sample, repeated scans of the same electrophorogram were identical. Therefore, if the baseline was not constant from gel to gel then the relative percent protein value of a band that apparently remains

constant may appear considerably increased. This could be partly controlled if a uniform thick gel could be formed and destaining conditions were standardized.

Besides some physical errors, such as drift of the densitometer, the chromoscan was very sensitive. In starch-gel media the instrument was capable of indicating each fraction that could be seen visibly. Therefore, it was useful for detecting any changes in fine bands (post albumins) separated in starch-gel. The instrument was unsuitable for rapid scanning of polyacrylamide columns. After tedious manipulations and scanning the column repeatedly, all bands separated by the differential disc electrophoretic procedure could be detected. However, a densitometer especially designed for scanning these gels, such as the model E microdensitometer (Canalco) would perhaps have given satisfactory scans of these columns.

The conventional disc electrophoretic procedure (132) was found unsatisfactory for the separation of bovine serum. The pore size of the separating gel was too small for the adequate separation of serum proteins. The simplicity with which the pore size of polyacrylamide gels can be varied (82, 116, 128, 129, 177) is a distinct advantage over starch-gels by which differential disc electrophoresis was possible.

Since human serum has been extensively employed in establishing separating conditions of most, if not all fractionation procedures, it served as a control in this study.

The separation of human serum by the standard procedure yields a large number of fine bands closely spaced that migrate only a short distance into the separating gel (gamma globulin zone). When bovine serum as electrophoresed in 7% gel, a wide, densely stained region, the "haze zone," appears a short distance from the origin. Leaving some of the protein at the point of origin is a common difficulty and crowds the slower moving components together (122).

The differential disc electrophoretic procedure allows the serum or any complex protein solution to migrate first in a large pore gel (4.75%) which separates the large molecules from the smaller or faster moving components. These smaller molecules, apparently "trapped" by the larger components in the 7% gel subsequently separate in the small pore size gel (10%). Similarly, the large molecular weight components which were trapped in stiffer gels because of almost identical molecular dimensions but differed slightly in charge separate further in the large pore gel.

The length of the upper separating gel (4.75%) was a critical feature. If the distance of migration was too short, protein molecules would pile up at the origin of the 10% gel, which masked other bands; and the post albumin bands were poorly resolved. A length of 3.5 cm was satisfactory for separation of human and bovine serum proteins.

Because large quantities of sample could be applied, bands detected only by the differential procedure may not have been "new" bands, but because of the increase in concentration, they now could be detected. However, no new bands could be detected when the same sample size was applied to 12 cm tubes containing only 4.75% or 10% separating gel.

Some of the new bands may have been "created" due to splitting or denaturation of certain proteins. Hjerten, Jerstedt, and Tiselius (83) hypothesized that photopolymerization of certain ionic mixtures could result in denaturation of the sample. When photopolymerized samples were compared with samples added directly to the column (49) identical patterns developed. Therefore, photopolymerization did not appear to alter the protein molecules in the serums tested. The possibility still exists that dissociation of the molecules into dimers and trimers occurred during electrophoresis.

Although, the separation of bovine serum was markedly improved, human serum gave the most striking results. In some cases, twice as many proteins could be detected. This increased resolution becomes extremely important when analyzing serums from diseased individuals. The reflection of disease in tuberculous cows was best demonstrated by the new procedure while the standard procedure revealed little except a change in the concentration of bands.

The differential system yielded greater separation of serum proteins from a number of different animals but was not suitable for all. The separation of mouse, dog, chicken and pig serum proteins was not improved. It is unlikely that any one combination of separating gels will adequately separate all the fractions of a complex mixture.

The elicitation of precipitating antibodies in the rabbit against 32 constitutents of bovine serum indicated that the serum proteins of cattle are as antigenically complex as that of man. A nomenclature was proposed by Heremans et al. (76) from their studies of mouse serum designating the antigenic components detected by immunoelectrophoresis analyses. It is based on the electric mobility of protein and the terminology employed in moving boundary electrophoresis. It is a simple, direct method by which a component can be readily identified but does not take precedence over specific naming procedures.

The sensitivity of immunoelectrophoresis (IE) is dependent on a number of factors not present with cellulose acetate, starch and acrylamide gel electrophoresis. These factors have been discussed by Crowle (46). The potential resolving power of IE is primarily dependent on the antiserum. Therefore, the different responses of individual animals to mixtures of antigen makes it difficult if not impossible to standardize antiserums. This was demonstrated in this study when three rabbits failed to produce antibodies specific for

albumin and two failed to produce anti-gamma globulin antibodies. This was also observed by Dardas and Mallmann (48) who suggested that unless a variety of animal species is used for antiserum production, all of the antigenic constituents of the mixture probably would not be detected.

The multiplicity of immunoprecipitates in the $alpha_1$ and $alpha_2$ regions makes analyses difficult. Absorbed antiserums would greatly facilitate such analyses. The identification of some of the components as lipoproteins and glycoprotein substantiated that many of the proteins in human serum have their counterparts in bovine serum.

The lipoproteins of human and bovine serum seem to be quantitatively similar. Apart from a fraction which penetrates poorly in the agar gel and presumably corresponds to chylomicrons, there are two main lipoproteins differentiated in the serum from both species by IE. The slow component carries the major part of protein bound lipids and lies between the alpha and beta zones in agar gels (a_2-13). The minor component, although not so consistent, is somewhat slower than albumin a_1-1. In sheep serum, the major lipoprotein is fast and the minor component slow, but the mobilities place these components in the same region as the human and bovine lipoproteins (40).

Serums from normal calves were separated into four main fractions by continuous flow electrophoresis. Although the resolution was poor by stained curtain, the gamma

globulin, beta globulin and albumin fractions were of unusually high purity as confirmed by cellulose acetate electrophoresis of the eluted concentrated fractions. The only disadvantage being, that only small dilute volumes could be collected. These concentrated fractions of gamma and beta globulins and albumin contributed to the identification of the bands separated by starch-gel, polyacrylamide and immunoelectrophoresis.

Each fraction was not isolated and characterized. The nomenclature assigned to cellulose acetate electrophorograms, immunoelectrophorograms, and starch-gel electrophorograms was based only on mobility.

Classification on the basis of mobility of the bands separated by differential disc electrophoresis would not be reliable due to the difficulty of correlating the numerous bands with those separated by the other three procedures. The mobility of specific protein can be quite different in these media. For example, the major serum lipoprotein and glycoprotein was a beta globulin in cellulose acetate and alpha$_2$ globulin in agar immunoelectrophoresis. This lack of correlation, especially with cellulose acetate and immunoelectrophoresis was a handicap in the analyses of serums from tuberculous cattle. Not until these bands have been isolated and characterized will the method be fully utilized.

Analyses of Serums from Tuberculous Cattle

Since it was desirable to examine and compare all serum samples for each experimental calf at the same time, samples were stored at -20 C and not analyzed by electrophoresis for at least two weeks after the last sample was collected. Freezing or "aging" mouse serums caused alterations in the immunoelectrophoretic properties of a $beta_2$ globulin (210). It was possible that freezing or "aging" of bovine serums might cause observable changes in the electrophoretic patterns. However, no changes were detected by the methods employed. If any changes did occur, they did not alter the comparative results.

The polymorphism in the beta globulin region of the electrophoretic patterns of bovine serums due to breed differences has been noted. It was possible to distinguish 3-6 beta bands, after starch-gel electrophoresis, in normal serums from the different breeds used in this study: serum from Guernsey cattle always contained 3 major beta fractions (6a, b, and c); 4-5 bands (6a, b, and c, and 7) were present in the serum from Jersey cattle; and Holstein cattle serum contained, in addition to the above, a 6 band, number 8. Therefore, it is necessary to establish the normal variations before evaluating serum protein changes that occur during disease. Such variations could be erroneously interpreted as resulting from the disease process.

The possibility of other genetically dependent serum variations that might be detected in immunoelectrophorograms also had to be considered. To minimize this problem, the inoculums used for antiserum production contained a pool of serums from all of the experimental cattle.

The most regular and quantitative alterations in the serum proteins in experimental bovine tuberculosis was increase in gamma globulin and decrease in albumin. These changes were as easily demonstrated by the cellulose acetate procedure which was more facile than starch-gel, differential disc electrophoresis and immunoelectrophoresis. The results by all four methods were in close agreement.

Since the most consistent and apparent alterations in protein fractions from tuberculous cattle were in the albumin and gamma globulin, the albumin-gamma ratio (A/G) had practical value. In normal calves (pre-inoculation sample) the average value was 1.4, and slightly lower 1.2, for normal adult cows. The higher gamma globulin concentration in the older animals is probably the result of various antigenic contacts during the longer life span, with resultant increased formation of gamma globulin.

The A/G values for experimental animals inoculated with M. bovis or 68C and naturally infected animals was consistently lower than the normal value indicating an increase in gamma globulin. The only exception to this finding was

the increase in the A/G values of post-inoculation serums collected from calf #81. This calf was inoculated subcutaneously (SC) with the same M. bovis culture (#81) that had produced classical tuberculosis in calf #20. Since tuberculosis did not develop in calf #81 as demonstrated by the absence of gross and microscopic lesions, three assumptions can be made: (1) the virulence of the organisms was reduced, (2) SC route of inoculation does not as readily induce infection, and (3) the resistance to infection in calf #20 was lower than calf #81. The latter is one of the more probable major factors. Some of the calves purchased for the earlier studies were younger and in poor condition. Perhaps because of some metabolic deficiency, the natural resistance mechanisms in calf #20 were less effective. This calf survived only 65 days post-inoculation. In other experimental laboratory animals, the intradermal route has generally been more favorable than the subcutaneous route for the sensitization and infection by either a low number of virulent organisms, or by less virulent organisms. (V. A. Mallmann, personal communication). This may be related to the longer retention of organisms at the site of inoculation and the development of delayed sensitivity.

Animals inoculated with M. avium (with the exception of calf #21) or 50B, and tuberculin negative cows had normal values or slightly increased. Calf #21, from the same lot as calf #20 and inoculated with M. avium, developed lesions

and a lowered A/G ratio. This was the only calf to develop these changes following the inoculation of M. avium, and is probably additional supportive evidence that this group of animals had lowered resistant mechanisms.

Animals inoculated with 51C gave inconsistent results and the cellulose acetate electrophorograms were classified into two types: Type I, the A/G ratio was normal or slightly increased and Type II, the ratio was below the normal value. The difference in response of the host to culture 51C could not be compared to the degree of infection as determined by presence and locations of lesions.

These differences may be due either to host resistance, age, and/or hypersensitivity. Older or adult cows appeared to be more resistant to infection as determined by extent of lesions. All calves with the exception of one (#108) had generalized lesions while the three experimental adult cows developed a primary complex without any signs of progressive disease. Host resistance and/or age possibly explains the variation in disease but still does not explain the variation in serum patterns. A possible explanation is that tuberculin caused these variations. This seems probable since an anamnestic-like response, as detected by a marked increase in the HA titer following injection of tuberculin, was observed inconsistently in the experimental animals (118).

Decreasing A/G ratios calculated from cellulose acetate electrophorograms did differentiate experimental infections of M. bovis or 68C which produced gross lesions between the infections of M. avium or 50B in which no gross lesions were detected. The difficulty in using the A/G ratio from cellulose acetate electrophorograms are the inconsistent values obtained from animals inoculated with 51C. In spite of its shortcomings, the determination of serum protein fractions is useful in the study of bovine tuberculosis and may serve as prognostic guides.

The most striking and consistent change detected by starch-gel electrophoresis was the marked increase in the a_2-V fraction of serums from calves after inoculation with M. bovis, 68C, or 51C, and all GL and some NGL animals from naturally infected herds. It may be possible that tuberculin positive and false negative animals infected with virulent mycobacteria can be differentiated from animals giving a false positive response to tuberculin due to the presence of avirulent mycobacteria, by this method. If thin layer starch-gel electrophoresis can be perfected so that the gel would not have to be sliced, reliable A/G values could be calculated. A larger sampling of serum from tuberculin negative cows to more accurately determine normal protein values must be determined before this method can be reliably recommended. Only four tuberculin negative cows were available for this study.

Immunoelectrophoresis of post-inoculation serums also demonstrated the marked increase in the a_2-V fraction which migrated as an a_2-1. This method was not better than starch-gel electrophoresis for the detection of specific or nonspecific changes. Accurate interpretations of immunoelectrophoretic results are difficult. Quantitative changes have numerous limitations. There is difficulty in standardization of antiserums and differences in displacement of precipitin arcs may be due to changes either in mobility of antigen or antigen concentration. Recently immunoelectrophoretic capabilities have been extended to quantitative determinations (161). Even if the quantitative values can be obtained, it would not be as practical as starch-gel electrophoresis or perhaps the differential disc electrophoresis. A combination of polyacrylamide electrophoresis and diffusion in agar gel may be more fruitful.

Only pooled rabbit-normal-bovine-antiserums were used to develop electrophoresed serums from tuberculous cattle. Presumably the same specific immunoprecipitates would be present if these serums were developed with antiserums produced against post-inoculation serum. In addition, these antiserums might detect mycobacterial antigens (either free or in the form of soluble antigen-antibody complexes) if these antigens were present in the serum used for antiserum production. Post-inoculation serum developed after

electrophoresis with anti-post-inoculation serum (containing specific anti-mycobacterial antibodies) could produce a specific precipitate not found in normal serum. Post-inoculation serums could also contain altered host tissue antigens. If so, immunoprecipitates could be formed.

The a_2-V fraction was not isolated and characterized. Certain of its properties can be inferred from its behavior during starch-gel electrophoresis and immunoelectrophoresis. The a_2-V fraction was a protein antigen which migrated in the $alpha_2$ globulin region in both starch-gel electrophoresis and immunoelectrophoresis. It stained with carbohydrate but not with lipid specific stains. It consistently formed a clear precipitate. It was the precipitate farthest from the antiserum trough, the $alpha_2$ (a_2-I). These data suggest that it might be macroglobulin but not a major lipoprotein and that it is present in relatively high concentrations in serums from tuberculous cattle. It also appears to be similar to the $alpha_2$-T precipitate described by Dardas and Mallmann (48) found in serums from tuberculous guinea pigs. The $alpha_2$-T was not characterized as a macroglobulin and $hyperalpha_2$-globulinemia always preceeded its detection. Therefore, the fraction characterized in this study is called $alpha_2$-V and not $alpha_2$-T. These may subsequently be demonstrated to be essentially identical. Dardas and Mallmann (48) also found that hypersensitivity did not stimulate the $hyperalpha_2$-globulinemia nor were consistent

changes found in the distribution of protein-bound carbohydrates during experimental tuberculosis. This was also found with serums collected from human tuberculosis patients (79) and from naturally infected cows (188). Increases in serum glycoprotein levels may result from many causes (171, 201). Therefore serum glycoprotein determinations cannot be recommended as a diagnostic supplement to tests for tuberculosis of cattle.

A simultaneous increase in the slow moving alpha$_2$ (Sa_2) globulin lipoprotein with the increase in gamma globulin and the a_2-V fraction occurred only in calves inoculated with M. bovis, 68C or 51C. A similar phenomenon was observed by Williams and Wemysis (210) in tuberculous mice, and in tuberculous guinea pigs by Dardas and Mallman (48).

The role and mechanism of increased gamma globulin in tuberculosis is not known. If the gamma globulin elevation is related to antibody formation, it is not associated with immunity. Tuberculoimmunity in man or animals has no significant rise in gamma globulin and the immunity can not be passively conferred with serum (44, 175). Many of the tuberculous calves which had high gamma globulin levels 14 days post-inoculation had low HA titers. An increase in gamma globulin may be induced by tuberculin as indicated by a fourfold increase in HA titer following tuberculin testing. The degree of specificity of the antibodies is not known.

The increase in titer was observed in 2 of 4 tuberculin negative cows. This phenomenon is just as difficult to explain as the variation in electrophoretic patterns in animals infected with 51C. The polysaccharides in the tuberculin might affect the amount of polysaccharide-specific antibodies or it may be a stimulation of a broad range such as that elicited by endotoxins. This phenomenon did not occur in animals exposed to an aersol of the organisms although the gamma globulin was markedly elevated. Because no increasing titer could be detected in these animals, immunologic paralysis may have resulted due to the large dose of organisms. Therefore, the increase in gamma globulin appears to be largely due to tissue destruction and not to the excess of circulating antibodies alone.

Increased gamma globulin occurs commonly in most hepatic diseases. Liver impairment occurs in advanced tuberculosis. Therefore the elevation in generalized disease may be due in part to liver alterations. It probably does not account for the increase in gamma globulin in animals which develop only enlargement of the cervical lymph nodes (primary complex). There is increasing evidence that excess gamma globulin is formed not only in the kupffer cells of the liver but by plasma cells and other reticuloendothelial cells in response to tissue destruction (147). This more probably accounts for the elevated gamma globulin in animals with only a primary complex.

The inability of liver cells to form albumin, possibly because of damage, does not account for the hypoalbuminemia present in animals with a primary complex. It is probable that the low albumin concentration is the result of preferential formation of gamma globulin from available amino acids over the formation of albumin (5).

A hyperalpha$_2$-globulinemia accompanied an increase in gamma globulin and a decrease in albumin in human tuberculosis (12, 172, 197) and tuberculous guinea pigs (48, 176). A hyperalpha$_2$-globulinemia was not detected in serums from tuberculous calves by cellulose acetate electrophoresis since the alpha band did not separate into two components. Apparently the alpha$_2$ globulins were masked by the albumin band. Albumin constitutes the major component of serum, therefore if the alpha$_2$ globulins were masked by albumin, changes in this fraction would not be detected. It was evident by starch-gel electrophoresis, differential disc electrophoresis and immunoelectrophoresis that changes in the alpha$_2$ globulin in serum from tuberculous calves did occur. The increase in only two alpha$_2$ globulins (Sa$_2$ and V fraction) is additional supportive evidence that these alpha$_2$ globulins were in low enough concentration to be masked by albumin in cellulose acetate electrophorograms.

The most widely accepted explanation for the increase in alpha$_2$ globulin that occurs during tuberculosis in man and guinea pigs is that it is mainly a reflection of

tissue destruction. Bovornkitti (20) proposed that the increase in alpha$_2$ globulin was due to the result of delayed hypersensitivity. It did not appear that hypersensitivity induced the increase in the a_2-V fraction and agrees with the assumption that the increase was due to tissue destruction.

Some investigators have reported an increase in the beta globulin during tuberculosis (172, 197). This was not demonstrated in bovine tuberculosis.

The drop in total serum protein concentration in serum from naturally infected animals following the injection of tuberculin is an interesting phenomenon and difficult to explain. It occurred inconsistently in some of the experimental animals. The decrease in total serum protein did not occur in serum from tuberculin negative cows following tuberculin testing. Apparently animals that had prior antigenic stimulation developed a mild form of shock to the colloidal osmotic regulatory mechanism due to the secondary stimulation by tuberculin. As a result excess fluid from the tissues entered the circulatory system and diluted the plasma proteins. The decrease in total proteins, however, did not have any effect on the relative protein values of the individual components. This mechanism is conjectural and further investigations are required to establish the significance of this phenomenon.

Because other chronic inflamatory diseases may mimic the serum protein changes demonstrated for tuberculosis, electrophoresis would not be adapted to routíne use as a laboratory test. The evaluation of the starch-gel electrophoretic patterns in some problem cases would have diagnostic significance, particularly when evaluated with the total clinical picture.

The inability to demonstrate circulating antibodies in a large number of cows with bacteriologic and clinical evidence of tuberculosis by a procedure as sensitive as the passive hemagglutination test is paradoxical. The concept that antibody activity associated with human serum protein may be inhibited by other proteins or substances present in the serum proved fruitful. Gerstl et al. (60, 61) using ethanol fractions of serum from humans infected with tuberculosis found antibody activity in one or several fractions which as a whole had failed to react. However the incomplete separation of proteins accomplished by this fractionation procedure did not permit exact localization of antibody activity. Fractionation of whole serum from humans infected with tuberculosis by continuous flow electrophoresis revealed antibody activity in the $alpha_2$ fraction (2). Turcotte et al. (192) used DEAE-cellulose to separate the antibodies responsible for the hemagglutinating activity of serums from healthy tuberculin positive individuals and

individuals with active pulmonary tuberculosis. The latter investigation revealed that false positive HA titers were possible.

The high degree of separation and resolution of serum proteins by starch-gel and differential disc electrophoresis makes it even more possible to detect mycobacterial antigens and anti-micobacterial antibodies in serums from individuals suspected of having tuberculosis. Such studies might detect separate fractions that contain diagnostic antibodies or antigen-antibody complexes. The possibility exists that the a_2-V fraction contains specific anti-mycobacterial antibodies. If any antibody is partially responsible for immunity in tuberculosis, it may occur in some fraction other than gamma globulin. The chance of uncovering these antibodies will depend largely on the sensitivity of the fractionation procedure. The more components that can be separated the greater the chance of finding a specific component. The differential disc procedure, although still experimental, may meet these requirements. Such studies can also be used to investigate extracts from white blood cells such as the transfer factor. Results from either or both can contribute to our understanding of the "ecology of tuberculosis," the interactions of the mycobacteria and their hosts.

SUMMARY

Modified techniques and apparatus for starch-gel electrophoresis and for the rapid staining and destaining of both starch and disc gels is described. The mechanical manipulation of starch-gels during the preparation and slicing procedures was facilitated. The need of handling either types of gels during the staining and destaining processes was eliminated.

A modified polyacrylamide electrophoretic procedure is described which separated human serums into 44-50 components and bovine serum into over 30 bands. In this procedure, a complex ionic mixture migrates through both a large and a small pore size gel in a single column. This procedure, tentatively named differential disc electrophoresis, is simple, facile, and affords improved separation and resolution of serum proteins.

Serums from normal cattle were analyzed by the electrophoretic methods of continuous flow, cellulose acetate, starch-gel, two-dimensional starch-gel, agar gel, immunoelectrophoresis, starch-gel immunoelectrophoresis, and differential disc electrophoresis. Differential disc electrophoresis was the most sensitive single procedure.

Thirty to 38 fractions of bovine serum were observed by this technique. Thirty-two antigens were found in normal serum by immunoelectrophoresis and a standard nomenclature was proposed. The antigenic heterogeneity of the majority of the protein bands separated by starch-gel electrophoresis was demonstrated. A few of the bands separated by these procedures were identified as lipoproteins and glycoproteins.

A hypergammaglobulinemia and a decrease in albumin was detected by all electrophoretic procedures in the serums from experimental calves inoculated with Mycobacterium bovis or 68C (virulent Group III) 7-14 days post-inoculation and naturally infected cows, gross lesion and no gross lesion, prior to tuberculin testing.

Inconsistent changes were detected in cellulose acetate electrophorograms of serums from calves inoculated with 51C (virulent Group III). No changes in the electrophorograms of serum from experimental animals inoculated with M. avium or 50B (avirulent Group III) or tuberculin negative cows were detected. The hypergammaglobulinemia and passive-hemagglutination anamnestic-like response to tuberculin did not consistently occur simultaneously.

The A/G ratio calculated from cellulose acetate electrophorograms was practical in differentiating experimental cattle inoculated with virulent strains or species (M. bovis, 68C) from cattle inoculated with avirulent

acid-fast bacilli (M. avium, 50B). However, cattle inoculated with 51C could not be differentiated by this method.

No consistent differences occurred in the protein-bound carbohydrates. An increase in the slow moving alpha$_2$-13 globulin lipoprotein (detected by immunoelectrophoresis and differential disc electrophoresis) occurred only in the serum from calves infected with virulent mycobacteria.

Starch-gel, differential disc electrophoresis and immunoelectrophoresis revealed an alpha$_2$ fraction that was always increased in serums from experimental animals inoculated with M. bovis, 68C or 51C and pretuberculin serums from gross lesion (GL) and no-gross lesion (NGL) cows from naturally infected herds. This fraction remained unchanged in serums from calves inoculated with M. avium or 50B and therefore has been tentatively named the a$_2$-V (virulence) fraction. This fraction was most reliably detected by the starch-gel procedure. Further definition and characterization of the individual fractions and a reliable densitometric procedure is needed before the full potential of differential disc electrophoresis can be fully appreciated in the analysis of serums from pathological disorders.

The hypergamma- and hyperalpha$_2$-globulinemia may result from tissue destruction, circulating antibodies and in a few cases from delayed hypersensitivity.

A total protein decrease was marked in serums from all GL and NGL cows from naturally infected herds following the injection of tuberculin. The total protein remained constant in serums from tuberculin negative cows. It was suggested that a secondary stimulation by tuberculin caused a mild form of shock to the colloidal osmotic regulatory mechanism so that excess fluid from the tissues entered the circulatory system diluting the plasma proteins.

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