PATHOLOGICAL SERUM PROTEINS WITH SPECIAL REFERENCE TO LIVER, HEART and RHEUMATOID DISEASES

A THESIS

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INTRODUCTION

For half a century immunology has accepted the famous expression of Ehlrich of "Horror Autoxicus", implying that an organism will not react immunologically against the introduction of its own constituents. It was later alleged by Metchnikoff (1905) however, that an animal can be immunized against its own spermatozoa, the immune serum produced being called cytotoxic as it caused cell destruction. To be precise the spermatozoan is an organism itself and it is questionable whether this can be regarded as a typical case of autoimmunity to a homologous organ. However, since then many investigators have studied cytotoxic sera produced by injecting experimental animals with constituents of various organs. In most cases the sera have been prepared by the injection of heterologous organs, ie. organs from animals of another species or from another animal of the same species and when these sera containing hetero antibodies are introduced into the animal against whose tissue they have been formed it has been shown that in certain cases they will be fixed mainly, but not solely, on the corresponding organ. Cytological examination of such organs shows that in many cases there is an appearance of lesions often very similar to those which occur naturally in animals and human subjects (Grabar 1957). On the basis of these experiments it has been suggested that in certain diseases of man and animals the underlying pathological process may be the formation of autoantibodies and such diseases have been described as "autoallergic". (Clough (1960); Dixon (1958); Burnet (1959)). Many attempts have been made to immunize experimental animals against their own organs in /

in order to evoke the production of true autoantibodies but in most cases successful immunization has only been achieved with organs of other animals (Grabar 1957).

Antibodies to a wide variety of tissues have been produced in experimental animals, generally with the aid of emulsified adjuvants containing killed bacilli (Freund and McDermott 1942). Among the tissues used to induce the formation of homo, hetero and autoantibodies are notably:-

(1) Thyroid tissue (Witebsky and Rose 1957)
(2) Central nervous system tissue (Rivers, Sprunt and Berry 1933)
(3) Peripheral nervous system tissue (Wakesman and Adams 1955)
(4) Testes (Freund, Lipton, and Thompson (1953)
(5) Uvea (Collins 1949)
(6) Lens (Freund, Thompson and Lipton 1955)
(7) Liver tissue (Gear 1955)
(8) Kidney tissue (Baxter and Goodman 1956)
(9) Heart, skeletal muscle and connective tissue (Cavelti 1947)

The precise mechanism of the autoimmune process in most cases is not understood. Serum factors possessing most or all of the generally accepted characteristics of autoantibodies have been demonstrated in serum in a variety of experimental and clinical lesions but due to the lack of specificity of the antigens observed in these instances, the exact nature of the antigenic components required to initiate the response has, in most cases, remained obscure. As the tissue preparations used for immunization are obviously mixtures of antigens causing multiple antibody responses, it is possible that the antibodies detected in the sera of these /

these experimental animals may be unrelated to the antibody or other pathological factor, reacting with and causing damage to, the tissue involved. This uncertainty has led to the alternative hypothesis that the appearance of these autoantibodies is indeed not related to an immunological response but that they are pathological proteins, produced by the patient or experimental animal, and represent a secondary manifestation of some primary disease process which happen to possess the property of reacting with various tissue components.

Never-the-less during the last two decades evidence for the occurence of auto-immunization in man has been accumulating and the question of its clinical and pathological significance has become extremely important.

(1) Autoantibodies in human sera.

In 1938 Dameshek described cases of acute hemolytic anaemia believed to be due to the presence in the blood serum of auto-hemolysins or autoantibodies directed against the patients own red cells, causing hemolysis *in vivo*. These autohemolysins have since been physically and chemically characterized and it appears that they are γ-globulins with many of the properties of antibodies (Franklin, Holman and Kunkel. 1957). Autoantibodies to platelets and leukocytes have also been detected in the sera of patients with thrombocytopenia (Evans 1951) and leukopenia (Dameshek 1953) respectively.

Several hypotheses for this autoimmunization mechanism have been put forward. Dacie (1959) suggested that a modification of the red cell occurs by bacterial, viral or enzyme action which renders it antigenic evoking the production of autoantibodies which cross react with the normal cells causing hemolysis in *vivo.*

Other workers have suggested that the basic lesion is a pathological change in the antibody forming tissue leading to a loss of tolerance to the normal blood cell antigens (Mackay, Larkin and Burnet 1957). Although hemolytic anaemia is a prime example of autoantibody formation in man, no one has yet produced an autoantibody induced hemolytic anaemia in experimental animals. Conversely there are many examples of experimentally produced autoantibodies in animals accompanied by tissue damage without concomitant evidence of similar autoantibodies occuring in the corresponding human pathological conditions.

(2) Experimental production of autoantibodies in animals.

It has been suggested that lesions of the central nervous system, peripheral nervous system, testes, uvea and lens are of an autoimmune nature on the basis of evidence which has arisen largely from auto, homo and hetero immune responses in experimental animals on injection of the appropriate tissues plus adjuvants. The clinical and pathological features involved in these experimental lesions resemble those found in certain human diseases of unknown but suspected immunological origin and circulating auto, homo or hetero immune antibodies have been demonstrated in the sera of these animals. It has thus been suggested that in the pathogenesis of the resulting lesion the hosts own tissues serve as target organs and are injured as a result of the immune reaction.

Autoimmune reactions have been postulated to operate, on this basis, in the human disorders involving the same organs or tissues. However, evidence for the support of the autoimmune pathogenesis of these lesions cannot be regarded as definitive /

definitive for two reasons. Firstly the diseases are produced in animals and it is necessary to assume an identity between the experimental lesions in the animals and the human disorder on the basis of clinical and pathological comparisons, which are not entirely satisfactory; secondly, whilst an autoimmune pathogenesis for these disorders seems probable, final demonstration of the responsible immunological agent is lacking and attempts to transfer these diseases passively by cells or serum of the affected animal have been unsuccessful.

The destructive demyelinating and inflammatory lesions of the central nervous system seen in experimental allergic encephalomyelitis have been produced in monkeys and other animals by immunization with heterologous, homologous and even autologous central nervous system tissue (Rivers, Sprunt and Berry 1933). The simultaneous development of circulating autoantibodies and of morphological changes in the central nervous system imply an immunological basis for these lesions but it has not, however, been possible to induce lesions of the central nervous system by transfer of serum, nor did the levels of circulating antibody correlate with the degree of tissue damage.

Demyelinating lesions of the peripheral nervous system have also been produced in experimental animals (Wakesman and Adams 1955). A condition known as experimental allergic neuritis was produced with the concomitant appearance in the sera of the experimental animals of complement fixing autoantibodies to the injected peripheral nerve tissue. The experimental lesion resembled in many ways acute infectious polyneuritis in man, suggesting that an autoimmune mechanism may operate in the human /

human disorder.

The antigenicity of homologous spermatozoa has long been recognized (Metchnikoff 1905) but only relatively recently has testicular damage been associated with an immune response to testicular tissue antigens (Freund, Lipton, and Thompson 1953). The effective antigen was found only in homologous testes and the levels of complement fixing antibodies to testicular tissue in experimentally immunized animals roughly paraleled the degree of testicular damage. The injection of homologous uvea plus Freunds adjuvant into experimental animals has in many cases produced an inflammatory reaction in the uvea (Collins 1949). The reactions were characterized by focal infiltrations of lymphocytes and epitheloid cells in the uvea, an experimental lesion resembling that of sympathetic ophthalmia of man, a disease suspected to be an autoimmune response to the antigens of the injured eye. The lens of the eye has also been suspected of autoantigenicity and lens tissue will produce antibody responses when injected with Freunds adjuvant into homologous hosts (Freund, Thompson and Lipton 1955).

(3) Experimentally produced autoantibodies resembling those found in man.

Experimental lesions similar to those found in man suffering from disorders of the thyroid, kidney and liver have been produced in animals by injection of homologous and heterologous tissue antigens. Not only have antibodies to the injected antigens been demonstrated in the sera of the experimental animals but similar autoantibodies have also been detected in the sera of patients suffering from the corresponding thyroid (Roitt 1956 and 1957), kidney (Cavelti 1945) and liver (Gear 1955 and Gajdusek 1958) disorders. These /

These autoantibodies react with human and in some cases animal tissue antigens of the organs involved. The inference is that the autoantibodies found in these patients may bear some relation to those produced in experimental animals and are believed to be either involved in the pathogenesis or produced as the result of tissue damage.

The immunological properties of thyroid tissue were recognized as early as 1927 when it was observed that antibodies appeared in the serum of a rabbit which had received injections of a thyroid tissue extract from another species (Hektoen 1927). Three decades later Witebsky and Rose (1956) attempted to produce isoantibodies by sensitizing a rabbit with the thyroglobulin of another rabbit. Sensitization was achieved by the injection of thyroid extract plus Freunds adjuvant and antibodies were detected by the agglutination of tanned red cells sensitized with thyroid extract (Witebsky and Rose 1956).

Subsequently these workers challenged one of the fundamental concepts of immunology, that an animal will not produce antibodies to its own tissue proteins. They injected rabbits with extracts of their own thyroid glands and showed that autoantibodies frequently appeared in their sera. It was also shown that the appearance of these autoantibodies was accompanied by damage to animals own remain- thyroid gland and photomicrographs of the injured thyroid tissue showed lesions which closely resembled those observed in Hashimoto's disease of the thyroid (Witebsky and Rose 1957). This finding led to a search for autoantibodies to thyroglobulin in the sera of patients with thyroid diseases and the role of autoimmune reactions in thyroid disorders in man was dramatically disclosed in 1956 when Roitt et al. showed by a precipitation technique that the serum from patients /

patients with Hashimoto's thyroiditis contained an antibody to a constituent of human thyroid tissue (Roitt, Doniach and Campbell 1956). Antibodies to thyroglobulin were also found in cases of subacute thyroiditis and primary myxodema. Precipitin reactions tended to be the highest in untreated patients and lower in patients treated with desiccated thyroid preparations, after thyroidectomy or myxodema had set in the precipitin reaction often disappeared. On the basis of these findings Roitt and Doniach suggested that some mechanism must initially permit the release of thyroglobulin from the follicles whereupon a lymphadenoid response occurs and the thyroid gland is infiltrated by lymphoid tissue. Antibodies to thyroglobulin are produced and a chain reaction is set in motion causing further epithelial damage and further escape of thyroglobulin (Roitt and Doniach 1957).

The evidence that autoimmunity is involved in certain renal disorders is indirect but never-the-less suggestive. In 1934 Masugi produced experimental nephritis in animals by the injection of nephrotoxic serum and found that a whole spectrum of renal lesions resulted, from those characteristic of a progressive nephritis to the apparently reversible glomerular changes associated with massive proteinuria. Cavelti (1945) observed that rats injected with mixtures of homologous or autologous kidney tissue with killed β -hemolytic streptococci developed morphological and functional renal changes suggestive of glomerular nephritis. He also demonstrated the presence of antibodies to the injected antigens in the sera of these animals. Acute glomerular nephritis tends to follow an injection with group A streptococci after an interval of about three days. Organisms are not found in the renal lesions and it has been shown that serum complement is low or absent during the /

the acute phase (Kellet and Thomson 1939), suggesting that some substance is released by the streptococci which is itself antigenic or reacts with some glomerular constituent rendering it antigenic; the interval between infection and onset of the disease representing the time required for antibody formation.

Liu and McCrory (1958) investigated a large number of human sera for antibodies to human kidney by the agglutination of tanned sheep cells coated with human renal antigen and reported significant titres in 71% of the cases of acute glomerular nephritis tested and in 89% of the cases of nephrotic syndrome. On the basis of these results it seems feasible to assume that some component of kidney tissue is rendered antigenic, by the streptococcal infection, invoking the production of antibodies which by their reaction with renal tissue _in vivo_ precipitate glomerular lesions and can be detected in the serum by _in vitro_ techniques.

In 1955 Gear observed that an extract of the liver of a monkey dead of yellow fever stimulated antibody formation in another monkey but liver extracts of a normal monkey did not, indicating that some alteration of the liver cells is required to render the liver tissue antigenic. He also showed that serum of a patient convalescent from viral hepatitis gave a positive precipitin reaction with the serum from one in the acute phase and showed that the antigen was not the virus but some derivitive of liver tissue. The inference here is that some component of liver tissue is rendered antigenic by virus interaction and on liberation into the circulation during the acute phase of hepatitis stimulates the production of antibodies which make their appearance during the convalescent phase.

Gajdusek (1958) in an extensive investigation of liver, kidney, the collagen diseases and certain para-proteinaemias in /

in man found positive complement fixation reactions between a variety of human tissue antigens including saline suspensions of normal liver, kidney, skeletal muscle and spleen tissue and the sera from patients suffering from acute viral hepatitis, lupoid hepatitis, primary non-obstructive biliary cirrhosis, disseminated lupus erythematosus and certain paraproteinaemias, diseases for which autoimmune mechanisms of pathogenesis have been proposed. He reported that the sera of certain patients with acute rheumatic fever and acute glomerular nephritis gave positive reactions with liver and kidney tissue. Sera from patients with disseminated lupus erythematosus, multiple myeloma and macroglobulinaemia also reacted in certain cases with liver and kidney extracts. Diverse hepatic diseases, including viral hepatitis and biliary cirrhosis, were reported to show a high incidence of positive autoimmune complement fixation reactions with liver extracts and kidney extracts, positive reactions in the first two months of acute viral hepatitis tended to be the most frequent. No strict organ specificity was observed in these reactions and certain hepatic sera also gave positive reactions with extracts of muscle, spleen and kidney tissue. Nor were the reactions species specific and high titre positive sera also reacted with rat organ antigens.

The non specificity of these reactions might seem to favour the view that the activity of these proteins is non-immunological in the light of classical immunological concepts but these authors (Gajdusek and Mackay 1958) believe that the cross reactions can more plausibly be explained by the supposition that the antigenic stimuli which evoke the production of the serum reagents are complex and common to a wide variety of tissue cells.

(4) The collagen diseases

The collagen diseases constitute an important group of disorders which possibly involve autoimmune mechanisms. In the rheumatism group two antibody-like substances, the lupus erythematosus (LE) factor and the rheumatoid arthritis (RA) factor have been studied immunologically and several workers have attempted to attribute to them a specific antigen (Bywaters 1959). The LE factor meets many of the criteria of antibody (Hargraves, Richmond and Morton 1948) and can be transmitted by intravenous injection of LE serum in both man and guinea pig. It migrates with the γ-globulins on electrophoresis and has the same sedimentation constant as the normal low molecular weight γ-globulins (Carlson and Mollenberg (1958). It is believed to consist of several related factors directed towards various nuclear components, including the desoxy ribose nucleic acid (DNA), nucleoprotein and histones. It also reacts with DNA of many species from bacteriophage to man (Deicher, Holman and Kunkel (1959) and Holborow (1957).

Friou et al (1958) using frozen tissue slices or white cell smears showed that the LE factor will deposit γ-globulin on exposed nuclei which can be detected by subsequent staining with fluorescent anti-human globulin. The relationship of the LE factor to the disease process is uncertain and it is not known whether it is involved in the aetiology of the lesions or if it is an epiphenomenon produced during the disease process.

The possibility that rheumatoid arthritis has an immunological basis has long been entertained but with insufficient evidence. Recently, however, evidence regarding its close relationship with lupus erythematosus has been obtained and it has been observed that one of the many manifestations of lupus is rheumatoid arthritis /

arthritis and a number of patients with rheumatoid arthritis possess the LE factor in their sera and give a positive LE cell test (Kunkel 1959). The rheumatoid factor has many of the properties of an antibody and has been characterized as a high molecular weight γ-globulin which will combine with partially denatured γ-globulin in several immunological reactions (Heimer, Federico and Freyberg 1958; Kunkel, Franklin and Muller-Eberhard 1959). Its role in the aetiology of rheumatoid arthritis has not been established but Parson (1956) has reported the production of an extensive polyarthritis in rats following injections of macerated homologous muscle in Freunds adjuvant. The arthritis most frequently involved the tarsal joints and left residual permanent arthritic lesions such as ankylosis. If this disorder could be established as an autoimmune reaction it would be an important development in the understanding of the aetiology of the rheumatoid diseases.

Cavelti (1945) demonstrated the presence of autoantibodies to human heart tissue in the sera of 75% of the patients tested with acute rheumatic fever. Experiments with rats showed that animals immunized with homologous tissues of heart, skeletal muscle and connective tissue in conjunction with killed streptococci developed cardiac lesions which resembled those seen in cases of rheumatic fever (Cavelti 1947). On the basis of these results he suggested that in cases of rheumatic fever autoantibodies are produced to heart muscle and connective tissue components which have been rendered antigenic by streptococcal infection and by combination with these antigens situated _in vivo_ are capable of acting as pathogenic agents leading to tissue damage. From the _in vitro_ studies it appeared that once the formation of the autoantibodies had been /

been invoked the streptococcal infection was no longer necessary for the ensueing reaction of these autoantibodies with the tissues, as the experimental autoantibodies reacted in vitro with the unmodified tissue component. This hypothesis would explain the relationship between the streptococcal infection and the rheumatic fever, the interval between the two corresponding to the time necessary for the formation of autoantibodies which represent the pathogenic agents of rheumatic fever. It has also been implied that immunological mechanisms are involved in cases of myocardial infarction when there is no evidence of a preceding streptococcal infection and Dornbusch (1957) demonstrated the presence of heart specific autoantibodies in the sera of certain patients following myocardial infarction.

Theories of the autoimmune phenomenon.

Many attempts to describe the fundamental processes involved in the autoimmune response have been made, especially during the last decade. Until comparatively recently the most widely accepted hypothesis reconciled classical immunological theory with the problem of autoimmunity (Grabar 1957). It has been suggested that under certain conditions, as a consequence of trauma or infection some normal constituents of an organ or cell are transformed, modified or denatured to such an extent that they become "foreign" to the organism which reacts by the formation of antibodies to them. If the modification is persistent ie. if there is a latent infection which provokes a continuous modification of normal components, a continuous formation of antibodies may result which react with the modified antigen and ultimately induce tissue lesions. On the other hand if the modification is only temporary, /

temporary, the antibody produced against the modified constituent may also react with the normal body component by a cross reaction due to the presence in the normal constituent of chemical groupings similar to those in the modified component which originally invoked production of the antibody.

An alternative hypothesis which has recently gained much support was put forward by Burnet (1959) in his "clonal selection" theory of antibody production. This hypothesis is based on an abnormality of the antibody producing cells and allows the antigen no part in impression of pattern on the antibody molecule as in the classical theories of immunology. Burnet postulates that the capacity to produce a given antibody is determined genetically by certain clones of mesenchymal cells, the function of the antigen being to stimulate the cells of these clones to proliferate and to produce antibody. The main premise of this theory is that contact of antibody producing cells with the corresponding antigen in embryonic life results in the elimination of these cells rendering the organism tolerant to such antigens. In the second half of embryonic life a range of residual clones remain which are capable of reacting only with potential foreign antigens which were not present in the organism during embryonic life. After birth, however, contact with antigenic material causes proliferation of the corresponding clones and stimulation of antibody production, but if during adult life random mutation occurs which results in the production of patterns reactive against body constituents a homeostatic mechanism results in the elimination of such "forbidden clones" by contact with small concentrations of the antigen. Burnet suggests that the autoimmune diseases may be regarded as examples of failure of such homeostatic mechanisms whereby "forbidden clones" are allowed to proliferate /

proliferate and to produce antibodies. Tissue damage is postulated to arise, on this basis, as the result of a viscious circle wherein the antigenic determinant is liberated from the target organ in increased amounts following antibody tissue interaction.

The existence of autoimmune reactions almost exclusively in diseases wherein self inflicted immunological damage has long been suspected to be of aetiological significance justifies further study of such reactions in all stages of illness especially in the collagen disease group and other entities suspected to be auto-aggresive phenomena.

One may speculate that any process leading to an abnormal destruction of tissue may give rise to conditions conducive to the evokation of such autoimmune responses and it is reasonable to suspect that pathologic autoimmune reagents may be found in the sera at some stage during the course of a wide variety of lesions such as those encountered in burns, radiation injuries, fractures or crush injuries, pulmonary embolism, myocardial damage, viral infections and malignant neoplasms, when damaged or partially denatured material is made accessible to the antibody producing cells and due to failure of some homeostatic mechanism invokes the production of autoantibodies.

PART I

INTRODUCTION

Introduction to the problem of autoantibodies in liver disease.

Since Gajdusek (1958) reported the presence of autoantibodies to extracts of human liver tissue in the sera of a large proportion of patients with hepatocellular lesions it has been suggested (Gajdusek and Mackay 1958) that the onset of the immune reaction may characterize the transition from an acute to a chronic stage of hepatitis and it is known that the low titre positive results found in infective hepatitis sera frequently become negative coincident with recovery.

Gajdusek did not define the precise antigenic components in the liver extracts but it is probable that several of the hepatic cell proteins may be rendered antigenic because of some modification of chemical pattern caused by viral, enzymatic or toxic action or because components normally inaccessible to immunological processes have been made available and elicited the production of autoantibodies.

The alternative explanation is that these autoimmune reactions observed are merely a manifestation of an adventitious reactivity of serum globulins and not the result of a specific response to a tissue antigen. The serum reagents described by Gajdusek had, however, many of the properties of antibodies. They were shown to be present in the slow γ- globulin fraction on paper electrophoresis; they are stable to heat at 56°C but have an increasing instability above 60°C and in antibody titrations they exhibit typical prozones (Gajdusek 1958).

On the assumption that these proteins which react with liver extracts are in fact true autoantibodies, it was proposed to search for the presence of an antibody to one specific liver cell protein which may have been formed by an analogous mechanism to the autoantibodies in Hashimoto's disease of the thyroid when auto/

autoantibodies to a specific protein of thyroid tissue, thyroglobulin, have been demonstrated in the serum (Roitt, Doniach and Campbell 1956). These workers demonstrated precipitin reactions and agglutination of tanned erythrocytes by the sera of patients who had undergone lymphadenoid infiltration of the thyroid gland and purified preparations of human thyroglobulin. They suggested that these patients were immunized against their own thyroglobulin and that destruction of the thyroid tissue occured as a result of the progressive interaction of the thyroglobulin in the tissue with autoantibodies in the serum (Doniach and Roitt 1957).

(ii)

There is evidence that there is a marked elevation of certain plasma enzymes in patients with acute hepatocellular lesions (Molander, Wroblewski and La Due 1955; West and Zimmerman 1959). Increases in the levels of glutamic-oxalo acetic transaminase, glutamic-pyruvic transaminase, lactic dehydrogenase, aldolase, isomerase and malic dehydrogenase were reported (West and Zimmerman 1959). Elevations of lactic dehydrogenase have been demonstrated in the sera of 33% of the patients with hepatic cirrhosis tested and in 66% of those tested with infective hepatitis (Zimmerman and Weinstein 1956). The elevations were found to occur predominantly in the early stages and to return to normal levels about three weeks after the onset, coincident with recovery.

On the hypothesis that virus multiplication in the liver cell may render such proteins antigenic and capable of initiating autoimmunization phenomena, liver lactic dehydrogenase (LDH) was selected as a model protein and antibodies to purified human liver lactic dehydrogenase were sought in the sera of patients suffering from acute and chronic hepatocellular lesions (Burton, Kerr and Frazer 1959). It was proposed to test for the presence of such an /

an antibody by biochemical and immunological procedures and if possible to study its properties.

Enzymes, like other proteins, may be antigenic and their catalytic action is generally reduced by the presence of the corresponding antibody. A plot of residual enzyme activity as a function of the quantity of antibody added to a constant amount of enzyme shows, at first a linear decrease, then a deviation from linearity and finally a residual level of activity which is only slightly influenced by further addition of antibody (Cinader 1957). Complete inhibition may be obtained in some cases but in the case of LDH between 45 and 98% inhibition of the enzyme activity has been brought about by a specific immune antiserum (Kubowitz and Ott 1943; Henion, Mansour and Bueding 1955; Wroblewski 1958). Wroblewski (1958) produced antibodies, which were inhibitory to rabbit muscle LDH, in roosters by intravenous and subcutaneous injection of the purified enzyme. He found that the anti-LDH activity appeared in the serum about three days after injection and declined subsequently as the precipitin titre was rising. The percentage inhibition of the LDH was measured by the addition of the antiserum to a given amount of enzyme of known activity and calculated from the equation:-

$$\%\ \text{Inhibition} = \left(\text{LDH activity of enzyme} + \text{LDH activity in anti-enzyme preparation}\right) - \frac{(\text{LDH activity of mixture})}{(\text{LDH activity of enzyme})} \times 100$$

Up to 98.7% inhibition of purified rabbit LDH was obtained with the purified antienzyme and it was found that there was an increase in precipitin activity of the sera with the enzyme whilst the anti-LDH inhibition titre was decreasing, suggesting that the inhibitory /

inhibitory antibody was different from the precipitating antibody (Najjar and Fisher 1956).

Henion (Henion, Mansour and Bueding 1955) showed that the sera of roosters injected with rabbit muscle LDH caused inhibition of the activity of rabbit and rat muscle LDH and also that antisera prepared against Schistosoma mansoni LDH caused a marked reduction in the activity of Schistosoma LDH. Mansour, Bueding and Stavitsky (1954) tested for precipitating and agglutinating properties of the rabbit muscle antienzyme by the method of Boyd (1947) and the tanned cell agglutination method of Boyden (1951) and modified by Stavitsky (1954) respectively. All attempts to demonstrate precipitation of the enzyme or agglutination of enzyme treated red cells with the antisera were, however, consistently negative.

In this investigation it was proposed to prepare human liver LDH in a highly purified form and to test for any inhibition of its activity following incubation with the sera of patients suffering from necrotic lesions of the liver by a method similar to that used by Wroblewski (1958).

In order to detect antibodies to human liver LDH which were not necessarily inhibitory to LDH activity such as the secondary antibody observed in Wroblewski's experiments, it was proposed to test the sera by the sensitive Boyden haemagglutination technique (Boyden 1951).

In order to test, experimentally, the hypothesis that the presence of circulating antibodies to liver cell components may exert a cytotoxic effect on the liver tissue resulting in necrotic lesions, it was proposed to immunize rats with homologous rat liver lactic dehydrogenase and to examine histologically the liver of /

of the rats after the appearance, if any, of antibodies had occured.

PART I

REAGENTS and METHODS

(1) Biochemical investigation of autoantibodies.

REAGENTS.

0.5M sodium d l-lactate.

BDH lactic acid was diluted with an equal volume of distilled water. To 10mls of the diluted solution 5N. sodium hydroxide was added until the solution was alkaline to phenol phthalein. The solution was heated to 80°C to hydrolyse the inner ester and more sodium hydroxide was added until the solution remained neutral. It was then diluted to 94mls with distilled water.

0.33M sodium d l-lactate.

5mls of 50% BDH lactic acid was titrated with sodium hydroxide until the PH = 8.0. The solution was then diluted to 140mls with distilled water.

2×10^{-2}M Diphosphopyridine nucleotide. (DPN)

26.6 mgms DPN (supplied by Böehringer) were dissolved in 1.0ml distilled water. N. sodium hydroxide was added until the PH = 6.0 and the solution was diluted to 2 mls and stored at 5°C.

1×10^{-2}M DPN.

A stock solution was made up by dissolving 6.65 mgms/ml DPN in barbiturate buffer PH 8.0. The solution was diluted to a 9×10^{-4}M solution for use.

0.1M glycine buffer PH 10.0

0.75 gms 'BDH Reagent Grade' glycine was dissolved up in 95 mls distilled water and the PH adjusted to PH 10.0 with 5N. sodium hydroxide. The volume was made up to 100 mls.

0.1M barbiturate buffer PH8.6

87.1 mls 0.1M 'Analar' sodium barbiturate and 12.9 mls 0.1M hydrochloric acid were mixed and the PH was adjusted to 8.6.

0.1M phosphate buffer PH 7.8

90 mls 0.1M 'Analar' sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$) and 10 mls 0.1M sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) were mixed and the PH adjusted to 7.8.

Optical density measurements were carried out in a 'Unicam' S.P.500 instrument.

Preparation of human liver lactic dehydrogenase.

The human liver lactic dehydrogenase used in these experiments was prepared by the method of Gibson et al (Gibson, Davisson, Bachhawat, Roy, Vestling 1953) by Dr. Lynda Kerr. The activity was assayed at each purification stage by the method of Gibson et al.

Assay of LDH prepared from human liver.

The LDH fractions obtained in the successive stages of purification were assayed by measuring the initial rate of formation of reduced diphosphopyridine nucleotide (DPNH) from diphosphopyridine nucleotide (DPN) by the enzyme preparation in the presence of lactate. The reaction was carried out in barbiturate buffer at room temperature and the rate of formation of DPNH was followed by measuring the change in optical density of the solution at 340 mμ over the first three minutes of the reaction at 30 second intervals.

The reaction mixture consisted of:-

1.0 ml 0.10M Barbiturate buffer PH 8.6
1.0 ml 0.33M Sodium d l-lactate
0.5 ml 9×10^{-4}M DPN
0.5 ml enzyme solution

The DPN was added at zero time and the optical density was read against a blank solution containing no enzyme. From the equation $\frac{d(DPNH)}{dt} = K = E/ml$ the activity was defined as the concentration per ml. of enzyme (E) which would effect a change in optical density of 0.005 unit in 5 seconds at the onset of the reaction when $\frac{0.005}{dt}$ was almost constant. The protein concentration (P) of the fractions was measured by the optical density at 280 mμ and the specific activities $\frac{(E)}{(P)}$ were calculated for each purification stage.

Electrophoretic analysis of human liver LDH.

A solution of purified human liver LDH prepared by the method of Gibson et al (1953) containing 1.2 mgms/ml protein was analysed on cellulose acetate paper at PH 8.6 in barbitone buffer. 10 μml of solution was applied to the strip and a current of 5m amps at 115 volts was applied for 1.75 hour. The strip was then cut longitudinally into two portions. One portion was stained with Lissamine green dye and the other portion was cut horizontally into three zones comprising, the origin ie. the line of application of the solution, the zone on the anode side of the origin and the zone on the cathode side of the origin. The protein was eluted from all three bands by barbitone buffer and the LDH activity was estimated on 0.5 ml aliquots by the method of Gibson et al (1953). The dye was eluted from the bands of the stained strip by 60% acetic acid and the percentage of the total protein present in each band was calculated from the optical densities at 630 mμ (N.B. This analysis was carried out by Dr. Lynda Kerr by the method of Kohn (1960) described later in this section).

Estimation of lactic dehydrogenase activity in human sera.

The method used for estimation of LDH levels in normal and pathological human sera was a modification of that used by Zimmerman and Weinstein (1956). It was based on the reduction of DPN in the presence of excess lactate by the serum at PH 10.0. The DPNH produced in a given time was measured by the change in optical density at 340 mμ

The reaction mixture consisted of:-

0.30 ml 0.1M glycine buffer PH 10.0
0.40 ml 0.5M sodium d l-lactate.
0.02 ml 2×10^{-2}M.DPN.
0.02 ml serum.

The DPN was added at zero time and the solutions were incubated at 37°C for 30 mins. The reaction was terminated by plunging the tubes into boiling water for exactly 3 mins. After cooling the optical densities of the solutions were read against a no substrate blank solution at 340 mμ.

The LDH activity was represented by the amount of DPNH produced in a system containing lactate minus the amount in a system without lactate. The moles DPNH formed were calculated from the measured optical density with the use of the molar extinction coeficient for DPNH. (Horecker and Kornberg 1948). The LDH activity of the serum was expressed as μmoles DPNH released per 100 mls serum and calculated from the expression:-

$$\log \frac{I}{I_0} = ecl \text{ moles/ml}$$

$$c = \frac{\text{optical density} \times 10^6}{6.22 \times 10^6} \ \mu \text{ moles/ml}$$

$$c = \frac{\text{optical density} \times 5000}{6.22} \ \mu \text{ moles DPNH/100 mls serum}$$

where $e = 6.22 \times 10^6$

c = concentration

l = length of light path.

<u>Inhibition test for antilactic dehydrogenase.</u>

The activity of a solution of purified LDH prepared from human liver by the method of Gibson et al (1953) was assayed by the method of Zimmerman and Weinstein (1956). The LDH levels in the sera of 8 patients with hepatocellular lesions were estimated. Equal volumes of enzyme solution and serum, diluted or undiluted, were incubated together for 2 hours at 37°C and placed in the fridge at 5°C overnight. The LDH activity of the final mixture was then estimated and compared with the theoretical value. The percentage error for the optical density readings was ± 2% and this was taken into account when comparing experimental and theoretical values.

If LDH activity of the enzyme = E units

and LDH activity of the serum = S units

Theoretical activity of a mixture containing

equal volumes of E and S = $\frac{E+S}{2}$ units.

In each case this value, $\frac{E+S}{2}$, was compared with the value obtained experimentally.

(2) Immunological investigation of autoantibodies.

REAGENTS.

Human Group O Rhesus negative blood.

This was collected in an acid citrate-dextrose solution (1 volume citrate-dextrose : 5 volumes blood) and supplied weekly by the Scottish National Blood Transfusion Association, Royal Infirmary, Edinburgh.

Tannic Acid.

A 1% solution of BDH tannic acid powder was made up daily in 0.9% sodium chloride and diluted to 1:40,000 with phosphate buffered saline Ph 8.0.

0.15M phosphate buffered saline PH 8.0.

1132.8 mls 0.15M sodium phosphate (anhydrous); 67.2 mls 0.15M potassium dihydrogen phosphate and 1200 mls 0.15M sodium chloride were mixed and the PH adjusted to 8.0 with M. sodium carbonate.

Human liver lactic dehydrogenase.

This was prepared by Dr. Lynda Kerr in this laboratory by the method of Gibson et al (1953).

Human serum Cohn FII

Supplied by the Scottish National Blood Transfusion Association (S.E. Region laboratory), Royal Infirmary, Edinburgh, through the courtesy of Dr. Cumming.

Bovine serum Cohn FII

Supplied by Armours Laboratories Ltd.

METHODS.

The investigation of antibodies in the sera of patients suffering from acute and chronic hepatocellular lesions was carried out by a modification of the haemagglutination technique of Boyden (1951) as described by Heller et al (1955).

The haemagglutination reaction.

The mechanism of this reaction depends on the agglutination of red blood corpuscles, coated or "sensitized" with a given protein antigen, by sera containing a specific antibody to, or an antibody capable of reacting with the sensitizing protein. The red cells are tanned by treatment with a dilute solution of tannic acid in order to render them receptive to the sensitizing antigen. These tanned and sensitized corpuscles are then added to serial dilutions of the test sera and incubated at 37°C. Agglutination of the sensitized cells occurs when an antibody capable of combination with the antigen on the surface of the cell is present in sufficiently high concentration in the serum.

The main advantage of the haemagglutination technique is its great sensitivity. It has been shown (Stavitsky 1954) to be more sensitive to the presence of antibody than the precipitin reaction (Martin 1943), the complement fixation reaction (Stavitsky, Stavitsky and Ecker 1949) and the agglutination of collodion particles (Cannon and Marshall 1946). On account of this sensitivity, however, particular attention must be paid to control reactions in which negative sera and positive sera of known titre must be included to ensure that non-specific agglutination does not occur.

The haemagglutination technique is capable of detecting non-precipitating antibodies (Borduas and Grabar 1953) in sera /

sera when the antibody is present in too low a concentration for a visible precipitin reaction or when the low avidity and non-precipitability of sera is due to a physical characteristic of the antibody, such as high solubility, when the solubility of the particulate antigen-antibody complex may be less than that of the soluble antigen-antibody complex and agglutination is favoured by conversion of the antigen to a particle.

METHOD

Human group O Rhesus negative blood cells were washed four times with 0.9% sodium chloride solution and once with phosphate buffered saline PH 8.0. The cells were suspended in buffered saline to give a 33$^{1}/_{3}$% suspension. 2 volumes of a 1:40,000 solution of tannic acid was added per 1 volume of packed red cells, the mixture was agitated and incubated at 38°C for 10 mins. The cells were washed twice with buffered saline and reconstituted to a 33$^{1}/_{3}$% suspension. 2 volumes of the sensitizing protein solution was added for each volume of cell suspension. The concentration of the sensitizing protein varied from 1-2 mgms/ml. The mixture was then incubated at 38°C for 30 mins, after which the cells were washed three times with buffered saline. The cell suspension was made up to 0.5% with buffered saline.

SERA:- Sera from patients suffering from a variety of hepatocellular lesions were tested. Positive control sera of known titre, from patients suffering from rheumatoid arthritis, and a Coombs antihuman globulin serum of known titre were used. Negative control sera were supplied by the Scottish National Blood Transfusion Association, Edinburgh and laboratory volunteers.

<u>ANTIGENS</u>:- (i) Human liver LDH in concentrations varying from 1.0 - 1.5 mgms/ml.

(ii) Human serum γ-globulin (Cohn FII) in concentrations of 1.0 - 1.5 mgms/ml.

(iii) Bovine serum γ-globulin (Cohn FII), partially denatured by heating a 1% solution in buffered saline at 63°C for 10 mins, and used in a concentration of 2mgms/ml.

<u>Titration</u>.

The sera were diluted 1 in 10 to 1 in 10,240 with buffered saline PH 8.0 in the cups of 'Salk' perspex plates. 0.5ml aliquots of the sensitized red cell suspension were added to 0.5 ml of the diluted sera, the plates were incubated at 38°C for 1 hour and kept in the refrigerator at 5°C overnight. The titres were read by placing the plates on a sheet of white paper. Maximal agglutination was characterized by a thin layer of cells forming a complete mat over the surface of the cup. In some very strongly agglutinating sera the edges of the layer of cells tended to curve inwards. The titre of the serum was recorded as the reciprocal of the highest dilution of the serum in which visible agglutination occured, negative agglutination being characterized by a small clump of cells in base of the cup.

The sensitized sheep cell test was carried out on cirrhotic and infective hepatitis sera by the method of Heller, Kolodny, Lepow, Jacobson, Rivera and Marks (1955). This test depends on the agglutination of sheep cells sensitized with rabbit sheep erythrocyte antibody by sera containing antibodies to the rabbit antibody γ-globulin.

(3) Immunization of rats with rat liver LDH.

Rats were injected with purified preparations of rat liver LDH prepared by the method of Gibson et al (1953) from the livers of rats of the same strain. Antibodies to rat liver LDH were sought in the sera of these rats by biochemical and immunological methods.

REAGENTS.

Adjuvant: contained arosil oil and killed tubercle bacilli in liquid paraffin. It was mixed to an emulsion with the LDH solution before use.

Heparin: Supplied by Evans Medical Suppliers Ltd. A solution containing 1mgm/ml in 0.9% sodium chloride was used.

METHODS.

Preparation of rat liver lactic dehydrogenase.

Rat liver LDH was prepared from the livers of male and female white rats by the method of Gibson et al (1953).

Estimation of LDH in rat serum.

The LDH levels in rat sera were estimated by the method of Zimmerman and Weinstein (1956). Great care had to be taken in order to obtain unhaemolysed rat blood as rat red blood corpuscles tended to be more fragile than human corpuscles. Unhaemolysed sera were obtained successfully by sharply cutting off the tip of the tail and dipping the cut end into a solution of heparin in order to prevent clotting. The blood was then collected dropwise in small polythene tubes. The LDH activity of the rat sera was estimated before and after the course of injections.

Injection of rats.

Two groups of white rats were used. One group was injected with a solution of purified rat liver LDH alone and the other group with LDH plus adjuvant.

Group I.

Three male white rats were anaesthetized with the minimum amount of ether and each was given an injection of 0.2 ml rat liver LDH, containing 5 mgms/ml protein in saline, in the right femoral vein. The following day each rat was given an intramuscular injection of 0.1 ml enzyme solution containing 1 mgm/ml protein. These intramuscular injections were repeated daily for a period of 8 days and subsequently every third day for fourteen days. The rats were then bled and the sera were tested for antibodies to rat liver LDH.

Group II

Three female white rats were anaesthetized with ether and injected subcutaneously in four sites with a solution of LDH containing 5 mgms/ml protein in adjuvant. The enzyme and adjuvant were mixed to a stiff, white, milky emulsion containing arosil oil, killed tubercle bacilli in liquid paraffin and enzyme solution in the proportion by volume of 2:2:1. The emulsion was injected by means of a Lewer No.20 needle.

Two control rats were injected with adjuvant plus saline. After a period of three weeks each rat was given an intravenous injection of LDH solution containing 2.4 mgms protein into the right femoral vein. After a further two weeks the rats were given intramuscular injections of 0.1 ml LDH solution containing 6 mgms/ml of protein. These intramuscular injections were repeated every third /

third day for eighteen days.

Test for antilactic dehydrogenase activity in the sera of the injected rats.

0.25 ml of purified rat liver LDH containing 0.02 mgms/ml of protein was mixed with 0.25 ml rat serum diluted 1 in 3 with saline. The mixture was incubated at 37°C for 2 hours and placed in the refrigerator overnight. The solution was then diluted 1 in 100 with saline. A solution of enzyme of the same final concentration was made up and the LDH activity of both solutions was estimated by the method of Zimmerman and Weinstein (1956). The LDH activity of the serum in the mixture was ignored as this had been diluted 1 in 600 and any activity would therefore be negligible.

Test for agglutination of LDH sensitized red cells.

The sera of the injected rats were tested for antibodies to rat liver LDH by a modification of the Boyden haemagglutination method (1951). Human group O Rhesus negative erythrocytes were tanned and sensitized with a solution of rat liver LDH containing 1 mgm/ml of protein.

(4) Physical and chemical characterization of the antiglobulin in sera of patients suffering from hepatocellular lesions.

REAGENTS.

Cellulose acetate paper.

Supplied by 'Oxoid' Ltd., London.

Lissamine Green dye.

A 2% solution of 'Gurrs' Light green powder was made up in 6% sulphosalicylic acid.

0.1M Barbitone buffer PH 8.6

12.5 gms 'Analar' sodium barbitone, 8.125 gms sodium acetate, 8.75 mls 0.99N. hydrochloric acid were dissolved in 1000 mls distilled water and the volume made up to 2000 mls.

Starch-Hydrolysed.

Supplied by the Connought Medical Research Laboratories, University of Toronto, Canada.

Batch Number: 139 was used.

Potato starch.

Unhydrolysed potato starch was supplied by British Drug Houses.
About 40 gms were washed with 250 mls acetone in a measuring cylinder. The suspension was allowed to settle for 2 mins and the supernatant poured off. This was repeated twice and the large starch grains so obtained were filtered free of excess acetone in a Buchner filter. The acetone was completely removed by evaporation at 50°C overnight.

Borate buffer PH 8.48.

(1) Electrophoresis buffer: consisted of 0.023M 'Analar' Boric acid and 0.0092M 'Analar' sodium hydroxide. 2.8446 gms boric acid and 0.7360 gms sodium hydroxide were dissolved in 2000 mls distilled water, and the PH was adjusted to 8.48 with conc. hydrochloric acid or conc. /

conc, sodium hydroxide.

(2) Electrode compartment buffer: contained 2% sodium chloride in the above borate buffer.

(3) Bridge buffer: contained 14.223 gms boric acid and 1.848 gms sodium hydroxide in 1000 mls distilled water and the PH was adjusted to 8.48.

Amido Svartz 10B.

Supplied by "Bayer" Leverkusen.

A saturated solution was made up in a methanol - water - glacial acetic acid solvent in the proportion by volume (50:50:10.)

Polyvinyl pyrrolidone (MW = 11.000)

was supplied by L. Light & Co.Ltd., Colnbrook, England.

A 15% solution in 0.9% sodium chloride was used.

0.1M Thioglycollic acid.

A 0.2M solution of 'BDH Reagent grade' thioglycollic acid solution was made up in phosphate buffered saline. Equal volumes of this solution and serum were mixed so that the final concentration was 0.1M.

0.5M cysteine.

A 0.5M solution of 'BDH Reagent grade' cystein - hydrochloride was made up in distilled water. Conc. sodium hydroxide was added until the PH of the solution equalled 8.5 to an outside indicator.

0.02M iodoacetic acid.

Made up by dissolving 'BDH Reagent grade' iodoacetic acid crystals in 0.15M phosphate buffer PH 8.0.

Folin and Ciocalteaus Reagent.

Made up by diluting 'BDH Reagent grade' Folin and Ciocalteaus Reagent 1 in 3 with distilled water immediately before use.

Coombs antihuman globulin rabbit serum.

Supplied by The Wellcome Foundation, Burroughs Wellcome Ltd., Kent, England.

Preparation of serum euglobulin fraction.

The euglobulin fraction of serum is defined as that fraction which is insoluble in water in the absence of salts and is precipitated on reduction of the ionic strength of the serum by dialysis against distilled water (Green 1938) or by one third saturation of serum with ammonium sulphate.

Serum samples were diluted 1 in 5 with distilled water and dialysed against distilled water for 48 hours at 5°C with two changes of water. The precipitate which formed was centrifuged off, washed twice with distilled water and dissolved up in 0.9% sodium chloride to a volume 5 times that of the original serum. The euglobulin fraction and the supernatant serum were tested for antiglobulin activity.

Salt fractionation with ammonium sulphate.

This was carried out at 5°C at the PH of blood. Three fractions were prepared by adjusting the ammonium sulphate concentration to 34% and 46%. The fractions precipitated by 34% saturation, between 34% and 46% saturation and the remaining supernatant sera were thus obtained.

1.0 ml serum was made 34% saturated with respect to ammonium sulphate and left at 5°C for 30 mins. The protein precipitate was then centrifuged off at 0°C at 3000 rpm. The supernatant serum was removed and the precipitate was dissolved up in 5 mls of 0.9% sodium chloride. The protein was reprecipitated twice from this solution and finally dissolved up in 2 mls of 0.9% sodium chloride./

chloride.

The supernatant serum from the first precipitation was made 46% saturated with respect to ammonium sulphate, the precipitate was centrifuged off and reprecipitated as before. The remaining supernatant serum was stored.

All three serum portions were tested for antiglobulin activity in the Boyden haemagglutination test using bovine γ-globulin and analysed electrophoretically on cellulose acetate paper at PH 8.6.

Starch gel electrophoresis of serum.

Preparative electrophoresis of the serum of a patient (P), with a clinical and pathological diagnosis of hepaticcirrhosis, was carried out by a modification of the method of Smithies (1955). The electrophoresis was carried out in a continuous borate buffer system at PH 8.48.

(i) Preparation of gel.

500 mls of a suspension of hydrolysed starch, containing 12.3 gms per 100 mls of borate buffer PH 8.48, was made up in a 1 litre Pyrex conical filter flask. The flask was heated over a gauze on a Bunsen flame with continuous swirling. Care was taken not to remove the flask from the flame and the heating was continued until the starch grains had ruptured and the liquid gel was on the point of boiling. The flask was then removed from the flame, stoppered and the pressure in the flask was reduced by attaching the side arm to a water suction pump. On reduction of pressure the gel began to boil and vigorous boiling was continued for 30 seconds. The air-free gel was then immediately poured into a perspex tray of dimensions (5x12x0.5 inches), covered with a sheet of polythene, which had been lightly smeared with liquid paraffin, and the excess gel was removed by pressing/

pressing a heavy glass plate on top of the sheet. The gel was left to set for several hours until it reached room temperature. Gels were always used the same day.

(ii) Insertion of sample.

5 mls of serum was mixed to a thick suspension with washed, unhydrolysed potato starch grains. A slot 0.6 cms by 10 cms was cut out of the gel about 10 cms from the negative end by means of a sharp scalpel and the serum-starch suspension was poured into the cavity. The slot was covered by means of a piece of perspex and the whole gel surface was lightly smeared with a film of liquid paraffin and a sheet of polythene.

(iii) Electrode vessels and filter paper connections.

The electrode vessels consisted of two compartments A and B. The A chambers contained borate buffer PH = 8.48 and the B chambers contained the same buffer plus 2% sodium chloride. Thick wads of filter paper soaked in the borate bridge buffer made the connections between chambers A and B and the A chambers and ends of the gel. A piece of narrow bore glass tubing connected the two A chambers.

(iv) Electrical connections.

A direct current of 40-45 m amps at a voltage of about 200 volts was applied for 22 hours. The gel was cooled during this time by circulating tap water in a coil of dialysis tubing which completely covered the surface of the gel.

(v) Staining of the gel.

At the end of the electrophoretic run the gel was removed and blotted free of excess oil. A strip 3 cms in width was cut longitudinally from one edge of the gel and sliced horizontally along its length by means of a taut wire saw supported on the edges of a perspex tray/

tray half the depth of the gel. One half of the sliced gel was stained by placing it in a saturated solution of Amido Svartz 10B in a methanol -water - glacial acetic acid solvent (50:50:10) for 2 minutes. Excess dye was then eluted by repeated washing with the methanol - water - glacial acetic acid solvent. The gels were preserved for periods of several months in this solvent. Before staining the gel strip was cross-hatched at 1 cm intervals along its length for comparison with the unstained section of the gel.

(vi) Elution of protein.

The remainder of the unstained starch block was sliced across its longitudinal axis into 1 cm zones. The protein was eluted from each segment by molar sodium chloride. 5 mls M.Nacl were added to each segment in a test tube and homogenized by hand with a mushroom ended glass rod. The pulp was then filtered through two layers of Whatman number 1 filter paper in a small Buchner filter funnel into a 100 ml filter flask. The pulp was returned to the tube and re-extracted with another 5 mls M.Nacl and re-filtered and the two filtrates were combined. A small, constant volume of each eluate was removed for protein estimations. The remaining zone eluates were concentrated to 0.6 ml by dialysis against 15% polyvinyl-pyrrolidone in 0.9% Nacl at 5°C. The concentrated solutions so obtained were centrifuged in order to remove any starch particles present. The clear supernatant solutions were tested for anti-globulin activity with bovine γ-globulin sensitized red cells and analysed electrophoretically on cellulose acetate paper at PH 8.6.

(vii) Estimation of protein in zone eluates.

This was carried out on 0.5 ml aliquots of the starch gel eluates with the Folin-Wu-Ciocalteau phenol reagent (Wu 1922).

To 0.5 ml of eluate 1.5 ml of 12.5% sodium carbonate was added /

added and the solutions were left at room temperature for 1 hour. 0.25 ml of Folin and Ciocalteaus reagent, diluted 1 in 3 with distilled water, was then added and the solutions were left for a further 30 mins. The optical density of the blue colour which developed was read against a no-protein reagent blank at 650 mμ. A plot of protein concentration (obtained from a calibration curve) and antiglobulin titre against zone number was made.

Electrophoretic analysis on cellulose acetate paper.

This was carried out by the method of Kohn (1960) to characterize the ammonium sulphate fractions of serum and the concentrated starch gel fractions.

(i) Apparatus

The electrophoresis tank used in these experiments consisted of two parallel perspex electrode vessels, one-third filled with 0.1M barbital buffer PH 8.6 and connected by means of a piece of narrow polythene tubing. Two platinum-mercury electrodes made contact at either end of the electrode vessels by means of glass funnels plugged with filter paper and two-thirds filled with buffer. Each electrode vessel was covered by a perspex lid with two narrow slots along its length. Strips of filter paper were allowed to dip into the buffer in electrode vessels through these slots and the edges of the saturated filter paper were pressed firmly down onto the edges of a central platform. Six 1 x 5 inch 'Oxoid' cellulose acetate paper strips, which had been soaked in buffer for several hours between porcelain plates and blotted free of excess buffer, were stretched over the central platform and supported at either end by the filter paper wicks dipping into the electrode vessels. Edge effects were minimized by placing two narrow strips of cellulose acetate paper at the extreme ends of the tank. The electrode /

electrode vessels and paper strips were then covered by means of a double celluloid lid, which was firmly screwed into position. A current of 12 m amps was applied for a period of 10 mins to allow equilibration of the system.

(ii) Insertion of sample.

The current was switched off and a narrow slot in the celluloid lid was exposed by removal of an adhesive tape. About 10μ ml of serum or serum fraction was applied in a narrow band on the strip about ¼ of the distance from the cathode end by means of a narrow capillary tube and allowed to dry. If the protein solution to be applied was dilute a second or third application was made. The slot was then covered by the adhesive strip and a current of 12 m amps was applied for 2.5 hours.

(iii) Staining of the strips.

At the end of the run the strips were removed from the tank and placed in a solution of Lissamine Green dye for approximately 2 hours. The excess dye was removed by several washings with 5% acetic acid. The strips were then dried between sheets of filter paper in a heavy press.

Ultra centrifuge fractionation of serum.

0.8 ml diluted serum (2 vols serum diluted with 1 vol saline) of patient (P) was fractionated in a partition cell by centrifuging at 59,780 r.p.m in an Analytical Ä rotor for 64 mins until all the 19S macroglobulin components had completely sedimented in the lower cell, leaving most of the light 7S γ-globulins, α and β globulins and albumin in the upper cell. The sedimented macromolecular components in the lower cell were dissolved up in 0.7 ml 0.9% sodium chloride. This solution, the supernatant serum in the upper cell and the unfractionated serum were tested for antiglobulin activity.

Treatment of sera with sulphydryl reagents.

The 19S antibodies, like the 19S proteins in general, (Dungern 1900) are very sensitive to sulphydryl reagents which cause dissociation into smaller units with marked loss of agglutinin activity. Fudenberg and Kunkel (1958) reported that there was a loss in agglutinin activity of the cold agglutinins of acquired haemolytic anaemia, the heterophile antibodies and the rheumatoid macroglobulin on treatment with these reagents. Similar treatment of the 'warm antibodies' of acquired haemolytic anaemia and the incomplete Rh_o antibodies resulted in little or no loss of activity. Fudenberg and Kunkel (1957) showed that when the dissociation of the cold agglutinins of acquired haemolytic anaemia, by treatment with mercapto-ethanol or cysteine, was followed in the ultracentrifuge low molecular weight proteins were produced. With this physical dissociation a concomitant loss of serological activity was also observed. Deutch and Morton (1957) showed that addition of sulphydryl reagents to macroglobulinemic sera resulted in the conversion of the 19S macro-globulins into a molecular entity possessing a sedimentation constant near that of the usual 6.5S γ-globulin on ultra centrifuge analysis. After treatment of the sera with mercaptoethanol the sulphydryl compound was removed by dialysis against phosphate buffer or phosphate buffer containing iodoacetate. They found that removal of the mercaptoethanol by dialysis against buffer resulted in the reaggregation of the macromolecules to a certain extent whereas removal of the sulphydryl reagent by dialysis in the presence of iodoacetate, a sulphydryl group blocking reagent, prevented the reassociation of the subunits. This indicated that depolymerization of the macro-globulins was the result of disruption of the disulphide bonds, /

bonds, the presumed site of action being the disulphide bonds bridging the units comprising the 19S macroglobulin.

METHODS

Sera from patients suffering from infective hepatitis and hepatic cirrhosis possessing agglutinin activity were treated with:-

(i) 0.5M cysteine.

(ii) 0.1M thioglycollic acid.

Treatment with 0.5M cysteine.

A 0.5M solution of cysteine hydrochloride was brought to PH 8.5 by the addition of conc. sodium hydroxide. Serum samples were diluted 1 in 5 with phosphate buffered saline PH 8.0 and dialysed against a solution of 0.5M cysteine for 18 hours at 5°C. The sera were then tested for antiglobulin activity.

Treatment with 0.1M thioglycollic acid.

Equal volumes of 0.2M thioglycollic acid in phosphate buffered saline PH 8.0 and serum diluted 1 in 5 were mixed and left at 5°C for 48 hours with occasional mixing. The solutions were then divided into two parts. One part was dialysed against 0.15M phosphate buffer PH 8.0 and one part was dialysed against 0.02M iodoacetate in phosphate buffer for 36 hours at 5°C. The sera were tested for antiglobulin activity. Control sera included sera from patients suffering from rheumatoid arthritis and a rabbit antihuman globulin serum.

Heat stability of antiglobulin.

Antibodies, like other proteins, are inactivated by heat and possess a high heat of activation (Boyd 1954). The complement fixing antibodies described by Gajdusek (1958) were reported to be stable at 56°C with increasing instability above 60°C.

METHOD

Sera from patients with hepatocellular lesions were diluted 1 in 10 with 0.15M phosphate buffered saline and heated for 30 mins at :-

(i) 55°C

(ii) 60°C

(iii) 65°C

The antiglobulin activity was tested in heated and unheated sera.

PART I

RESULTS.

I Biochemical investigation of antibodies in the sera of patients suffering from hepatocellular lesions.

(1) Electrophoretic analysis of human liver LDH. on cellulose acetate paper at PH 8.6

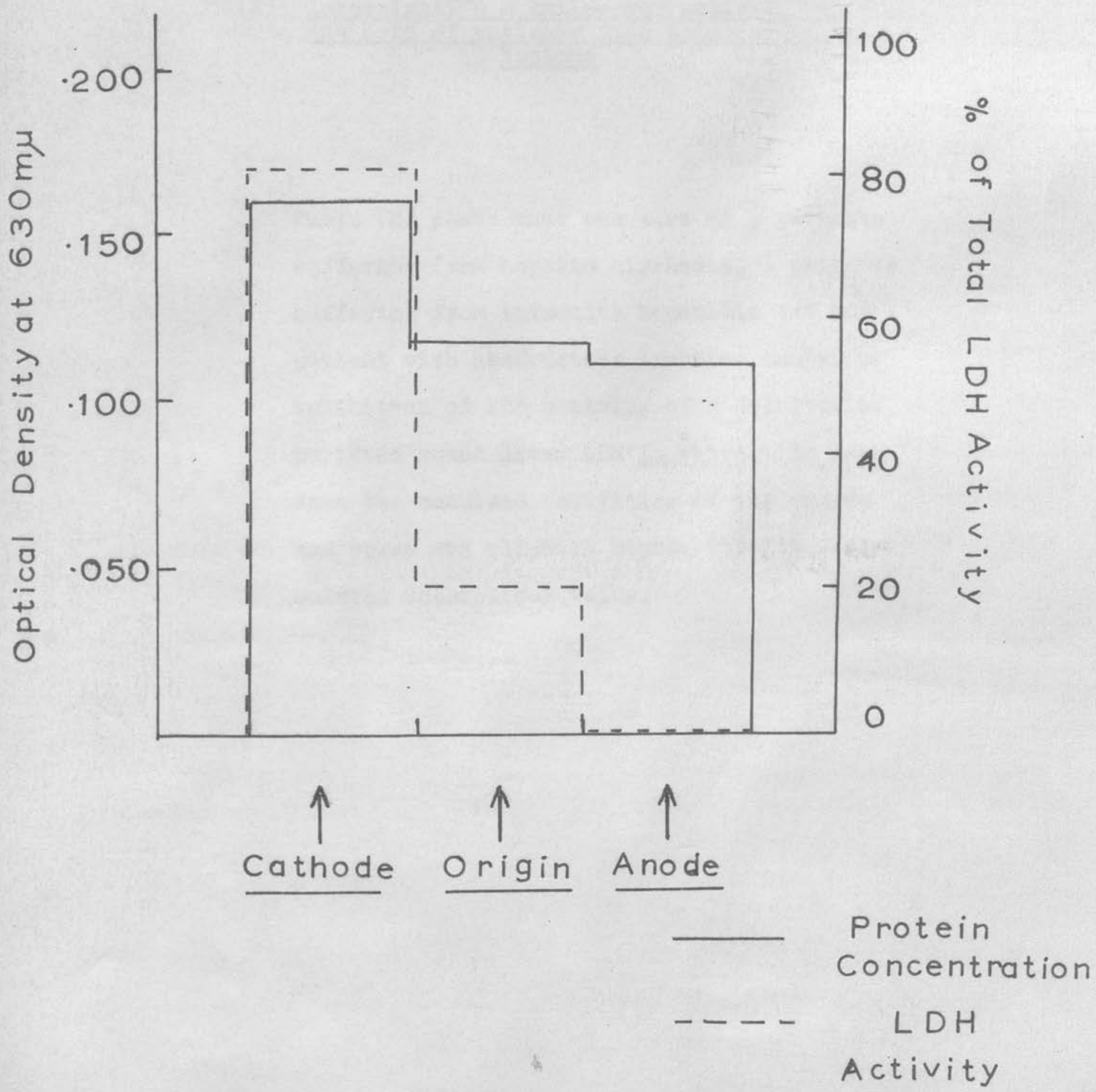
Table (1) shows that on electrophoresis of a solution of purified LDH, of protein concentration 1.2 mgms/ml and activity 10×10^3 units, 100% of the enzyme activity was associated with 67% of the total protein and migrated with the mobility of γ-globulin at PH 8.6.

TABLE (1)

ZONE	% of TOTAL PROTEIN	% of TOTAL LDH ACTIVITY
Cathode	39	80
Origin	28	20
Anode	27	0

Electrophoretic mobility of

Lactic Dehydrogenase at PH 8·6



(2) Investigation of antienzyme activity in the sera of patients with hepatocellular lesions

Table (2) shows that the sera of 4 patients suffering from hepatic cirrhosis, 3 patients suffering from infective hepatitis and one patient with obstructive jaundice caused no inhibition of the activity of a solution of purified human liver LDH in vitro. In each case the combined activities of the enzyme and serum was slightly higher than the calculated theoretical value.

TABLE (2)

Clinical Diagnosis	Conc.of LDH mgms/ml	Units of Activity of LDH	Units of LDH Activity of Serum	Combined LDH Activity of Enzyme Serum	Theoretical LDH Activity of Enzyme + Serum	% Inhibition of LDH Activity
Hepatic cirrhosis case (1)	0.140	310	352	409	331	0
Hepatic cirrhosis case (2)	0.090	251	612	721	432	0
Hepatic cirrhosis case (3)	0.090	251	357	337	304	0
Hepatic cirrhosis case (4)	0.090	251	630	563	440	0
Infective hepatitis case (1)	0.020	837	664	867	750	0
Infective hepatitis case (2)	0.001	430	426	480	428	0
Infective hepatitis case (3)	0.001	430	794	676	612	0
Obstructive jaundice case (1)	0.001	430	365	445	398	0

II <u>Immunological investigation of antibodies in the sera of patients suffering from acute and chronic hepatocellular lesions.</u>

Table (3) shows that the sera of 7 patients with a clinical diagnosis of hepatic cirrhosis and 2 patients with infective hepatitis agglutinated, to low titres, human erythrocytes sensitized with purified human liver LDH whereas the sera from 11 normal laboratory control subjects caused no agglutination.

Table (4) shows that the sera of 3 patients with a clinical diagnosis of hepatic cirrhosis and one patient with a diagnosis of infective hepatitis agglutinated, to low titres, erythrocytes sensitized with human serum Cohn FII (γ -globulin) whereas again the sera from 11 normal control subjects gave negative titres.

Table (5) shows that the sera of 6 patients with a clinical diagnosis of hepatic cirrhosis and of 5 patients with a diagnosis of infective hepatitis gave positive titres with bovine serum Cohn FII (γ -globulin) sensitized erythrocytes. The sera from 5 blood donor control subjects gave negative titres.

Table (6) compares the titres obtained by the sera of patients suffering from hepatocellular lesions with bovine FII sensitized human erythrocytes and sensitized sheep cells. In this test a titre of 0-64 was considered negative.

TABLE (3)

Clinical Diagnosis	Number of Sera Tested	Number with Titres between 20 and 160	Number with Negative Titres
Hepatic cirrhosis	9	7	2
Infective hepatitis	3	2	1
Normal controls	11	0	11

TABLE (4)

Clinical Diagnosis	Number of Sera tested	Number with Titres between 20 and 160	Number with Negative Titres
Hepatic cirrhosis	6	3	3
Infective hepatitis	2	1	1
Normal controls	11	0	11

TABLE (5)

Clinical Diagnosis	Number of Sera Tested	Number with Titres between 20 and 640	Number with Titres between 1280 and 10,240	Number with Negative Titres
Hepatic cirrhosis	7	2	4	1
Infective hepatitis	6	3	2	1
Normal controls	5	0	0	5

TABLE (6)

Clinical Diagnosis	Titre with Bovine γ-Globulin-sensitized Human Cells	Titre with Sensitized Sheep Cells
Hepatic cirrhosis (case lw)	320	64
Hepatic cirrhosis (case (P))	5120	64
Hepatic cirrhosis (case (E))	320	64
Hepatic cirrhosis (case (M))	10,240	64
Hepatic cirrhosis (case D)	2560	128
Infective hepatitis (case L)	40	64
Infective hepatitis (case T)	1280	64

III Physical and chemical characterization of antiglobulin.

(1) Activity of euglobulin fraction of serum.

Table (7) shows that in 4 or the 5 cases of hepatic cirrhosis and in the 2 cases of infective hepatitis not more than 17% of the agglutinin activity of the serum was associated with the euglobulin fraction. In one patient (case D), 50% of the agglutinin activity was found in the euglobulin fraction. It will be observed that there was partial loss of activity of the serum on dialysis and the activity of the euglobulin fraction is expressed as the % of the total remaining activity.

(2) Activity of ammonium sulphate fractions.

From table (8) it is seen that over 90% of the agglutinin activity in the serum of a patient (P) with a clinical and pathological diagnosis of hepatic cirrhosis, was found to be associated with the fraction precipitated by 34% saturation with ammonium sulphate. Plate (1) shows that this fraction migrated with the mobility of γ-globulin on cellulose acetate paper at PH 8.6.

TABLE (7)

Clinical Diagnosis	Titre of Serum	Titre of Euglobulin Fraction	Titre of Supernatant Serum	% of Total Activity Associated with Euglobulin Fraction.
Hepatic cirrhosis (case P)	5120	544	2560	17
Hepatic cirrhosis (case C)	1280	68	640	10
Hepatic cirrhosis (case M)	10,240	160	2560	6
Hepatic cirrhosis (case D)	5120	320	320	50
Hepatic cirrhosis (case Mo.)	320	0	160	0
Infective hepatitis (case T)	1280	10	160	3
Infective hepatitis (case S)	320	0	80	0

TABLE (8)

Serum Protein Fraction Tested	Titre
Serum (case P)	5120
Euglobulin fraction	544
Supernatant serum	2560
Fraction precipitated by 34% saturation with ammonium sulphate	2560
Fraction precipitated between 34% and 46% saturation with ammonium sulphate	160
Supernatant serum	20

(3) Analysis of starch gel electrophoresis fractions.

Tables (9) and (10) and graph (1) show the protein and activity distribution obtained on electrophoresis of the serum of a patient (case P) with a clinical and pathological diagnosis of hepatic cirrhosis.

It is observed that the antiglobulin activity in this serum was found to be associated with two protein fractions; one of which migrated with the mobility of a slow γ-globulin and the second migrated in the fast γ-globulin region at PH 8.48.

Plate (1) shows that on electrophoretic analysis of these fractions on cellulose acetate paper at PH 8.6, zone (3) containing the first activity peak migrated with the mobility of a slow γ-globulin and zone (6) containing the second activity peak migrated with the mobility of a fast γ-globulin bordering on the β-globulin area.

TABLE (9)

Zone Number	Protein Concentration in mgms/ml
1	0.090
2	0.025
3	0.250
4	0.430
5	0.450
← ORIGIN	
6	0.210
7	0.120
8	0.090
9	0.120
10	0.100
11	0.090
12	0.070
13	0.080
14	0.110
15	0.110
16	0.109
17	0.170
18	0.270
19	0.268
20	0.310
21	0.190
22	0.310
23	0.190
24	0.160
25	0.090
26	0.060

TABLE (10)

Zone number	Titre
Unfractionated serum (case P)	2560
1	0
2	0
3	160
4	20
5	80
←ORIGIN	
6	320
7	80
8	40
9	0
10	0

GRAPH (1)

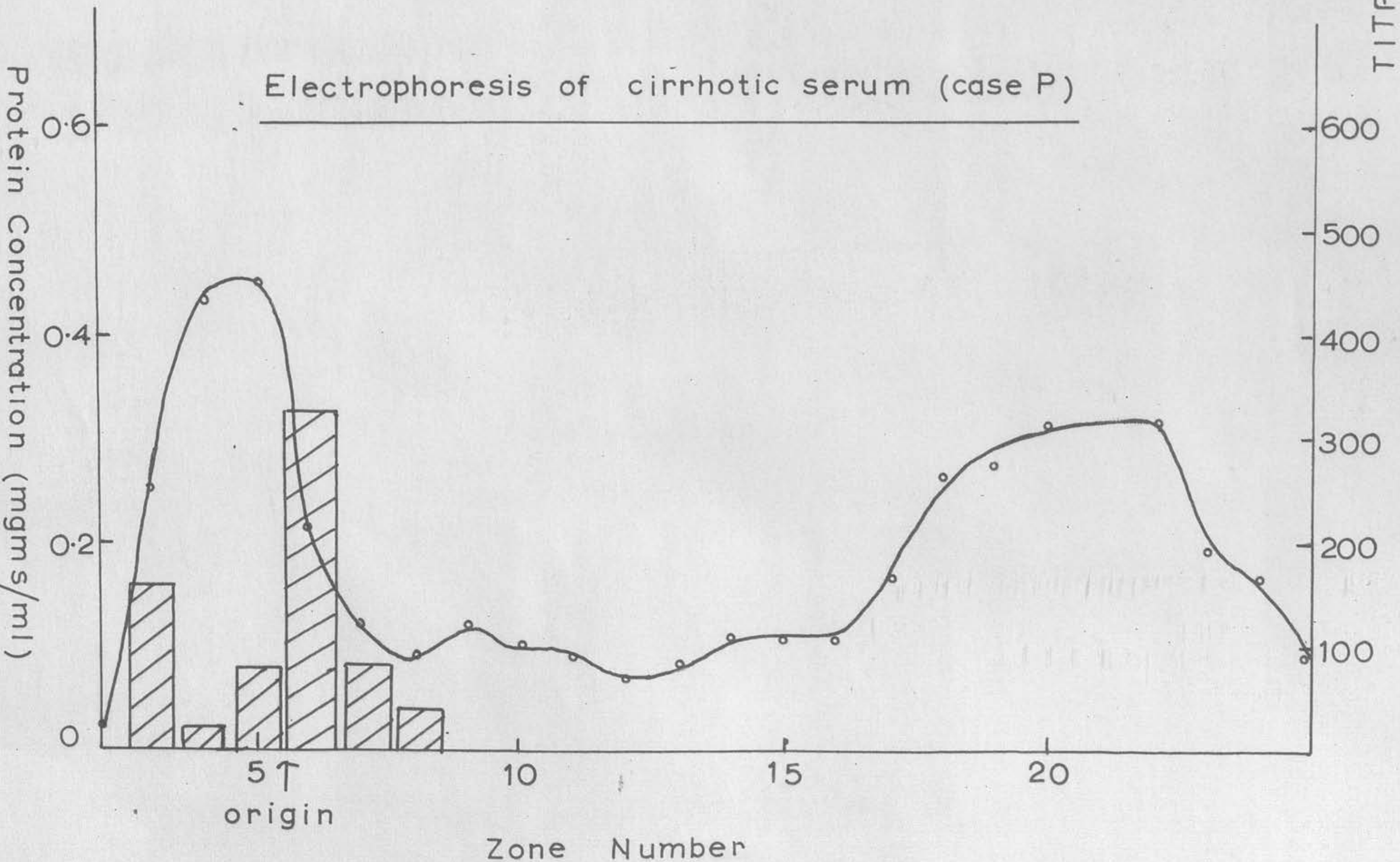
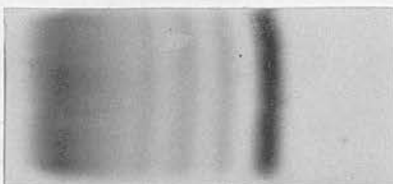


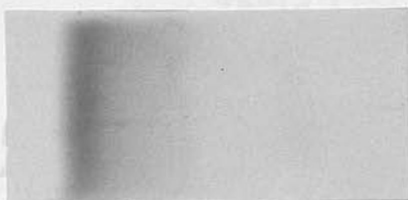
PLATE (1)

Electrophoretic analysis of ammonium sulphate and starch gel fractions of serum (case P) on cellulose acetate paper at PH 8.6.

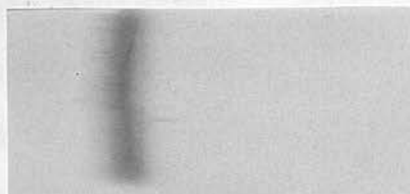
Serum.



34% Ammonium sulphate fraction

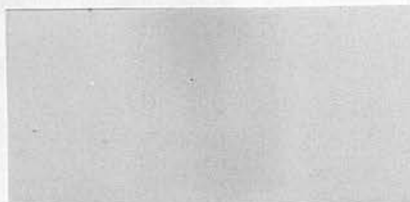


Starch gel zone (5)

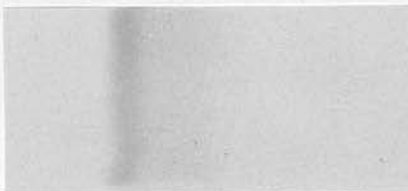


34% to 46% Ammonium sulphate fraction

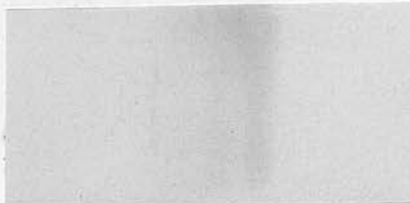
Starch gel zone (6)



Starch gel zone (3)



Starch gel zone (7)



Starch gel zone (4)

Starch gel zone (8)

(4) Activity of ultra centrifuge fractions.

Table (11) gives the antiglobulin activity of the upper and lower cell fractions obtained by ultra centrifuge fractionation of the serum of a patient (case P) with a clinical and pathological diagnosis of hepatic cirrhosis. It appears that all the agglutinin activity is associated with the macroglobulin fraction in the lower cell, the upper fraction being totally inactive. There had been some loss of activity on centrifugation.

TABLE (11)

Serum Fraction Tested	Titre
Unfractionated serum (case P)	2560
Lower cell fraction containing all 19S macroglobulin	640
Upper cell fraction	0

(5) Action of sulphydryl reagents.

Table (12) shows that treatment of the sera of 5 cases of hepatic cirrhosis and 3 cases of infective hepatitis, with 0.1M thioglycollic acid, resulted in the loss of over 90% of the agglutinin activity. It is also observed that in cases P and C there was partial regeneration of activity on removal of the thioglycollic acid by dialysis against phosphate buffer.

Similar treatment of rheumatoid arthritis serum also resulted in over 90% loss in activity but treatment of the rabbit antiserum with thioglycollic acid caused no loss of agglutinin activity.

It is seen from table (13) that treatment of sera of patients with hepatic cirrhosis and infective hepatitis with 0.5M cysteine PH 8.5 resulted in 75-90% loss in agglutinin activity. Treatment of rheumatoid arthritis serum with this reagent caused over 90% loss of activity whereas the activity of the rabbit antiserum was again unaffected.

TABLE (12)

Clinical diagnosis	Titre of serum	Titre after treatment with 0.1M thioglycollic acid and 0.02M iodoacetate	Titre after treatment with 0.1M thioglycollic acid
Hepatic cirrhosis (case P)	5120	20	160
Hepatic cirrhosis (case C)	1280	0	80
Hepatic cirrhosis (case M)	10,240	0	0
Hepatic cirrhosis (case D)	5120	0	20
Hepatic cirrhosis (case E)	320	0	20
Infective hepatitis (case T)	1280	0	10
Infective hepatitis (case S)	320	0	0
Infective hepatitis (case 1w)	320	0	0
Rheumatoid Arthritis	10,240	10	10
Coombs rabbit antiserum	2560	2560	2560

TABLE (13)

Clinical diagnosis	Titre	Titre after dialysis against 0.5M cysteine
Hepatic cirrhosis (case M)	10,240	320
Hepatic cirrhosis (case D)	5120	1280
Infective hepatitis (case T)	1280	20
Infective hepatitis (case S)	320	20
Rheumatoid Arthritis	10,240	20
Coombs rabbit antiserum	2560	2560

(6) Heat stability.

Table (14) shows that the antiglobulin factor present in the sera of 4 patients with hepatic cirrhosis and 4 patients with infective hepatitis was stable on heating at 55°C for 30 minutes. There was a partial loss of activity in 3 of the hepatic cirrhosis sera and 2 of the infective hepatitis sera on heating to 60°C. At 65°C the antiglobulin activity of all the sera was unstable, with the exception of case (1w), which appears to be stable to heat up to 65°C.

TABLE (14)

Clinical diagnosis	Titre	Titre after heating at 55°C	Titre after heating at 60°C	Titre after heating at 65°C
Hepatic cirrhosis (case P)	5120	5120	5120	1280
Hepatic cirrhosis (case E)	1280	1280	640	160
Hepatic cirrhosis (case M)	10,240	10,240	5120	2560
Hepatic cirrhosis (case D)	5120	5120	2560	320
Infective hepatitis (case T)	640	640	320	320
Infective hepatitis (case S)	320	320	40	40
Infective hepatitis (case lw)	160	160	160	160
Infective hepatitis (case B)	2560	2560	2560	640

IV Injection of rats with rat liver lactic dehydrogenase

Antibodies to rat liver LDH could not be detected in the sera of rats which had been injected with homologous LDH by biochemical or immunological techniques. In spite of the fact that the serum LDH levels had increased as a result of the injections, these sera did not inhibit the activity of purified rat liver LDH in *vitro*; neither were tanned human erythrocytes sensitized with rat liver LDH. agglutinated by the sera of these rats.

DISCUSSION.

Attempts to demonstrate inhibition of human liver lactic dehydrogenase by the sera of patients with acute and chronic hepatocellular lesions were unsuccessful in all the cases tested. It was observed, in fact, that there was a slight enhancement of the LDH activity by the presence of the serum, probably due to the stabilizing effect of the serum proteins. These results thus suggest that antibodies to human liver LDH are not present in these sera or that any antibodies present do not possess antienzyme activity and the antigenic sites on the surface of the molecule do not coincide with the enzyme active centres. This would be contradictory to the results of Wroblewski (1958) and Henion, Mansour and Bueding (1955) who showed that antibodies to rabbit muscle LDH caused a marked reduction in enzyme activity. However, in order to detect the presence of an antibody to human liver LDH which did not possess antienzyme activity sensitive immunological procedures were employed, namely a modification of the Boyden haemagglutination technique (1951). Preliminary results showed that human erythrocytes sensitized with a solution of purified human liver LDH were agglutinated to low titres by the sera of certain patients with hepatic cirrhosis and infective hepatitis, whereas sera from normal control subjects gave negative titres. Subsequent experiments, however, showed that the sera from these patients also caused agglutination of erythrocytes sensitized with preparations of human and bovine serum Cohn FII (γ-globulin). It thus became evident that the agglutinating reagent present in these sera was not specific for LDH but reacted also with serum γ-globulins. It is significant that electrophoretic analysis of the purified human liver LDH /

LDH used in the agglutination experiments showed that 100% of the enzyme activity was associated with a protein which behaved electrophoretically at PH 8.6 as a γ-globulin. An antigenic similarity to γ-globulin would explain its reaction with the antiglobulin factor. This demonstration of antibodies with a broad specificity range in the sera of patients suffering from hepatocellular lesions is not unique. False positive results for the rheumatoid agglutinating factor have been reported in cases of hepatic cirrhosis and infective hepatitis (Svartz 1958; Ziff 1957) and Dresner (1960) found a high incidence of "false positive" results for the rheumatoid factor, using FII-coated latex particles, in hepatocellular lesions, including portal and biliary cirrhosis and viral hepatitis. He postulated that the agglutinating activity in these sera was independent of the type of lesion or the presence of jaundice; the important factor being hepatocellular damage and when the factors causing liver injury cease to operate during a clinical remission the agglutinating activity disappears. He stressed that agglutinating activity in such sera accurately reflects the clinical course of the disease and whereas on recovery from hepatocellular disease many of the indexes of hepatic function may take months to return to normal the latex fixation test appears to be a more subtle test for the presence of active disease. Electrophoretic and ultra centrifuge analysis of the sera studied by Dresner showed that the agglutinating activity was associated with a slow moving γ-globulin with a sedimentation constant of 19 or 22 S_{20} (Dresner 1960).

Gajdusek (1959) reported the presence of serum reagents, which gave positive autoimmune complement fixation reactions with a variety of tissue extracts from human and animal species, in the/

the sera of patients with liver damage. He maintained that these reagents exhibited many of the properties of antibodies and in spite of their non-specificity alleged that they were true auto-antibodies to components of damaged liver tissue.

In view of these reports it thus seemed pertinent to inquire whether the non-specific agglutinating reagents reacting with human liver LDH and γ-globulin were also of antibody nature. A physical and chemical study of these factors was consequently undertaken. Preparation of the euglobulin fraction of the sera showed with one exception (case D) not more than 17% of the agglutinin activity was associated with this fraction. In case (D), 50% of the activity was found in this fraction and it may be significant that of the 7 sera tested for the sheep cell factor only one, case (D), gave a positive titre. This suggests that the activity in the euglobulin fraction of this serum was due in part to the rheumatoid agglutinating factor which is known to be associated with this fraction (Ziff 1956).

Ammonium sulphate fractionation of the serum of a patient (P) with a clinical and pathological diagnosis of hepatic cirrhosis showed that over 90% of the agglutinin activity was associated with the fraction precipitated by 34% saturation with ammonium sulphate. On electrophoresis at PH 8.6 on cellulose acetate paper this fraction migrated as a γ-globulin.

Starch gel electrophoresis of this serum at PH 8.48 showed that the activity was associated with two protein fractions, one of which migrated in the slow γ-globulin region and the other in the fast γ-globulin region. Ultra centrifuge fractionation of the serum of case (P) showed that all the agglutinin activity of the /

the serum was associated with the macroglobulin fraction. This result was confirmed chemically by treatment of sera from patients suffering from hepatic cirrhosis and viral hepatitis with sulphydryl reagents.

On treatment of the sera of 5 patients suffering from hepatic cirrhosis and 3 patients suffering from viral hepatitis with 0.1M thioglycollic acid followed by dialysis against 0.02M iodoacetate in phosphate buffer PH 8.0, over 90% of the serological activity of all the sera was lost, whereas similar treatment of a rabbit antihuman globulin serum resulted in no loss of agglutinin activity. The antibodies of rabbit serum are known to belong almost entirely to the low molecular weight 7S γ-globulin class (Kabat 1939) and are therefore not affected by sulphydryl reagents.

Dialysis of these sera against 0.5M cysteine also resulted in a marked loss of agglutinin activity but the rabbit antiserum was again unaffected.

These results clearly show that the antiglobulin activity in the sera of patients suffering from hepatocellular lesions is associated with a macroglobulin fraction which is destroyed by sulphydryl reagents by virtue of their destructive action on the disulphide bonds which maintain the integrity of the molecule. (Deutch and Morton 1957; Franklin, Holman and Kunkel 1957).

There thus appears to be a physical resemblence between the serum factors reported here which react with human group O rhesus negative erythrocytes sensitized with human liver LDH, human and bovine Cohn FII γ-globulin and the latex particle agglutinating reagents described by Dresner (1960) which were also shown to be γ-globulins of the 19S class. Although the latex particle /

particle agglutinator was found to be associated with the slow moving γ-globulin fraction on electrophoresis, whereas the FII human red cell agglutinator was shown to be associated with proteins in the slow and fast γ-globulin region, with the bulk of the activity in the fast γ-region, it is quite possible that they are identical or closely related to each other.

It was concluded, in the light of the results presented, that specific autoantibodies to the human liver cell protein lactic dehydrogenase, were not detectable in the sera of patients suffering from hepatocellular lesions by biochemical or sensitive immunological techniques. Less specific serum reagents of antibody nature, were shown to be present, however, and these reacted immunologically not only with purified human liver LDH but also with human and bovine γ-globulin but not with sensitized sheep cells.

It is suggested that these reagents are produced as a general response to tissue damage and not to the specific antigenic stimulus of any one liver tissue protein.

No conclusions could be drawn from the animal experiments in which rats were injected with homologous rat liver LDH. Antibodies could not be detected in the sera of these rats by biochemical or immunological techniques. The sera exerted no inhibition on the activity of purified rat liver LDH, prepared from the livers of other rats of the same strain, in vitro. Human group O rhesus negative erythrocytes which were tanned and sensitized with the same rat liver LDH were not agglutinated by the sera of the injected rats. Presumably the LDH used for injection was not sufficiently "foreign" to the organism to elicit the formation of antibodies. It was therefore concluded that neither agglutinating antibodies nor antibodies /

antibodies possessing antienzyme activity were produced in the two groups of rats on injection of purified preparations of homologous rat liver LDH with or without adjuvants by intravenous, intra muscular and subcutaneous routes.

SUMMARY.

(1) Human liver lactic dehydrogenase was prepared and shown to migrate with the electrophoretic mobility of γ-globulin at PH 8.6.

(2) Antibodies to human liver lactic dehydrogenase were not detected in the sera of patients with hepatocellular lesions by their ability to inhibit the activity of purified human liver LDH <u>in vitro</u>.

(3) A factor was demonstrated, in the sera of patients suffering from hepatocellular lesions, which caused agglutination of human group O rhesus negative erythrocytes sensitized with preparations of purified human liver LDH, human and bovine serum γ-globulin but did not agglutinate sensitized sheep cells.

(4) The agglutinating factor was characterized as a γ-globulin, not more than 17% of which, was associated with the euglobulin fraction of serum. Starch gel electrophoresis at PH 8.48 showed that the serological activity in the serum of a patient suffering from hepatic cirrhosis was associated with two γ-globulin fractions in the slow and fast γ-regions respectively. Ultra centrifuge fractionation of the serum and reaction with sulphydryl reagents established the agglutinin activity to be associated with the 19S macroglobulin fraction.

(5) No antibodies were detected, by biochemical or immunological methods, in the sera of rats which had been injected with purified preparations of rat liver LDH without adjuvants by intravenous, intramuscular and subcutaneous routes, or with adjuvants by subcutaneous routes.

PART II

"The characterization of an antiglobulin present in the sera of certain patients following myocardial infarction; its comparison with and differentiation from the rheumatoid factor."

INTRODUCTION.

In 1945 Cavelti demonstrated the presence of autoantibodies to heart muscle tissue in the sera of 75% of the patients tested with active rheumatic fever by the agglutination of collodion particle sensitized with saline extracts of human heart. He showed that the autoantibodies were present during the active stage of the disease and disappeared when the rheumatic process became inactive. In subsequent experiments (Cavelti 1947) it was shown that rats immunized with homologous tissues of heart, skeletal muscle and connective tissue developed cardiac lesions and he suggested that these lesions resulted from an interaction between the autoantibodies and connective tissue occuring <u>in vivo</u>. He found that there was a large degree of overlapping of the antigens <u>in vitro</u> and suggested that this was due to the fact that connective tissue is present in both heart and skeletal muscle and a number of different antibodies will be formed against the various constituents of crude heart emulsion, connective tissue and skeletal muscle. In cases where a more pronounced reaction occured the structure of the cardiac lesions seemed to resemble that seen in cases of rheumatic fever in humans and on the basis of these results it was suggested that during or succeeding the streptococcal infection, which precedes a rheumatic attack by two to three weeks, an antigenic substance is formed by a reaction in which streptococcal substances or products combine with components of the heart tissue, probably present in the connective tissue, inciting the formation of autoantibodies which then precipitate the rheumatic lesions by reacting in <u>vivo</u> with the antigen situated in the tissues.

The implication of heart specific autoantibodies in the pathogenesis of myocardial lesions was substantiated by the work of Kaplan (1959). He found that the sera of patients suffering from rheumatic fever frequently exhibited immuno-fluorescent staining of tissue sections of normal human heart. This activity was attributed to the presence in these sera of two γ-globuilns. The first was demonstrated only in the sera of certain patients with rheumatic diseases of the heart and the staining capacity could be specifically absorbed with human heart tissue homogenate. The second type of γ-globulin was shown to be present in the sera of rheumatic patients and in certain other pathological sera and was found to be associated with the complement fixing and flocculating activity of the sera with alcoholic extracts of normal human heart.

Dornbusch (1957) reported the presence of autoantibodies to homologous cardiac tissue in the sera of patients following myocardial damage. Kleinsorge and Dornbusch (1957) examined the sera of patients after myocardial infarction by means of the sensitive Boyden haemagglutination technique (1951) and found that 34% of the patients tested possessed in their sera autoantibodies to heart muscle extracts. They occured at certain intervals only in low titres and direct relationships of any kind between the appearance of the autoantibodies and the degree of myocardial damage could not be found. These autoantibodies, unlike those reported by Cavelti (1945) and Kaplan (1959) were found in the sera of patients following a purely aseptic infarction excluding a previous bacterial influence, although positive agglutination reactions were also obtained with the sera of patients suffering from inflammatory myocarditis.

Gery, Davies and Ehrenfeld (1960) reported the presence of heart specific autoantibodies in the sera of patients following /

following myocardial infarction, acute rheumatic fever, myocardial insufficiency, anginal syndrome and one patient following mitral valvotomy. They demonstrated the autoantibodies by means of the Boyden haemagglutination technique using extracts of human heart as the antigen. These workers also produced autoantibodies to heart muscle antigen in the sera of rabbits which were similar to those present in human sera but they did not result in pathological lesions of the rabbit heart suggesting that they were a consequence rather than a cause of the lesions observed in man.

It thus appears that autoantibodies to some component of human heart muscle tissue, which has been released by bacterial attack or as the result of an aseptic degenerative process, are formed and appear in the sera of patients several weeks after the myocardial damage has occured.

It has been shown, during the course of the present investigation, that an antiglobulin factor was present in the sera of 50% of the patients tested three or more weeks following myocardial infarction, and it was suggested that it may be related to the heart muscle autoantibodies reported by Dornbusch (1957), and Gery, Davis and Ehrenfeld (1960) or the γ-globulins described by Kaplan (1959), reflecting an immune response to some component of heart muscle or connective tissue released as a result of myocardial damage.

This factor in many ways resembles the rheumatoid agglutinating factors present in the sera of over 75% of patients tested with active peripheral rheumatoid arthritis (Heller, Kolodny, Lepow, Jacobson, Rivera and Marks (1955); Ziff, Brown, Laspalluto, Badin and McEwen (1956)).

The possibility that rheumatoid arthritis has an immunological basis has long been entertained (Kunkel 1959; Bywaters 1957; Clough 1960) and the most convincing evidence for this is the presence in the sera of such patients of the rheumatoid factor which is responsible for a wide variety of serological tests employed in the diagnosis of rheumatoid arthritis. These tests include, the sheep erythrocyte agglutinin test, the FII γ-globulin tanned cell test and the latex particle fixation test (Heller, Kolodny, Lepow, Jacobson, Rivera and Marks (1955); Ziff (1957)) all of which are dependent on the agglutination of particulate bodies coated with γ-globulin by the rheumatoid factor.

A number of workers have isolated and characterized the rheumatoid factor and it has become evident that it possesses many of the chemical, physical and antigenic properties of an antibody (Heimer, Federico, Freyberg (1958); Lospalluto and Ziff (1959); Kunkel, Franklin and Muller-Eberhard (1959)). On electrophoresis of rheumatoid serum the serological activity was found to be associated with the γ-globulin fraction (Rose, Ragan and Pearce (1948); Svartz (1958 (1)); Kunkel, Franklin and Muller-Eberhard (1959)). Ultra centrifuge patterns of the purified rheumatoid factor are particularly characteristic and show that it is a macroglobulin with a sedimentation constant of 19 or $22S_{20}$, and a molecular weight of one million (Svartz 1958 (1)); Kunkel, Franklin and Muller-Eberhard (1959)) and like other 19S antibodies (Franklin, Holman and Kunkel (1957)) it is susceptible to the action of sulphydryl reagents with complete loss of serological activity (Kunkel, Franklin and Muller-Eberhard (1959)). The true antigen involved in the production of the rheumatoid factor has not been precisely defined but it will react in serological /

serological reactions with partially denatured or aggregated γ -globulin which may be derived from a variety of species (Glynn and Holborrow 1957).

This investigation has shown that the sera of certain patients following myocardial infarction also contain a factor which reacts serologically with γ -globulin preparations and gives "false positive" results for the rheumatoid factor. It was consequently proposed to study the physical, chemical and immunological properties of this antiglobulin and if possible to differentiate it from the rheumatoid factor.

PART II

REAGENTS and METHODS.

I Physical characterization.

REAGENTS.

Bovine γ-globulin (Cohn FII)

Supplied by Armours Laboratories Ltd.

Human γ-globulin (Cohn FII)

Supplied by the Scottish Blood Transfusion Association, The Royal Infirmary of Edinburgh by the courtesy of Dr. Cumming.

Cellulose acetate paper.

Supplied by 'Oxoid' Ltd., London.

Lissamine Green dye.

Contained a 2% solution of 'Gurrs' Light green powder in 6% sulphosalicylic acid.

Carboxymethyl cellulose ion-exchange resin.

Whatman CM-30 carboxymethyl cellulose cation exchange powder was used. A fresh sample of resin was made up for each column.

Starch - Hydrolysed.

Supplied by the Connought Medical Research Laboratories, University of Toronto, Canada. Batch number 139 was used.

Unhydrolysed Potato Starch.

Supplied by British Drug Houses Ltd.

Amido Svartz 10B.

Supplied by 'Bayer' Leverkusen. A saturated solution in a methanol-water-glacial acetic acid solvent (50:50:10) was used.

Polyvinyl Pyrrolidone (Molecular weight = 11,000)

Supplied by L. Light & Co.Ltd. A solution containing 15 gms in 100 mls of phosphate buffered saline PH 8.0 was used.

Coombs Rabbit antihuman globulin serum

Supplied by The Wellcome Foundation, Burroughs Wellcome Ltd., Kent.

Rheumatoid serum

Supplied by the Rheumatic Unit, Northern General Hospital, Edinburgh, by the courtesy of Dr. Duthie.

Post myocardial infarct sera

Obtained from patients in the Royal Infirmary, Edinburgh.

Control sera

Normal non-agglutinating control sera were supplied by The Blood Transfusion Service, Royal Infirmary, Edinburgh and obtained from blood donors.

All sera were stored at -10°C.

BUFFERS.

0.15M phosphate buffered saline PH 8.0

1132.8 mls 0.15M 'Analar' sodium phosphate (anydrous), 67.2 mls 0.15M 'Analar' potassium dihydrogen orthophosphate and 1200 mls 0.15M 'Analar' sodium chloride were mixed and the PH adjusted to PH 8.0 with M. sodium carbonate.

0.10M Barbitone buffer PH 8.6

12.5 gms of 'BDH Reagent grade' sodium barbitone, 8.125 gms of 'Analar' sodium acetate and 8.75 mls 0.99N hydrochloric acid were dissolved in 1000 mls of distilled water and the volume was made up to 2000 mls.

Phosphate buffers for cation-exchange chromatography.

(1) 0.05M phosphate buffer PH 5.2.

0.05M sodium phosphate ($Na_2HPO_4.7H_2O$) was added to 0.05M sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) until PH = 5.2.

(2) <u>0.075M phosphate buffer PH 6.0</u>

448.5 mls 0.075M sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) were added to 61.5 mls 0.075M sodium phosphate ($Na_2HPO_4.7H_2O$).

(3) <u>0.10M phosphate buffer PH 6.5</u>

342.5 mls 0.10M sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) were added to 157.5 mls sodium phosphate ($Na_2HPO_4.7H_2O$).

<u>Borate buffer PH 8.48</u>

Consisted of 0.023M 'Analar' Boric acid and 0.0092M 'Analar' sodium hydroxide. The electrode vessels contained 2% sodium chloride in this buffer. The bridge buffer used for filter paper connections contained 14.223 gms boric acid and 1.848 gms sodium hydroxide in 1000 mls of distilled water.

<u>Titration of sera and serum fractions for the post myocardial infarct factor and the rheumatoid factor.</u>

The antiglobulin present in the post myocardial infarct sera and the rheumatoid factor were estimated by titration of the sera or serum fractions with human group O rhesus negative erythrocytes sensitized with bovine γ-globulin (Cohn FII) by a modification of the Boyden haemagglutination technique, described in Part I, unless otherwise specified. Pooled rheumatoid arthritis serum was used and the sera of 58 patients who had suffered a myocardial infarction three or more weeks previously were tested for antiglobulin activity.

<u>Preparation of the euglobulin fraction</u>

It has been reported (Svartz and Schlossman 1954) that the rheumatoid factor is completely precipitated in the "cold globulin" fraction of rheumatoid serum obtained by dilution with 14 volumes of distilled water at 4°C. Ziff et al (1956) prepared the "euglobulin" fraction of rheumatoid serum by dialysis against a dilute citrate-phosphate /

citrate-phosphate buffer PH 5.8 and reported that the rheumatoid factor was present in this fraction in 90% of the patients tested with rheumatoid arthritis and in only 1.9% of the non-rheumatoid controls. Franklin, Kunkel and Muller-Eberhard (1957) also reported that the rheumatoid factor could be concentrated in the euglobulin fraction of serum and as this fraction is rich in the high molecular weight 19S γ-globulins suggested that the rheumatoid factor was associated with these macroglobulins.

METHOD

Serum was diluted 1 in 5 with distilled water and dialysed against distilled water at 4°C for 48 hrs. with two changes of water. The precipitate was then centrifuged down and washed twice with distilled water. The precipitate was dissolved up in 0.9% sodium chloride and tested together with the supernatant serum for antiglobulin activity.

Ammonium sulphate fractionation

There has been some discrepency between workers using different fractionation techniques as to which fraction of serum the rheumatoid factor activity is associated. Using salt fractionation Waaler (1940) found the agglutinin activity in the globulin fraction. Robinson et al (1954) showed that the agglutinating activity was precipitated by sodium sulphate most consistently in a fraction rich in β-globulin. Using electrophoretic techniques Rose et al (1948) found the activity in the β-γ-fraction. Heller et al (1955) showed that the haemagglutinating activity of the serum fractions separated by alcohol fractionation was all found in FIII which contained mainly α and β-globulins with some γ-globulin and assumed that the rheumatoid factor was associated with the β-lipoprotein fraction but admitted /

admitted the possibility that the activity was associated with the small but measurable quantities of γ-globulin known to be present in FIII.
Svartz (1958 (i)) established that the rheumatoid factor could be precipitated specifically in the γ-globulin fraction prepared by 29% saturation of the serum with ammonium sulphate.

METHODS

Sera were made 34%, 46% and 50% saturated with respect to ammonium sulphate by addition of saturated solutions of ammonium sulphate solution to undiluted serum at 4°C. Each protein fraction was reprecipitated twice from a large volume of 0.9% sodium chloride solution. The final fractions were dissolved up in 0.9% sodium chloride solution to the original serum volume and tested for anti-globulin activity. The fractions were analysed electrophoretically on cellulose acetate paper at PH 8.6.

Cation exchange chromatography.

Svartz (1958 (ii)) subjected the dissolved 'cold precipitate' of rheumatoid serum to ion-exchange chromatography on a carboxymethyl cellulose cation exchange column, using a modification of the method of Sober and Peterson (1956). She eluted two protein fractions with phosphate buffer of increasing ionic strength, a major peak at PH 5.8 and a minor peak at PH 6.6.
They found that the bulk of the agglutinin activity was associated with the first fraction eluted at PH 5.8. Using a continuously increasing PH gradient they also obtained two peaks one at PH 5.8 and a second at PH 8.8, the bulk of the agglutinin activity again being associated with the first fraction eluted at PH 5.8. On /

On ultra centrifuge analysis the active first peak was found to possess a sedimentation constant of 18.7 swedbergs (Svartz 1958 (2)).

Fallet and Lospalluto (1958) fractionated sera containing the lupus erythematosus (LE) agglutinating factor and the rheumatoid arthritis (RA) factor on a carboxy-methyl cellulose cation exchange column and showed that the sheep erythrocyte agglutinin activity of the RA factor was eluted in a major protein peak at PH 6.5 whereas the LE factor was found to be present in this peak and a minor protein peak eluted at PH 7.0. Lospalluto and Ziff (1959) carried out chromatography of rheumatoid serum on a carboxy-methyl cellulose cation-exchange column and showed that all the sheep cell agglutinin activity was associated with a major γ-globulin peak eluted at PH 6.5 with 0.075M phosphate buffer. The second minor γ-globulin peak eluted at PH 7.0 with 0.15M phosphate buffer showed no sheep cell agglutinin activity but precipitated with γ-globulin and agglutinated γ-globulin sensitized latex particles.

METHOD

The γ-globulin fraction of serum was subjected to chromatography on a carboxy-methyl cellulose cation exchange column by a modification of the method of Sober and Peterson (1956). The fractions so obtained were tested for antiglobulin activity, analysed electrophoretically on cellulose acetate paper and analysed in the ultra centrifuge.

(i) Preparation of sera

The γ-globulin fraction of serum was prepared by 40% saturation of 5 mls serum with ammonium sulphate and reprecipitated twice. The precipitate was dissolved up in 5 mls 0.9% sodium chloride and dialysed against 0.9% sodium chloride overnight at 4°C in order to remove the ammonium sulphate. The solution was then diluted to /

to 50 mls with 0.05M phosphate buffer PH 5.2.

(ii) Preparation of column.

4 gms of carboxymethyl cellulose powder were mixed to a suspension with 500 mls 0.05M phosphate buffer PH 5.2 and allowed to settle. The PH of the supernatant was tested and decanted off. Fresh buffer was added to the resin and well stirred. This was repeated until the PH of the supernatant buffer equalled 5.2. The bulk of the supernatant buffer was then decanted leaving a slurry which was poured in small amounts into a glass column plugged with glass wool. The column which was 30 cms in length and 1.3 cms in diameter was drawn out into a narrow tube at the base and closed by means of a piece of rubber tubing and a screw clip. A small piece of glass tubing drawn out to a capillary was attached to the rubber connecting tube. The slurry was allowed to settle under gravity and gently packed with a mushroom ended glass rod. A resin column of 16 cms in height was used.

(iii) Application of sample.

The γ-globulin solution was poured onto the column in small aliquots and the solution was allowed to pass through at the rate of 3 mls/hour, the rate of flow being adjusted by means of the screw clip. Excess protein was then eluted from the column by passing 0.05M phosphate buffer PH 5.2 through until no more protein could be detected in the eluates by measurement of the optical density at 280$m\mu$. 0.075M phosphate buffer PH 6.0 was then passed through the column and 3 ml fractions were collected hourly until no more protein was eluted. The optical density of the 3 ml fractions was then read at 280$m\mu$ against phosphate buffer. The buffer was then replaced by 0.10M phosphate buffer PH 6.5 and 3 ml fractions were again collected hourly until no more protein was eluted. /

eluted. A plot of optical density at 280 mμ and antiglobulin activity against fraction number was made.

(iv) Concentration of eluates.

The protein fractions comprising each peak were pooled and concentrated by ultra filtration by the method of Recht (1960). The solutions were concentrated to 0.5 ml in a cellophane dialysis sack by the application of 150 p.s.i. pressure from a nitrogen cylinder at room temperature. After concentration the solutions were centrifuged to remove particulate matter and the protein was precipitated from the clear supernatant by 50% saturation with ammonium sulphate. The precipitate was then dissolved up in the minimum volume of 0.15M phosphate buffered saline PH 8.0 or 0.9% sodium chloride.

(v) Analysis of chromatographic fractions.

The concentrated chromatographic fractions were tested for antiglobulin activity, analysed electrophoretically on cellulose acetate paper at PH 8.6 and analysed in the ultra centrifuge.

Ultra-centrifuge fractionation.

Svartz (1957 (i) and (ii)) showed that the active 'cold precipitate' of rheumatoid serum on fractionation in a partition cell in the ultra-centrifuge was separated into upper and lower fractions. The upper fraction contained chiefly γ-globulins of the low molecular weight 6-7S class which gave negative haemagglutinating tests whilst the lower fraction gave strong haemagglutination and contained a high percentage of γ-globulins of the 19-22S class. It was thus concluded that the haemagglutinating factor was associated with the macroglobulin fraction of serum. Franklin et al (1957) subjected rheumatoid serum to ultra centrifuge separation into two fractions and found two protein peaks in the lower fraction and showed that rheumatoid factor was associated with the 19S peak.

METHOD

3 mls serum obtained from a patient (B) who had suffered a myocardial infarction was spun in a partition cell in the ultra centrifuge in three 1 ml portions at 60,000 rpm for 2 hours until all the 19S macroglobulin had been sedimented into the lower cell. The substance in the lower cell was dissolved up in 1 ml 0.9% sodium chloride. This fraction and the supernatant serum in the upper cell were tested for antiglobulin activity.

Starch gel electrophoresis.

Svartz (1957 (ii)) carried out starch gel electrophoresis of the 'cold precipitate' of rheumatoid serum and showed that the haemagglutinating activity was present in the γ-globulin fraction and in that part of the β-globulin area bordering on the γ-region. Kunkel, Franklin and Muller-Eberhard (1959) also located the rheumatoid factor in a broad band in the fast γ-globulin region on starch gel electrophoresis in barbital buffer at PH 8.6.

METHOD

Serum was subjected to electrophoresis on starch gel in borate buffer at PH 8.48 by a modification of the method of Smithies (1955) as described in Part I (methods).

5 mls of serum was mixed to a thick suspension with unhydrolysed potato starch and poured into 6 mm x 7 cm cavity approximately 7 cms from the negative end of the gel. A current of 40 m amps at 200 volts was applied for 22 hours. At the end of the run a 3 cm strip was cut from one edge of the gel and cross-hatched at 1 cm intervals. The strip was then sliced longitudinally into upper and lower sections and stained with a saturated solution of Amido Svartz 10B in methanol-water-acetic acid (50:50:10). The remaining block was divided into 1 cm segments and the protein eluted from each zone by extraction /

extraction with 2 x 5 ml aliquots of M. sodium chloride. Protein concentration of the eluates was estimated with the Folin-Wu-Ciocalteau phenol reagent (Wu 1922) as described in Part I. The eluates were concentrated to 0.6 ml by dialysis against 15% polyvinyl pyrrolidone in 0.15M phosphate buffered saline PH 8.0 and subsequently tested for antiglobulin activity, analysed electrophoretically on cellulose acetate paper at PH 8.6 and characterized antigenically by immuno-electrophoresis on cellulose acetate paper.

Immunoelectrophoresis.

The method consists of two basic stages, an initial electrophoretic separation of the proteins on cellulose acetate paper, followed by an immunological reaction when an antiserum to the components of human serum is allowed to diffuse towards the electrophoretically separated proteins. In that area of the paper where each diffusing antigen meets its homologous antibody and where the relative concentrations of these reagents are optimal an opaque arc shaped precipitate arises. The location of these arcs on the long axis of the paper strip reflects the electrophoretic positions of the antigens which have given rise to the precipitate.

Immunoelectrophoresis provides for more sensitive criteria between proteins and will distinguish between components which are apparently electrophoretically identical but antigenically distinct and conversely between antigenically related proteins which are electrophoretically different. When immunoelectrophoresis is applied to a protein solution containing two antigens which are immunologically different but electrophoretically identical it is highly probable that the precipitation arcs formed will occupy different positions and appear as two distinct and separate arcs /

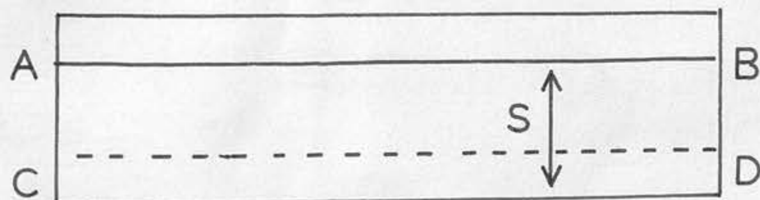
arcs with the same diffusion centre. If two components are antigenically related but electrophoretically disimilar a precipitation arc with two humps, resulting from the confluence of two arc shaped precipitates or an extended precipitation arc may be formed. This phenomenon is known as the reaction of identity. If on the other hand the two components are both electrophoretically and antigenically disimilar, two arc shaped precipitates which overlap are obtained and this is known as the reaction of non-identity.

It was proposed to employ the immunoelectrophoretic technique in the present investigation in order to study the antigenic and electrophoretic relationships between the antibody-like γ-globulins present in the pathological sera studied which had been previously isolated by starch gel electrophoresis.

METHOD

The initial electrophoretic stage was carried out on cellulose acetate paper at PH 8.6 by the method previously described, the only modification being the inclusion of thymol in the barbital buffer to prevent bacterial growth.

The protein band (S) was applied over 2/3 of the width of strip only.



A current of 12 m amps was applied for 2.5 hours as before. At the end of the run the strips were carefully removed from the tank with forceps and a narrow strip CD was cut from the edge of each strip and placed in lissamine green for 2 hours. The remaining portion of the strip was replaced in the tank and supported as before by means of the perspex bars. A line of Coombs antihuman globulin serum was then applied along AB by means of a capillary tube. The band of antiserum was applied perpendicular to and almost touching the protein application band (S). After drying the strips were placed in liquid paraffin for 2 days to allow diffusion to take place. The strips were then washed free of oil by washing with several changes of petroleum ether and placed under running tap water overnight to remove the unprecipitated soluble protein. The strips were then blotted dry and placed in lissamine green for 2 hours and subsequently washed in 5% acetic acid.

(2) Chemical characterization.

REAGENTS.

0.002M Acetic anhydride

'Analar' acetic anhydride was diluted with 0.15M phosphate buffered saline PH 8.0.

0.002M 1:fluoro 2:4 dinitrobenzene

'BDH Reagent Grade' 1:fluoro 2:4 dinitrobenzene was dissolved up in 0.5M sodium carbonate solution.

0.2M Thioglycollic acid

'BDH Reagent Grade' thioglycollic acid was diluted with phosphate buffered saline PH 8.0.

0.5M cysteine

A 0.5M solution of 'BDH Reagent Grade' cysteine hydrochloride was made up in distilled water and adjusted to PH 8.5 with 5N. sodium hydroxide.

0.2M sodium sulphite.

'Analar' sodium sulphite was dissolved in phosphate buffered saline PH 8.0.

6M urea

'BDH Reagent Grade' urea was dissolved in distilled water.

0.02M Iodoacetate

'BDH Reagent Grade' iodoacetic acid crystals were dissolved in 0.15M phosphate buffered saline PH 8.0.

Phosphate buffered saline

0.15M phosphate buffered saline was made up at PH'S 6.5, 7.0, 7.5 and 8.0 from 0.15M 'Analar' sodium phosphate (Na_2HPO_4), 0.15M 'Analar' potassium dihydrogen orthophosphate (KH_2PO_4) and 0.15M sodium chloride./

chloride. The PH'S were adjusted with M. Na_2CO_3 or dilute Hcl.

Chemical groups involved and mechanism of the agglutination reactions.

Heimer, Federico and Freyberg (1958), in a study of the PH dependence of the sensitized sheep cell test, showed that with rheumatoid serum maximal agglutination occured above PH 7.0 with inhibition below PH 7.0, whereas sera from patients suffering from other collagen diseases would agglutinate as low as PH 5.2. This suggested that free uncharged - NH_2 groups were required for agglutination by rheumatoid serum and in support of this hypothesis they showed that agglutination was inhibited by certain amino group blocking reagents such as acetic anhydride, carbobenzoxychloride and fluorodinitrobenzene.

METHODS

(i) PH optimum

The FII tanned cell agglutination reaction was carried out at PH 6.5, 7.0, 7.5 and 8.0 in phosphate buffered saline. In each case the specified buffer was used for the serum and sensitized red cell diluent.

(ii) Effect of amino group blocking reagents

Sera were treated with the amino group blocking reagents acetic anhydride and 1: fluoro 2:4 dinitrobenzene and tested for agglutinating activity before and after treatment.

The sera were diluted 1 in 10 with phosphate buffered saline PH 8.0 and an equal volume of 0.002M acetic anhydride or 0.002M 1: fluoro 2:4 dinitrobenzene was added dropwise over a period of 4 hours at 4°C with occasional agitation of the reaction mixture. The sera were then dialysed against 0.15M phosphate buffer PH 8.0 at 4°C for 2 days in order to remove excess acetic anhydride or fluorodinitrobenzene. Control sera diluted to the same volume with /

with buffered saline were also dialysed for the same period of time. After dialysis the PH of the sera was tested to ensure that the PH equalled 8.0.

Stability of the agglutinating factors.

Kunkel, Franklin and Muller-Eberhard (1959) showed that the rheumatoid agglutinating factor was unstable in the presence of sulphydryl reagents such as mercaptoethanol with loss of activity in the latex particle fixation test, sensitized sheep cell test and precipitin test. The Waldenstrom macroglobulin (Deutch and Morton 1957), the cold agglutinins of acquired hemolytic anaemia (Fudenberg and Kunkel (1957)) and the heterophile antibodies (Fudenberg and Kunkel (1958)) are also destroyed by these reagents which are believed to cause disruption of the macroglobulin molecules by reduction of essential disulphide bonds. Deutch and Morton (1957), as reported in Part I, showed that addition of sulphydryl reagents to macroglobulinemic sera resulted in the conversion of these macromolecules to small units of the 6.5S type and that dialysis against buffer resulted in a partial reaggregation of these subunits whereas dialysis against 0.02M iodoacetate, a sulphydryl group blocking reagent, prevented this reaggregation.

Putnam (1959) showed that disaggregation of the macroglobulins in a number of macroglobulinemic sera could be accomplished by mercaptoethanol but not by temperature, ultra sonic vibration or urea indicating that the forces involved in maintaining the structure of the molecule were covalant disulphide bonds and not only electrostatic linkages.

METHODS

Treatment with 0.5M cysteine

Sera were diluted 1 in 5 with 0.15M phosphate buffered saline /

saline PH 8.0 and dialysed against a solution of 0.5M cysteine PH 8.5 for 18 hours at 4°C.

<u>Treatment with 0.1M thioglycollic acid</u>

Sera were diluted 1 in 5 or 1 in 10 with phosphate buffered saline and an equal volume of 0.2M thioglycollic acid in buffer was added. The solutions were mixed and incubated at 4°C for 24 hours. They were then divided into two portions, one portion was dialysed against 0.15M phosphate buffer PH 8.0 and the other portion was dialysed against 0.02M iodoacetate in phosphate buffer for 48 hours at 4°C.

<u>Treatment with 0.1M sodium sulphite</u>.

Sera were diluted 1 in 10 with phosphate buffered saline and an equal volume of 0.2M sodium sulphite in buffered saline was added and mixed. The solutions were incubated at 4°C for 24 hours and subsequently dialysed against 0.15M phosphate buffer PH 8.0 for 48 hours.

<u>Treatment with 6M urea</u>

Sera were diluted 1 in 10 with buffered saline and dialysed against a solution of 6M urea for 24 hours at 4°C and subsequently against 0.15M phosphate buffer PH 8.0 for 48 hours.

<u>Heat stability</u>.

Sera were diluted 1 in 10 with phosphate buffered saline and heated at 55°C, 60°C and 65°C for ½ hour.

All sera were titrated for antiglobulin activity after the above procedures and the titres compared with those of untreated sera.

(3) Immunological studies.

Reaction with extracts of human heart.

Autoantibodies to heart muscle tissue were sought in post myocardial infarct sera by a modification of the Boyden haemagglutination technique (1951) as used by Dornbusch (1957). Sera were also absorbed with human red cells sensitized with extracts of human heart or bovine γ-globulin (Cohn FII).

METHODS

(i) Agglutination reaction with heart extract sensitized red cells.

A saline extract of fresh human heart tissue was prepared. 5 gms of myocardial tissue was washed with water to remove excess blood and chopped finely in ice-cold saline. The finely chopped tissue was then suspended in 50 mls of ice-cold saline and homogenized at full speed for 2 mins in a Waring blendor surrounded by a jacket containing crushed ice and water.

The homogenate obtained was then centrifuged for 3 hours at 3,500 rpm at 4°C in order to remove particulate matter. The clear reddish supernatant was then decanted and used as the red cell sensitizing antigen.

Human group O rhesus negative erythrocytes were sensitized with 1 in 5 or 1 in 10 dilutions of the saline extract in 0.15M phosphate buffered saline PH 8.0.

Sera obtained from patients several weeks after myocardial infarction were titrated with both heart extract sensitized and bovine γ-globulin sensitized erythrocytes.

(ii) <u>Absorption of sera with heart muscle extract sensitized cells, bovine γ-globulin sensitized erythrocytes and unsensitized erythrocytes</u>

Human red cells were sensitized with extracts of human heart and bovine γ-globulin (Cohn FII) and sera were absorbed with these cell suspensions and a suspension of unsensitized cells.

0.2 ml serum was diluted with 0.3 ml phosphate buffered saline and to each sample 0.5 ml of a 10% suspension of sensitized or unsensitized cells was added. The tubes were shaken and allowed to stand at room temperature for 1 hour with occasional mixing of the contents. They were then centrifuged at 2000 rpm for 10 mins. 0.7 ml of the supernatant sera was removed from each tube and added to 0.7 ml of a 10% cell suspension in another tube. The solutions were incubated at 4°C overnight and then centrifuged at 2000 rpm at 4°C for 10 mins. The supernatant sera were removed and titrated for antiglobulin activity.

<u>Specificity of antiglobulin factors.</u>

The reactant γ-globulin in the FII tanned cell test for the rheumatoid factor is believed to be a 7S molecule which has been partially denatured or aggregated by heating (Kunkel 1959). The rheumatoid factor is specific for γ-globulin and will not react with other proteins but it is not species specific and will react with human and bovine γ-globulin and rabbit antibody γ-globulin (Glynn and Holborrow 1957).

Winblad (1952) observed that dilute rabbit serum added to a system containing sensitized sheep cells and rheumatoid serum inhibited agglutination. Subsequently Heller et al (1954) established that human sera had the same effect and the inhibitory substance was found to be present entirely in the γ-globulin fraction. They postulated that γ-globulin had an affinity for the rheumatoid/

rheumatoid factor and inhibited agglutination by competing with the sensitized sheep cell for this factor.

Experiments were carried out to compare the specificities of the rheumatoid and the post myocardial infarct factor for different γ-globulin preparations from the same and from different species. The ability of γ-globulin preparations to inhibit agglutination by the two factors was also studied.

METHODS

(i) Agglutination of erythrocytes sensitized with different γ-globulin preparations.

The ability of sera to agglutinate the following types of sensitized erythrocyte was studied and the titres compared:-

(i) Bovine γ-globulin (Cohn FII)

(ii) Partially denatured bovine γ-globulin

(iii) Human γ-globulin (Cohn FII)

(iv) Partially denatured human γ-globulin

(v) Sheep cells sensitized with rabbit sheep erythrocyte antibody.

The γ-globulin preparations were partially denatured by heating a 1% solution in phosphate buffered saline PH 8.0 at 63°C for 10 mins. The sensitized sheep cell test was carried out by the method of Heller et al (1955).

(ii) Inhibition of agglutination by γ-globulins.

The agglutination reaction was carried out in the presence of human and bovine γ-globulin preparations both undenatured and partially denatured by heating. A solution of γ-globulin containing 5 mgms/ml of protein in phosphate buffered saline was used as the serum diluent in the first cup.

PART II

RESULTS

TABLE (1)

Summary of antiglobulin titres of post myocardial infarct sera.

Number of sera tested	Number of titres of 20-160	Number of titres of 320-1280	Number of titres of 2560-10,240	% of sera positive
58	12	13	4	50%

Table (1) indicates that 50% of the post myocardial infarct sera tested gave positive antiglobulin titres. Titres of 20 or greater were considered positive in this test and all the sera were tested not less than 3 weeks after the infarction had occured.

(1) Physical Characterization

Activity of euglobulin and ammonium sulphate fractions

Table 2 records the activity of the euglobulin and supernatant fractions of the sera of 9 patients following myocardial infarction and one aliquot of pooled rheumatoid serum.

In 8 of the 9 post-myocardial infarct sera not more than 11% of the antiglobulin activity was found in the euglobulin fraction. In one serum (case S2) 50% of the activity was found to be associated with the euglobulin fraction. 100% of the agglutinin activity of the rheumatoid serum was present in this fraction. As there was a certain loss of activity of the sera on dialysis the activity of the euglobulin fraction was expressed as a percentage of the total remaining activity.

Table 3 shows that over 90% of the antiglobulin activity in all the post-myocardial infarct sera and the rheumatoid serum was present in the fraction precipitated by 46% saturation with ammonium sulphate.

TABLE 2

Diagnosis	Titre of serum	Titre of euglobulin fraction	Titre of supernatant serum	% of total activity in euglobulin fraction
Rheumatoid Arthritis	2560	2560	10	100
Post myocardial infarct (case R)	640	0	40	0
Post M.I. (case B)	40	0	20	0
Post M.I. (case E)	320	10	80	11
Post M.I. (case S1)	320	10	160	6
Post M.I. (case S2)	1280	160	160	50
Post M.I. (case BG)	1280	0	640	0
Post M.I. (case D)	640	10	320	3
Post M.I. (case T)	320	0	80	0
Post M.I. (case McK.)	1280	0	640	0

TABLE 2

Diagnosis	Titre of serum	Titre of euglobulin fraction	Titre of supernatant serum	% of total activity in euglobulin fraction
Rheumatoid Arthritis	2560	2560	10	100
Post myo-cardial infarct (case R)	640	0	40	0
Post M.I. (case B)	40	0	20	0
Post M.I. (case E)	320	10	80	11
Post M.I. (case S1)	320	10	160	6
Post M.I. (case S2)	1280	160	160	50
Post M.I. (case BG)	1280	0	640	0
Post M.I. (case D)	640	10	320	3
Post M.I. (case T)	320	0	80	0
Post M.I. (case McK.)	1280	0	640	0

TABLE 3

Diagnosis	Titre of serum	Titre of 34% $(NH_4)_2SO_4$ fraction	Titre of 34-46% $(NH_4)_2SO_4$ fraction	Titre of 46-50% $(NH_4)_2SO_4$ fraction	Titre of supernatant serum
Rheumatoid Arthritis	2560	1280	160	10	0
Post myo-cardial infarct (case H)	160	80	80	40	0
Post M.I. (case B)	80	40	10	0	0
Post M.I. (case R)	640	80	80	0	0
Post M.I. (case S1)	320	80	160	10	0
Post M.I. (case T)	320	160	40	0	0
Post M.I. (case BG)	640	160	320	20	0

Carboxymethyl cellulose cation-exchange chromatography.

(1) Rheumatoid serum

Graph 1 shows that chromatography of the globulin fraction of rheumatoid serum prepared by 40% saturation with ammonium sulphate gave rise to two protein peaks. A major protein peak (FI) was eluted at PH 6.0 and a minor one (FII) was eluted at PH 6.5. Table 4 shows that the specific activity of FI was found to be over 6 times as great as that of FII.

Plate No. (1) shows that on electrophoretic analysis on cellulose acetate paper at PH 8.6 both these chromatographic fractions behaved as electrophoretically homogeneous γ-globulins.

Ultra-centrifuge analysis of FI, as seen in table 5, revealed the presence of 2 components, a major component with a sedimentation constant of 5×10^{-13} svedberg units and a minor component with a sedimentation constant of 13×10^{-13} svedberg units.

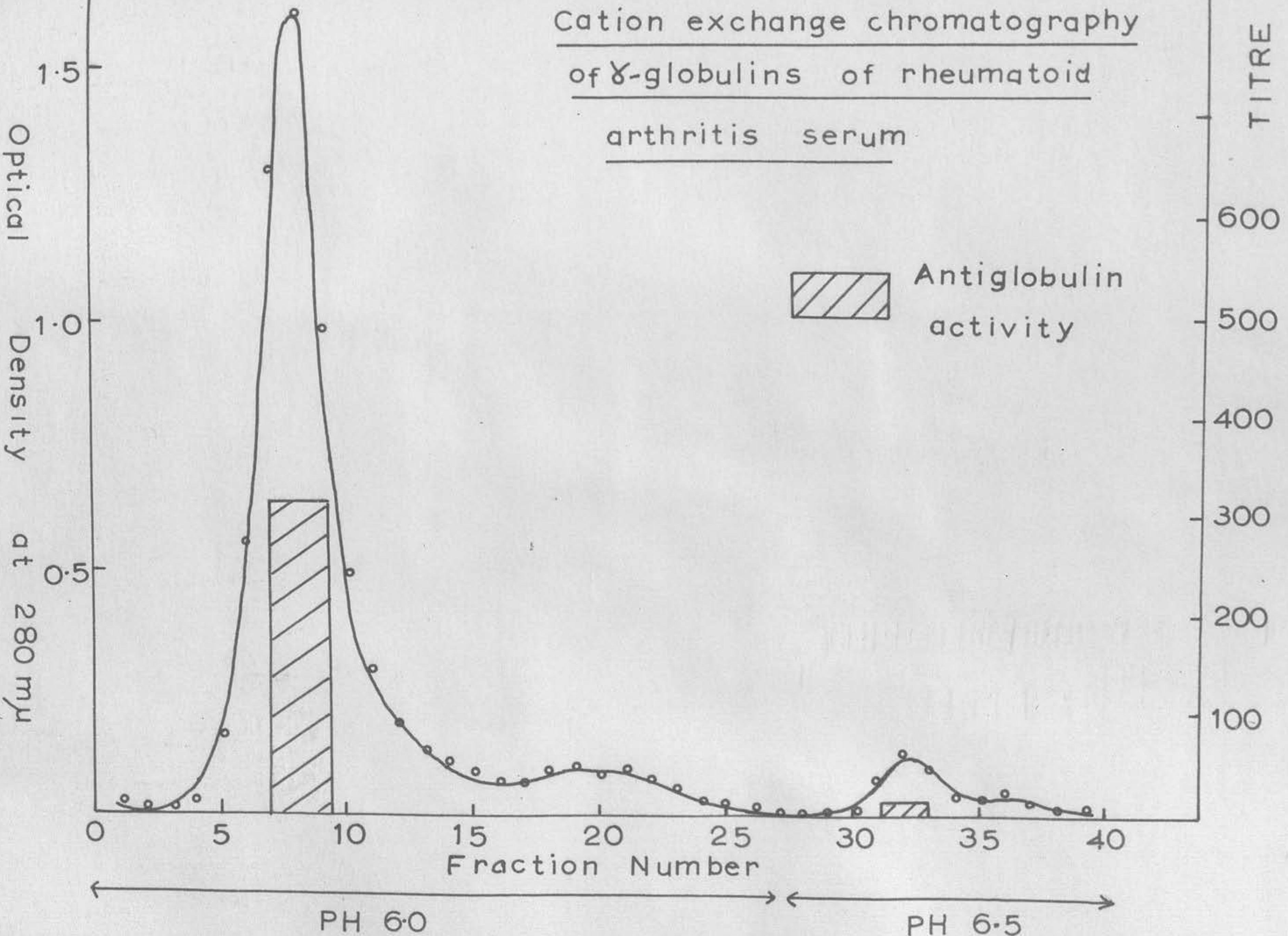
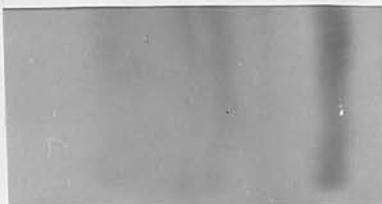

GRAPH (1)
Cation exchange chromatography of γ-globulins of rheumatoid arthritis serum
Antiglobulin activity
Optical Density at 280 mμ
1·5
1·0
0·5
TITRE
600
500
400
300
200
100
0
5
10
15
20
25
30
35
40
Fraction Number
PH 6·0
PH 6·5

PLATE (1)

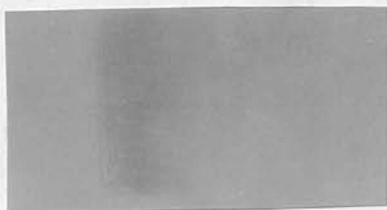
Electrophoretic analysis of rheumatoid serum and γ-globulin fractions on cellulose acetate paper at PH 8.6.

(1) Rheumatoid serum

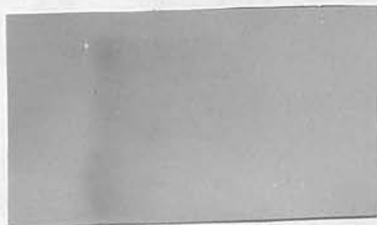


(2) Fraction precipitated by 34% saturation with ammonium sulphate.

(3) Fraction precipitated between 34% and 46% saturation with ammonium sulphate.



(4) Chromatographic peak FI



(5) Chromatographic peak FII

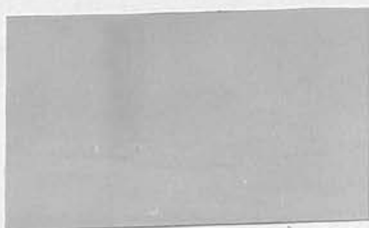


TABLE 4

Chromatographic Peak No.	Titre (A)	Protein conc.gms % (P)	Specific Activity $\frac{(A)}{(P)}$
Peak (FI)	320	2.0	160
Peak (FII)	10	0.4	25

TABLE 5

Details	Protein conc. gms %	Sedimentation constant (svedbergs)
Chromatographic peak (FI) eluted at PH 6.0	0.75	(1) Major component $S = 5x10^{-13}$ (2) Minor component $S = 13x10^{-13}$

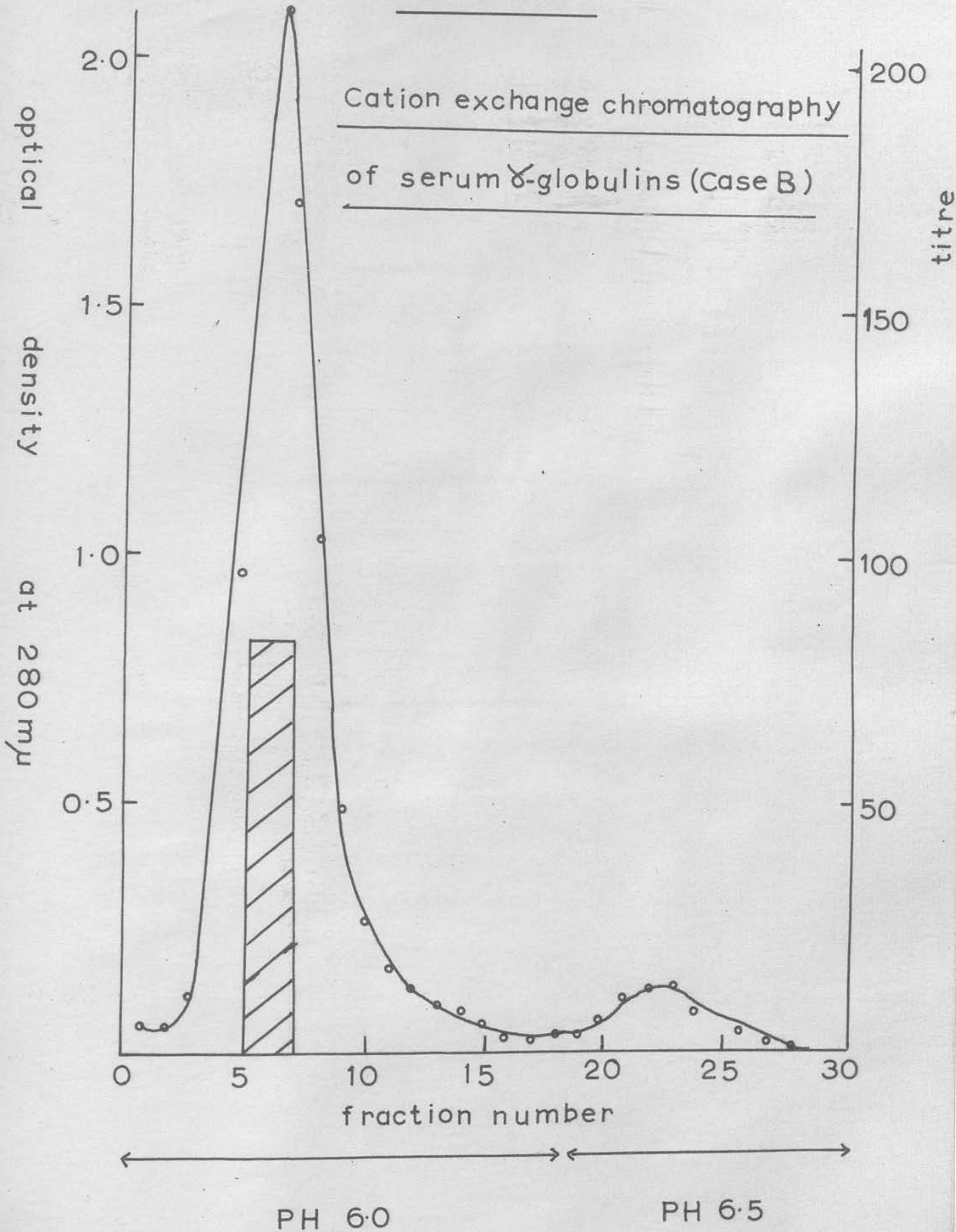
(2) Post myocardial infarct serum (case B.G.)

Graph (2) shows that ion-exchange chromatography of the 40% ammonium sulphate fraction of the serum of patient (BG), obtained 7 weeks after a myocardial infarction, gave rise to two peaks FI and FII, eluted at PH 6.0 and 6.5 respectively.

It is seen from Table 6 that all the antiglobulin activity was associated with FI and FII was totally inactive.

Electrophoretic analysis of FI and FII on cellulose acetate paper at PH 8.6 indicates that both these fractions behaved electrophoretically as γ -globulins (Plate (2)).

Ultra centrifuge analysis of FI showed that 2 components were present (Table 7), a major component with a sedimentation constant of 5.7×10^{-13} svedbergs and a minor component with an S value of 16×10^{-13}. Table 7 also gives the results for the ultra centrifuge fractionation of (BG) serum in a partition cell. It is observed that the antiglobulin activity is associated with both upper and lower fractions.

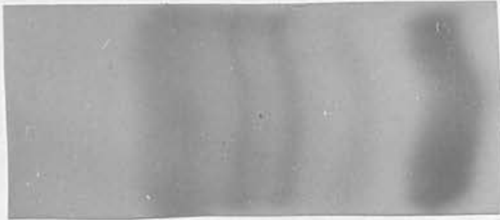


GRAPH (2)
Cation exchange chromatography
of serum γ-globulins (Case B)
optical density at 280 mμ
titre
2·0
1·5
1·0
0·5
200
150
100
50
0
5
10
15
20
25
30
fraction number
PH 6·0
PH 6·5

PLATE (2)

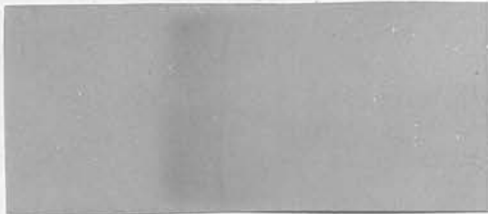
Electrophoretic analysis of case (BG) serum and γ-globulin fractions on cellulose acetate at PH 8.6.

(1) SERUM (BG)

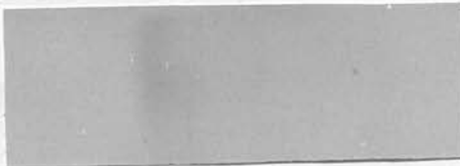


(2) Fraction precipitated by 34% saturation with ammonium sulphate

(3) Fraction precipitated between 34% and 46% saturation with ammonium sulphate.



(4) Chromatographic peak FI



(5) Chromatographic peak FII

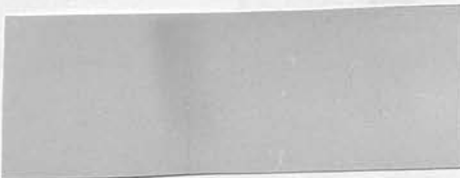


TABLE 6

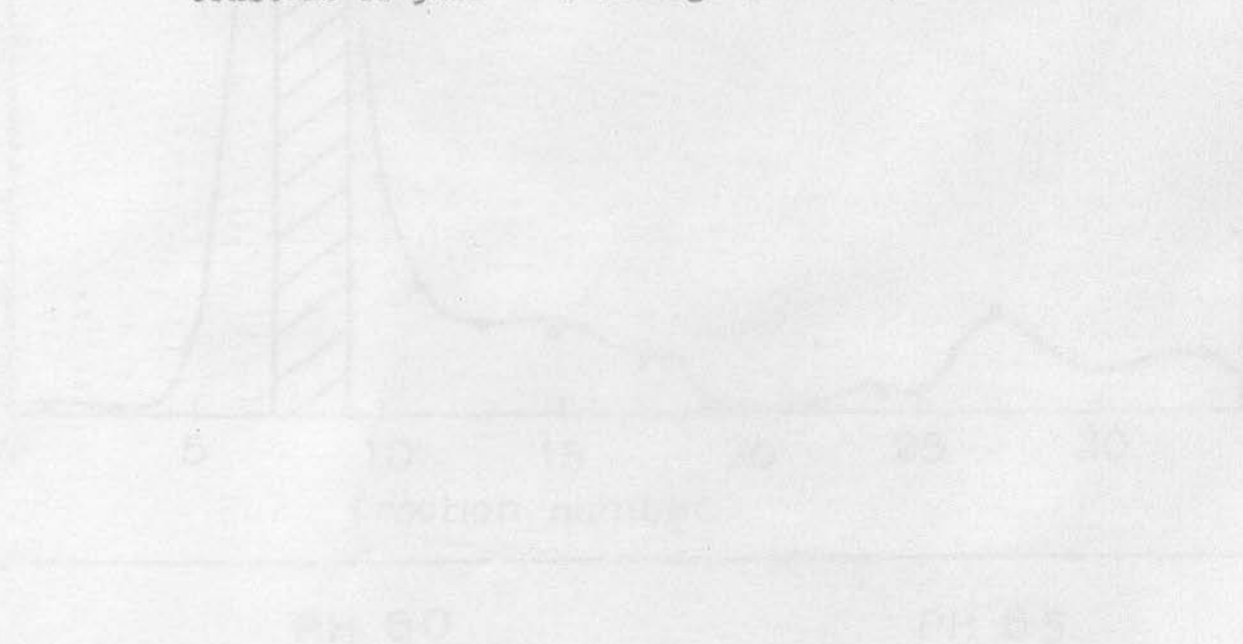
Serum Fraction	Titre
Serum (BG)	1280
Chromatographic (FI) peak	80
Chromatographic (FII) peak	0
Lower ultra centrifuge fraction	640
Upper ultra centrifuge fraction	320

TABLE 7

Details of fractions	Sedimentation constants in svedberg units
Chromatographic peak (FI) eluted at PH 6.0	(1) Major component $S = 5.7 \times 10^{-13}$ (2) Minor component $S = 16 \times 10^{-13}$
γ-globulin components of serum before fractionation in ultra centrifuge	(1) Major component $S = 7 \times 10^{-13}$ (2) Minor component $S = 20 \times 10^{-13}$
γ-globulin components of lower cell fraction after spinning at 60,000 rpm for 2 hrs.	(1) Major component $S = 7 \times 10^{-13}$ (2) Minor but greatly enhanced component $S = 20 \times 10^{-13}$

(3) Post myocardial infarct serum (case S)

Graph 3 shows that ion-exchange chromatography of the 40% ammonium sulphate fraction of the serum of patient (S) obtained 5 weeks after a myocardial infarction, gave rise to two peaks FI and FII eluted at PH 6.0 and 6.5 respectively. As in case (BG) all the antiglobulin activity was associated with FI and FII was inactive. (Table 8). It is seen from plate 3 that on electrophoretic analysis on cellulose acetate paper at PH 8.6 both these fractions behaved as electrophoretically homogeneous γ-globulins.
Ultra centrifuge analysis of FI showed that only one component was present with a sedimentation constant of 5×10^{-13} svedbergs (Table 8).



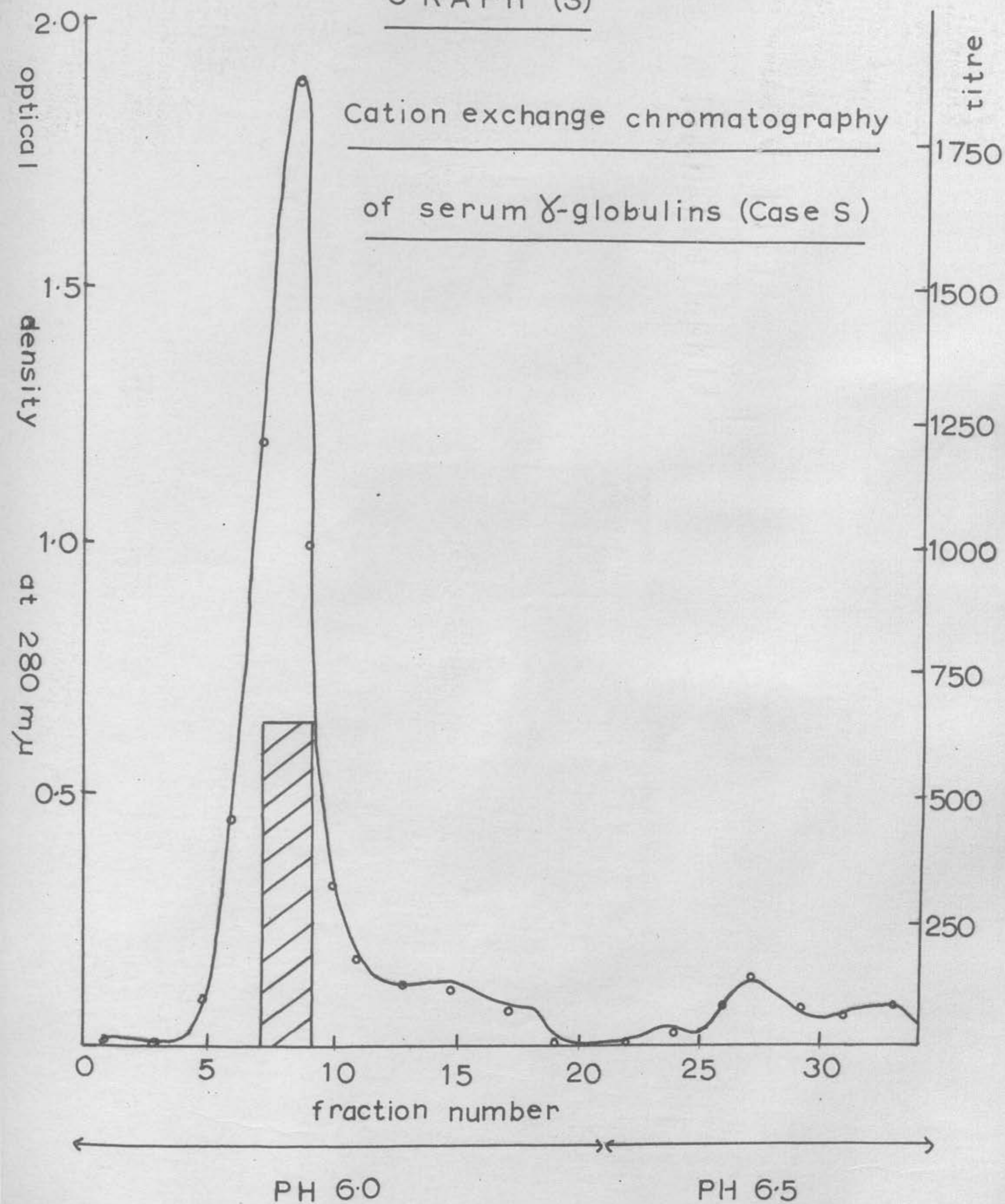

GRAPH (3)
Cation exchange chromatography
of serum γ-globulins (Case S)
optical density at 280 mμ
titre
2·0
1·5
1·0
0·5
1750
1500
1250
1000
750
500
250
0
5
10
15
20
25
30
fraction number
PH 6·0
PH 6·5

PLATE (3)

Electrophoretic analysis of case (S) serum and γ-globulin fractions on cellulose acetate paper at PH 8.6.

(1) SERUM (S)

(2) Chromatographic peak FI

(3) Chromatographic peak FII

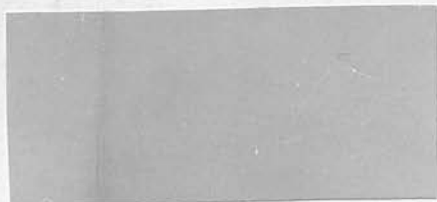


TABLE 8

Protein tested	Protein Concentration gms %	Titre	Sedimentation constant (svedbergs)
Serum (case 8)	-	640	-
Chromatographic peak (FI) eluted at PH 6.0	3.65	640	$5x10^{-13}$
Chromatographic peak (FII) eluted at PH 6.5	1.28	0	-

Starch gel electrophoresis of serum

(1) Rheumatoid serum

Graph No.(4) shows that the activity peak of the rheumatoid serum was associated with a protein fraction which migrated in the fast γ-globulin region towards the anode at PH 8.48. The activity peak did not coincide with the main γ-globulin protein peak.

The photographs on Plate (5) show the corresponding mobilities of the starch gel fractions on cellulose acetate paper at PH 8.6. It is seen that the fraction containing the activity peak, zone (7), migrated as a fast γ-globulin bordering on the β-region.

PLATE (4)

Starch Gel Electrophoresis Patterns.

(1) Analytical electrophoresis of normal serum.

(2) Preparative electrophoresis of rheumatoid serum.

(3) Preparative electrophoresis of post-myocardial infarct serum (case G)

(4) Preparative electrophoresis of post-myocardial infarct serum (case BG)

TABLE 9

Zone Number	Titre
1	0
2	0
3	0
4	0
5	0
6	10
←ORIGIN	
7	80
8	10
9	0
10	0
11	0
12	0
13	0

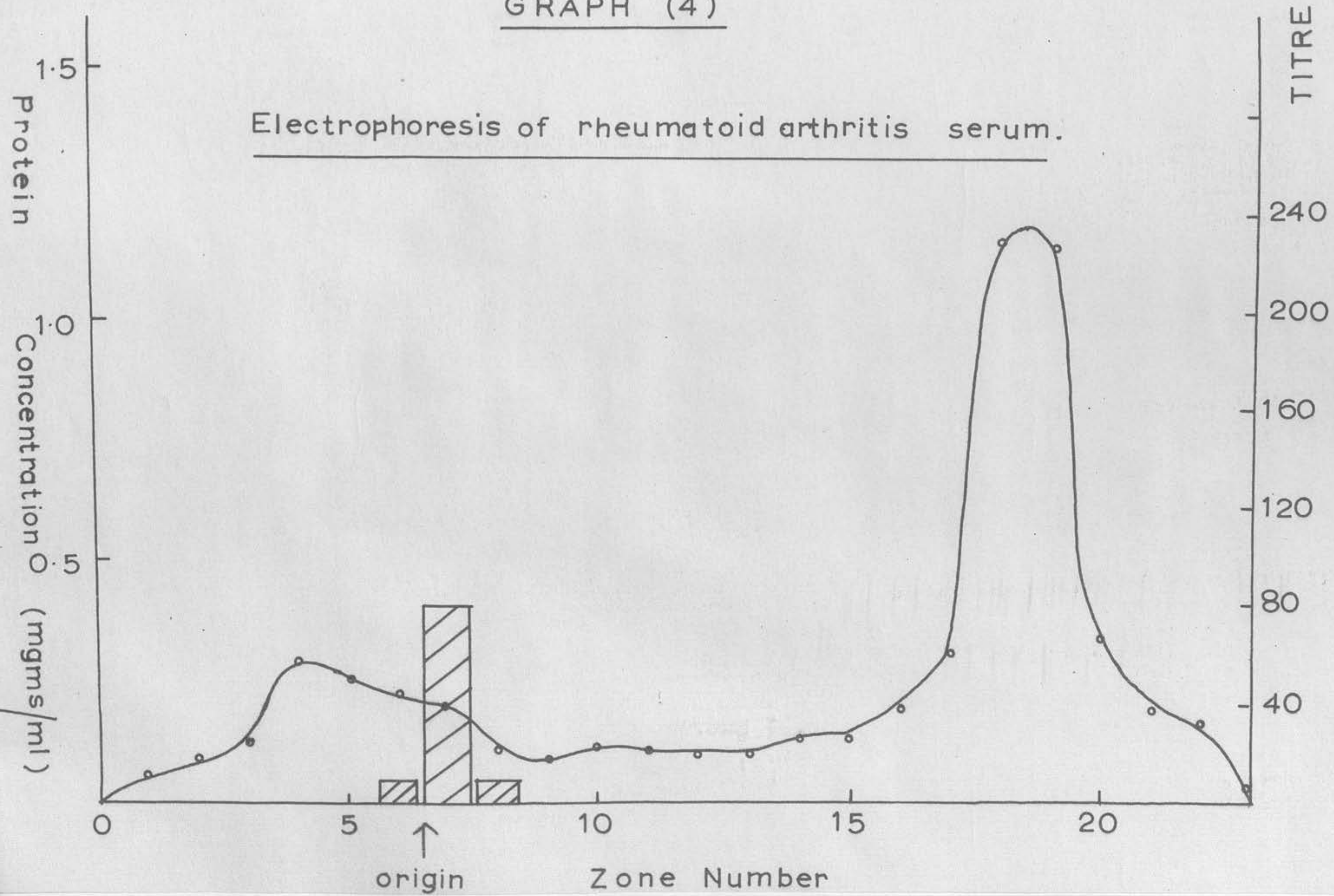
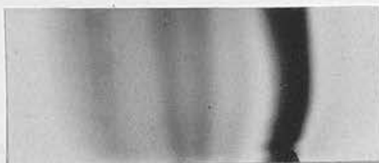

GRAPH (4)
Electrophoresis of rheumatoid arthritis serum.
Protein Concentration (mgms/ml)
1·5
1·0
0·5
TITRE
240
200
160
120
80
40
0
5
10
15
20
origin
Zone Number

PLATE (5)

Electrophoretic analysis of starch gel fractions of rheumatoid serum on cellulose acetate paper at PH 8.6.

(1) Rheumatoid serum



(2) Starch gel zone (4)

(3) Starch gel zone (5)

(4) Starch gel zone (6)

(5) Starch gel zone (7)

(6) Starch gel zone (8)

(2) <u>Post myocardial infarct serum (case G)</u>

Graph (5) shows that the activity peak of this serum, from patient (G) obtained 3 weeks after a myocardial infarction, was associated with a fraction which migrated in the mid γ-globulin region and migrated towards the cathode at PH 8.48. The activity peak actually coincided with the main γ-globulin protein peak in this serum. The photographs on plate (6) show that the active fraction zone (6) migrated in the mid γ-globulin region on cellulose acetate paper at PH 8.6.

TABLE 10

Zone Number	Titre
1	0
2	0
3	0
4	0
5	20
6	160
← ORIGIN	
7	40
8	20
9	0
10	0
11	0

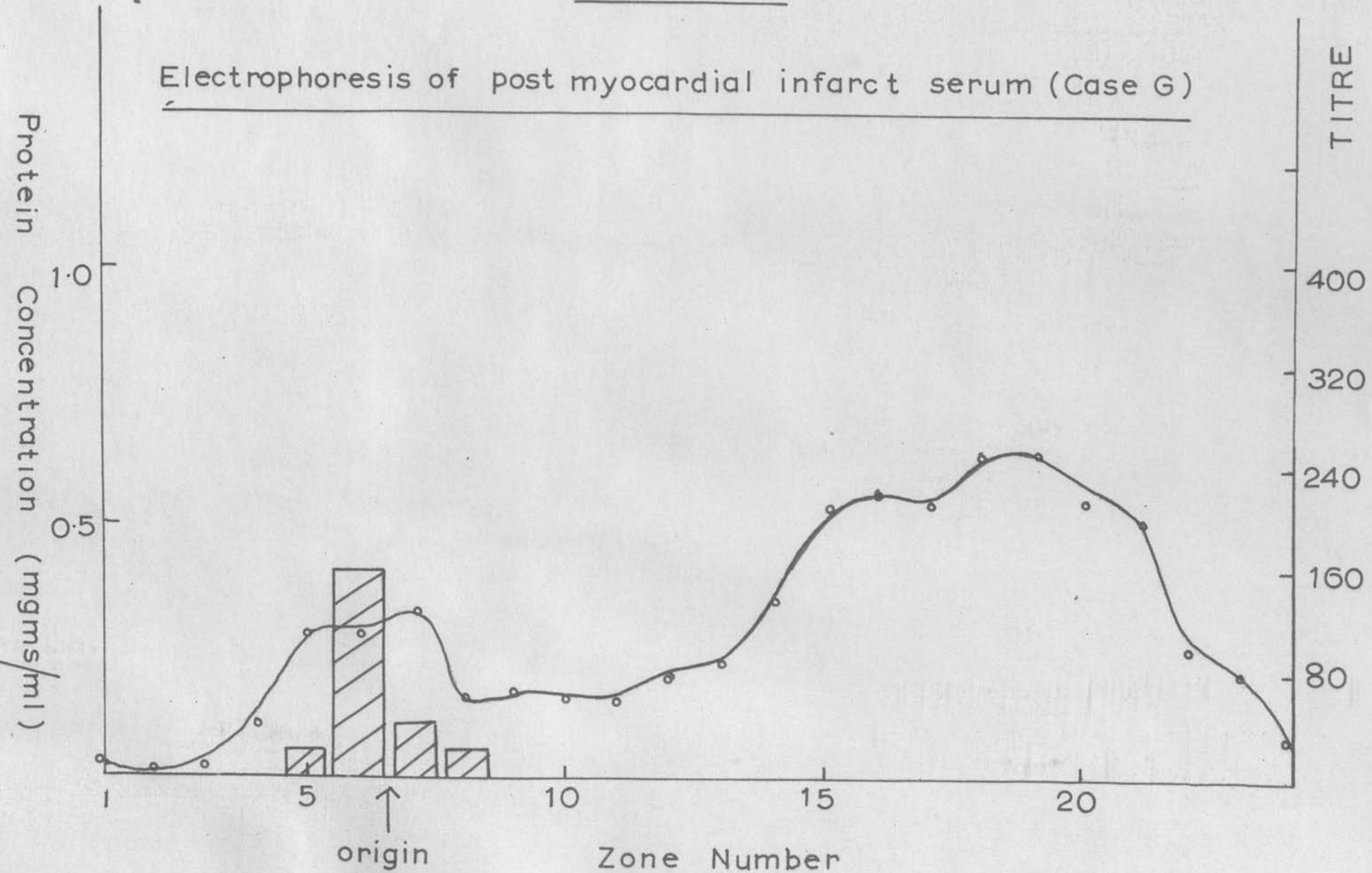
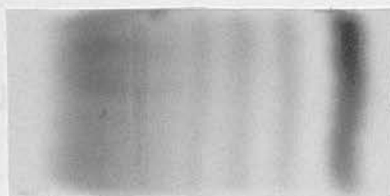

GRAPH (5)
Electrophoresis of post myocardial infarct serum (Case G)
Protein Concentration (mgms/ml)
1·0
0·5
TITRE
400
320
240
160
80
1
5
10
15
20
origin
Zone Number

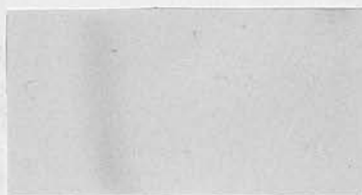
PLATE (6)

Electrophoretic analysis of starch gel fractions of post-myocardial infarct serum (case G) on cellulose acetate paper at PH 8.6.

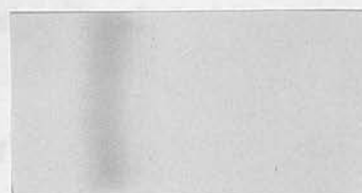
(1) SERUM (case G)



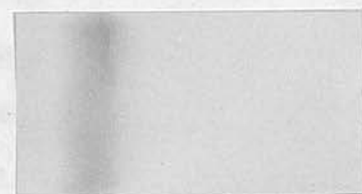
(2) Starch gel zone (4)



(3) Starch gel zone (5)



(4) Starch gel zone (6)



(5) Starch gel zone (7)

(6) Starch gel zone (8)

(3) Post myocardial infarct serum (case BG)

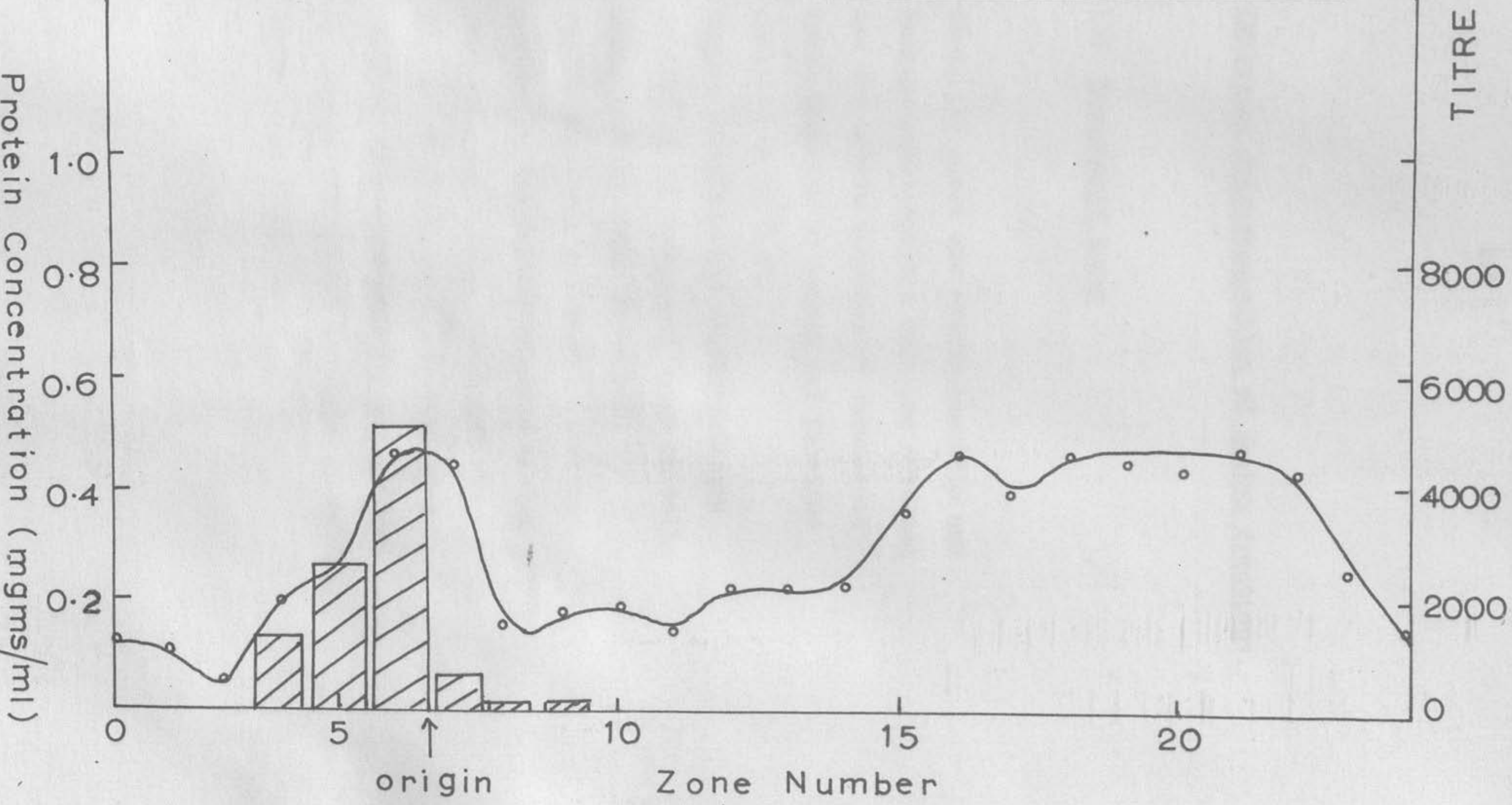
It is observed from graph (6) that the activity peak of the serum of patient (BG), tested 7 weeks after a myocardial infarction, was associated with a fraction in the mid γ-globulin region and migrated towards the cathode at PH 8.48. The activity peak coincided with the main γ-globulin protein peak as in serum (G). Electrophoretic analysis of the starch gel γ-globulin fractions on cellulose acetate paper at PH 8.6 indicated that zone (6) containing the activity peak migrated in the mid γ-globulin region.

TABLE 11

Zone number	Titre
1	0
2	0
3	0
4	1280
5	2560
6	5120
←ORIGIN	
7	640
8	20
9	20
10	10
11	0
12	0

GRAPH (6)

Electrophoresis of post myocardial infarct serum (Case B)



Antigenic characterization of active fractions.

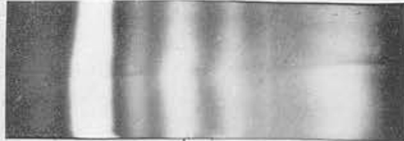
(1) Rheumatoid serum

Plate (7) gives the electrophoretic and immunoelectrophoretic patterns obtained for the active fraction of rheumatoid serum obtained by starch gel electrophoresis at PH 8.48.
Figure (1) gives the electrophoretic pattern of the reference serum on cellulose acetate paper at PH 8.6.
Figure (2) gives the electrophoretic pattern of the active fraction eluted from zone (7) of the starch gel and figure (3) shows the precipitin arcs obtained when this fraction was allowed to diffuse against an antihuman globulin serum.

PLATE (7)

Antigenic characterization of starch gel zone (7) of rheumatoid serum.

(1)



Electrophoresis of serum

(2)

Electrophoresis of zone (7)

(3)

Immunoelectrophoresis of zone (7)

(2) Post myocardial infarct serum (case G)

Plate (8) gives the electrophoretic and immunoelectrophoretic patterns obtained for the active fraction of post myocardial infarct serum (case G) obtained by starch gel electrophoresis at PH 8.48.

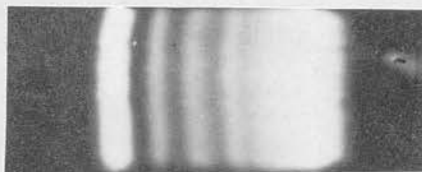
Figure (1) gives the electrophoretic pattern of the serum at PH 8.6 on cellulose acetate paper.

Figure (2) gives the electrophoretic pattern of the fraction eluted from zone (6) of the starch gel and figure (3) shows the precipitin arc obtained when this fraction was allowed to diffuse against an antihuman globulin serum.

PLATE (8)

Antigenic characterization of starch gel zone (6) of post-myocardial infarct serum (case G)

(1)



Electrophoresis of serum

(2)

Electrophoresis of zone (6)

(3)

Immunoelectrophoresis of zone (6)

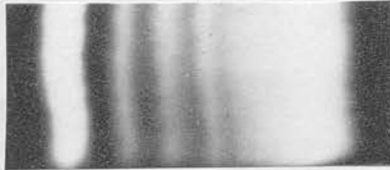
(3) Post myocardial infarct serum (case BG)

Plate (9) gives the electrophoretic and immunoelectrophoretic patterns obtained for the active fractions of post myocardial infarct serum (case BG) obtained by starch gel electrophoresis at PH 8.48.
Figure (1) gives the electrophoretic pattern of the reference serum. Figure (2) gives the electrophoretic pattern of the fraction eluted from zone (6) of the starch gel and figure (3) shows the precipitin arc obtained when this fraction was allowed to diffuse against an antihuman globulin serum.

PLATE (9)

Antigenic characterization of starch gel zone (6) of post-myocardial infarct serum (case BG)

(1)



Electrophoresis of serum

(2)

Electrophoresis of zone (6)

(3)

Immunoelectrophoresis of zone (6)

(2) Chemical Characterization

Effect of amino group blocking reagents on agglutinin activity.

Table 12 shows that on exposure to 0.002M acetic anhydride or 0.002M 1: fluoro 2:4 dinitrobenzene 100% of the agglutinin activity of both the post myocardial infarct sera was lost. Slightly less activity was lost on exposure of rheumatoid serum to the same conditions but this difference in percentage loss of activity can be considered to be negligible.

TABLE 12

Diagnosis	Titre of control serum	Titre of serum treated with 0.002M acetic anhydride	Titre of serum treated with 0.002M fluorodinitro benzene	% inhibition of activity
Rheumatoid Arthritis	2560	40	40	98%
Post myo-cardial infarct (case C)	1280	0	0	100%
Post myo-cardial infarct (case G)	1280	0	0	100%

PH optimum of agglutination reaction.

Table 13 shows that the agglutinating activity of both aliquots of rheumatoid serum was PH dependent, the titres reaching a maximum at PH 8.0. The agglutinating activity of the four post myocardial infarct sera tested was not PH dependent and the same titres were obtained between PH 6.5 and PH 8.0.

TABLE 13

Diagnosis	Titre at PH 6.5	Titre at PH 7.0	Titre at PH 7.5	Titre at PH 8.0
Rheumatoid Arthritis (1)	320	640	1280	5120
Rheumatoid Arthritis (2)	1280	1280	2560	5120
Post myocardial infarct (case 1)	320	320	320	320
Post myocardial infarct (case 2)	80	80	80	80
Post myocardial infarct (case 3)	5120	5120	5120	5120
Post myocardial infarct (case 4)	160	160	160	160
Negative control	0	0	0	0

Treatment with 0.5M cysteine

Table 14 shows that over 90% of the agglutinin activity of rheumatoid and post myocardial infarct serum was lost after dialysis against 0.5M cysteine PH 8.5. Similar treatment of the rabbit antiserum caused no loss in activity.

Treatment with 0.1M thioglycollic acid.

Table 15 shows that treatment of rheumatoid and the 4 post myocardial infarct sera tested with 0.1M thioglycollic acid followed by dialysis against 0.02M iodoacetate resulted in 97-100% loss in serological activity. On dialysis of the thioglycollic acid treated sera agains 0.15M phosphate buffer PH 8.0 there was a slight regeneration of activity and in case (1) the activity was completely restored. The activity of the rabbit antiserum was not affected by this treatment.

Treatment with 0.1M sodium sulphite.

Table 16 shows that treatment of rheumatoid serum with 0.1M sodium sulphite resulted in over 90% loss of the agglutinin activity. Simi treatment of the post myocardial infarct serum resulted in 50% loss i agglutinin activity whereas the activity of the rabbit antiserum was not affected at all.

Treatment with 6M urea.

Table 17 shows that 96% of the activity of rheumatoid serum was lost after dialysis against 6M urea. 75% of the agglutinin activity of t post myocardial infarct serum was lost whereas there was a considerab enhancement of the activity of the rabbit antiserum.

Heat stability.

Table 18 shows that all the sera were stable at 55°C. At 60°C there was some loss of activity of the rheumatoid serum and 3 of the post myocardial infarct sera. At 65°C approximately 90% of the agglutin activity of all the sera was lost.

TABLE 14

Clinical Diagnosis	Titre	Titre after dialysis against 0.5M cysteine	% inhibition
Rheumatoid Arthritis	5120	0	100
Post myocardial infarct (case 1)	640	40	94
Post myocardial infarct (case 2)	40	0	100
Post myocardial infarct (case 3)	640	40	94
Rabbit antihuman globulin serum	2560	2560	0

TABLE 15

Clinical Diagnosis	Titre	Titre after treatment with 0.1M T.G.A.	% inhibition	Titre after treatment with 0.1M T.G.A. and 0.02M iodoacetate	% inhibition
Rheumatoid Arthritis	5120	160	96	20	99
Post myocardial infarct (case 1)	640	640	0	20	97
Post myocardial infarct (case 2)	2560	0	100	0	100
Post myocardial infarct (case 3)	2560	0	100	0	100
Post myocardial infarct (case 4)	2560	320	87	0	100
Rabbit anti-human globulin serum	2560	2560	0	2560	0

TABLE 16

Clinical Diagnosis	Titre	Titre after treatment with 0.1M Na_2SO_3	% inhibition
Rheumatoid Arthritis	5120	320	93
Post myocardial infarction	640	320	50
Rabbit anti-human globulin serum	2560	2560	0

TABLE 17

Clinical Diagnosis	Titre	Titre after dialysis against 6M urea	% inhibition
Rheumatoid Arthritis	5120	160	96
Post myocardial infarction	640	160	75
Rabbit anti-human globulin serum	2560	20,480	-

TABLE 18

Clinical Diagnosis	Titre	Titre after 55°C	Titre after 60°C	Titre after 65°C	% inhibition at 65°C
Rheumatoid Arthritis	10,240	10,240	5120	160	98
Post myocardial infarct (case Sm)	2560	2560	1280	20	99
Post myocardial infarct (case H)	40	40	40	0	100
Post myocardial infarct (case Hc)	2560	2560	1280	20	99
Post myocardial infarct (case Su)	2560	2560	1280	320	87

(3) Immunological characterization

Titration of post myocardial infarct sera with heart-extract sensitized erythrocytes.

Table 19 shows that none of the 15 post myocardial infarct sera tested agglutinated human erythrocytes sensitized with extracts of human heart. 5 sera which gave positive titres with γ-globulin sensitized cells gave negative titres with the heart extract.

Absorption of sera with unsensitized erythrocytes and erythrocytes sensitized with human heart extract and γ-globulin.

Table 20 indicates that absorption of the rheumatoid and post myocardial infarct sera with unsensitized red cells and cells sensitized with extracts of human heart resulted in no reduction of serological activity. Absorption of rheumatoid serum with γ-globulin sensitized erythrocytes resulted in a partial loss of activity whereas absorption of post myocardial infarct sera resulted in 87% and 100% losses in activity respectively.

TABLE 19

Clinical Diagnosis	Titre with bovine γ-globulin sensitized cells	Titre with heart-extract sensitized cells
15 post myocardial infarct cases	-	0
Rheumatoid Arthritis	5120	0
Post myocardial infarct (case S)	1280	0
Post myocardial infarct (case D)	160	0
Post myocardial infarct (case McK)	40	0
Post myocardial infarct (case N)	320	0

TABLE 20

Clinical Diagnosis	Titre	Titre after absorption with heart extract	Titre after absorption with bovine γ-globulin	Titre after absorption with unsensitized erythrocytes
Rheumatoid Arthritis	10,240	10,240	5120	10,240
Post myocardial infarct (case S)	1280	1280	160	1280
Post myocardial infarct (case T)	320	320	0	320

Specificity of agglutinating factors.

Table 21 shows that the rheumatoid serum and all the post myocardial infarct sera gave positive agglutination titres with denatured and undenatured bovine γ-globulin sensitized erythrocytes.

Only the rheumatoid serum and the post myocardial infarct serum (case H) gave positive titres with denatured human γ-globulin and all the sera gave negative titres with undenatured human γ-globulin.

Only the rheumatoid serum agglutinated sensitized sheep cells. A positive titre in this test is greater than 1 in 64.

TABLE 21

Clinical Diagnosis	Titre with bovine γ-globulin	Titre with denatured bovine γ-globulin	Titre with human γ-globulin	Titre with denatured human γ-globulin	Titre with sensitized sheep cells
Rheumatoid Arthritis	10,240	10,240	0	10,240	1024
Post myocardial infarct (case B)	320	320	0	0	16
Post M.I. (case C)	160	320	0	0	8
Post M.I. (case G)	320	320	0	0	16
Post M.I. (case Su)	1280	1280	0	0	64
Post M.I. (case H)	5120	5120	0	5120	8
Post M.I. (case Sm)	640	640	0	0	8

Inhibition of agglutination by γ-globulin.

Table 22 shows that the agglutinating activity of both the rheumatoid and post myocardial infarct sera was inhibited by the presence of undenatured or denatured bovine γ-globulin. One aliquot of rheumatoid serum (2) was inhibited by denatured and undenatured human γ-globulin whereas aliquot (1) was inhibited by denatured human γ-globulin only. All the post myocardial infarct sera tested were inhibited by denatured human γ-globulin and 3 of these sera were also inhibited by undenatured human γ-globulin.

Table 23 summarizes these results.

TABLE 22

Clinical Diagnosis	Titre	Titre in presence of bovine γ-globulin	Titre in presence of denatured bovine γ-globulin	Titre in presence of human γ-globulin	Titre in presence of denatured human γ-globulin
Rheumatoid Arthritis(1)	20,480	0	0	20,480	2560
Rheumatoid Arthritis(2)	2560	0	0	0	40
Post myocardial infarct (case (1))	80	0	0	0	0
Post M.I. (case (2))	160	20	0	0	0
Post M.I. (case (3))	20,480	0	0	20,480	5120
Post M.I. (case (4))	160	0	0	0	0
Post M.I. (case (5))	2560	0	640	10,240	0

TABLE 23

Clinical Diagnosis	Number of sera tested	Number of sera inhibited by bovine γ-globulin	Number of sera inhibited by denatured bovine γ-globulin	Number of sera inhibited by human γ-globulin	Number of sera inhibited by denatured human γ-globulin
Post myocardial infarction	5	5	5	3	5
Rheumatoid Arthritis	2	2	2	1	2

DISCUSSION

Physical characterization of the antiglobulin present in the sera of 50% of the 58 patients studied after myocardial infarction showed it to be a γ-globulin with many of the properties of an antibody. A comparison of its physical and immunological properties with those of the rheumatoid factor established several distinct differences between the two factors and these will be emphasised in the present discussion.

Precipitation of the euglobulin fraction of post myocardial infarct sera showed that in approximately 90% of the cases only between 0 and 11% of the agglutinin activity was found to be associated with this fraction whereas 100% of the agglutinin activity of rheumatoid sera was found in the euglobulin fraction. In one serologically active post myocardial infarct serum, however, 50% of the activity was present in the euglobulin fraction. This serum gave a low positive sheep cell test suggesting that part of the activity was due to the presence of the rheumatoid FII agglutinating factor.

Ammonium sulphate fractionation of both rheumatoid and post myocardial infarct serum followed by titration of the fractions indicated that approximately 90% of the agglutinin activity was associated with the fraction precipitated by 46% saturation with ammonium sulphate. Analytical electrophoresis of these fractions on cellulose acetate paper at PH 8.6 showed that the agglutinin activity was associated with the γ-globulin fraction in both sera.

Ion-exchange chromatography of the serum fractions precipitated by 40% saturation with ammonium sulphate on a carboxymethyl cellulose cation exchange resin effected a resolution into /

into two γ-globulin components in both sera. A major protein peak was eluted at PH 6.0 with phosphate buffer and a minor peak at PH 6.5. In the case of the rheumatoid serum the specific activity of the first peak was over six times that of the second peak whereas all the activity of the post myocardial infarct sera was associated with the first chromatographic fraction, the second being totally inactive. It was found, however, that the protein binding capacity of the resin was low at PH 5.2, the PH used for buffering the column, and a large proportion of the protein applied was washed off with the equilibrating buffer. The final protein concentrations of the eluted and concentrated fractions were consequently low and it is conceivable that any differences in activity distribution in the two sera may well be due to loss of agglutinin activity in the fraction eluted at PH 6.5 reducing the activity to undetectable amounts, rather than to differences in their ion exchange characteristics.

Electrophoretic analysis of the two chromatographic fractions of the post myocardial infarct sera on cellulose acetate paper at PH 8.6 showed that both fractions migrated as γ-globulins and appeared to be electrophoretically homogeneous. Ultra centrifuge analysis of the first active chromatographic fraction of rheumatoid serum eluted at PH 6.0 showed that it consisted of two molecular species; a major component with a sedimentation constant of 5.0×10^{-13} svedbergs and a minor component with a sedimentation constant of 13×10^{-13} svedbergs. Ultra centrifuge analysis of the first chromatographic fraction of one post myocardial infarct serum (case BG) also revealed the presence of two components with sedimentation constants of 5.7×10^{-13} svedbergs and 16×10^{-13} svedbergs /

16×10^{-13} svedbergs for the major and minor components respectively.

Analysis of the first chromatographic fraction of a second post myocardial infarct serum (case S) showed that only one component was present which had a sedimentation constant of 5×10^{-13} svedbergs. These S values are not corrected for concentration effects due to the limited amount of material available but it is obvious that the 5S and 13-16S components correspond to the normal 7S γ-globulin and 19S macroglobulin components of human serum respectively (Svedberg and Pedersen 1940).

Preparative ultra centrifuge experiments, in which post-myocardial infarct serum was fractionated in a partition cell into heavy and light components so that the lower cell contained all the macromolecular 19S material, showed that the agglutinin activity was associated with both fractions. These results are however equivocal and the activity of the lower fraction may well be due to the presence of the light 7S components which are inevitably present.

Chemical evidence regarding the nature of the antiglobulin molecule in post myocardial infarct sera was more convincing. Treatment of sera with 0.1M thioglycollic acid (TGA) followed by dialysis against 0.02M iodoacetate in phosphate buffer PH 8.0 resulted in 97-100% loss of serological activity. Similar treatment of rheumatoid serum resulted in 99% loss of activity, suggesting that the post myocardial infarct antiglobulin, like the rheumatoid factor, is susceptible to destruction by sulphydryl reagents. In certain cases a lower loss in agglutinin activity was obtained when the TGA treated sera were dialysed against buffer alone. It has been shown (Deutch and Morton 1957; Fudenberg and Kunkel (1957 and 1958) that sera containing antibodies of the high molecular weight 19S class are inactivated by sulphydryl reagents, due to destruction/

destruction of the disulphide bonds maintaining the integrity of the molecule, with the formation of serologically inactive subunits. The iodoacetate, which is a sulphydryl group blocking reagent, prevents reconstitution of the disulphide bonds on dialysis against buffer. Similar treatment of a rabbit antihuman globulin serum resulted in no loss of agglutinin activity due to the fact that the antibodies of rabbit serum belong almost entirely to the low molecular weight 7S class (Kabat 1939) and are therefore not affected by sulphydryl reagents.

The results obtained on treatment of sera with cysteine and sodium sulphite substantiate this evidence and both reagents caused inactivation of rheumatoid and post myocardial infarct serum but had no effect on the rabbit antiserum. The evidence presented thus strongly suggests that the agglutinin activity of post myocardial infarct sera is associated, at least in part, with the macroglobulin fraction of serum. It is possible that the activity is associated with both the high and the low molecular weight γ-globulins. The Wasserman antibodies or syphilitic reagins of human origin have been shown to consist of two components with sedimentation constants of 7S and 19S (Davis, Moore, Kabat and Harris 1945) and the warm and cold autoantibodies of acquired hemolytic anaemia belong to the 7S and 19S class respectively (Fudenberg, Kunkel and Franklin 1958).

It appears that the agglutinins of rheumatoid and post myocardial infarct sera are susceptible to urea and on dialysis against 6M urea 96% of the activity of rheumatoid serum and 75% of the activity of the post myocardial infarct serum was lost. This is in contrast to the results of Putnam (1959) who reported /

reported that the macroglobulins of a number of macroglobulinemic sera could be disrupted by sulphydryl reagents but not by temperature, ultra sonic vibration or urea and suggested that the forces involved in maintaining the integrity of the molecule were covalant disulphide bonds and not merely electrostatic linkages. The results obtained in this investigation, however, suggest that both covalant disulphide bonds and electrostatic linkages are involved in the structure of the rheumatoid and post myocardial infarct agglutinins. The unusual effect of urea on the agglutinin activity of rabbit anti-human globulin serum has not been satisfactorily explained but it is possible that unfolding of the globulin molecule, due to breakage of electrostatic linkages, causes exposure of latent antibody sites which are then available for combination with antigen.

The heat stability of the rheumatoid and post myocardial infarct agglutinating factors conforms with that of antibody heat stability (Boyd 1947) and it was found that whereas both factors were stable at 55°C, there was increasing loss of activity at 60°C and at 65°C over 90% of the serological activity of all the sera was lost.

Starch gel electrophoresis of rheumatoid and post myocardial infarct sera revealed a difference in the electrophoretic properties of the two factors at PH 8.48. Electrophoresis of rheumatoid serum showed that 90% of the agglutinin activity was associated with a fraction which migrated in the fast γ-globulin region towards the anode. Analytical electrophoresis of the globulin fractions eluted from the gel, on cellulose acetate paper at PH 8.6, showed that the fraction containing the activity peak migrated in the fast γ-globulin region bordering on the β-area./

β -area. No β -globulin band was visible in the starch gel segment from which this fraction was eluted but it is likely that some trailing of the fractions occurs in amounts not sufficiently large to be observed as a visible protein band but detectable on cellulose acetate paper.

On starch gel electrophoresis of post myocardial infarct serum it was found that the activity peak was associated with a fraction in the mid γ -globulin region which migrated towards the cathode. Analytical electrophoresis of the globulin fractions of these post myocardial infarct sera on cellulose acetate paper showed 75% and 92% of the activity of the two sera respectively to be associated with a fraction which migrated in the slow to mid γ -globulin region, although it was difficult to distinguish in this system between fractions which migrated towards the cathode in the mid and slow γ -globulin region.

The serologically active fractions of rheumatoid and post myocardial infarct sera prepared by starch gel electrophoresis were shown to be antigenically as well as electrophoretically distinct. When the active fraction of rheumatoid serum was subjected to immuno-electrophoresis on cellulose acetate paper against a rabbit anti-human globulin serum, two antigenically distinct, intersecting, precipitation arcs were obtained. When compared with the electro-phoretic pattern obtained for this fraction it was observed that the first precipitation arc ie. that nearest the origin extended over a broad area in the fast γ -globulin and β-globulin region. The second arc coincided with a band in the α_2 -globulin region bordering on the β-region. It is evident that the agglutinin activity of rheum-atoid serum is distributed over a wide range of fractions from the fast γ

the α_2-globulin region. This finding agrees with the results of Svartz (1957 (ii) and Kunkel, Franklin and Muller-Eberhard (1959) who located the haemagglutinin activity in a broad peak in the fast γ-globulin region bordering on the β-region.

Immunoelectrophoretic analysis of the active fractions of the post myocardial infarct sera revealed the presence of only one precipitation arc, the apex of which coincided with the axis of a protein band in the mid γ-globulin region. The serological activity of the post myocardial infarct sera is thus clearly associated with a protein fraction which behaves electrophoretically and antigenically as a γ-globulin.

It must be emphasised that individual genetic variations in the electrophoretic mobility of these proteins may occur and these results apply only to the sera investigated. Fudenberg and Kunkel (1957) showed, for example, that the haemagglutinin activity of the cold autoantibodies of acquired hemolytic anaemia sera was associated with protein fractions ranging from the fast γ to the β-globulin region in different patients.

It is apparent from this study of the physical characteristics of the post myocardial infarct antiglobulin that it possesses certain distinguishing features from the rheumatoid factor although, serologically, it behaves identically in the FII agglutination test. It has not hitherto been reported that false positive results for the rheumatoid factor are obtained in this test with post myocardial infarct sera, although it is well known that FII sensitized erythrocytes are agglutinated by the sera of a small percentage of patients with rheumatoid-like lesions such as disseminated lupus erythematosus, ankylosing spondylitis, psoriasis, periarteritis nodosa, /

nodosa, dermatomyositis, as well as certain hepatocellular lesions such as hepatic cirrhosis and infective hepatitis and by syphilitic sera (Ziff 1957; Svartz 1958 (ii)). In a systematic study of the "false positive" tests Dresner et al (Dresner and Trombly 1959) showed that the sera from patients with hepatocellular lesions, certain collagen diseases and syphilis caused agglutination of latex particles coated with FII. His results showed that there is a fundamental difference between the factors responsible for latex agglutination and agglutination of sheep cells sensitized with rabbit antibody and whereas in hepatic diseases, certain viral infections and syphilis the latex fixation test was commonly positive the Rose-Waaler sheep cell test was almost invariably negative.

Although the majority of these "false positive" results belong to three distinct groups of diseases which should rarely lead to confusion with rheumatoid arthritis, they may themselves be of considerable diagnostic value.

Possible interference of the naturally occuring blood group iso agglutinins in this system was ruled out by the absorption experiments, in which it was shown that no loss of serological activity occured on absorption of post myocardial infarct sera with unsensitized erythrocytes. It is significant that only one of the sera investigated gave a positive sheep cell test and there is thus a clear immunological distinction between the two factors. It seems that the true rheumatoid factor has a wider range of specificity with regard to the modified γ-globulins with which it reacts than the post myocardial infarct factor. The rheumatoid factor is believed to consist of at least two factors and Heller and Kolodny (1955) found that several reactant variants of the rheumatoid agglutinating factors /

factors were present in Cohn FIII preparations of rheumatoid serum. Some of the more marked variants in serum produced positive reactions in the sheep erythrocyte agglutinin test (SEA test) and negative reactions in the FII agglutinin test and vice versa indicating the wide heterogeneity of the rheumatoid factors. It was also found that positive sera absorbed with the SEA reagent remained positive to the FII cell reagent. The post myocardial infarct antiglobulin thus appears to resemble serologically the FII agglutinating reagent present in rheumatoid serum but not the sheep cell factor.

Although rheumatoid sera would agglutinate erythrocytes sensitized with denatured bovine and human Cohn FII, only one of the post myocardial infarct sera tested agglutinated cells sensitized with human γ-globulin but this serum gave a negative sheep cell test. This does not, however, negate the possibility that the reactant antiglobulin was identical to the rheumatoid factor and this serum may be similar to those studied by Heller et al (1955) which gave positive FII tests but negative sheep cell tests. Inhibition tests, on the other hand, showed that the agglutinin activity of both rheumatoid and post myocardial infarct sera was inhibited by the presence of denatured human and bovine γ-globulin, indicating that both these proteins fall within the specificity requirements of the two antiglobulins. The failure of post myocardial infarct sera to agglutinate human γ-globulin sensitized cells may be due to a difference in the degree of purity of the two preparations as it appears that partial modification or denaturation by heating or ageing of the γ-globulin is required for maximal activity in the FII test (Glynn and Holborrow 1957).

The possibility that post myocardial infarct sera may /

may contain autoantibodies to components of heart muscle tissue was investigated but yielded negative results. The sera did not agglutinate human group O Rhesus negative erythrocytes sensitized with saline extracts of human heart tissue nor did absorption of the sera with heart extract sensitized cells result in any loss of agglutinin activity.

These results suggest that either the autoantibodies reported by Dornbusch (1957) and Gery, Davies and Ehrenfeld (1960) were not present in these sera or that the erythrocytes were inadequately sensitized. This second possibility must not be excluded as an appreciable degree of hemolysis was observed following sensitization with the heart extract.

Absorption of the post myocardial infarct sera with FII sensitized erythrocytes resulted in a large or total loss of agglutinin activity whereas absorption of rheumatoid sera with these cells brought about a negligible loss of activity. This difference in absorption behaviour is interesting and indicates that the avidity of the rheumatoid agglutinin for its antigen is lower than that of the post myocardial infarct factor. This suggests that whereas the latter participates in the typical antigen-antibody linkages of the H-bond and electrostatic type (Landsteiner 1946), the former may participate predominantly in weaker electrostatic linkages of the Van der Waals type. This hypothesis is further substantiated by the PH dependence of the rheumatoid factor agglutination reaction. It appears that agglutination is inhibited below PH 8.0 and increases from PH 6.5 to PH 8.0, reflecting a requirement for free uncharged - NH_2 groups possessing a "lone pair" of electrons; whereas agglutination by post myocardial infarct sera is not PH dependent and occurs equally well between PH 6.5 and PH 8.0. Heimer, Federico and Freyberg (1958) also observed this PH /

PH dependence for the sheep cell test and showed that maximal agglutination occured above PH 7.0 with inhibition below PH 7.0 whereas sera from patients with other collagen diseases were shown to be active as low as PH 5.2.

It appeared, however, that free amino groups are involved in both agglutination mechanisms are completely inhibited by amino group blocking reagents such as 1: fluoro 2:4 dinitrobenzene and acetic anhydride.

Extensive speculation on the pathological significance of the post myocardial infarct antiglobulin is not justifiable on the basis of this investigation but it is conceivable that it bears some relation to the heart specific autoantibodies reported by Dornbusch (1957) and Gery, Davis and Ehrenfeld (1960) and reflects an immune response to some component of heart muscle tissue which has been modified and rendered antigenic as a result of tissue damage. This hypothesis, however, was not substantiated by the agglutination and absorption experiments using human heart extracts and it is more plausible to assume, in light of the evidence presented, that they represent a secondary manifestation of some primary immune response resulting from a breakdown of the autoimmune homeostatic mechanism postulated by Burnet (1959) in his "clonal selection" theory.

It is worthy of note that in a patient with active Hashimotos thyroiditis antibodies to thyroglobulin and γ-globulin were present in the serum which gave a titre of 1:10,240 with purified human thyroglobulin and 1:640 with bovine γ-globulin in the tanned cell test. Whereas the serum of another patient suffering from Hashimotos thyroiditis gave a positive antithyroglobulin and anti γ-globulin titre before thyroidectomy but after thyroidectomy the anti/

antithyroglobulin titre fell to zero whereas the anti γ-globulin titre persisted.

These results would appear to support the theory of Burnet (1959) that the production of autoantibodies is the result of the general failure of some homeostatic mechanism which allows the proliferation of certain "forbidden clones" of cells capable of producing antibodies to a spectrum of chemical patterns so that antibodies are produced not only to the circulating thyroglobulin but also to other body constituents including the patients own γ-globulin, the latter persisting after removal of the damaged thyroid tissue when the antigenic stimulus of thyroglobulin is no longeraccessible.

There have been other reports of the occurence of two or more circulating autoantibodies in one patient in support of this theory. Wasasljerna (1959) reported the case of a patient suffering simultaneously from severe haemolytic anaemia and Hashimotos disease, when the serum gave a strongly positive direct Coombs test for cold autoantibodies and also possessed a high titre for thyroid autoantibodies. The existence of haemolytic anaemia and thrombocytopenia in the same patient has also been reported (Evans 1951) and of haemolytic anaemia and leukopenia (Dameshek (1953). Leukopenia and thrombocytopenia as well as haemolytic anaemia are sometimes found in cases of disseminated lupus erythematosus and Dameshek (1958) considers this disease to be a complex autoimmune disorder.

On the basis of these findings the presence of antibodies to γ-globulin in the sera of patients following necrotic lesions of the myocardium can be feasibly explained if one accepts the premise that myocardial infarction involves autoimmune responses engendered by a breakdown of homeostatic control.

It may be argued that the non specificity of these γ-globulins denies them antibody status and although this may be true according to classical immunological theories, in the light of the clonal selection theory of Burnet (1959) an antibody is less specifically definable and "any population of globulin molecules in a body fluid which can unite to a specifically limited range of chemical patterns can justifiably be called antibodies."

SUMMARY

(1) A pathological protein with the properties of an antiglobulin has been demonstrated in the sera of 50% of the patients tested following myocardial infarction and has been differentiated from the 'rheumatoid factor.'

(2) It reacts with human and bovine FII γ-globulin but not with sensitized sheep cells and not with human heart extract.

(3) Physical characterization has shown it to be a γ-globulin less than 11% of which is associated with the insoluble euglobulin fraction of serum, over 90% of the agglutinin activity being associated with a fraction precipitated by 46% saturation with $(NH_4)_2SO_4$.

(4) Starch gel electrophoresis of post myocardial infarct sera at 8.48 has shown that the activity is associated with a fraction which migrates in the mid γ-globulin region.

(5) Antigenic characterization of the active fraction by immunoelectrophoresis on cellulose acetate paper has shown that it consists of single antigenic species behaving electrophoretically as a γ-globulin.

(6) Ultra-centrifuge fractionation of the serum suggests that the agglutinin activity is associated with both high and low molecular weight γ-globulins. Destruction of serological activity by sulphydryl reagents and sulphite however, supports the view that the activity is associated with the high molecular weight components of serum.

(7) The factor is inactivated by dialysis against 6M urea indicating that H-bonding and electrostatic linkages in addition to disulphide bonds are involved in the structure of the active molecule.

(8) The heat stability of the agglutinating factor conforms with that of other antibodies. It is stable at 55°C with some inactivation at 60°C and at 65°C over 90% of the activity is lost.

(9) The agglutination mechanism in which the antiglobulin participates is not PH dependent but inactivation by amino group blocking reagents, fluorodinitrobenzene and acetic anhydride indicates that $-NH_2$ groups are involved in the agglutination.

(10) It is suggested that antiglobulin reflects an autoimmune response to the antigenic stimulus of the modified components of damaged tissue due to the failure of some autoimmune homeostatic mechanism.

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REFERENCES

Baxter J.H. and Goodman H.C. (1956). J.exp.Med.104.467

Borduas A.G. and Grabar P. (1953) Ann.Inst.Past.84 903

Boyd W.C. (1947) Fundamentals of Immunology (2nd Ed.N.Y.)p.420

Boyd W.C. (1954) The Proteins (Vol.IIB) by Neurath H. and Bailey K. p.790

Boyden S.V. (1951) J.exp.Med. 93 107

Burnet M. (1959) Brit.J.Med. (ii) 645

Burton P.M. Kerr.L. and Frazer S.C. (1959) Biochem.J. 72 12P.

Bywaters E.G.L. (1959) Proc.Roy.Soc.Med.52 437

Cannon P.R. and Marshall E.E. (1940) J.Immunol.38 365

Cavelti P.A. (1945) Proc.Soc.exp.Biol.and Mech.60 379

Cavelti P.A. (1947) Arch.Path.44 1

Cavelti P.A. and Cavelti E.S. (1945) Arch.Path.39 148

Cinader B. (1957) Ann.Rev.Microbiol.II 371

Clough P.W. (1960) Annals Int.Med.52 930

Collins R.C. (1949) Am.J.Ophthalmology 32 1687

Dacie J.V. (1957) Proc.Roy.Soc.Med.52 437

Dameshek W. (1953) J.Suisse de Med.48 1037

Dameshek W. (1958) Ann.Int.Med.48 707

Dameshek W. and Schwartz S.O.(1938) New.Eng.J.Med.218 75

Davis B.D. Moore D.H. Kabat E.M. and Harris A.(1945)J.Immunol.50 1

Deicher H.R.G. Holman H.R. and Kunkel H.G. (1959).J.exp.Med.109 97

Deutch H.F. and Morton J.I.(1957) Science 125 500

Dixon F.J.(1958) Ann.Rev.Med.9 257

Dornbusch S.(1957) Int.Archives Allergy and Applied Immunol.11 206

Dungern E.(1900) Münch.med.Wschr. 962

Dresner E. and Trombly P.(1959) New.Eng.J.Med.261 981

Evans R.S. (1951) Arch.Int.Med. 87 48

Fallet G. Lospalluto J. and Ziff M.(1958) Arth.and Rheum.1 419

Franklin E.C. Holman H.R. and Kunkel H.G.(1957) J.exp.Med.105 425

Franklin E.C. Kunkel H.G. Muller-Eberhard H.J. and Holman H.R. (1957) Ann.Rheum.Dis.16 315

Freund J. and McDermott K. (1942) Proc.Soc.exp.Biol.Med.49 548

Freund J. Sterer E.R. and Pisani T.M.(1947) J.Immunol.57 179

Freund J. Thomson G.E. and Lipton M.M.(1955) J.exp.Med.101 591

Friou G.J.(1958) J.Immunol.80 476

Fudenberg H.H. and Kunkel H.G.(1957) J.exp.Med.106 689

Fudenberg H.H. Kunkel H.G. and Franklin E.C.(1958) Proc.VIIth Congress Intern.Soc.Blood Trans. 522

Gajdusek D.C. and Mackay I.R.(1958) Arch.Int.Med.101 29

Gear J. (1955) Acta Med.Scand.152 Supp.306. 39

German J.L.(1958) J.exp.Med.108 179

Gery J. Davis A.M. and Ehrenfeld E.N.(1960) The Lancet (i) 471

Gibson D.M. Davisson E.O. Bachhawat B.K. Roy B.R. Vestling C.S. (1953) J.Biol.Chem.203 397

Glynn L.E. and Holborrow C.J.(1952) Lancet (ii) 449

Glynn L.E. Holborrow C.J. and Johnson G.D. (1957) Proc.Roy.Soc.Med. 50 469

Grabar P. Texas Reports on Biol & Med. 15 (1957)

Green A.A. (1938) J.Am.Chem.Soc. 60 1108

Hargraves M.M. Richmond H. and Morton R.(1948) Proc.Mayo clinic 23 2

Heimer I. Federico O.M. and Freyberg R.H.(1958) Proc.Soc.exp.Biol. and Med.99 381

Hektoen L. (1927) J.Infect.Dis. 40 641

Heller G. Jacobson A.S.H. Kolodny M.H. and Schuman R.L. (1952) J.Immunol. 69 27

Heller G. Kolodny M.H. Lepow I.H. Jacobson A.S.H. Rivera M.C. and Marks G.H. (1955) J.Immunol. 74 340

Heller G. Jacobson A.S.H. Kolodny M.H. and Kammerer D.H.(1954) J.Immunol. 72 66

Henion W.F. Mansour T.E. and Bueding E. (1955) J.exp.Parasitol 4 40

Holborrow E.J. (1957) Brit.Med.J. 2 732

Horecker B.L. and Komberg A. (1948) J.biol.Chem.175 385

Kabat E.A. (1939) J.exp.Med.69 103

Kaplan M.H.(1959) Fed.Proc.18 (Supp.3) 576

Kellet E.C. and Thomson J.G.(1939) J.path.Bact.48 519

Kleinsorge H. and Dornbusch S.(1957) Medizinische Nr.14 475

KOHN. J. (1960) "CHROMATOGRAPHIC & ELECTROPHORETIC TECHNIQUES" VOL II PP.56.

Kunkel H.G. Franklin E.C. and Muller-Eberhard H.J.(1959) J.Clin. Invest.38 424

Kunkel H.G.(1959) J.Chronic Dis.10 418

Landsteiner K.(1946) Specificity of Serological reactions

Lange K. (1951) Arch.Int.Med.88 433

Liu C.T. and McCrory W.W. (1958) J.Immunol.81 492

Mackay I.R. Larkin L. and Burnet F.M. (1957) Lancet (ii) 122

Mansour T.C. Bueding E. and Stavitsky A.B.(1954) Brit.J.Pharmacol. and Chemotherapy 9 182

Martin D.S.(1943) J.Lab. and Clin.Med. 28 1477

Masugi M. (1934) Beitr.path.Anat.92 429

Metchnikoff E. (1905) Immunity in Infectious Diseases (Camb.Univ. Press 1905)

Najjar V.A. and Fisher J.(1956) Biochim et biophys acta 20 158

Parson C.M.(1956) Proc.Soc.exp.Biol.and Méd.91 95

Peterson E.A. and Sober H.A. (1956) J.Am.Chem.Soc.78 756

Putnam F.W.(1959) Arch.Biochem.and Biophys. 79 67

Recht F.A. (1960) 'Protides of Biological Fluids.' Proc.8th colloquim. Bruges 1960. P.50

Rich A.R.(1956) Bull.John Hopkins Hosp. 98 120

Rivers T.M. Sprunt D.H. Berry G.P. (1933) J.exp.Med.88 39

Robinson A.R. Steilberg and Kuyper A.C.(1954)Proc.Soc.exp.Biol.and Med.85 4

Roitt I.M. and Doniach D. (1957(i)) Proc.Roy.Soc.Med.50 958

Roitt I.M. and Doniach D. (1957(ii)) J.Clin.End.17 1293

Roitt I.M. Doniach D. and Campbell P.N. (1956) Lancet (ii) 820

Rose H.M. Ragan C. Pearce E. and Lipmann M.O. (1948) Proc.Soc. exp.Biol. and Med. 68 1

Smithies O.(1955) Biochem.J. 61 629

Stavitsky A.B. (1954) J.Immunol. 72 360

Stavitsky A.B. Stavitsky R. and Ecker E.E. (1949) J.Immunol.63 389

Svartz N. (1957(i)) Acta Med. Scandinav.158 163

Svartz N. (1957(ii)) Ann.Rheum.Dis.16 441

Svartz N. (1958(i)) Acta Med. Scandinav.162 (Supp.341) 118

Svartz N. (1958(ii)) Acta Med. Scandinav. 160 90

Svartz N. and Schlossman K. (1954) Acta Med.Scandinav.149 83

Svedberg T. and Pedersen K.O. (1940) The Ultra centrifuge. (Oxford Univ.Press 1940) P.375

Svensson H. (1941) J.biol.Chem.139 805

Thulin K.E. (1955) Acta rheumat. Scandinav.1 22

Waaler C. (1940) Acta Path.et Microbiol.Scandinav.17 172

Wakesman B.H. and Adams R.D. (1955) J.exp.Med.102 213

Wasasljerna C. (1959) Acta Med.Scandinav. 165 299

West M. and Zimmerman H.J.(1959) Medical Clinics of N. America 43 (2) 371

Winblad S. (1952) Acta Med.Scandinav.142 458

Witebsky E. and Rose N.R. (1956) J.Immunol.76 408

Witebsky E. and Rose N.R. (1957) J.A.M.A.164 1439

Wroblewski F. (1958) J.Immunol.81 359

Wu H. (1922) J.biol.Chem. 51 33

Ziff M. (1957) J.Chronic Dis.5 644

Ziff M. Brown P. Lospalluto. Badin J.and McEwen C.(1956) Am.J. Med.20 500

Zimmerman H.J. and Weinstein B.S. (1956) J.Lab.and Clin.Med.48 607