Ph.D. Experimental Medicine

Douglas C. Heiner, M.D.

BIOLOGICAL, IMMUNOLOGICAL AND PHYSICOCHEMICAL PROPERTIES OF γD IMMUNOGLOBULIN (IgD)

ABSTRACT

The recently discovered and relatively unstudied immunoglobulin, γD, was investigated to further elucidate its nature. It was found to be virtually absent from umbilical cord blood and to increase until age 6 when adult serum levels were attained. The mean serum level of γD was found to be elevated in cystic fibrosis of the pancreas, in Laennec's cirrhosis and in infants with the visceral larva migrans syndrome, but was decreased in hypoagammaglobulinemia. Small quantities were found in normal saliva, colostrum and breast milk. Evidence was found of γD antibody activity following diphtheria toxoid booster immunizations, of γD antibody to bovine gamma globulin in a milk-sensitive child, and of γD anti-nuclear antibodies in disseminated lupus erythematosis. Antiserum to γD was shown to induce blastogenesis in human lymphocyte cultures.

A D-myeloma protein was found to have a $S^{\circ}_{20,w}$ value of 6.7 - 7.0, a molecular weight of 200,000, a high carbohydrate content (16%) and a low proline content. It is unusually labile on exposure to heat, low pH, or high molarity, and is quite susceptible to spontaneous degradation.

BIOLOGICAL, IMMUNOLOGICAL AND PHYSICOCHEMICAL PROPERTIES OF 7D

BIOLOGICAL, IMMUNOLOGICAL AND PHYSIOCHEMICAL PROPERTIES OF YD IMMUNOGLOBULIN (IgD)

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

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II. INTRODUCTION

In the summer of 1966 when a project was to be selected for investigational study leading to the degree of Ph. D. in Experimental Medicine, four classes of immunoglobulins were recognized. These were YG, YA, YM and YD. The most recently recognized was YD and this was also the immunoglobulin about which least was known. This investigator had recently received 1 ml of antiserum specific for the heavy chain of YD from Dr. John L. Fahey, co-discoverer with Rowe (1964) of the immunoglobulin. Shortly before embarking on this study, it was possible through the use of the anti YD antiserum to identify a subject with YD myeloma and to obtain a moderate amount of serum from this subject. Initial attempts to induce the formation of anti D-myeloma antibodies in rabbits were successful, and with this encouragement the current study was undertaken. This preliminary work was completed by the author at the University of Utah before coming to McGill.

The purpose of selecting YD as the object of study was threefold. Firstly, it was hoped that it would be possible to increase the reported knowledge concerning YD and thereby contribute to the body of scientific information about immunoglobulins. Secondly, it was the intention of the investigator to acquire as much facility as he could in handling immunoglobulins by immunochemical and physicochemical means in order to study their biological role, particularly as they relate to diseases seen in humans. Thirdly, it was hoped that a number of immunological techniques could be adapted for use in the study of small quantities of antigens and antibodies. The writer is a Pediatrician who has many times been confronted with children with perplexing problems for

whom information of value was at least theoretically obtainable by immunological means but studies were limited by the very small quantities of serum or other body fluids which could be obtained for study. Hence an important goal of these studies has been the adaptation of as many investigative techniques to a micro scale as possible without decreasing the accuracy or the reproducibility of the findings. The modifications of techniques which have been made in this regard will be indicated in the section on experimental work.

At the inception of this study it was not known that YD was a highly labile protein nor was it known that this in itself had prevented several other laboratories from completing a number of investigative studies on this protein. This fact was indicated to me during personal conversations with Dr. Fahey in 1968. As it turned out, the YD of the initial patient with D-myeloma was exceedingly labile, but with persistent searching and with assistance from colleagues who sent myeloma sera for testing, a second subject with YD myeloma was identified and admitted to the Royal Victoria Hospital for workup. It was possible to obtain moderately large quantities of serum from this subject, and his serum YD proved easier to work with than that of the first subject, enabling the study to proceed to a degree that otherwise would have been impossible. Nevertheless, frequently repeated attempts at isolation were necessary because exposure to relatively mild changes in pH or molarity or repeated freezing and thawing often was associated with molecular breakdown or denaturation. This marked lability of YD was of sufficient interest to study in itself. These results were reported at the American Association of Immunologists' meeting in Atlantic City (Heiner, Saha and Rose, 1968). During the course of the current investigations, very little was written by other

workers about YD. The reasons for this appear to be at least three-fold: the unusual lability of YD making it difficult to study, the scarcity of D-myeloma and the low serum YD in healthy subjects making very limited quantities of material available for study, and the lack of recognition of a comparable immunoglobulin in experimental animals.

Since little is known concerning the biological role of YD, those functions and properties commonly ascribed to other immunoglobulins will be reviewed as a reminder of its possible actions. This of course does not close the door to a search for new and unique roles for this immunoglobulin. Those historical aspects of research on immunoglobulins which are of particular interest to the writer have been emphasized and no attempt has been made to include every contribution to the field.

III. HISTORICAL REVIEW

A. General Remarks

The history of immunoglobulins or antibodies begins with the history of resistance to disease. It probably had its earliest seeds in the recognition of immunity following recovery from many infectious diseases, an observation almost as old as recorded history. The Greek historian, Thucydides, who lived in the 4th century B.C. and whose History of the Peloponnesian War is a most important historical book, indicated that the sick and dying people in Athens would have received no nursing care at all had it not been for the devotion of those who had already recovered from the plague, since it was known that no one ever had it a second time. According to Major (1945) there has been some dispute for many centuries concerning the exact nature of the plague of Thucydides, it having been identified by various historians as the plague, measles, yellow fever, scarlet fever, typhus fever, typhoid fever, small pox and even ergot poisoning. However, most scholars are of the opinion that the description indeed represented glandular plague, or as it is more commonly called, the Bubonic Plague. Regardless of whether or not the pestilence described by Thucydides represented Bubonic Plagus it was recognized at the time that recovery from certain infectious diseases conferred immunity.

Not until 1890 was it clearly demonstrated that a factor in serum could play a major role in this protection following recovery from infection. In that year von Behring and Kitasato demonstrated that animals immunized with tetanus toxin produced a serum factor which they called anti-toxin and that the

anti-toxin could be transferred to another animal protecting it passively from the effects of tetanus toxin (Humphrey and White, 1963). Soon thereafter von Behring demonstrated that a similar protection was afforded by immune serum prepared against diphtheria toxin. These findings were rapidly confirmed by many workers and the substances which appeared in the serum in response to immunization became generally known as antibodies, a term which is almost synonymous with the term immunoglobulins. The specific properties of antibodies will be briefly reviewed in subsequent sections but at this point it is worthwhile to trace the development of the more modern concept of immunoglobulins.

B. Development of the Immunoglobulin Concept

In 1936 Tiselius separated the serum proteins into albumin, α, β and γ globulins according to their electrophoretic mobility. The term gamma globulin (or γ globulin) was used to designate those proteins of slowest electrophoretic mobility. Soon thereafter Tiselius and Kabat (1938) demonstrated that the majority of antibodies are contained in the gamma globulin fraction of serum and for many years following this it became customary to associate antibodies with gamma globulins. However, as methods for the separation and identification of serum proteins improved it became evident that some antibodies migrated in the β globulin region on electrophoresis, and after the development of immunoelectrophoresis by Grabar and Williams (1953) it became evident that several classes rather than a single class of serum protein were involved in antibody activity. Therefore the term immunoglobulin came into use to apply to that group of serum proteins which were associated with immunity or antibody

activity. It was hoped this would avoid some of the errors which resulted from identifying antibody activity with a particular electrophoretic mobility. In May 1964 a committee sponsored by the World Health Organization met in Prague to make recommendations concerning the nomenclature of immunoglobulins since many different and sometimes confusing designations had appeared in the world literature. At that time three immunoglobulin classes were recognized, the most prevalent having been designated by different workers as Y, 7SY, 6.6SY, Y2, YSS, or simply gamma globulin. It was proposed that this class of immunoglobulins be called either YG or IgG. The immunoglobulin present in the next greatest concentration had been termed β2A, or Y1A, and the designation recommended was YA or IgA. The third immunoglobulin class was previously called Y1M, β2M, 19SY, or gamma macroglobulin. The recommendation for this third class of immunoglobulin was that it be called YM or IgM. It was proposed that future immunoglobulin classes, once properly demonstrated, should be identified as YD or IgD, then YE or IgE, etc. It was not envisioned by early workers that perhaps an immunoglobulin class would be recognized which may not have demonstrable antibody activity. Nevertheless, precisely this problem arose the following year, when Rowe and Fahey (1965) described a new class of serum protein which met all the criteria for immunoglobulins except that antibody activity had not been demonstrated. As a matter of fact, up to the present time, no convincing evidence has been presented in the literature to suggest that this immunoglobulin has antibody activity. Thus the possibility exists and has apparently been accepted by investigators in the field that a normal immunoglobulin need not necessarily have demonstrable antibody activity. It must, however, have other major attributes of immunoglobulins such as

production by plasma cells or related cell lines; it should be found in increased amounts in certain subjects with myeloma, macroglobulinemia, or related disorders and it must have immunologically distinctive heavy chains and light chains common to other immunoglobulins. It also seems to be a requirement that any new immunoglobulin class should represent a protein which is demonstrable in the majority of normal subjects. A historically oriented description of the biological role of immunoglobulins and of their immunology and physical chemistry will be presented below.

C. Biological Role of Immunoglobulins

1. General

Immunoglobulins are known to function largely as the result of their property of combining with specific antigens or allergens. They are vital in the immunity of normal subjects and their deficiency has been associated with profound susceptibility to infections caused by microorganisms of various kinds. Thus there have been many different primary immunoglobulin deficiencies described ranging from severe inability to form any of the immunoglobulins to partial deficiencies and to the group of disorders now recognized as primary dysgammaglobulinemias. These disorders are called primary because the immunoglobulin deficiency is not secondary to any known disease process such as multiple myeloma, other malignancy, nephrosis, protein losing enteropathy, infection, etc. In addition to this, there are a fairly large number of causes of so-called secondary immunoglobulin deficiencies. A third group of immunoglobulin disorders are the paraproteinemias in which pathologically increased amounts of a specific class of immunoglobulin apparently serving no

useful function are produced. Beyond these disorders, all of which may manifest an impaired resistance to infection, it is now apparent that immunoglobulins are intimately involved in hypersensitivity phenomena. It appears that they may also play a role in lymphocyte reactivity, in graft rejection, in the regulation of antibody synthesis and in certain forms of immunosuppression. Each of these roles will be discussed briefly, since it is possible that YD may be found to be involved in several or many of these functions and the recognition of such possibilities could be helpful in future studies.

2. Immunity

(a) Toxin neutralization. Sewall (1887) demonstrated that pigeons could be immunized against the venom of rattlesnake and thereby protected from its lethal effects. This was the first experimental demonstration of toxin neutralization although the involvement of antibodies was not recognized. Roux (1894) demonstrated that the serum of horses immunized with diphtheria toxin led to a cure of diphtheria when injected into a patient suffering from this disease. This was the first demonstration that serum factors were capable of neutralizing the effect of toxins in human subjects. Such factors later came to be known as "anti-toxins" (Humphrey and White, 1963). Although the findings of Roux were confirmed by others and demonstrated many times, it was not possible to find such a salutory effect on other infectious disease processes with passively administered antibody once the disease had been established in the patient. However, the findings in regard to diphtheria stimulated a great deal of interest in antibodies as therapeutic agents. The value of tetanus anti-toxin as a prophylactic (as opposed to therapeutic) measure to prevent the

development of tetanus after wounds were sustained was dramatically proved during the first World War.

(b) Bacteriolysis and bactericidal antibodies. In 1894 Pfeiffer and Issaeff demonstrated that immune sera prepared against the cholera vibrio caused the death and destruction of this microorganism and that this property of immune serum could be transferred passively to a second animal. Antibodies that were instrumental in bringing about bacterial cell destruction were called lysins or bacterial lysins. In 1895 Bordet demonstrated that bacteriolytic and bactericidal activities in immune sera frequently depended on two distinct factors, one being thermolabile and present in normal serum and the other thermostable and specific, being present only in immune serum. The former substance, which was called alexin (Ehrlich, 1897), has proved to be the complex of macromolecules which is now known as serum complement. The thermostable factors, bactericidal antibodies or lysins, are now known to be specific immunoglobulins directed towards the bacteria in question.

(c) Agglutination. In 1889 Charriu and Roger demonstrated that immune serum would cause agglutination of bacteria and in 1896 Widal applied this knowledge to produce a laboratory technique which has become helpful in the diagnosis of typhoid and paratyphoid fevers (Boyd, 1956). Such antibodies are called agglutinins. In vivo agglutination and immobilization of microorganisms is thought to be a helpful defense mechanism.

(d) Precipitation. In 1897 Kraus demonstrated that cell-free filtrates of bacterial cultures frequently induced antibody production in an

immunized animal and that the immune serum thereafter precipitated substances in culture filtrates. This was the first demonstration of antibodies with the property which led to their becoming known as precipitins (Kabat, 1961). Initially it was uncertain whether or not the antigen actually entered into the precipitate or whether it in some way simply altered the colloidal state of the serum globulins by denaturing them and rendering them insoluble. This question was answered by von Dungern (1902) when he demonstrated that crab hemocyanin induced antibodies and the precipitate turned blue on exposure to air, a known property of the hemocyanin antigen which therefore was an integral part of the precipitate.

The precipitin reaction has probably been the most thoroughly studied of all the modes of antibody activity. So much so in fact that it is regularly utilized by nearly all immunologists in their investigative work. It is now generally recognized that precipitating antibodies can be produced against most proteins and against some polysaccharide antigens (Heidelberger, 1956). Precipitation is believed to be a secondary phenomenon following an interval of time needed for the primary union of antigen and antibody. It appears necessary that the reacting antigen and antibody molecules form a latticework of complexes sufficiently large or sufficiently hydrophobic that they become insoluble and precipitate from solution.

(e) Opsonization. Antibodies which promote phagocytosis of bacteria or other specific antigens are called opsonins (Ward and Enders, 1933).

(f) Complement fixation. Some antibodies have the property of fixing complement when they react with specific antigen and these have been termed

complement-fixing antibodies (Osborne, 1937). The important role of complement components in chemotaxis, bactericidial antibody activity, and in the release of the chemical mediators of immune reactions has recently been reviewed by Muller-Eberhard (1968). The fixation of complement is frequently used in serologic tests for certain antigen-antibody reactions as a diagnostic measure.

(g) Virus neutralization. Some antibodies are termed protective or neutralizing (Kabat, 1961) because they neutralize the effect of viruses either in intact animals or in tissue cultures.

(h) Immune elimination. It is a rather general property of antibodies that they increase the speed of elimination of a foreign substance from the body, an effect called "immune elimination" (Dixon et al, 1959). Not only does this play an obvious protective role in removing foreign macromolecules from the body in many circumstances but it is used as a sensitive index of the vigor of the immune response. For example, animals with immunologic paralysis or specific immune tolerance toward an antigen do not demonstrate immune elimination, but when the paralysis or tolerance is lost, the capacity for immune elimination returns (Dietrich and Weigle, 1964).

(i) Autoantibodies. Some antibodies are directed towards constituents of the host producing the antibodies and these are called autoantibodies. Examples of this are seen in auto-immune hemolytic anemia where antibodies to red cell antigens are produced and are found coated on the patient's red cells (Boorman, 1946). Another example is rheumatoid factor, a YM antibody directed towards the YG molecules of the same individual (Vaughan, 1959). Closely akin

to this are mixed cryoglobulins wherein YM antibodies combine with YG globulins of the host to produce high molecular weight complexes with high viscosity and the property of precipitation on lowering the temperature of the serum (Lo-Spalluto et al, 1962). Another example of autoantibodies is found in the disease disseminated lupus erythematosis in which antibodies to DNA (Deoxyribonucleic acid) and other cellular constituents may be present (Holman, 1966). It is believed that these antibodies may play a role in intravascular immune-complex formation with subsequent deposition in the kidney, resulting in renal disease. It is also possible that autoantibodies may constitute a protective immune mechanism in the body, eliminating unwanted body cells such as those which have undergone malignant transformation.

(j) Discussion. It should be mentioned that several or many of the above functions may be properties of a single antibody species or of a given immunoglobulin class. For example, precipitating antibody can also be hemagglutinating antibody and at the same time may increase the rate of immune elimination of an antigen from an animal host or may participate in other forms of antigen-antibody interaction. There are circumstances, however, where a particular immunoglobulin class is more efficient at a certain antibody function than another immunoglobulin class. Illustrations of this are hemagglutination and bacterial agglutination which are accomplished more effectively by YM antibodies than by other immunoglobulin classes. Antibodies to polysaccharides such as dextran or levan are almost exclusively of the YG variety, and more particularly of the YG_2 subclass (Kabat, 1968). YG and YM antibodies fix complement whereas YA does not. The bulk of the toxin neutralizing activity in hyperimmune tetanus antiserum residues in the YG immunoglobulin class.

Antisera produced in animals early in the course of immunization tend to be YM in nature and later to be YG and YA. On secondary immunization a much greater booster effect occurs among the YG antibodies than the YM. Obviously, to be thorough in one's search for the biological significance of an immunoglobulin such as YD careful investigation must be made into as many potential roles as possible.

3. Normal serum immunoglobulin levels

The serum levels of the different immunoglobulins vary considerably at different ages and also the absolute levels found in different laboratories seldom correspond precisely. Some of the reasons for the variations in the findings of one laboratory as compared to another are as follows. Almost all laboratories use immunochemical methods of assay which depend on specific antisera, and each usually employs antisera prepared in different animals, under different conditions and using slightly different preparations of antigens. Frequently some antisera are partially directed towards different structural groups than other antisera. For example, Seligman outlined in 1967 a number of different areas on immunoglobulin molecules which could induce antibody formation including various sites on the heavy chain, the light chain, and areas involving both chains such as in the Fab region. Also antisera may exhibit different degrees of avidity for the immunoglobulin to which they are directed. One laboratory may use anti-YA produced by injecting one A-myeloma protein into rabbits while a second laboratory may use a different A-myeloma protein for immunizing purposes and may produce antiserum with somewhat different specificity, particularly to idiotypic determinants on the Fd part of

the heavy chain (see page 43 for a discussion of idiotypic determinants). Slight variations in assay techniques may also play a role, as may the accuracy of standards used in computing the results. Such limitations in reproducibility must be kept in mind when one evaluates the results reported by different investigators.

A comparison of immunoglobulin levels found by a number of investigators is given in Table I. Determinations performed by this investigator are included for comparative purposes. Differences in the results of various laboratories are apparent. Although part of the variation may be due to differences in the population samples, at least some of them are due to differences in antisera and laboratory techniques (Stiehm and Fudenberg, 1966). The variations in reported levels of the normal level of YD are noteworthy.

TABLE I

Mean Serum Immunoglobulin Levels in Healthy Adults (mg%)

Author	YG	YA	YM	YD	YE
Heremans 1960	1200	112	72		
Claman & Merrill 1964	995	177	187		
McKelvey & Fahey 1965	1240	280	120	3	
Fahey 1965	1240	390	120	3	
Steihm & Fudenberg 1966	1158	200	99		
Allansmith 1966	1045	169	89		
Kohler & Farr 1967	1350	236	83		
Buckley & Dees 1967	1080	280	78		
Buckley et al 1968	1061	266	76		
Norberg 1967	1143	204	74		
Johansson 1967	1323	158	88	11.7	0.03
Heiner & Evans 1967	1400	235	80	8	
Heiner, current study	1210	205	65	5	

4. Immunoglobulins in other body fluids

It should be noted that immunoglobulins have been detected in cerebrospinal fluid, saliva, intestinal secretions, stools, joint fluids, urine, tears and nasal secretions. As part of the current investigation, some of these fluids have been analyzed and will be discussed in the experimental section. Selected findings of one group of investigators are listed as Table II because they contain information on YD.

TABLE II

Average immunoglobulin concentrations in serum, colostrum and saliva (mg%)*

	No. tested	YG	YA	YM	YD
serum	12	790	310	110	3.1
colostrum	12	37.5	1116	89.5	ND
% of serum value		4.9	376	81	
serum	28	1075	375	65	4.95
saliva	28	.50	5.4	.078	ND
% of serum value		.047	1.44	.12	

* Adapted from Rowe (1968)
ND = not detectable

It is worth noting that Rowe and co-workers reported the above YD data as % of a standard. The lower limit of detectability of YD by their technique was stated to be between 2.5 and 5% of the standard which had a level of .33 mg/ml. Thus their technique could detect only amounts in excess of approximately 0.01 mg/ml or 1 mg%. Since the sera of the subjects from

whom colostrum was obtained contained only 9.4% of the standard of 3.1 mg% the technique could not have detected anything less than 25% of the serum value. In the case of YG only 4.9% of the serum level was present in colostrum. If a similar proportion of the serum YD were present in colostrum it would have been completely missed by the technique. Hence these authors should have pointed out this fact since one can easily gain the impression that YD is probably not present in colostrum. The same reasoning holds in regard to salivary YD. These points are brought out because evidence bearing directly on the matter has been accrued as part of the current investigation.

It is likely that there is a potential biological role of antibodies in body fluids and differences between intravascular and extravascular distribution of the various immunoglobulins are therefore of interest. YD and YM for example, are restricted in their extravascular distribution (approximately 25-30%) whereas in the case of the other immunoglobulins 50-60% is in the extravascular compartment (Table III, p. 19). It is of interest in this regard that the molecular weight of YD is nearer to that of YG and YA than it is YM, suggesting that the extravascular distribution of immunoglobulins depends on more factors than molecular size alone.

5. Rates of synthesis and catabolism of immunoglobulins

The rates of synthesis and catabolism of the various immunoglobulins have been summarized by Rogentine et al (1966). These authors have done recent studies which include determinations of the synthetic and catabolic rates of YD. No one has yet done complete studies of this kind on YE but Johansson reported that the fractional catabolic rate of YE was

approximately 16% in 3 subjects and that approximately 50% was intravascular (1968). The proportion of the total immunoglobulin which is present in the intravascular compartment may be determined by the method of Sterling (1951). Radiolabelled immunoglobulin is injected intravascularly and an adequate number of spaced blood samples are obtained from which a curve of the plasma radioactivity is constructed. The labelled molecules (counts per minute per ml) decrease rapidly at first but after 1 to 3 days the rate of disappearance is much slower and becomes steady. The slope of the latter part of the curve represents the catabolic rate expressed as a fraction of the total labelled protein and this line projected back to the ordinate indicates the fraction of the labelled protein which ordinarily is intravascular (Fig. 1). Care must be taken to assure that the protein is not denatured by the radiolabelling process and is therefore eliminated at a normal rate. The extravascular proportion is calculated by simple subtraction of the intravascular from the total radiolabelled pool.

The currently available data on human immunoglobulins are summarized in Table III from which it can be seen that YG is synthesized in the greatest quantities in normal subjects and also has the slowest rate of catabolism, the two combining to give it the highest serum level. YA and YM are degraded at roughly the same speed but there is a higher rate of synthesis of YA and hence the serum concentration is greater for this immunoglobulin. YD is synthesized at a much lower rate than the three major immunoglobulins and it also has the highest catabolic rate of any immunoglobulin studied so far.

TABLE III

Metabolism of Immunoglobulins

	YG*	YA*	YM*	YD*	YE**
Mean serum level (mg%)	1070	250	77	2.3	.03
% Intravascular	44	40	70	73	50
Synthetic rate (mg/kg/day)	42	21.1	4.13	.396	
Fractional catabolic rate (% intravascular pool catabolized daily)	6.3	23.7	17.9	37	16
Biologic half life (days)	22	6	5	2.8	

* From Rogentine et al, 1966

** From Johansson, 1968.

Rogentine et al suggested that the fractional catabolic rate of YD (the fraction of the intravascular pool catabolized daily) is influenced by the serum YD concentration in a way opposite to that for YG. In the case of YD, high serum levels appear to be associated with low fractional catabolic rates whereas in the case of YG high serum levels are associated with rapid catabolism. It is quite clear that both the synthesis and catabolism of YD are largely independent of the other immunoglobulins.

6. Primary Immunoglobulin Deficiencies

(a) General. The clinical importance of gamma globulins in relation to immunity was strikingly demonstrated by Bruton's discovery of agammaglobulinemia in 1952. A four year old boy was found to have severe recurrent

infections, many of which involved pneumococcal septicemia. Attempts were made to immunize him with diphtheria and tetanus toxoids but no antibody response was attained on numerous occasions. When electrophoretic studies were done, it was expected that the child would be found to have hypergammaglobulinemia, but to Dr. Bruton's surprise he had no detectable gamma globulin. This, in conjunction with his failure to make antibodies in response to immunizations, prompted the case report and was the beginning of intensive studies in several laboratories into the disorders which came to be known as agammaglobulinemia.

It is of interest that from 1952 until the present most workers have considered the Bruton type of agammaglobulinemia to represent a sex-linked congenital defect with inheritance similar to that of hemophilia. The reason for this is that nearly all of the early subsequent cases which were discovered did have sex-linked inheritance. However, in retrospect and on review of the original findings, there are a number of facts which were different about the original patient from the more classical patients with sex-linked agammaglobulinemia. Bruton's original patient had large tonsils which were removed. He did not have difficulty with infection until he was four years of age and he had no family history of troubles with infection. A particularly interesting point about him is that his mother said he was normal in all respects until he had a severe case of red measles at four years of age, from which time he began to have frequent infections (Bruton, 1968). In the light of current knowledge, as summarized in a recent book (Bergsma, 1968), it is highly likely that this boy actually had acquired agammaglobulinemia since in this form more frequently there is a delayed onset of symptoms, the tonsils become enlarged, there is no family history of severe infections, and there may be an onset following

viral infections. Since 1952 an impressive variety of types of hypoagammaglobulinemia have been described. It has been the custom of many investigators to label a condition agammaglobulinemia if there is less than 100 mg% of total gamma globulin in the serum and hypoagammaglobulinemia if there is between 100 and 400 mg%.

The production of gamma globulin is associated with the plasma cell line of mononuclear cells in the body, and patients with congenital agammaglobulinemia have an absence or marked reduction of plasma cells along with an inability to make specific antibodies. They may or may not have other disorders of the immune response such as impaired graft rejection and delayed hypersensitivity reactions. The severity of the disorders seems to depend on the nature of the stem cell which is defective.

(b) Reticular dysgenesia. With involvement of the most primitive cell type so far described it appears that a stem cell involving all white cells is deficient or defective. Almost no polymorphonuclear leucocytes, lymphocytes, or plasma cells were found in the body in the disorder as it was described in two twins of a single family by de Vaal (1959). The children had no lymph nodes, tonsils, or Peyer's patches in the intestinal tract at post mortem examination, and only a very hypoplastic rudimentary thymus. Both children died within eight days of birth. The disorder was termed reticular dysgenesia with congenital aleukia.

(c) Swiss-type agammaglobulinemia. The next most severe kind of agammaglobulinemia is that now generally recognized as the Swiss-type. This was originally described by Glanzmann and Riniker (1950) and later by Hitzig

et al (1958). The defect is due to involvement of a lymphoid stem cell which appears to be not quite so primitive as that involved in reticular dysgenesia. These patients do not have plasma cells or circulating antibody and are deficient in lymphocytes as well as having a marked deficiency or absence of all immunoglobulins. Polymorphonuclear leucocytes, however, are usually normal. These patients are unable to develop delayed hypersensitivity or to reject homografts which in all likelihood is related to their lymphocyte deficiency and to their rudimentary thymuses.

(d) Classical sex-linked agammaglobulinemia seems to involve a less primitive cell type affecting only the plasma cell series. The lymphocyte, polymorphonuclear leucocyte population and the thymus usually are quite normal. These subjects do not have an absence of delayed sensitivity and they are able to reject grafts although graft rejection in some may be somewhat slow (Good et al, 1968). This immunologic disorder as well as those of a more profound nature have a marked depletion or absence of the four major immunoglobulins in most instances. This includes YD which supports the validity of including YD in the classification of immunoglobulins even though its role as an antibody has not been clarified up to the present.

(e) Additional variants. There are additional variants of hypo- and agammaglobulinemia. Some instances of congenital agammaglobulinemia are not sex-linked (Good, 1968). In other subjects, marked immunoglobulin deficiency is clearly acquired and it now appears that this may follow viral infections such as rubella or measles, and perhaps others (Good, 1968). Disorders of thymic function and delayed hypersensitivity have been described without

involvement of the immunoglobulins (DiGeorge, 1968, Nezelof et al, 1964). In one syndrome with thymic dysplasia and immunologic incompetence but normal immunoglobulin levels there are absent or very primitive parathroid glands; these glands arise from the same branchial cleft as the thymus during embroygenesis (DiGeorge, 1968). Milder forms of a Swiss-like agammaglobulinemia have also been described, some of which are sex-linked and some not. One large category is the secondary hypogammaglobulinemias which includes diseases associated with excessive loss of immunoglobulins (nephrosis, protein-losing enteropathy, draining fistulae, etc.) and diseases which interfere with normal immunoglobulin synthesis such as leukemia, multiple myeloma, etc. The number of possible variations of congenital and acquired immunologic deficiencies seems to be almost limitless and in the future it is likely that these disorders will be grouped into rather broad categories helpful in understanding their basic pathophysiology.

7. Dysgammaglobulinemias

The term dysgammaglobulinemia has been used to describe two different kinds of disorders. In the first, immunoglobulins are present in normal or increased quantities, but are non-functional in the sense that it is difficult to induce antibodies to a particular antigen or microorganism, and there is decreased resistance to certain infections (Giedion and Scheidegger, 1957). There are probably many variations of this kind of defect, one of which appears to occur in subjects with measles who develop giant cell pneumonia (Mitus et al, 1959), and another in patients who develop progressive vaccinia but who have normal immunoglobulin levels (Kempe, 1968). The latter subjects are helped markedly by the administration of specific anti-vaccinial gamma globulin. A

more common use of the term, dysgammaglobulinemia, however, applies to conditions in which one or two of the immunoglobulin classes are absent or markedly diminished while the others are in normal concentration or even increased. Such defects have been summarized by Hobbs (1968) and unfortunately have been given different designations by various authors in the literature. It would appear to this writer that the most satisfactory solution would be to designate each disorder by the manifest immunoglobulin defects and not to use numbers such as dysgammaglobulinemia type III or IV, etc., since there are conflicting numerical designations which already are proving to be confusing. Thus, a subject who has an absence of YA might best be recognized as "Dysgammaglobulinemia with an Isolated Absence of YA", or a subject with what is usually called type I dysgammaglobulinemia (Rosen and Bougas, 1961) could more easily be understood if called "Dysgammaglobulinemia with deficient YA and YG but with normal levels of YM".

8. Immunoglobulin Paraproteins

(a) General. It is important to define what is commonly meant by paraproteinemias that involve immunoglobulins and to indicate the importance of this group of disorders in the development of knowledge about antibodies and immunoglobulins. Certain diseases, particularly multiple myeloma but also Waldenstrom's macroglobulinemia, lymphomas, and a few miscellaneous disorders may be associated with the production of extremely large amounts of homogeneous or "monoclonal" immunoglobulins. In a given patient the appearance of a monoclonal or "M" peak represents the proliferation to an excessive degree of cells producing a particular kind of immunoglobulin. These immunoglobulins have most,

if not all, physico-chemical characteristics of antibodies except that they represent a much more homogenous group of molecules than do the usual antibody populations. Paraproteins, for example, usually consist of a population of a single type of immunoglobulin molecule all with the same kind of heavy chain and the same kind of light chain, with identical genetic markers, and belonging to the same immunoglobulin subclass. The very high serum levels of these proteins makes them relatively easy to isolate from other proteins and permits their chemical study in a manner which would otherwise be impossible.

(b) Properties of monoclonal immunoglobulin paraproteins. Monoclonal paraproteins contrast sharply with the spectrum of immunoglobulin of a given class found in a given normal serum. Monoclonal immunoglobulins tend to migrate as a single dense band on electrophoresis because the molecules have an identical amino acid composition and a similar electric charge. Normal immunoglobulins on the other hand, have a wide range of electrical charge and may migrate in much of the region between the $\alpha 2$ and β globulins and the slowest gamma globulins. Antibodies to a given antigen tend to migrate in a somewhat more homogenous manner which bears some relationship to the molecular charge and conformation of the antigen which induced its formation (Sela, 1968). Since many antigens are involved in the induction of the antibodies that comprise the normal gamma globulin population it is perhaps easy to see why the electric charge and electrophoretic migration varies from molecule to molecule. It has also become apparent that it is very difficult to isolate precisely similar specific antibodies from two different individuals because the individuals produce antibodies of slightly different specificity and avidity in response

to the same antigenic stimulus. It is similarly true that no two myeloma proteins from different individuals are identical, each appearing to represent the product of a specific clone of cells and having its own unique amino acid sequence and molecular charge. It is the virtual homogeneity in molecular composition, conformation, and functional activity (if any) of a given monoclonal paraprotein and its ease of separation that distinguishes it and makes it particularly valuable to the research worker.

(c) Paraproteins and classifications of immunoglobulins. It is possible to prepare antisera to specific myeloma proteins and thereby to study the immunologic relationships of one to the other (Korngold and Lipari, 1956; Slater et al, 1955). Over the last thirteen or fourteen years comparative analyses of antigenic relationships of many myeloma proteins have established what is now regarded as five major immunoglobulin classes, each being represented by a distinctive heavy chain and having light chains shared by the other classes (Bull. World Health Org., 1964; Ibid., 1968). There are two major subclasses of light chains, kappa and lambda, each of which can combine with any of the five heavy chains, γ, α, μ, δ or ε. In addition, enzymatically produced subunits have been produced and studied in detail and a uniform classification prepared (Table IV).

TABLE IV

Nomenclature of Immunoglobulins

Substance	Current Nomenclature	Previous Nomenclature
Immunoglobulin	γG, IgG	γ_2, γSS, 7Sγ, 6.6Sγ
	γA, IgA	γ_1A, β_2A
	γM, IgM	γ_1M, β_2M, 19Sγ
	γD, IgD	
	γE, IgE	γE globulin, IgND
Polypeptide chains	heavy chain	H, A
	light chain	L, B
Heavy chain of γG	γ (γ1, γ2, γ3, γ4)	γ2b, γ2a, γ2c, γ2d
" " " γA	α (α1, α2)	
" " " γM	μ (μ1, μ2)	
" " " γD	δ	
" " " γE	ε	
Light chains (common to all immunoglobulins)	kappa, or k	I, B
	lambda, or λ	II, A
Papain fragments	Fab	I, II, A, C, S
	Fc	III, B, F
	Fd	A piece
Peptic fragments	$F(ab)_2$	5S divalent fragment
	Fab	univalent fragment
Molecular formulae γG	γ2k2, γ2λ2	
" " γA	α2k2, α2λ2, $(\alpha 2k2)_2$ T*, $(\alpha 2\lambda 2)_2$ T*	
" " γM	$(\mu 2k2)_5$, $(\mu 2\lambda 2)_5$	
" " γD	δ2k2, δ2λ2	
" " γE	ε2k2, ε2λ2	

Adapted from Cohen and Milstein (1967) and Tomasi et al (1968).

* T = transport piece associated with secretory γA.

In addition to this, all normal sera contain a variety of immunoglobulin subgroups. Perhaps the best studied of these are the subgroups of YG globulin. They are most easily illustrated in tabular form (Table V).

TABLE V

Subgroups of human YG*

Current name	Previous name	% of total YG	% with k chains	% with λ chains
Y1	$Y_{2}b$, We, C	77	54.5	22.5
Y2	$Y_{2}a$, Nc	11	5.8	5.2
Y3	$Y_{2}c$, Vi, Z	9	4.7	4.2
Y4	$Y_{2}d$, Ge	3	2.6	0.5
		100	67.6	32.4

* Adapted from Cohen and Milstein (1967) and Tomasi et al (1968).

These subgroups were discovered through studies on myeloma proteins and have been found to have definite relations to the Gm genetic types (see section on immunology of immunoglobulins). Subclasses of YA have been reported by Kunkel and Prendergast (1966), Feinstein and Franklin (1966), Vaerman and Heremans (1966) and Terry and Robert (1966). The subgroups of the first three of these reports are identical. Subgroups of YM and of λ immunoglobulin chains have also been described, and undoubtedly additional subgroups will be found.

Certain genetic markers are found in association with specific immunoglobulin classes and subclasses. For example, the Inv genetic factor is associated only with kappa type light chains and the Gm factors are associated only with gamma type heavy chains. This will be discussed in more detail in the section on the immunology of immunoglobulins.

(d) Amino acid sequence studies. Within the past two years a number of monoclonal light chains, or Bence-Jones proteins, have been studied in sufficient chemical detail to enable a comparison of their amino acid sequences (Putnam et al, 1967). These studies have demonstrated the presence of what has been termed constant and variable portions of each light chain. In other words, all λ light chains are very similar or identical in the amino acid sequence of approximately half of the molecule, which happens to be the carboxy-terminal half. The other half of the molecule varies considerably from one Bence-Jones protein to another. The same is true of kappa Bence-Jones proteins, which are similar to one another in half the molecule. A precisely analogous situation is being found with respect to the amino acid sequences of immunoglobulin heavy chains although the heavy chains are approximately twice as long as light chains and seem to have three areas of homology, one in each of the three quarter-molecule segments of the c-terminal end (Hill et al, 1966b; Putnam, 1969; Edelman, 1969). Thus it may become apparent after further studies of heavy chain sequences have been completed, that the usual immunoglobulin molecule is composed of two heavy and two light chains, each light chain and heavy chain containing a so-called constant segment which is common to all polypeptide chains of that particular class

or subclass, and a variable region in the N-terminal part of the chain. The variable region involves the area of the molecule responsible for its antigen-binding specificity.

There also appears to be significant homology between the constant portion of the light chain, each half of the Fc fragment and the c-terminal half of the Fd fragment, suggesting an ancestral link between these areas (Fig. 2) and indicating that they may have originated from a common gene (Hill et al, 1966; Putnam et al, 1967). In addition it is known that the COOH-terminal ends of rabbit, human and horse γ chains are nearly identical in their final 11 residues whereas the c-terminal ends of α and γ chains from the same or unrelated species differ markedly (Tomasi et al, 1968). This suggests that an important functional difference between γG and γA immunoglobulins has been preserved throughout evolution.

(e) Discussion. Were it not for the occurrence of paraproteins and methods to study them, knowledge concerning the immunologic relationships among immunoglobulins, their subgroups and their subunits would be much less secure. Detailed chemical and structural analyses would be extremely difficult and often impossible. Even many theories of immunoglobulin function depend on studies of paraproteins. For example, one current theory states that the function of the constant regions of immunoglobulin molecules is to provide stability to the variable region and an advantageous configuration to the molecule at the same time enhancing the mutual affinity of heavy and light chains and the accessibility of antibody combining sites (Putnam, 1969). The functional advantage of the variable

portion of the immunoglobulin molecule in all likelihood relates to the unique antigen-binding specificity of immunoglobulin molecules and the wide variety of antigenic determinants with which different antibodies must be capable of reacting. Probably variable regions permit complementary conformation to antigenic determinants (Putnam et al, 1967). It is likely that from sequence analyses of the heavy as well as light chains of a sufficient number of myeloma proteins including those with antibody activity, it will be possible to learn what amino acid sequences are responsible for specific binding of antigen. Also studies of the Fc part of the heavy chain should indicate the structural conformity needed for homocytotropic skin attachment such as occurs with human YE antibodies in Prausnitz-Kustner reactions and for heterocytotropic activity such as occurs with human YG in guinea pig passive cutaneous anaphylaxis (PCA) reactions. It will also be of considerable interest to learn what sequences in Fc fragment are concerned with class-specific antigenic determinants, for complement fixation and for the placental transmission of YG. All of these seem within the realm of understanding.

9. The Role of Immunoglobulins in Hypersensitivity

(a) General. Since there is considerable evidence that immunoglobulins are intimately involved in hypersensitivity and since evidence was accrued during the course of the current investigations to suggest that YD may at times be associated with antibody activity in hypersensitive subjects, the history of the concept of allergy and secondly the role of immunoglobulins will be reviewed briefly.

(b) The concept of allergy and atopy. That allergic disorders have been present on the earth for many centuries seems quite certain. It has been reported, for example, that several centuries B. C. the Greeks described symptoms resembling asthma (Feinberg, 1944). Another early description of asthma in which a primitive form of treatment is found is in the Ebers Papyrus (Sobhy, 1934). The transient, recurrent, and seasonal occurrence of asthma appears to have been first clearly reported by Helmont in 1607. In 1698 Floyer observed that asthmatic attacks could be precipitated by climate or by dietary indiscretions, and excellent detailed clinical descriptions and observations on asthma were recorded in 1859 in a book on asthma (Salter, 1859).

The first description of hay fever was made by Botallus in 1565 wherein symptoms were described as resulting from the inhalation of the odor of roses. The eponym "rose fever" which is synonymous with hay fever had its origin here. About a century later another author, Mather, remarked on the ill effects of inhaled substances in his book called "Remarkable Providence". In part, he described it this way: "Some men also have strange antipathies in their natures against that sort of food which others love and live upon. I have read of one that could not endure to eat either bread or flesh of another animal who fell into a swooning fit at the smell of a rose. There are some who if a cat accidentally came into the room, though they neither see it or are told of it, will presently be in a sweat and ready to die away."

In 1828, Bostock presented a very detailed description of his own symptoms and proposed the term hay fever for the first time. In 1873, Blackley wrote an even more detailed description of hay fever and of experiments

relating to it. He demonstrated that hay fever in his own instance was due to the pollens of grass and that he could artificially reproduce these symptoms by the nasal inhalation of grass pollen during the season when he was not having symptoms. He was the first person to do a scratch skin test with pollen, producing a severe local reaction on himself by the procedure. He also performed pollen counts and showed a close correlation of his symptoms with outdoor pollen counts. He was the first to demonstrate a causal effect from the inhalation of mould spores in upper respiratory tract allergy. There were few advances of major significance in the understanding of hay fever from Blackley's time until the past few years. Wyman, however, in 1872 published a book "Autumnal Catarrh" (hay fever) in which he demonstrated that ragweed pollen was an important cause of autumn hay fever. His findings have since been confirmed by many investigators. It is of note that the antigens of ragweed and the antibodies which they induce in allergic subjects have been very important in modern studies of allergy and in the role played by immunoglobulins (Sehon et al, 1955; Yagi et al, 1963; Goodfriend, Perelmutter and Rose, 1965; Osler, Lichtenstein and Levy, 1968; Ishizaka and Ishizaka, 1968; and many others).

Von Pirquet had introduced the term "allergy" in 1906 to indicate an altered or abnormal response to foreign substances and he included in this an increased sensitivity to tuberculin on which he reported. The term was subsequently used by many workers in describing different forms of hypersensitivity including hay fever and asthma. In 1923, Coca and Cooke introduced the appellation "atopy" to distinguish from other hypersensitivities the group of disorders exemplified by asthma and hay fever which depended on an

hereditary predisposition more than on unusual antigenic exposure. These disorders were characterized by immediate urticarial reactions in the skin on exposure to antigen, and were considered by Coca to be associated with positive dermal passive transfer reactions or Prausnitz-Kustner reactions.

(c) Anaphylaxis - early history. Although the symptoms which could result from injection of foreign proteins had been noted earlier, the first good experimental work on what is now called anaphylaxis was reported by Magendie in 1839. He noted that dogs injected repeatedly with egg albumin often died suddenly after an injection. In 1902 Portier and Richet named the sudden death which followed second inoculations of eel serum into animals (dogs) "anaphylaxis". In 1907 Otto demonstrated that anaphylaxis depended on serum antibody by producing the syndrome in guinea pigs which had been passively transfused with serum from another sensitized animal. Similar findings in many different animals were soon reported by a number of workers. At about the same time it was shown by Otto and independently by Besredka and Steinhart (1907) that a sensitized animal could be made refractory to anaphylaxis or desensitized by giving repeated small injections of the antigen.

(d) Arthus phenomenon. Arthus reported in 1903 that repeated subcutaneous injections of horse serum into rabbits at several day intervals gave rise to edema, sterile abscesses, hemorrhage and necrosis at the site of inoculation. This phenomenon became apparent 4 - 24 hours after the injection and has since been termed the Arthus phenomenon. It has more recently been thought to be associated with immunoglobulins of the YG variety.

(e) Serum sickness. The name serum sickness was applied to symptoms which von Pirquet and Schick (1905) described in children 5 to 10 days after therapeutic injections of diphtheria anti-toxin in the form of horse serum. Many studies into the nature of serum sickness have subsequently been reported and it is now believed that most of the manifestations are due to antigen-antibody complexes. The pathogenesis has been very well summarized by Dixon et al (1959). The additional importance of reaginic antibodies in serum sickness as well as a close correlation between hemagglutinating titers to horse serum antigens and the appearance of clinical symptoms has been reported. The evidence for this has been summarized by Arbesman (1965).

(f) The concept of reaginic antibody. In 1919, Ramirez transfused blood from a horse-sensitive individual into a subject suffering from pernicious anemia. The recipient developed symptoms of asthma when he was next exposed to horses, whereas he had previously not had any difficulty from such exposure. This suggested that passive transfer of allergic sensitivity with serum was possible. In 1921, Prausnitz and Kustner showed that injection of serum from a subject allergic to fish into the skin of a healthy non-allergic individual could result in passive sensitization of the skin and that a local inflammatory reaction would develop in the skin site when the recipient ate fish or when extracts of fish were injected into the site. Coca introduced the term "reagin" in 1923 to describe this skin-sensitizing factor in serum and the term has persisted to the present time.

(g) The chemical nature of reaginic antibody. Scherrer in 1930 demonstrated that reaginic antibodies were present in the pseudo-globulin rather

than the euglobulin fraction of patients' serum. These antibodies were concentrated in fraction III by the Cohn ethanol fractionation procedure indicating that they are different from the bulk of the gamma globulins, being more similar to certain β globulins in this regard (Vaughan et al 1952). On zone electrophoresis reaginic activity was found largely in the β and γ1 globulin region by Sehon et al (1955) and by Cann and Lovelace (1957). Fractionation of reaginic serum on DEAE-cellulose columns revealed that the earliest peak containing largely γG globulin usually lacks reaginic activity (Humphrey and Porter, 1957). These findings were confirmed by Stanworth (1959) and this has since been the experience with many workers. Upon sedimentation in the ultracentrifugal field activity was found to reside somewhere between the 7S gamma globulins and the 19S fraction. An average sedimentation coefficient of approximately 7.8S was recorded by Anderson and Vannier (1964). These findings suggested that reagins differed from γG and γM in physico-chemical properties but did not separate them out from a third major immunoglobulin, γA, which has many characteristics in common with reagin. Fireman et al (1963) and Vaerman et al (1964) both presented evidence suggesting that reagin may be associated with γA globulin and Ishizaka et al (1963) suggested that a γA globulin fraction of serum but not γG or γM fractions will block passive sensitization by human reagins. The first important evidence against the concept of reagins belonging to the γA class of antibodies was presented by Lovelace (1964) who described the presence of reagin in a subject with no detectable γA globulin. Ishizaka et al (1962) had previously shown that γA globulins are not necessarily skin sensitizing. Ragweed binding antibodies were found to be present in γA, γM and γG immunoglobulins of sensitive individuals by Yagi et al (1963). This

finding, however, constituted no proof for an association of reagin with any immunoglobulin class.

Ishizaka and Ishizaka (1966) and Goodfriend et al (1966) independently deduced that reagin must belong to an immunoglobulin class different from YA, YG, YM or YD. These conclusions were largely based on the behavior of reaginic antibodies on DEAE ion-exchange and Sephadex columns since the presence of reagin in eluates did not parallel that of any of the four major immunoglobulins. Similar conclusions were also suggested by the studies of Reisman et al (1965) who showed that the presence of YA antibody did not correlate with the skin sensitizing activity of reaginic serum. Ishizaka et al (1966) then reported a close correlation between reagin and a new immunoglobulin class which was called YE globulin. Subsequent work by Ishizaka and by Johansson and co-workers (see section C 9 j) has confirmed and strengthened the view that a separate immunoglobulin class is associated with most of the reaginic activity in human serum.

(h) Studies of an E-myeloma protein. The finding of a subject with E-myeloma by the Swedish workers Johansson and Bennich (1967 and 1968) has permitted studies which also have reinforced the view that reaginic antibodies are associated with a fifth immunoglobulin class, YE. Johansson (1968) demonstrated that there are elevated levels of YE or as he originally called it, IgND, in subjects with extrinsic asthma but that levels are normal in subjects with intrinsic asthma (inciting allergens not recognizable and few if any positive skin tests). Elevated levels of YE were found not only in cases of asthma and hay fever but also in cases of Wiskott-Aldrich

syndrome, atopic eczema and in subjects with parasitic diseases (Johansson, 1968). Johansson and co-workers also indicated that patients with multiple allergies have higher levels of YE than patients in whom only single allergens provoke symptoms (Wide, Bennich and Johansson, 1967). It was of interest that elevated levels of YE were found in three subjects with absent YA but with obvious atopic allergy.

The suggestion has been made by Johansson (1968) that YE antibody to specific allergens may constitute the major part of the YE moiety since a significant amount of YE could be removed by absorption with single insolubilized allergens. Also a very high correlation was found between the serum level of YE antibodies to specific allergens and provocation tests using the allergens to induce symptoms in the same patient (Wide et al, 1967). Furthermore, it was possible to inhibit skin fixation of reaginic antibodies with an E-myeloma protein (Stanworth et al, 1967). Levels of IgE do not appear to be elevated in chronic or severe infections in contrast to the levels of YG, A, M and D which often are.

All of the above findings strongly indicate the importance of the YE class of antibodies in the group of disorders which constitute atopic allergy. It should be noted that nearly all descriptions of E-myeloma have been discussed under the term IgND or myeloma-ND rather than IgE or YE; it is only upon recent recommendations (Bull. World Health Org., 1968) that the final designation YE has been given to this myeloma protein since it has now been conclusively shown that the heavy chain of myeloma protein ND is antigenically identical with that of the heavy chain of the YE discovered by Ishizaka. As Ishizaka has pointed out, the possibility still exists that

perhaps immunoglobulins other than YE may at times possess reaginic activity. As yet, there has been no evidence that a sixth undiscovered immunoglobulin class might be present which could also contain reaginic activity, although the possibility has not been ruled out.

(i) The relationship between immunoglobulins and hyposensitization. As previously indicated, Otto and others demonstrated in 1907 that an animal sensitized for anaphylaxis could be de-sensitized by repeated small injections of the antigen. At about that time, the suggestion was made by Wolff-Eisner that human hypersensitivity in the form of hay fever was probably related to anaphylaxis in animals (1906). In 1910, Meltzer suggested that asthma was also a phenomenon of anaphylaxis and the following year, Noon (1911) and Freeman (1911) both reported controlled experiments on active pollen immunization suggesting that de-sensitization in humans to pollen antigens was a possibility. Even prior to this time, Curtis (1900) reported that he was able to get excellent results in the treatment of hay fever by oral administration of an extract from flowers. These results, however, were apparently not well documented and were not taken very seriously since they were not tested for many years. As is well known, hyposensitization is now considered by most clinical allergists to be helpful therapy in many forms of atopy, particularly those related to inhaled allergens. The efficacy of hyposensitization has been ascribed to several mechanisms, the most logical of which is the production of "blocking" antibodies, first described by Cooke et al in 1935. Such antibodies are thought to compete with reagin or other mediator-releasing immunoglobulins for sites on allergens which

cause symptoms. Other authors have suggested that hyposensitization may induce a degree of immunologic tolerance (Sheldon, Lovell and Mathews, 1967) but if this occurs its mechanism is poorly understood as it relates to patients with allergy.

(j) The stimulation of antibody responses by ingested or inhaled allergens. In order for specific antibodies or immunoglobulins to be formed and participate in the common forms of atopic hypersensitivity the antigen must stimulate antibody forming cells either directly or indirectly. Bruner and Walzer (1928) demonstrated that small amounts of undigested proteins are normally absorbed from the gastrointestinal tract. They did this by showing that the ingestion of fish would cause local reactions in skin sites of 98% of subjects sensitized by local injection of fish reagin from a sensitive subject. By a reverse technique of first feeding the offending food and thereafter injecting reagin at intervals, Walzer showed that absorbed antigen reached its highest level in the circulation at one and one-half to two hours after ingestion of the food since at this time injected reagin would elicit a maximal reaction. Circulating antigen remained demonstrable for about 48 hours after ingestion as judged by this technique.

While these studies as well as many others (e.g. Ascoli, 1902; Crofton, 1908; Schloss and Worthen, 1916; Haltever and Krez, 1925; Lippard et al, 1936; Gruskay and Cooke, 1955) have shown the absorption of antigenically active protein moieties from the gastrointestinal tract, they do not in themselves prove the immunogenicity of absorbed proteins or peptides.

Ingested food protein could, for example, stimulate local antibody production in the gastrointestinal tract independently of systemic absorption. In any event, it is a common clinical experience to observe instances of marked sensitivity to food proteins when patients have been exposed to these foreign substances only through the gastrointestinal tract.

There is evidence to suggest that antigens may be absorbed in sufficient degree after inhalation to cause the appearance of reaginic antibodies in the blood and to sensitize the subject (Feinberg, 1939). Saltzberger and Vaughn (1934) demonstrated the production of skin sensitivity following passive nasal application of silk antigen and Kohn et al demonstrated the same thing following the application of ragweed pollen to the nose (1930). In addition, there is abundant evidence that ingested foods can lead to the production of antibodies to food proteins (Gunther et al, 1962; Heiner et al, 1962; Rothberg and Farr, 1964). In studying YD, therefore, one would do well to search for specific YD antibodies to food proteins in sensitive subjects. This has been done in the present study.

(k) Cell-mediated hypersensitivity. In addition to the antibody-mediated immune responses discussed above there is a large literature concerning cell-mediated hypersensitivity of the delayed or tuberculin type. Serum antibodies are apparently not involved directly since passive transfer is effected by cells or cell constituents but not

by serum. This subject has been reviewed in detail by others (Cowdle, 1962; Mackaness, 1968; Bloom and Chase, 1967; Uhr, 1968) and will not be discussed here. However, if cell-mediated responses involve structures on the surface of small lymphocytes which are similar to or identical with immunoglobulin molecules as suggested by a number of workers (Sell and Arofsky, 1968; Mitchison, 1967) then YD or a YD-like structure could be indirectly involved along with other immunoglobulins. Some evidence in this regard has been reported from this laboratory by Heiner et al (1968.) who found that antiserum specific to the heavy chain of YD causes blastogenic transformation of washed human lymphocytes suggesting that YD-like structures do indeed exist on the surface of small lymphocytes as do receptors bearing the antigenic determinants of other immunoglobulins (see experimental section).

10. Regulatory role of antibody on the immune response

In order to more completely understand the possible mechanisms of action of YD another feature of immunoglobulin activity should be mentioned as a reminder that YD could also conceivably be involved in such activity. It has been demonstrated by a number of workers that immunoglobulins are involved in the regulation of antibody synthesis, the evidence for which has been summarized recently by Uhr and Moller (1968). In particular it has been demonstrated that passive administration of specific antibody to animals inhibits the formation of new antibody to the corresponding antigen. In a sense this is a form of immunosuppression

and it may have important implications in regard to cancer immunology, autoimmunity,defects in the regulation of antibody synthesis, induction of immunological tolerance, etc.

D. The Immunology of Immunoglobulins

The common immunoglobulin nomenclature was briefly discussed in the section on paraproteins. Antigenic differences responsible for the major heavy chain classes and subclasses are determined by separate genes and are referred to as isotypic specificities. A second type of antibody or immunoglobulin specificity has been called allotypic and refers to antigenic determinants which are determined by allelic genes and hence are not present in all members of a given species. The Inv and Gm determinants are in this category. A third kind of antigenic specificity amongst immunoglobulins is called idiotypic. This is found in specific isolated antibodies and in myeloma proteins and refers to the fact that each of these has determinants which are different from all other antibodies or myeloma proteins, even of the same class (Cohen and Milstein, 1967; Pernis, 1967). Such variations in immunoglobulins always appear to occur on the Fab fragment and can be localized either to the Fd part of the heavy chain, to the light chain or to determinants that require both of these structures. Oudin (1966) described idiotypic specificity in isolated antibodies. Antisera detecting these specificities react only with the antibody resulting from immunization with a specific

antigen and may react with similar specific antibody raised in other animals, but not with normal immunoglobulins or specific antibodies directed towards different antigens. It thus appears that idiotypic specificity is associated with the antigen-combining specificity of the antibodies which in turn is determined largely by the variable segments of F(ab) fragments. A fourth type of specificity is species specific. This is characteristic of each species and involves both heavy and light immunoglobulin chains (Tomasi et al, 1968). Cross reactions of rabbit antiserum to bovine γG with human γG provide an example of this kind of specificity. When the cross-reacting antibodies are absorbed out, species-specific antibodies remain.

There are over 20 Gm allotypes of γG heavy chains known at the present time. These originally were designated by alphabetical letters but the current terminology (Table VI) is one which labels them 1, 2, 3, 4, etc. (Bull World Health Org., 1965). Gm specificities are located only on the γ variety of H chains, most of them being present on the Fc part of the chain but others, i.e. Gm 4 and Gm 17, on the Fd fragment (Steinberg and Polmar, 1965; Litwin and Kunkel, 1966). Some Gm factors are present only on γ1 heavy chains (Gm 1, Gm 2, Gm 4, Gm 17 and Gm 22), whereas others, Gm 5, Gm 6, Gm 14, Gm 15 and Gm 16, are found only on γ3 heavy chains (Martensson, 1966). Another, Gm 24, is associated only with γ2 molecules (Kunkel et al, 1966. One factor, Gm 4, is dependent on the quarternary structure of the molecule and cannot be detected when the light and heavy chains are separated one from another by reduction with mercaptoethanol (Polmar and Steinberg, 1964).

TABLE VI

Human Gm and Inv Allotypes

Specificity	Current Name	Original Name	Current Name	Original Name
Gm (on Y chains)	1	a	13	b^3
	2	x	14	b^4
	3	b^w, b^2	15	s
	4	f	16	t
	5	b, b'	17	z
	6	c, Gm-like	18	
	7	r	19	
	8	e	20	
	9	p	21	g
	10	b^a	22	y
	11	b^g	23	
	12	b^Y	24	n
Inv. (on k chains)	1	1		
	2	a		
	3	b		

From Cohen and Milstein, 1967.

Because each Gm factor is confined to one subclass of YG heavy chains, it has been suggested by several investigators that there are four genetic loci which direct the synthesis of Y heavy chains. According to this theory the Y1 locus controls the synthesis of the Y chains of a certain variety, the Y2 locus controls the synthesis of another variety having particular Gm allotypes, etc. (Cohen and Milstein, 1967).

Inv activity, the other well-studied form of immunoglobulin allotype, is present only on kappa chains and only YG subclasses YG1 and YG3 are associated with the light chains carrying Inv determinants. A single amino acid substitution at position 191 of kappa light chain determines the Inv allotype of the molecule. When amino acid 191 is valine the genetic factor is Inv (3+) and when it is leucine it becomes Inv (2+) (Easley and Putnam, 1966; Baglioni et al, 1966; Putnam et al, 1967).

One immunoglobulin, YM, is composed of five 7S units linked by inter heavy-chain disulfide bonds. In some lower vertebrates immunoglobulins analogous to YM are not cross-linked in this fashion and remain in a 7S monomer form. This has also been described in occasional humans (Rothfield, 1965; Stobo and Tomasi, 1966). A specialized form of another immunoglobulin, secretory or exocrine YA, is an antigenically distinct molecule found in the exocrine secretions which possesses a specialized fragment called the transport piece. The molecular weight of human secretory YA is approximately 510,000 and its structure has been postulated to consist of 3 7S YA units combined with one transport fragment (Hong et al, 1966). The function of the so-called "transport piece" has not been clearly demonstrated, although it has been thought to play a role in the process of secretion of exocrine YA.

E. Heterogeneity of Antibodies

As suggested in a previous section, when an animal is immunized with a purified protein it produces antibodies which belong to several immunoglobulin classes. It also produces antibodies of one immunoglobulin class that frequently extend over a range of electrophoretic mobilities. Some of the antibodies appear to be directed towards one portion of the antigen molecule and others are directed towards different antigenic sites. Some have higher affinity for the antigen than others. Thus, it seems to be characteristic of the immune response that a large family of antibodies are developed (Sela, 1968).

It is possible to isolate antibodies with different binding affinity for antigens and with different sizes of combining sites by sequential precipitation of a portion of the antibody molecules from a serum using an insoluble antigen added in increments, followed by elution of antibody from each precipitate obtained. Antibodies with the highest binding affinity are precipitated by the least additions of antigen, and many populations of antibodies with decreasing binding affinity are obtained as successive additions of antigen are made (Kabat, 1968). One would expect heterogeneity to be a characteristic of YD just as it is of the other classes of immunoglobulins although only minimal evidence is available for this at present, e.g. the differences in electrophoretic migration of D-myeloma proteins.

F. Physical Chemistry of Immunoglobulins

1. General

Physical chemical studies on immunoglobulins have been performed in a large number of laboratories and some of these studies have been discussed briefly in previous sections of this review. The isolation of gamma globulins in large quantities by ethanol fractionation (Cohn et al, 1950) and the later development of techniques to recognize and isolate myeloma proteins each provided a basic impetus for physical chemical studies on immunoglobulins. Initially the molecular weight of YG globulins was considered to be 160,000 although more recent studies indicate it to be closer to 145,000 (Noelken et al, 1965). The molecular weight of serum YA is about 180,000, that of YM about 900,000 and that of YE about 195,000 (Johanssen and Bennich, 1967). The sedimentation constant of YG previously was thought to be 7.2 or 7S and is now known to be nearer to 6.7 $S_{20,w}$ (Svedberg units) as noted by Hansson et al (1966). The only published report on the molecular weight of YD suggests a value near 180,000 (Heiner et al, 1968).

The original evidence that YG molecules measure approximately 235 x 44 Å (Oncley, 1949) has been updated as a result of electron microscopical studies. The current estimation is that these molecules are approximately 105 - 120 Å in length, 80 Å wide at the base and 35 Å in height, being somewhat triangular in shape (Feinstein and Rowe, 1965).

2. Solubility in ammonium sulfate

γG globulin has been shown to be precipitated from solution best with ammonium sulfate concentrations between 1.49 and 1.64 molar whereas γM precipitates best at between 1.64 and 2.05 molar (Olesen et al, 1965). It is of interest that γD has been found in this laboratory to require a higher saturation of ammonium sulfate for maximal precipitation than any of the other immunoglobulins, being most similar to γM in this regard (see experimental section, Isolation of D-myeloma Protein).

3. Carbohydrate constituents

The total carbohydrate content of γG is less than of the other immunoglobulins as indicated in Table VIa (Heimberger et al, 1964; Johansson and Bennich, 1967). The content of the individual carbohydrate moieties reported for each of the immunoglobulin classes are also listed. It will be seen that hexoses generally comprise just under half the total carbohydrate. Acetylhexosamine has a representation approximating that of total hexoses in γG but is less than total hexose in each of the other immunoglobulins. Fucose surprisingly is in approximately equal amounts in γG and γA but is a proportionately greater part of each of the other immunoglobulins. Sialic acid is low in γG.

TABLE VI a

Carbohydrate Content of Immunoglobulins

	YG	YA	YM	YD*	YE
Hexose %	1.2	3.3	5.4		5.5
Acetylhexosamine %	1.3	2.3	4.4		3.64
Fucose %	0.2	0.22	0.7		0.56
Sialic acid %	0.3	1.8	1.3		1.0
Total carbohydrate %	2.9	7.5	11.8		10.7

Data for YG, YA and YM from Heimberger et al (1964).
Data for YE from Johansson and Bennich (1967).
* No reports to date.

The above data are of interest as they relate to the carbohydrate analyses made on IgD in the course of this investigation (see section on Experimental Results).

A word is in order concerning the current concepts of linkage of carbohydrate to immunoglobulin molecules. It is known that carbohydrates are linked to immunoglobulin polypeptide chains through the beta-COOH group of aspartyl residues (Melchers and Knopf, 1967). Evidence has been presented by these authors that there is sequential addition of carbohydrate moieties to immunoglobulin polypeptide chains, glucosamine being attached to nascent peptide chains as they are being formed on polyribosomes and additional glucosamine, mannose and galactose apparently being added after a short time lag. The final groups of carbohydrate

residues to be incorporated are galactose, fucose and sialic acid. There is suggestive evidence that the latter residues are added during or after secretion of the immunoglobulin from the cell. These authors suggest that the attachment of carbohydrate residues may be a pre-requisite for transport of the protein across the plasma cell membrane into interstitial fluid and thence into the blood stream. Their findings were partially confirmed by Moroz and Uhr (1967) who demonstrated that some carbohydrate moieties are added after protein synthesis has been stopped by puromycin. Sialic acid residues appear to be attached last and varying degrees of attachment of this moiety have been shown to be responsible for the heterogeneity of migration observed in certain isolated monoclonal immunoglobulin polypeptide chains on urea-starch gel electrophoresis (Melchers et al, 1966). The effect of carbohydrate on the functional activity of immunoglobulin molecules after they are secreted from the plasma cells in which they are formed is quite unknown at present. Data obtained in the current investigation suggest that carbohydrate residues may possibly be related to the lability of immunoglobulins. This will be discussed in the experimental section.

4. Amino Acid Composition

Since the amino acid composition of each of the immunoglobulins varies within a given immunoglobulin class, only limited information can be obtained from comparing amino acid analyses. Nevertheless, after analyses of several immunoglobulins of one class are compared to those of

another class, certain information of interest is obtained such as the number of half cysteine residues which indicate the potential number of disulfide bonds per molecule. Also the number of proline residues per mole may suggest conformational characteristics since the usual α-helix configuration is interrupted at proline residues. This has proved of interest in our studies of YD. More meaningful information is available from comparisons of the amino acid sequences of immunoglobulins, but work on YD has not progressed to this point in any laboratory and the subject will thus not be discussed further.

G. Current Status of Knowledge about YD

1. General

Very little is known about the biological role of YD although some inferences can be drawn from studies of serum levels in a variety of clinical disorders. In the original descriptions of YD by Rowe and Fahey (1965) it was shown that YD may be the only immunoglobulin elevated (in rare cases of D-myeloma). They showed that YD was present in most normal adult sera but was not detectable in 18% of the subjects by the technique used because the concentration was less than 0.3 mg%, the lower level of sensitivity of their method. The average serum value was 3 mg%, and the top normal was about 40 mg%. Conflicting findings have been reported in regard to YD levels in allergic subjects. Kohler and Farr (1967) reported elevated levels of YD in atopic subjects whereas

Johansson (1967) found no difference in YD levels in atopics and controls. Rowe and Fahey found the levels of YD very low or undetectable in sera from patients with agammaglobulinemia and reduced in multiple myeloma (other than D-myeloma) and in macroglobulinemia. They also stated that elevated levels of YD were found in some patients with chronic infectious diseases. Hence the inferential biological evidence is quite good that this protein belongs among the immunoglobulins as its chemical association with both kappa and lambda light chains suggests it does.

A rather unusual feature of YD is the broad range of serum levels found in the normal population. This involves at least a 100-fold variation from low normal to high normal which is a much wider range than is observed in the case of YG, YA, YM or YE. The independent synthetic and catabolic rates of YD were outlined in detail in a previous section.

2. <u>D-myelomas</u>

Hobbs et al (1966) described studies on six cases of D-myeloma, which were found among a total of 204 myelomas or about 3% of the myelomas in their series. They noted certain pecularities about D-myeloma, namely that it was likely to be found in what previously had been thought to be myelomas with only Bence-Jones proteinemia or proteinuria, without monoclonal (or "M") protein peaks. There appeared to be a particular propensity towards renal failure and proteinuria. In each of their cases the other immunoglobulins were reduced in concentration. Frequently the percentage of plasma cells in the bone marrow was not as great as in

other myelomas and they felt that there was less reduction in the non-myeloma immunoglobulins than in cases of G- or A-myeloma. All six of their cases had lambda-type Bence-Jones proteinuria and they noted that only two of 17 YD myelomas reported up to that time were of the kappa variety, a reversal of the unusual predominance of kappa over lambda types of myeloma protein.

Hansson et al (1966) studied 1300 sera with "M" peaks and found that four were due to D-myeloma proteins. Thus one out of 325 sera with "M" peaks were due to YD or about 0.3%. Sedimentation constants for YD were found to be similar to those of normal YG, all three of their YD's were of the lambda variety. The Gm allotype determinants were missing from each of the D-myeloma proteins.

The most recent report on D-myelomas is that of Fahey et al (1968) in which certain characteristics of 24 patients were presented. The serum YD concentrations varied from 80 mg% to 3300 mg%. The very low levels encountered in some patients were ascribed to rapid catabolism of YD. This feature can make the detection of D-myeloma more difficult than that of A- or G-myeloma or Waldenstrom's macroglobulinemia since 80 mg% YD can be buried amongst the other immunoglobulins. One patient was described who had both G- and D-myeloma proteins in the serum. Patients were diagnosed as having multiple myeloma by Fahey et al on the basis of the presence of more than 10% malignant plasma cells in the bone marrow or jaw tumor and other symptoms and signs of myeloma. Total serum proteins were elevated in only 5 of 15 patients. Abnormal protein peaks were

detectable on careful examination in 10 out of the 15 patients on paper electrophoresis. In each of the 15 sera it was possible to identify an elevated YD by immunoelectrophoresis and in each instance this YD had restricted mobility and had either kappa or lambda light chains but not both. The authors pointed out that D-myeloma protein is easily altered in stored serum and may demonstrate multiple components on immunoelectrophoresis. Sixteen of 20 patients were found to have lambda-type light chains and the remainder kappa light chains, confirming the reversed ratio of kappa/lambda in the case of D-myeloma proteins. There was no cell morphology in the bone marrow unique to D-myeloma and it was impossible to make a specific diagnosis of D-myeloma by ordinary histologic study. They commented that the most striking finding was the presence of Bence-Jones proteinuria in all of the patients reported. In this connection, it is of interest that one of the two subjects reported in the present study did not have detectable Bence-Jones proteinuria, whereas the other had both Bence-Jones proteinuria and Bence-Jones proteinemia of the lambda variety.

3. Biologic implications of studies on YD to date

It was shown both by Ishizaka et al (1966) and by Goodfriend et al (1966) that YD was not associated with the antibody activity commonly known as atopic reagin. A different role in hypersensitivity phenomena has not been ruled out. Pernis et al (1966) demonstrated the presence of YD immunoglobulins in human spleen cells in a ratio of approximately

.13 YD-containing cells per 100 cells staining for YG. In two additional patients they found .45 and .30 YD cells per 100 YG cells. Their conclusion was, therefore, that the serum concentration of YD depends largely on the number of YD producing cells present. (One notes, however, that the data of Rogentine et al (1966) suggest rapid catabolism of YD also plays an important role in the low serum levels of this immunoglobulin.) Mature as well as immature splenic plasma cells contained YD and in addition it was seen in the cytoplasm of some large lymphocytes. The YD-containing cells in the spleen were not clustered together but were scattered randomly amongst the other cells, suggesting to these authors that YD-producing cells or their precursors can readily be displaced by other cell lines.

Rowe et al (1968) studied YD levels in serum and body fluids. They stated that YD was rarely found in umbilical cord plasma and usually was not detectable in serum during the first six months of life. They found elevated levels of YD in certain chronic infections but no elevation in auto-immune diseases and it was usually absent in hypogammaglobulinemia. They concluded that lymphoid cells containing YD were rare in lymph nodes, spleen and intestinal mucosa in comparison with cells containing other classes of immunoglobulins. They stated that YD-containing cells were relatively more frequent in the adenoids where they were similar in numbers to cells containing YM. YD was not detected in colostrum, saliva, urine, lacrimal secretions, bile, jejunal secretions, human milk or cerebro-spinal fluid. The authors report that they were unable to show YD antibody activity in subjects who were immunized against diphtheria and tetanus

toxoids using the technique of radioimmunoelectrophoresis. They found that YD levels were not elevated in subjects who had rheumatoid arthritis, Sjogren's syndrome, Hashimoto's throiditis, nor experimental malarial infections, although other immunoglobulins were increased in each of these conditions. They did, however, find high levels of YD in a child with chronic osteomyelitis, in five subjects with leprosy, and in a child with recurrent skin infections. In addition, they stated that high levels were sometimes found in children with kwashiorkor and stated that elevations were frequent in children in a "primitive African community". Crabbe and Heremans (1966) demonstrated the presence of a large number of plasma cells containing YD in the rectal mucosa of a patient with ulcerative colitis.

Heiner and Evans (1967) measured the immunoglobulin levels in commercial preparations of gamma globulin and found that YD was absent from some but was concentrated to a moderate degree in others, the highest levels being found in a commercial preparation made by 45% ammonium sulfate fractionation of pooled plasma. The suggestion was made that labels indicating the individual immunoglobulin concentrations of various commercial preparations might be of value since only YG was similar in the different products on the market. It was felt that certain clinical trials of gamma globulin therapy could be better evaluated if more precise compositions were known. Contradictory reports, for example, have appeared in regard to the efficacy of gamma globulin in allergic disorders (Bernton, 1958; Abernathy, 1958; Redner and Markow, 1963; Hilman, 1969).

4. Areas of relative lack of knowledge

There is virtually no direct information in the literature concerning the functional role of γD although it is increased in certain infections, liver diseases and specific myelomas, and is usually decreased in hypogammaglobulinemias. It appears to be less frequently implicated in antibody responses than is any other immunoglobulin class including γE which is present in even smaller quantities.

There have been no papers published dealing with the specific molecular weight of γD, its amino acid composition, carbohydrate content, ammonium sulfate precipitability, heat and acid lability, or structural conformation. It is believed, therefore, that information pertinent to the above points should contribute to the understanding of this protein and its role in human immunobiology.

IV. EXPERIMENTAL STUDIES

A. Isolation of D-myeloma Protein

1. Introduction

The immunoglobulin about which least is known at the present time is γD. The desirability of learning more about it is obvious. In order to carry out studies of its properties and physicochemical nature it is necessary to isolate the protein in a sufficient degree of purity that one can be confident the results obtained represent γD rather than contaminating proteins. This has been a particular challenge in the case of γD, a very labile protein which was not available to us in serum having more than a faint suggestion of a monoclonal or ''M'' protein peak. Most detailed studies on specific classes of immunoglobulins in the past have been made on myeloma proteins of the monoclonal variety. The invaluable nature of ''M'' proteins is especially evident in the case of the immunoglobulin classes or subclasses which are present in small quantities in normal serum as are γD and γE. Such a protein peak was present in the D-myeloma serum originally studied by Rowe and Fahey (1964) and in the E-myeloma reported by Bennich and Johansson (1967). Ishizaka has had notable success in working immunochemically with non-monoclonal γE, but even his work has been restricted to investigations which can be carried out on minute amounts of protein not of an optimal

degree of purity. For example, it has not been possible for him to do molecular weight determinations, amino acid analyses or assays of the carbohydrate content of YE.

In the case of the two D-myeloma sera without "M" peaks reported here, initial concentrations of YD which approached the concentrations of YG made it possible to further isolate YD until it was of 90% or greater purity and to prepare this in sufficient amounts to pursue these investigations.

2. Materials and methods

(a) Identification of YD.

(i) Micro-Ouchterlony. One ml of antiserum specific to the heavy or δ-chain of YD was kindly supplied by Dr. John Fahey, National Institute of Health, as was 1 ml of a standard serum containing 0.33 mg/ml of YD. It was possible to demonstrate a distinct specific precipitate line in micro-Ouchterlony analyses using three or four-fold dilutions of each of these reagents. Upon further dilution of the reference serum it was possible to show that the tip of a reference YD precipitate line produced by these reagents was bent by as little as 20 ug/ml or 0.02 mg/ml of YD placed in an adjacent reservoir. The kind of precipitate patterns produced by this technique are illustrated in Fig. 3. When serum in reservoir 3 contains 0.02 rather than 0.12 mg/ml of YD the precipitate line forms immediately adjacent to reservoir 3 giving the appearance of bending the tip of the reference line. This principle is further

illustrated in the section on plexiglas micro-Ouchterlony and in Figure 10.

Initially one hundred sera including 15 from subjects with multiple myeloma were studied. One myeloma serum bent the standard precipitate line when diluted 128 fold suggesting a concentration of γD in excess of 2 mg/ml. An aliquot of this serum was sent to Dr. Fahey who confirmed the diagnosis of γD myeloma. All the other 99 sera had less than 0.4 mg/ml of γD and one third had less than 0.02 mg/ml. The micro-Ouchterlony technique has been used both for qualitative identification of γD and as a method of rough quantitation of this immunoglobulin using serial dilutions of sera, column eluates or other fractions. The technique involves the application of 2 ml of 1% Noble agar in 0.1 M borate buffer, pH 7.5, to a clean microscopic slide precoated with 0.1% agar. Reservoirs in the agar are 2 mm in diameter and 2 mm apart and are filled with 3 microliters (3λ) of serum or antiserum using a Hamilton microliter pipette and narrow gage polyethylene tubing extending beyond the needle.

(ii) Immunoelectrophoresis. In order to identify contaminating proteins in purified fractions of γD, and to establish the lack of unwanted antibodies in absorbed antisera, immunoelectrophoresis was used in addition to micro-Ouchterlony analyses. National Instrument Company immunoelectrophoresis cells, slide frames and agar cutters were employed. Three reservoirs and two troughs were cut from the agar on each microscopic slide. Two ml of 2% Noble agar in 0.15 M barbital buffer, pH 8.5 was applied to each of eight

slides placed on a level table and 2λ of serum or serum fraction (concentrated to 5-50 mg protein/ml) was placed in each reservoir, after which the slides were immediately electrophoresed for 50 minutes using 40 volts and 50 milliamperes. Following electrophoresis the slides were removed, the troughs filled with 30λ of appropriate antiserum, and the slides incubated overnight in a moist chamber at room temperature. Unprecipitated proteins were washed from the slides by placing them in 500 ml of 0.1 M borate buffer, pH 7.5 for 2 days with agitation from a magnetic stirrer and one buffer change at 24 hours. Following this electrolytes were washed away in distilled water for 2-4 hours. The slides were then stained with .1% Thiazine Red-R in 1% acetic acid, rinsed in acetic acid and distilled water, and dried overnight in an incubator at 37°C. A drop of Permount cement was placed on each slide and coverslips affixed for permanent preservation.

In searching for serum proteins in column eluates or ammonium sulfate fractions, the electrophoresed fractions were developed with two different antisera to whole human serum, one containing antibodies to YD as well as to other serum proteins, and the other containing antibodies to more than 15 serum constituents. In order to identify individual precipitate arcs, specific absorbed antisera were used, as were purified proteins, when available. The absence of significant amounts of unwanted antibodies in specific antisera was confirmed by immunoelectrophoresis of whole serum and developing with the absorbed antiserum placed in a trough.

(b) Preparation of antisera. Initially whole serum from the subject with D-myeloma was diluted 1:5 with 0.15 M sterile saline and emulsified in an equal quantity of complete Freund's adjuvant. Two ml of the emulsion was administered intraperitoneally and two ml subcutaneously into each of two New Zealand white rabbits. An additional two ml of the emulsion was given subcutaneously one month later, and at two months serum was harvested for study. It was shown both by immunoelectrophoresis and by Ouchterlony analysis that antibodies had been produced to YD. By absorbing aliquots of the antisera with one fourth quantities of several normal sera containing less than 10 ug/ml of YD one serum was found which completely absorbed out all antibodies to other immunoglobulin heavy or light chains and to other serum proteins while having little effect on the titer of precipitins to YD as demonstrated by Ouchterlony analysis and immunoelectrophoresis. Thus this one serum had a virtual lack of YD which made it valuable for purposes of absorbing antiserum to YD in order to obtain specific anti δ-chain serum (Fig. 4). The initial absorbed anti-YD serum was indeed specific but the antibodies were in low titer and the antiserum had to be used full strength for gel diffusion studies. Subsequently it was possible to produce antiserum of considerably higher titer by using YD-rich fractions for immunization of rabbits. These were prepared by ammonium sulfate precipitation of the D-myeloma serum followed either by G-200 Sephadex gel filtration or DEAE-column chromatography. It was also possible to produce antiserum to the YD of a normal subject who had a high serum level of this immunoglobulin.

Antiserum to YG was produced by immunizing rabbits with highly purified pooled normal YG in adjuvant, the YG being obtained from The Protein Foundation, Jamaica Plain, Mass. The resultant antiserum was absorbed with isolated kappa and lambda Bence-Jones proteins.

Antiserum to YM was prepared by injecting into rabbits an adjuvant emulsion of 15 mg of washed YM euglobulin precipitate obtained by exhaustive dialysis against distilled water from the serum of a macroglobulinemic subject. The antiserum was absorbed with umbilical cord serum devoid of YM and was shown by the usual techniques to have antibodies directed only to the μ chain of YM.

Antiserum to YA was prepared by injecting into rabbits 15 mg of an adjuvant emulsion of isolated YA from a subject with A-myeloma. The resulting antiserum was absorbed with one tenth volume of serum from a subject having an absence of serum YA, and the absorbed antiserum was shown by Ouchterlony analyses and immunoelectrophoresis to be specific for the α chain of YA.

Antiserum to the Fc fragment of YE was kindly supplied by Drs. S.G.O. Johansson and H. Bennich of Sweden as was a reference serum containing 88 ug/ml YE.

Antisera to kappa and lambda light chains were produced by repeatedly injecting 10 mg quantities of urinary Bence-Jones proteins of the appropriate type isolated by DEAE-column chromatography into rabbits as adjuvant emulsions. Antisera to kappa chains were absorbed with whole urinary proteins from two subjects with lambda Bence-Jones proteinuria.

Antisera to lambda chains were similarly absorbed with an excess of urinary proteins from two subjects with kappa Bence-Jones proteinuria.

The specificity of all antisera was confirmed by immunoelectrophoresis and Ouchterlony double diffusion techniques against whole normal serum as well as against isolated myeloma and Bence-Jones proteins. Each antiserum (with the exception of antisera to YD and YE) was furthermore shown to have identical specificity to mono-specific antisera obtained from Hyland Laboratories, California, and the Boehringwerke Ag, Germany. Antiserum to YD was shown to be specific by Ouchterlony analyses comparing it with anti-YD supplied by Dr. Fahey.

(c) Quantitation using micro-Oudin tubes. Experience with quantitation of immunoglobulins by the dilution method in Ouchterlony plates and using purchased Hyland Immunoplates suggested that a more precise technique was desirable. After experimenting with several modifications of Mancini's (1964) radial diffusion technique as well as with Huntley's (1963) modification of Gitlin's (1954) micro-Oudin technique, the latter was selected because of its reproducibility, economy of antisera and ease of performance. When employed to quantitate YD, this technique consists of incorporating appropriate concentrations of specific rabbit anti-YD into melted agar at 55°C, mixing thoroughly, and filling capillary tubes (3" long with an internal diameter of 1.5 mm) half full of the antiserum-agar solution by capillary attraction. Immediately after filling each tube, the end is sealed with plastic clay. After the agar has been allowed to

solidify for half an hour solutions to be tested are overlaid on the agar by means of a capillary tube of 1.2 mm external diameter with care to avoid the formation of an air bubble. The tubes which are now 3/4 filled are left for 16 hr in a vertical position in a constant temperature chamber at 37° to permit migration of YD into the gel. By using three sera of known YD concentration as standards each time tests are done, it is possible to construct a line on semi-log paper indicating the concentration of YD for any observed distance of migration of precipitate from the liquid-gel interface into the agar (Fig. 5). By adjusting the concentration of anti-YD in the agar downward until a relatively faint but still distinct zone of precipitation forms it is possible to make the test sensitive to levels of 5 mg% of YD (.05 mg/ml). The test is performed in duplicate. Using the same technique and other specific antisera it is possible to detect as little as .05 mg/ml of YG (Fig. 6) or YA (Fig. 7), and .15 mg/ml of YM (Fig. 8). This technique has been used routinely in this laboratory to determine YG, YA, YM and YD immunoglobulin levels in serum or other fluids. New standard curves are prepared for each immunoglobulin whenever a new batch of antiserum-in-agar tubes is prepared. The same standard sera were used throughout as were the same pools of specific antisera. The technique will be referred to as "micro-Oudin" in this thesis.

(d) <u>Quantitation using plexiglas reservoirs</u>. It was found that still smaller quantities of each of the immunoglobulins could be detected by a different method of micro-Ouchterlony analysis utilizing a special

plexiglas matrix modified from those described by Crowle (1958). Experimentation demonstrated that at least a ten-fold increase in sensitivity over the most sensitive micro-Ouchterlony, micro-Oudin or Hyland Immunoplate techniques could be attained by utilizing reservoirs with centers 3 mm apart (Heiner and Evans, 1967). The essential item of equipment is the plexiglas reservoir. This is shown in Fig. 9. By this technique it is possible to reliably detect 1 ug/ml of YD, YG or YA, or 5 ug/ml YM by the method outlined in Fig. 10. This sensitive technique is somewhat more difficult to perform than the other methods of quantitation because of skill required in filling the small holes and the tendency for agar to dry under the plexiglas and cause leakage from below the reservoirs. Nevertheless, it is reliable in experienced hands and is routinely used when dealing with immunoglobulin levels below 30-40 ug/ml. Studies with this method are regularly done in duplicate and occasionally in triplicate or quadruplicate if necessary. The technique is referred to as plexiglas micro-Ouchterlony. Details of the method are as follows:

Six peripheral reservoirs were arranged around a central reservoir in a 1" square of plexiglas which was 3 mm thick. The reservoirs were 1.5 mm in diameter at point of contact with the agar and were counter-sunk from the top to a depth of 2.2 mm and a diameter of 2.5 mm. The holes were 3 mm apart, center to center, Double thickness layers of electrical tape were affixed to glass slides precoated with .2% Noble agar and the plexiglas squares were placed across the tape strips as shown in Fig. 9. One percent agar in 0.1 M borate buffer was introduced beneath each plexiglas square to

fill the space between the plexiglas and the slide. Practice is required in introducing the proper amount of agar but with experience this can be readily done. The reactants are then placed in the appropriate reservoirs and allowed to develop overnight. After this the plexiglas squares are gently lifted from the agar surface and the slides are examined for precipitate lines in the agar. They are then washed for 2 days to remove unprecipitated protein and are stained with thiazine red R. Following this the slides are dried, a drop or two of mounting fluid is placed on the surface of each, and coverslips are affixed to produce permanent mounted slides.

(e) Fractional ammonium sulfate precipitation. Two ml of D-myeloma serum (L.P.) were diluted with 2 ml 0.15 M NaCl and made up to 20% saturated ammonium sulfate using the nomogram of Dixon (1953) to determine the amount of ammonium sulfate which should be added. The small amount of precipitate was labelled 20% AS and was saved for study as indicated below. The supernatant was then made up to 35% ammonium sulfate again employing Dixon's nomogram. The resulting precipitate was washed twice with 35% saturated ammonium sulfate and dissolved in 1 ml 0.1 M tris-HCl buffer, pH 8.0. This solution was dialyzed for 24 hours in the cold against 100 volumes of 0.1 M tris-NaCl, pH 8.0 which was changed twice. The fraction was made up to 2 ml and was called 20-35% AS. The supernatant from the 35% precipitation was made up to 45% ammonium sulfate saturation, the resulting precipitate was twice washed with 45% saturated ammonium sulfate, dissolved in 0.1 N tris-NaCl, pH 8.0, dialyzed as above, reconstituted to 2 ml and called 35-45% AS. The

supernatant from the 45% saturated ammonium sulfate precipitation was dialyzed exhaustively with 0.1 M tris-NaCl and was called >45% AS.

Preliminary micro-Ouchterlony analyses of the above fractions indicated that only YG was present in 20% AS, predominantly YG in 20-35% AS, and predominantly YD in the 35-45% AS fraction. A number of non-immunoglobulin serum proteins were also present in small amounts in this fraction. Negligible amounts of YD and YG were present in >45% AS. On the basis of these results a 50 ml aliquot of D-myeloma serum (L.P.) was fractionated in precisely the same manner except for the larger volumes of reagents employed. Later a second D-myeloma serum (J.M.) was fractionated by this technique, and subsequently additional aliquots of each myeloma serum and sera from several non-myeloma subjects were similarly subjected to this procedure, generally as a preliminary step to gel filtration or ion-exchange chromatography.

(f) Sephadex G-200 gel filtration. The 35-45% AS fraction from 50 ml of L.P. serum was dissolved in 10 ml tris-NaCl, pH 8.0, dialyzed, and made to a final volume of 15.5 ml. The total protein concentration was 10.1 mg/ml, the concentration of YD was 6.4 mg/ml and of YG 1.7 mg/ml as measured by micro-Oudin. The remainder of the protein represented other immunoglobulins and non-immunoglobulin serum proteins. Sephadex G-200 (Pharmacia) was prepared by swelling the gel beads in distilled water for 48 hours, removing slowly sedimenting fines three times, then washing with 0.1 M NaCl, 0.05 M tris-HCl, pH 8.0. A 7 x 75 cm column was prepared (20 cm water pressure)and equilibrated with the same buffer

overnight. Fifteen ml of the YD-rich 35-45% AS fraction was then applied. This represented 151.5 mg protein of which approximately 96 mg was YD. Filtration was allowed to proceed at the rate of 10 drops per minute and 10 ml fractions were collected at 4°C using an LKB automatic fraction collector. The optical density of the solution in each tube was read in a Beckman DU Spectrophotometer at 280 mμ and aliquots from individual tubes were tested by micro-Ouchterlony for approximate immunoglobulin content. The protein peaks were then pooled and labelled I, II, III and IV. The 20-35% AS fraction of L.P. was prepared and applied to a second Sephadex G-200 column in a manner identical to that just described. Whole serum from non-myeloma subject K.G. was also fractionated in this manner.

(g) DEAE-cellulose column chromatography. DEAE-cellulose was prepared by initial suspension in distilled water followed by washing alternately with 2 N HCl and 2 N NaOH for three washes each, the final wash being NaOH. The suspension was then washed four times with 0.015 M sodium phosphate, pH 8.2, and was considered ready for use. A 2 x 25 cm column was packed under mild air pressure with freshly prepared DEAE-cellulose and was equilibrated overnight with 0.015 M phosphate buffer. Four hundred and fifty mg of an ammonium sulfate fraction of L.P. D-myeloma serum (35-45% AS) was dissolved in 0.015 M sodium phosphate buffer, pH 8.2, exhaustively dialyzed against the same buffer and brought to a volume of 15 ml. This was applied to the column and was eluted in 15 ml aliquots using stepwise increases in buffer molarity at constant pH. Several

subsequent column chromatographic separations were performed in similar manner using 35-45% AS fractions of J.M. D-myeloma serum or similar preparations of non-myeloma serum.

3. Results

(a) Fractions obtained by ammonium sulfate precipitation were tested by the micro-Oudin technique after dialysis against 0.15 M sodium chloride. This revealed that 50-70% of the total protein in the 35-45% AS fractions was YD in each fractionation of D-myeloma serum whereas only 10-30% was YG. The remainder was YM, YA, α_2 macroglobulin and several other serum proteins in small quantities, as demonstrated by immunoelectrophoresis of concentrated fractions. The 20% AS fractions were exclusively YG and the 20-35% AS fractions contained 8-10 times as much YG as YD. This indicated that fractional ammonium sulfate precipitation was a useful procedure for concentrating YD relative to YG in serum samples.

(b) Sephadex G-200 gel filtration of a 150 mg aliquot of the 20-35% AS fraction of L.P. myeloma serum is shown in Fig. 11. The elution rate was 0.5 ml/min and the buffer was 0.1 N tris in 0.1 N NaCl, pH 8.0 throughout. The fraction eluted as two minor peaks and one major peak, one minor peak appearing as the ascending shoulder of the major peak. The first peak consisted almost exclusively of YM as determined by micro-Ouchterlony, micro-Oudin and immunoelectrophoretic analyses both before and after 100-fold concentration by pervaporation. Peak II contained largely YD but also contained YA, whereas the larger peak, III, was almost exclusively YG.

The 35-45% AS fraction eluted as three major and one minor peak on Sephadex G-200 gel filtration (Fig. 12). Peak I represented a small amount of YM and a larger amount of α_2M, peak II was composed largely of YD and peak III contained YD and a somewhat smaller amount of YG. Peak IV was found to contain, among other things, fragments of YD which on immunoelectrophoresis migrated as Fab and Fc fragments. YD was thus shown to fragment to a significant degree under the relatively mild conditions of ammonium sulfate precipitation and Sephadex G-200 gel filtration, an evidence of its fragility which has been frequently seen in this laboratory. YG has been observed to fragment but to a lesser degree. This fragility was also demonstrable in the concentrated fraction IV on immunoelectrophoresis.

Sephadex G-200 gel filtration of a 45% ammonium sulfate precipitate of a reaginic non-myeloma serum was analyzed for comparative purposes (Fig. 13). In this serum the YD concentration was 0.015 mg/ml and the YE concentration (kindly determined by Dr. S.G.O. Johansson) was 0.0073 mg/ml (or one half the YD level). The YD and YE each were assessed in concentrated gel filtration eluates pooled as indicated in the figure. YA was present in 100 times greater concentration than YD, which is probably the reason it was detectable in fraction 1, even though it peaked in fraction 3. There was slightly more YD in pool 2 than in pool 3 whereas the reverse was true for YE suggesting that in this serum YD was retarded less by the gel and hence may be a slightly larger molecule than YE. Passive transfer skin reactions (PK tests) were also done on representative unconcentrated fractions and are indicated at the bottom of the figure as an additional point of reference.

(c) DEAE-cellulose column chromatography of .L.P. myeloma serum fraction 35-45% AS is shown in Fig. 14. While YG was the only protein eluted in peak I, and both YG and YD were eluted in peaks II and III, peak IV was found to contain YD:YG in a ratio of 18:1 both by micro-Ouchterlony analysis of unconcentrated aliquots and by micro-Oudin tests on the concentrated peak. Immunoelectrophoresis of peak IV using anti YD as well as anti whole human serum revealed a high concentration of YD, a minimal concentration of YG and no other detectable proteins (Fig. 15). This peak was used for amino acid analysis and ultracentrifugal studies even though YG was present as a contaminant in an amount of about 10%. An additional fractionation of L.P. D-myeloma serum was made in a manner similar to this and precisely similar results were obtained. The peak containing YD in highest proportion was calculated to be 90% pure by micro-Oudin and this was confirmed by immunoelectrophoretic analyses. This peak was used for carbohydrate determinations. In neither fraction was YM or YA found to be present in the YD-rich peak in an amount exceeding 2% of the total protein. Further fractionation of YD by Sephadex G-200 gel filtration was attempted but was not employed at this stage because of the excessive losses of YD which occurred.

D-myeloma serum (J.M.) was fractionated using a 35-45% AS fraction as starting material but a slightly different stepwise buffer elution scheme as shown in Fig. 16. Only YG was eluted in peak I and YD was eluted largely in peak II. A small amount of YA was present in each of the last two peaks. Three additional chromatograms have been made of

35-45% fractions of J.M. myeloma serum, each with similar results to those shown here. Peaks found to contain YD in 90% or greater purity were used for amino acid analysis, ultracentrifugal studies and carbohydrate determinations (one peak on each of 3 columns proved to be suitable).

Column chromatography on DEAE-cellulose of a non-myeloma serum containing a high concentration of YE was carried out and is included for purposes of comparing elution patterns of the five immunoglobulins. It is seen that YD was eluted in two regions with a peak slightly preceding that of YA but following that of YE (Fig. 17). Column chromatography of the same atopic serum was also carried out on DEAE-Sephadex using a slightly different buffer and including PK titres which are seen to correlate better with the presence of YE than with YD or any other immunoglobulin though the correlation was imperfect even with YE (Fig. 18).

4. Discussion

During the process of isolating YD for immunologic and physicochemical studies the molecule was found to precipitate best at greater ammonium sulfate concentrations than YG and this property of YD was exploited in preparative procedures. It was also shown to elute earlier from Sephadex G-200 than YG, being recovered at almost the same location as YA and YE but peaking slightly before either, suggesting it may be a somewhat larger molecule than YG, monomer YA, or YE, but smaller than YM. In addition, fragments of both YD and YG containing heavy and light chain determinants were found by gel filtration, the molecular breakdown being

most marked in the case of YD. Using fractional ammonium sulfate precipitation and DEAE column chromatography it was possible to prepare YD of approximately 90% purity from the sera of two D-myeloma subjects.

While this degree of purity is not optimal it is the best that could be attained after repeated attempts. Whenever an additional step was employed to attain a greater degree of purity the loss of YD was so great that insufficient remained for analytical studies. The justification for using the 90% pure material rests on the reasonable assumption that the 10% of impurities in all likelihood would not significantly alter the results of amino acid and carbohydrate analyses nor of ultracentrifugal studies. Proof that this assumption is correct must await the availability of larger amounts of starting material and effective methods to prevent YD breakdown so that additional purification will become possible. Each of the immunoglobulins had a unique pattern of elution upon anion exchange chromatography with considerable overlap in many fractions.

B. Isolation of lambda Light Chains from L.P. Urine

1. Introduction

Most subjects with D-myeloma have lambda Bence-Jones protein in the urine, a pecularity that suggests either a unique affinity of δ-heavy chains for λ-light chains, or the production of a special variety of λ chain in subjects with D-myeloma, or both. In an attempt to gain some insight into the reason for the high incidence of λ Bence-Jones proteinuria and into the nature of the λ chains themselves, the urines of subjects L.P. and J.M. were studied. Urine L.P. was found to have a high concentration of λ Bence-Jones protein whereas urine J.M. had only very small (normal) amounts of both k and λ light chains. Urine L.P. was therefore subjected to further study.

2. Materials and methods

Two liters of L.P. urine containing approximately 5 grams of protein per liter were placed in 2.5 cm diameter Visking tubing and dialyzed for two days against cold tap water. The dialyzed urine was shell frozen in 300 ml aliquots and lyophilized.

Initially 5 ml of a solution of 47 mg/ml urinary protein (235 mg) was dialyzed against 0.05 M sodium phosphate buffer, pH 7.6 and applied to a 3 x 40 cm column of regenerated DEAE cellulose equilibrated with the same buffer. Step-wise elution was carried out as indicated in Figure 19.

Three additional aliquots of L.P. urinary protein (100 mg each) were chromatographed separately under almost identical conditions. For the last two chromatograms urine was used which had been stored for several months at 4°C in the presence of 1:10,000 sodium azide as a preservative.

Antiserum to L.P. urinary proteins was prepared by emulsifying 50 mg of dialyzed and lyophilized urine in 2 ml complete Freund's adjuvant and injecting half of this into each of two white New Zealand rabbits. Booster injections were given at 1 month and antiserum was harvested 2-3 times monthly beginning at 2 months. Precipitating antibodies to urinary proteins were demonstrated by immunoelectrophoresis against concentrated urinary proteins and antibodies to serum proteins by immunoelectrophoresis against undiluted L.P. serum.

3. Results

Representative tubes comprising each protein peak were analyzed as obtained from the columns for k, λ, γ and δ chains. Only λ chains were found in unconcentrated fractions tested by micro-Ouchterlony and these were distributed as indicated in Fig. 19. The last protein peak was judged to be pure λ chains since no other protein was detected when this peak was concentrated to 30 mg/ml and tested by immunoelectrophoresis, and the λ chains accounted for all the protein according to quantitative gel diffusion tests. This protein peak was saved and used for the subsequent studies on L.P. λ chains reported herein.

The next DEAE column chromatograms performed on L.P. urine were of interest in that the second chromatogram had similar protein peaks in response

to buffer changes but intact λ chains were restricted to the final peak except for trace amounts in a small preceding peak (Fig. 20). An identical optical density elution pattern was obtained from the third chromatogram except no λ chains could be identified in any peak even though λ Bence-Jones protein was present in high concentration in the urine before lyophilization. The fourth chromatogram resulted in a protein elution pattern similar to the others but again no λ chain was detectable immunochemically in any of the peaks. This was interpreted as demonstrating marked lability of the λ-chain antigenic determinants in this subject's urine.

4. Discussion

Chromatography of L.P. urine has demonstrated an unusual pattern of L-chain elution with late emergence of a sharp λ peak. At the same time evidence was obtained that L.P. λ chains are highly labile and may lose precipitating λ-chain antigenic characteristics after mild preparative procedures. Re-study of the original urines and eluates revealed that in the later fractionations the antigenic determinants became denatured during the lyophilization procedures rather than while on the columns. In neither instance did the denaturation affect the elution pattern of the proteins. This lability is similar to that observed for whole YD molecules and suggests that labile λ chains may be responsible in part for the lability of YD. It also brings up the possibility that YD heavy chains may have a specific affinity for uniquely labile λ light chains.

C. Studies on the Lability of YD

1. Introduction

In view of the ease of fragmentation of YD during storage, upon repeated freezing and thawing, during lyophilization or during the mild separative procedures utilized for isolating the protein, an attempt was made to gain more insight into this behavior. It was found that not only did fragmentation occur which was evident on immunoelectrophoresis but also the apparent concentration of YD in the serum or chromatographic fractions was frequently found to decrease with storage and handling. In other words, there appeared to be deterioration or perhaps masking of δ-chain antigenic determinants much more readily than was the case with YG, YA or YM.

An important objective was to study immunoglobulin lability in terms of resistence to denaturation on exposure to controlled heat. An equally important aim was to learn what ranges of pH and molarity could be applied to solutions of YD without causing significant denaturation. The latter information was also helpful in choosing conditions for elution from immunosorbents used in the isolation of YD.

2. Materials and methods

(a) Denaturation on freezing and thawing. Sera from two subjects with D-myeloma were repeatedly frozen and thawed over a six month period during which times small aliquots were removed for routine studies. New

Pasteur pipettes were used to remove each aliquot in order to minimize the chance of problems resulting from an accidental lapse in glassware cleaning technique. Immunoglobulin concentrations were determined at the beginning and the end of the six month period.

(b) Fragmentation during ammonium sulfate precipitation. Sera from both D-myeloma subjects were fractionated by ammonium sulfate as previously described. Paired aliquots of the serum specimens were not fractionated. Prior to quantitation, all samples were dialyzed against 0.1 M barbital buffer, pH 8.5 and tested by immunoelectrophoresis under identical conditions. Evidence of molecular fragmentation was recorded and selected reactions were photographed.

(c) Heat stressing. Undiluted sera from two D-myeloma subjects and from two non-myeloma subjects were heated to various temperatures as were dilutions in 0.1 M phosphate buffer, pH 7.4 adjusted to contain approximately equivalent amounts of γD (0.05 to 0.10 mg/ml). One of the non-myeloma subjects was healthy and had a serum level of 0.35 mg/ml, the other had hepatic cirrhosis and a level of 0.90 mg/ml. Heating was accomplished by placing six 0.3 ml aliquots of each serum and serum dilution in a water bath at 52°C for 1 hour, after which one aliquot of each was removed. Another aliquot of each was removed at 4 hours. The remaining tubes were then heated to 56°C for 1 hour and 4 hours, after which additional aliquots of each preparation were removed. The final aliquots were heated to 60°C for four hours. A fourth control aliquot of each preparation was kept unheated at 25°C and was analyzed for immunoglobulin content along with the heated samples.

(d) Alterations in pH and molarity. Aliquots of 1/2 ml of serum from each of the two D-myeloma subjects and from the same two non-myeloma subjects as in (c) were placed in 1/4" diameter Visking dialysis tubing and dialyzed for 22 hours in 500 ml of buffer having the composition indicated in the table below. Buffers were changed at 16 hours and were kept in constant agitation by a magnetic stirrer

TABLE VII

Buffers evaluated for effect on YD integrity

Exper No.	Molarity of Added Salt	Added Salt	0.1 M buffer	Final pH
1	2.0	KI	phosphate	7.4
2	2.0	KI	Tris	9.0
3	2.0	KI	Tris	10.0
4	2.0	KI	Tris	11.0
5	2.0	KCl	Gly-HCl	4.5
6	2.0	KCl	Gly-HCl	3.0
7	-	-	Gly-HCl	2.2
8	8 M urea	-	phosphate	7.4
Control	-	-	phosphate	7.4

3. Results

(a) Spontaneous breakdown. The effect of storage and freeze-thawing on the apparent concentration of YD as determined by micro-Oudin analysis on sera before and after a 6-month observation period was as follows:

Serum	YG	YA	YM	YD
		mg%		
L.P. Initial	550	40	20	500
" Stored	500	38	17	100
J.M. Initial	500	25	5	660
" Stored	520	25	5	300

These results suggest the lability of YD under conditions as mild as the freezing and thawing and removal of aliquots from test tubes which occur with careful everyday laboratory activities.

An illustration of the splitting of YD precipitate arcs which often occurred following freezing and thawing of L.P. serum is shown in Fig. 21. An aliquot of J.M. serum exposed to the same degree of freezing and thawing showed no detectable fragmentation (Fig. 22).

(b) Ammonium sulfate. Fragmentation of a small to moderate proportion of the YD fractionated by ammonium sulfate precipitation at 35% and 45% saturation was evidenced by breaking of molecules into Fc and Fab-like fragments. Such fragmentation varied considerably from one ammonium sulfate precipitation to the next and the reasons for the variation were not evident. Sometimes as little as 10-15% of YD was

fragmented and at other times as much as 80-90% as judged by immuno-electrophoresis and by ultracentrifugation. Often there was some fragmentation upon ammonium sulfate precipitation and further fragmentation after DEAE-cellulose chromatography. Particularly striking examples of the fragmentation which occasionally occurred are shown for L.P. YD in Fig. 23 and for J.M. YD in Fig. 24.

(c) Heat. The effect of heat on the precipitability of YD in the 4 sera studied is indicated in Fig. 25. These values were averaged and compared in Fig. 26 to the simultaneous effect of heating on the other immunoglobulins. It is seen that early denaturation of YD occurred at 52°C with a progressive increase in the proportion denatured as temperatures were increased to 60°C. Of the four major immunoglobulins tested by this technique, YM was next to YD in lability, YG was least heat-labile and YA was intermediate between YM and YG. Serum from a subject with a high titer of reaginic antibodies was heated in the same manner and the effect on passive transfer tests of the P-K variety included for comparison although the Prausnitz-Kustner method of indicating YE lability is difficult to relate to the method of precipitability by specific antiserum.

(d) pH and molarity. The effect of variation in pH and molarity on the precipitability by specific antisera of YG, YA, YM and YD and on the P-K activity of reaginic serum is shown in Fig. 27. All sera were restored to pH 7.4 and 0.15 M saline-phosphate before testing. The diagram has been arranged to indicate increasing degrees of denaturation of YD.

It is seen that YD again is more labile than YG, YA or YM and is perhaps similar in lability to reaginic antibodies or YE, allowing for the difference in the P-K assay method used for reagins. It may be noted that YD alone appears to be labile in 2 M KI at pH 4.5. Eight molar urea in 0.1 M phosphate, pH 7.4, completely denatured YD but had no effect on YG. The effects on YA and YM of 8 M urea were not studied and therefore this solution is not included in the figure.

4. Discussion

The marked lability of YD is a finding not anticipated at the beginning of this study but one which has now been commented upon by workers in at least five separate communications (Heiner and Evans, 1967; Skvaril and Radl, 1967; Heiner, Saha and Rose, 1968; Johansson, 1968; Fahey et al, 1968). The current studies indicate that this lability involved both myeloma YD and non-myeloma YD. It obviously increases the difficulty of working with the small quantities of YD ordinarily available for study. It is perhaps possible, though unproved at present, that the very high catabolic rate of YD in the body is related to its lability in the test tube. Possible causes of the lability of YD include a molecular configuration making it particularly susceptible to attack by a serum proteolytic enzyme such as plasmin, or a configuration making it easy to unfold but difficult to refold. The former is perhaps suggested by Figure 24 wherein J.M. YD acquired a more anodic migration (smaller number of positive charges) after fragmentation. This could be explained on the basis of a loss of predominantly positively charged peptides or amino acids

as a result of enzymatic activity. Heat and acid lability would suggest that molecular folding and refolding may also be important.

D. Attempts to Isolate Serum YD from a Healthy Subject

1. Introduction

Preliminary attempts to isolate YD from non-myeloma sera in this laboratory by the methods outlined in section IV (a) were unsuccessful. This was thought to be due to the relatively small amounts of YD in non-myeloma sera, to the difficulty of separating YD from that proportion of serum YG which has similar charge and chromatographic characteristics, and to the lability of YD and resulting loss during fractionation. An illustration of the problem is provided by one serum which had YD in average normal amounts (0.05 mg/ml) and which was fractionated first by precipitation with 45% ammonium sulfate and thence by DEAE-cellulose chromatography to learn if the normal YD could be purified significantly by these two procedures alone. Most of the YD-containing fractions also contained two or three other immunoglobulins in greater amount and all fractions were shown to have more than 25 times as much YG and YD (Fig. 18).

Therefore, a different approach to the isolation of non-myeloma YD using immunosorbents was tried.

2. Materials and methods

(a) Preliminary immunosorbents. Since only small amounts of YD and anti-δ chain antiserum were available initially and large amounts of YG and anti-γ chain were on hand, the first immunosorbents prepared involved YG. These served as models to gain experience. In the first experiment, a highly purified preparation of normal human YG (obtained from the Protein Foundation, Jamaica Plain, Mass.) was copolymerized with ethylene maleic annhydride (EMA) by the method of Centeno and Sehon (1966), a procedure adapted from the original work of Levin et al (1964). This immunosorbent was used to selectively remove antibodies to YG from a rabbit antiserum containing antibodies to YG, YD and several other serum constituents. All antibodies directed to YG were selectively removed and immunoelectrophoretic studies indicated no significant alteration in the concentration of other antibodies. The procedure for preparing the YG immunosorbent was as follows:

Ethylene maleic anhydride copolymer resin D x 840 - 31 (Monsanto, Canada, Ltd.) was dissolved in acetone to make a 1% solution wt/vol. Ten ml of this solution was added dropwise to 50 ml of a solution of purified YG, 4 mg/ml in 0.1 M phosphate buffer, pH 5.5, and the reaction mixture was constantly agitated by a magnetic stirrer. The copolymerized YG separated as a flaky white precipitate which was washed in 0.1 M phosphate, pH 7.4 until the supernate had an optical density of less than 0.020. The precipitate was then washed with 2 M KCl, pH 3.0 and again with 0.1 M phosphate, pH 7.4

after which it was ready for use as an antigen immunosorbent for removal of antibodies to γG from rabbit antisera.

Next, rabbit antiserum to γG was fractionated by precipitation with 35% saturated ammonium sulfate to separate most of the rabbit antibodies in a somewhat purified form. The antibody globulins were then copolymerized with EMA by the technique mentioned above to make an antibody immunosorbent which was used in an attempt to remove γG from a chromatographic serum fraction containing equal parts of γG and myeloma γD. It was possible by means of micro-Ouchterlony analyses to show that γG indeed could be selectively removed from the reaction mixture (Fig. 28). However, very large quantities of anti-γ chain immunosorbent were needed to remove small amounts of γG in comparison with the relatively small amounts of γG immunosorbent which would effectively remove anti-γG from antisera. One contributing reason for this is probably that much of the protein copolymerized in the anti-γG immunosorbent was non-specific and only a fraction of the final product was specific antibody to γG. Also it is probable that some antibody combining sites were buried and no longer available for combination with antigen after the copolymerization procedure. When purified γG was used as an antigen immunosorbent, on the other hand, all protein employed for the immunosorbent contained antigenic sites, and perhaps more than two antigenic sites per original molecule were available for combination with antibody (Kabat, 1961), hence a greater immunosorbent efficiency.

In the preliminary experience with immunosorbents the preparations were made in conformance to the procedure outlined by Centeno and Sehon. The method of immunosorbent preparation was thereafter modified when the author learned about the publication of Avrameas and Ternynck (1967) describing the copolymerization of antigens and/or antibodies using ethyl chloroformate, a technique which held promise of being more easily performed.

(b) Copolymerization of a serum lacking in YD. In order to obtain insoluble serum proteins (except for YD) for use in absorbing antisera to provide anti-YD without soluble complexes, serum was obtained from a healthy subject (A.U.) known to have an isolated absence of YD. Twenty milliliters were dialyzed against 50 volumes of 0.2 M sodium acetate, pH 5.0, for 20 hours with one change of buffer. 0.6 ml of ethyl chloroformate (ecf), obtained from Eastman Kodak Co., Rochester, New York, was added to the dialyzed A.U. serum and slowly mixed with a magnetic stirrer. The pH was checked every 2 minutes and was readjusted to pH 5.0 by the addition of 1 N NaOH whenever the pH decreased to 4.5, keeping the pH between 4.5 and 5.0 throughout the procedure. After 20-30 minutes a heavy precipitate had formed and at 30 minutes 75 ml of 2 molar sodium acetate pH 5.0 was added to maintain the desired pH and allow the reaction to go to completion for another half hour. The precipitate was separated by centrifugation and was washed with 0.1 M sodium phosphate buffer, pH 7.4 until the O.D. of the supernate was 0.025 or less. This required

seven or eight washes using 5 - 10 volumes of buffer per wash. Determination of the protein content of the first supernatant indicated that less than 5% of the serum proteins had remained soluble.

The copolymer was washed twice with 2 M KI, pH 10, to remove any adsorbed soluble proteins and then brought back to pH 7.4 with 0.1 M phosphate. It was then allowed to sit for two days to allow any unreacted active groups to combine after which it was washed again with 0.1 M sodium phosphate pH 7.4 and was ready for use. It was called ecf-AU.

(c) Preparation of complex-free antiserum to YD. One half the washed ecf-AU was mixed with 20 ml of unabsorbed whole rabbit antiserum to YD. The antiserum used had large amounts of anti YD and small amounts of contaminating anti-YG and other antibodies. It could be completely absorbed with one twentieth volume or 1 ml of fluid serum AU. About 5 times this amount of ecf-AU was required for absorption but double the needed amount was used to be certain small amounts of undetected antibodies to serum proteins other than YD did not remain. The mixture was stirred at room temperature for one hour and left at 4°C overnight after which the supernate was removed following centrifugation at 5,000 rpm for one half hour. The supernate was tested by micro-Ouchterlony analysis and by immunoelectrophoresis to demonstrate the complete removal of antibodies to YG, k, λ, and other serum proteins. It was called complex-free anti-YD, since the absorbed antiserum contained no soluble antigen-antibody complexes.

(d) Copolymerization and testing of complex-free antiserum to YD. The above 20 ml of complex-free antiserum was copolymerized with 0.6 ml ecf, according to the technique described above, was washed with 0.1 M phosphate, pH 7.4 until the O.D. was 0.015 and was then treated successively with 2 M KL-tris, pH 11, 0.1 M phosphate, pH 7.4, 2 M KCl in 0.1 M glycine HCl, pH 4.0, and finally with three washes of 0.1 M phosphate, pH 7.4. The anti-YD immunosorbent was called ecf-anti-YD. It was tested for effectiveness by preparing a slurry of the entire precipitate made to 20 ml with 0.1 N phosphate, pH 7.4 and placing 1 ml of the slurry in a separate test tube. To this was added 1 ml of a L.P. myeloma serum fraction containing 0.03 mg/ml YG and 0.03 mg/ml YD. The mixture was inverted several times, centrifuged, and the supernate tested for its content of YG and YD. Since YD was removed and YG was not according to micro-Ouchterlony analyses the immunosorbent was considered ready for use.

(e) Removal of YD from normal serum. To ten ml of anti-YD slurry (known to be capable of removing at least 0.03 mg/ml or .3 mg of YD) were added 10 ml of K.S. serum containing 0.35 mg/ml YD or 3.5 mg YD. This was stirred slowly at room temperature for 1 hour, then overnight at 4°C. The immunosorbent was centrifuged to the bottom of the tube at 3,000 rpm for 1/2 hour and the supernate saved for testing. The YD concentration in the supernate was found to have decreased from 0.35 mg/ml to 0.02 mg/ml indicating most of it had been removed by the immunosorbent.

The YG concentration had decreased from 12 mg/ml to about 7.5 mg/ml which could be accounted for by dilution. The immunosorbent and adsorbed proteins were then washed with 0.1 M phosphate, pH 7.4, until the O.D. was under 0.020 and an attempt was made to elute the YD by stepwise addition of different buffers (see Results).

(f) Regeneration of immunosorbents. After exposing the immunosorbent-YD complex to the desired buffers and separating the eluted proteins by centrifugation it was possible to regenerate each immunosorbent. This was accomplished by successive double additions of 0.1 M glycine-HCl, pH 3.0 and 2 M KCl in 0.1 M tris, pH 11.0, followed by washes with 0.1 M phosphate, pH 7.4, until neutrality was reached. The immunosorbents could be kept frozen as a wet precipitate or lyophilized and most of their activity retained. However, treatment with glycine-HCl buffer at pH ranges below 3.0 caused permanent loss of combining activity of the antibody immunosorbents, the inactivation being complete when final pH was 2.2 or below.

3. Results

The definitive experiments partially confirmed the validity of this method for isolating non-myeloma YD from human serum. However, the success was associated with problems, not all of which were solved. The antigen immunosorbent (whole serum lacking in YD) was very effective since its capacity in removing all unwanted antibodies from rabbit antiserum to

YD was repeatedly demonstrated. It was also possible to copolymerize the resulting specific anti-YD without concern that unwanted antigen-antibody complexes might interfere with its specificity. It was demonstrated on several occasions using different immunosorbents prepared as described above that YD could be removed from normal serum. However, it was very difficult to prove that YD was the only protein removed from the serum. Table VIII illustrates the results of the experiment in which the specific anti-YD immunosorbent was charged with 10 ml of normal serum (containing 3.5 mg of YD) and was eluted with successive additions of two volumes each of three different buffers. The eluates obtained with each buffer were dialyzed against 0.1 M phosphate, pH 7.4 and then studied by the sensitive plexiglas micro-Ouchterlony technique to detect immunoglobulins.

TABLE VIII

Elution of YD from immunosorbent

Buffer added	Final pH of eluate	mg protein in eluate	mg native YD eluted	mg YG	other native proteins
(1) 2 M KCl, 0.05 M acetate, pH 5.5	6.1	.57	0*	0.2	0
(2) 2 M KCl, 0.1 M glycine-HCl, pH 4.0	4.5	4.6	0.48	0	0
(3) 0.1 M glycine-HCl, pH 2.2	2.5	2.1	0*	0	0

* YD is completely and irreversibly denatured at pH 2.2, therefore, it would not be detected if eluted.

It can be seen that some protein was removed from the immunosorbent with each buffer, most being released at pH 4.5. Only this eluate contained detectable native YD. However, each of the other two eluates inhibited the precipitation of YD by specific antiserum in micro-Ouchterlony slides suggesting that they may have contained small amounts of partially denatured YD. It is also evident from the above that about twice as much total protein was eluted from the immunosorbent (7.27 mg) as the amount of native YD in the serum to which it was exposed (3.5 mg). Thus, one of three possibilities probably occurred:

(1) The serum contained denatured YD which was not detected by the original YD quantitation but which retained sufficient antigenic identity to be removed from the serum by the immunosorbent, and subsequently from the immunosorbent by the solutions of high molarity.

(2) There was non-specific adsorption of serum proteins other than YD to the immunosorbent which were not removed by the washes but were by acid buffers. This possibility was investigated by micro-Ouchterlony testing of the eluates with full strength, 1:4 and 1:16 dilutions of two different antisera to whole human sera, and by searching for YG, YA or YM by plexiglas micro-Ouchterlony. No serum protein other than YD could be detected in eluate No. 2. The most likely proteins to be involved would be components of complement , many of which would not have been detected by the antisera used.

(3) A certain amount of the immunosorbent may have auto-digested and the shed protein fragments might have caused an increase in the optical density

of the eluates from which the total protein concentration was determined. It has been noted that the optical density of suspending buffers of certain carefully washed ecf-immunosorbents increase slightly on standing for several days at 4°C. This suggests the possibility that serum proteolytic enzymes (e.g. plasmin) may be active in immunosorbents prepared from whole serum, and perhaps may account for the excess protein eluted from the anti-γD immunosorbent.

Subsequent attempts were made to reproduce this exclusive elution of normal γD from the immunosorbent. Similar problems as well as others were encountered. In one experiment γD and γG were eluted in approximately equal amounts with both buffers employed with the elution scheme outlined above. In another an attempt was made to elute with 1.5 M KI, pH 7.5, followed by elution with 0.1 N NaOH in 2% Na_2CO_3, pH 12.2. The 1.5 M KI, pH 7.5 resulted in elution of 1.2 mg protein containing 0.06 mg of native γD, the pH 12.2 buffer released 0.4 mg of protein of which 0.08 mg was γG but no native γD. Collateral tests indicated that dialysis against pH 12.2 denatured all γD.

In addition to the above, an attempt was made to remove γD from normal serum using a different preparation of anti-δ chain immunosorbent employing a larger quantity and 2 M KI at pH 10 for elution. It was not possible to attain elution of γD free of γG but on several occasions they were eluted in approximately equal quantities, thus attaining a considerable degree of purification over what existed in whole serum.

4. Discussion

Ammonium sulfate precipitation and DEAE chromatography in themselves were not adequate for purification of normal γD. Perhaps with larger volumes and with the sequential use of recycling Sephadex G-200 gel filtration this goal could have been realized but the procedures would be lengthy and probably would have failed because of the losses which usually occur with γD.

The poor correlation of γD with reaginic activity in the serum studied and the better correlation of reagins with γE is in agreement with the findings of most other investigators.

The preliminary studies with γG and anti-γG immunosorbents indicated the feasibility of using copolymerized whole serum lacking in γD as an immunosorbent to prepare complex-free specific anti-γD. In addition, these experiments demonstrated that by using large amounts of anti-γ chain immunosorbent γG could be selectively removed from a mixture containing γG and γD in equal proportions, leaving much of the γD in solution. This in turn suggested the possibility of selectively removing γD from whole serum in one step using an anti-δ chain immunosorbent, hopefully permitting many of the usual steps needed to isolate normal γD to be by-passed. This however was only partially successful in that on only one occasion could γD be directly removed from normal serum using an anti-γD immunosorbent without the concomitant removal of γG. In addition there was real difficulty in eluting γD from immunosorbents without significant denaturation. Reference to Table VI

indicates that an average of 60% of YD molecules are denatured and are no longer precipitated by specific antiserum to YD after exposure to 2 M KCl at pH 4.5. Therefore, if one assumes this degree of denaturation as a result of the first two buffer elutions indicated in Table VIII, one would estimate that 1.20 mg of YD was eluted by this buffer which is believed to be approximately correct. In order to gain more insight into the nature of the 4.6 mg of protein or peptide eluted from the immunosorbent at pH 4.5, it was analyzed for amino acid content. The details of the technique were the same as those presented in a subsequent section. The results are presented in Table IX along with amino acid analyses on pooled normal YG and one D-myeloma protein. They suggest that some proteins or peptides other than YD or YG were released from the immunosorbent since the eluted protein contained more lysine, half cystines, and isoleucine than would be expected from immunoglobulins alone.

TABLE IX

Comparison of amino acid analyses of protein eluted from an anti-YD immunosorbent with normal YG and D-myeloma protein.

Amino Acid	Normal YG	Eluted "YD"	myeloma YD
lys	89	107	51
his	29	17	15
arg	44	45	58
asp	112	149	131
thr	109	141	155
ser	152	149	197
glu	136	175	195
pro	104	94	58
gly	100	119	133
ala	81	124	133
cys/2	26	32	24
val	126	127	131
met	19	15	20
ileu	38	60	40
leu	105	141	179
tyr	68	62	71
phe	51	65	77

It is quite possible that human complement was fixed by the rabbit antibody-immunosorbent-human YD complex and that both complement and YD was eluted. Such a possibility was not tested in this instance because of the small amount of YD available and the need to use it for other studies. However, it has been shown (Heiner, 1968) that complement does participate in immunosorbent antigen-antibody reactions and can be detected in concentrated eluates using specific antisera to C_3 and C_4.

It may be desirable in future attempts to isolate "normal" YD with immunosorbents to use purer fractions obtained by preliminary

fractional ammonium sulfate precipitation, DEAE-cellulose column chromatography, and G-200 Sephadex gel filtration.

E. Serum Levels of YD and other Immunoglobulins

1. Introduction

Immunoglobulin serum levels were discussed briefly in the historical section. Little has been published in regard to levels of YD in different age groups or the variations which may be encountered in specific diseases. Prior to the writer's present postgraduate studies and research he began a study of serum YD levels. The studies were completed during the current investigation and all are included for the sake of completeness since none have been previously published.

2. Materials and methods

Serum samples were collected from 19 pregnant women on the day of delivery, usually within a few minutes of delivery, and from the cord blood of the 19 infants. Samples were also obtained from 13 infants 6 - 16 weeks of age, from 9 infants 4 - 12 months of age and from 15 children 1 - 5 years of age in a well baby clinic. All were healthy. Serum was also obtained from 89 children 5 - 6 years of age during preschool physical examinations, from 25 healthy children 7 - 10 years of age and from 9 who were 11 - 15 years of age. Twenty healthy adults were similarly studied, comprising medical students, research laboratory workers, medical school faculty and secretaries.

Sera from subjects with a variety of diseases were also studied. Most were collected by, or sent to, the writer for studies of immunoglobulin levels or for specific immunologic tests. They were stored frozen at -20°C until used.

Levels were determined by the micro-Oudin technique in all instances except for levels of YD under 5 mg% or 0.05 mg/ml. These were determined by plexiglas micro-Ouchterlony which is more sensitive.

The mean immunoglobulin levels in each age group were used to construct graphs indicating the most likely mean values for all ages from birth to adults assuming an infinite sampling had been possible. These graphs then were used to determine normal values at any age. The ages of the subjects with each disease were averaged as were their immunoglobulin levels. The average of the measured values is compared with the expected value for that age derived from the appropriate graph and the normal is expressed in parentheses in the tables.

3. Results

(a) Mean serum immunoglobulin levels of healthy subjects are given in Table X and are graphed to produce the theoretical mean values for all ages in Figures 29-32. It will be seen that cord blood levels of YG exceeded those of the mother obtained at the time of birth. Umbilical cord blood levels of all other immunoglobulins are markedly reduced at birth, as is well known, and reflect the lack of their placental transmission.

TABLE X

Mean Serum and Salivary Ig's in Healthy Subjects

Age	No.	YG mg% Ser	YA mg% Ser	YM mg% Ser	YD mg% Ser
Maternal	19	1.41	227.3	98.7	16.4
Cord blood	19	1.38	0.3	9	0.1
6-16 wks	13	.66	17	32	0.2
4-12 mo	9	.61	45	46	0.7
1-4 yr	15	1.00	102	53	3.4
5-6 yr	89	1.37	146	67	8.3
7-10 yr	25	1.33	154	69	8.3
11-15 yr	9	1.22	202	71	8.2
Adult	20	1.40	235	80	8.0

It is quite clear that the placenta transmits YG from the mother to the infant, perhaps even against small concentration gradients, and it has been reported that this characteristic depends on the Fc fragment of the Y-heavy chain (Gitlin et al, 1965).

(b) Mean immunoglobulin levels in selected diseases are shown in Table XI. Although in this study a number of variations from the expected normal value of each immunoglobulin were observed, this investigation was concerned largely with variations in the observed levels of YD. Thus, YD levels were found to be considerably elevated in rheumatic fever in children, in cystic fibrosis, in the visceral larva migrans syndrome and in cirrhosis. They were mildly elevated in milk-induced gastrointestinal bleeding, in children with precipitins to antigens in cow's milk, in asthma and in recurrent or chronic otitis media. The high mean level in rheumatic fever was largely due to three children whose sera had levels of 75-125 mg%. More recently the sera of 20 adults with chronic rheumatic heart disease have been studied for their YD level and have been found to have an average value very close to that of healthy adults. Thus, the elevated levels in rheumatic fever were restricted to a few children in the writer's experience, and the significance of the finding is uncertain.

TABLE XI

Mean Serum Ig's in Selected Disease and Healthy ( ) Controls

Disease	No.	gm % YG	mg % YA	mg % YM	mg % YD
Celiac disease	44	1.32 (1.15)	203 (148)	88 (56)	4.7 (6.5)
Pulm. hemosiderosis	13	1.53 (1.01)	161 (120)	82 (54)	7.4 (5.9)
Milk-induced gastro-intestinal bleeding	16	0.70 (0.65)	77 (56)	56 (53)	2.2 (1.0)
Pos. milk precipitins	74	1.20 (0.90)	106 (87)	66 (57)	5.0 (2.6)
Rheumatoid arthritis	10	2.13 (1.21)	302 (175)	96 (78)	5.6 (6.8)
Child. rheumatic fever	19	1.30 (1.15)	284 (203)	85 (61)	29.8 (6.2)
Cystic fibrosis	18	1.80 (1.17)	324 (148)	85 (71)	19.5 (5.5)
Asthma	37	1.10 (1.03)	137 (122)	71 (57)	8.1 (5.5)
Urticaria	15	1.30 (1.10)	210 (175)	95 (71)	5.5 (7.0)
Recurrent otitis media	27	0.94 (0.92)	105 (96)	65 (55)	4.8 (3.1)
Cirrhosis	18	2.57 (1.21)	880 (205)	308 (80)	20 (8)
Visceral larva migrans	5	2.02 (1.10)	121 (100)	131 (54)	11 (3)
Agammaglobulinemia*	10	0.15 (1.1)	2 (100)	18.7 (54)	1 (4)

* One subject had an elevated YM (165 mg%) and another had a normal level of YD (8 mg%).

Children with cystic fibrosis and adults with cirrhosis frequently have rather marked elevations of all immunoglobulins, including γD. To the writer's knowledge information regarding γD levels in these disorders has not yet appeared in the literature. Significantly low levels of γD were found in most subjects with agammaglobulinemia and in those with multiple myeloma other than D-myeloma. The latter patients appear to have low levels of γD whether or not the serum contains a γG or γA monoclonal "M" protein peak.

4. Discussion

At the present time the significance of a mild elevation or a depression in the serum γD level in a given person is quite uncertain since healthy individuals have levels which vary from undetectable (under 1 μg/ml) to 0.4 mg/ml or ten times the adult mean value. Such 400-fold normal variations are unique to γD. Nevertheless, some insight into the possible biological role of γD may be gained from studying levels in groups of subjects with the same diagnosis. γD levels are particularly valuable in identifying D-myeloma subjects among the myelomas not associated with significant elevations in γG, γA, γM or γE. In such patients a level of γD in excess of 1 mg/ml makes the diagnosis of D-myeloma very likely. Certainly D-myeloma must be strongly suspected in a subject with any provisional diagnosis if one finds a serum level in excess of 3 mg/ml (300 mg%).

F. Detection of YD in Body Fluids

1. Introduction

As pointed out in the historical section, YD was not identified in body fluids other than serum by Rowe et al (1968). However, the sensitivity of the technique used by these authors was such that it would not detect YD in similar proportional amounts to YG when each was related to the serum level. Since the functional role of YD is largely unknown, its presence or absence in body fluids becomes an important consideration.

2. Materials and methods

Sera, colostrum, breast milk, mixed saliva, stools, and urines were collected from normal individuals; gastric and duodenal juices were obtained from small children with iron deficiency anemia, and spinal fluids from divers patients receiving diagnostic lumbar punctures. One obviously cannot be certain that the gastric and duodenal fluids or the spinal fluids are representative of the normal situation. In addition, mixed saliva was obtained from the two subjects with D-myeloma as was urine from each. The latter was concentrated 200 fold whereas the saliva was tested without concentration. In all instances, the plexiglas micro-Ouchterlony technique was used and duplicate determinations were made.

3. Results

The findings are listed in Table XII. Only adult serum and one specimen of colostrum contained in excess of 2 mg% YD. Five sera contained 0.1 to 2.0 mg% YD. In each of the other fluids which contained YD, the amount present was between 0.1 - 2.0 mg% except for urine which had been concentrated 200 fold in each instance before this amount could be detected.

TABLE XII

Body Fluid Immunoglobulins (mg %)

	YG	YA	YM	YD	Number Tested	Number Pos. for YD *
Adult serum	1400	235	80	8	20	18
Cord blood serum	1400	0.5	9	0.06	19	3
Colostrum	500	1800	200	3	3	2
Breast milk	20	100	0	0.3	5	4
Adult saliva	3	8	0.5	0	8	1
Gastric juice	0.2	0.5	5	0.04	10	1
**Stool	0.3	6	2	0	3	0
Duodenal juice	not done				2	1
Urine	"				7	0
Spinal fluid	"				7	0
D-myeloma saliva	"				2	2
D-myeloma urine	"				2	2

* YD detectable in a concentration of 0.1 mg% or greater.

** Stools were diluted with equal parts of 0.1 M borate buffer.

4. Discussion

It is quite clear that YD is present in small amounts in some cord bloods, colostrum, breast milk, and mixed saliva in normal adults. It may also be found in gastric juice and duodenal juice in iron deficiency anemia, and appears to be readily detected in mixed saliva from subjects with D-myeloma as well as in their urine. The implication is that YD probably is present in many body fluids in amounts which may be proportional to the serum YD level. In other words, it seems to be present in a concentration of about 1% the serum level in many fluids which is very similar to the situation with YG.

If YD has an important functional role in bodily immune mechanisms, it is entirely possible that these body fluid concentrations of YD are of significance. Though low, they are in the range of concentration of YE in the sera of allergic individuals. The latter is believed to be of considerable physiologic importance with respect to hypersensitivity phenomena. Therefore, one should consider YD as also being a potentially functional protein in body fluids and should seek methods to elucidate whether or not this is so.

G. YD Antibody Activity Detected by Radioimmunodiffusion

1. Introduction

Since there had been no previous identification of antibody activity attributable to YD it was felt that a careful investigation into the possibility was in order. The first attempt in this regard was made by immunizing a healthy subject who had a relatively high level of YD (.35 mg/ml) with a variety of antigens, then selectively removing YD from his serum using a specific anti-δ chain immunosorbent prepared as described above to see if any reduction in antibody titre occurred. The results are indicated in Table XIII. It is evident that removal of YD with the immunosorbent did not lower antibody levels detectably in the YD-free serum. It should be pointed out, however, that since YD comprised only 2% of the immunoglobulins in the subject's serum, if only 2% of the antibody activity was removed this would not be detected. It can only be said that a majority of the antibodies were not of the YD variety.

TABLE XIII

Effect of Specific Removal of YD on Antibody Titres*

	Reciprocal titres		
	before immunization	after immunization	YD removed
Diphth. toxin hemagglutinins	0	1280	1280
Tetanus " "	0	0	0
Influenza A hemagglut. inhib.	128	64	32
Influenza B " "	8	32	32
S. typhosa "O" agglutinins	0	160	272
S. typhosa "H" "	0	40	136
Paratyphoid A "H" "	0	160	272
Paratyphoid B "H" "	0	40	136
Polio type 1, neutralizing Ab.	128	256	256
Polio type 2, " "	8	128	128
Polio type 3, " "	8	256	256

* Most determinations performed in hospital diagnostic laboratories.

The next procedure employed was the testing for antibody activity of a small amount of YD from the immunized subject after isolation and elution from an ecf anti-YD immunosorbent by the method previously described. The eluted YD was present in a concentration of 0.07 mg/ml or 7 mg% as measured by serial dilution in micro-Ouchterlony. This was equivalent to about 0.5% of the total immunoglobulin in the whole serum and if YD had a full proportionate share of hemagglutinating antibody activity for diphtheria toxin the solution should have caused hemagglutination in a titre of 1:6. However, no hemagglutinating activity was observed, suggesting either than YD did not contain anti-diphtheria hemagglutinating activity in significant amount, or that the

YD had been altered by the isolation procedure even though it was antigenically intact.

It was therefore considered necessary to employ a more sensitive technique for the study of the antigen-combining capacity of YD. Radioimmunodiffusion employing the micro-Ouchterlony technique was selected because of its sensitivity and the need for only small amounts of reagents.

2. Materials and methods

(a) Sera and antisera. Sera were obtained from 6 individuals who had recently been immunized with diphtheria and tetanus toxoids. These were analyzed by micro-Ouchterlony analysis to learn whether they contained sufficient YD to produce a good precipitate in gel. Sera from 4 of the subjects proved satisfactory in this regard. Later sera from 50 subjects who were known to have high levels of antibodies to one or more specific antigen were studied for evidence of antibodies of the YD variety. Antisera were produced locally and were the same as those used throughout the current investigation; the only one obtained elsewhere was antiserum to YE.

(b) Radiolabelling of antigens. The technique of Hunter and Greenwood (1962) was used for ^{125}I labelling of tetanus toxoid (Connaught Laboratories), diphtheria toxoid (University of Montreal), bovine serum albumin (Armour), bovine gamma globulin (Pentex), alpha casein

(T.L. McMeekin, USDA) and wheat α-gliadin (USDA). The essentials of the technique were the same for each protein and the labelling of diphtheria toxoid will be described as an example. The toxoid was concentrated by pervaporation and then dialyzed against two changes of 0.2 M sodium phosphate buffer, pH 7.5. The final protein concentration was 3.0 mg/ml. Fifteen microliters of toxoid (approximately 45 ug) were added to 2.38 mC ^{125}I as NaI dissolved in 20 microliters distilled water to which 25 microliters of 0.5 M sodium phosphate, pH 7.5, had been added.

To this mixture was added 25 microliters of chloramine-T at a concentration of 4 mg/ml. The mixture was agitated for 30 seconds and the reaction stopped by adding 100 microliters of sodium metabisulfite (2.5 mg/ml) after which 200 microliters of potassium iodide (10 mg/ml) were added. The mixture was then passed through a 1 x 20 cm column of Sephadex G-25 which had been pretreated with 0.5 ml of 20% human serum albumin (HSA) and washed with 15 ml of 0.1 M phosphate buffer, pH 7.5. Eluates of the labelled mixture were collected in 3 ml aliquots in test tubes containing 0.3 ml 20% HSA and were counted in a Packard automatic well-type gamma counter. The counts emerging in the protein peak (1,537,353) divided by the counts in all tubes (1,688,472) gave the fraction of ^{125}I bound to protein. This proved to be 0.91 or 91%. The specific activity of the labelled toxoid was therefore approximately .91 x 2.38 / 45 = 0.048 millicuries or 48 microcuries/microgram of protein.

(c) Radioimmunodiffusion. Either the micro-Ouchterlony or the plexiglas micro-Ouchterlony technique was employed depending on the serum concentration of YD. D-myeloma protein in a concentration of .1 mg/ml was placed in the central reservoir and specific anti-YD rabbit serum was placed in reservoirs 2 and 5 for the micro-Ouchterlony slides. Sera to be tested were placed in reservoirs 3, 4 and 6. Diffusion was allowed to proceed overnight after which the slides were washed in 0.1 M borate buffer with constant agitation and daily buffer changes for 3 days to remove unprecipitated protein. Then ^{125}I diphtheria toxoid was placed in reservoirs 2 and 5 and allowed to diffuse overnight. Following this, the slides were washed in borate buffer for an additional 3 days, dried, stained with thiazine red-R, dried again and radioautographed for 4 - 8 days by direct application of the dried gel surface to Kodak type RB-2 commercial X-ray film. The films were developed by hand using commercial developer and fixer according to standard procedures.

None of the four immunized subjects showed YD toxoid-binding activity by this technique whereas all four showed good YG toxoid-binding when tested by the same technique using a 1:100 serum dilution resulting in a YG concentration of approximately .1 mg/ml (similar to the YD concentration in the undiluted sera).

Therefore, the following modifications were made. The subject with the highest YD was given a second booster of diphtheria and tetanus toxoids (six months after initial booster). This time he had considerably more swelling and tenderness than before at the site of the diphtheria

toxoid injection as well as malaise and mild fever for 48 hours. There was much less tenderness at the site of the tetanus toxoid injection. This subject had received both diphtheria and tetanus shots and boosters as a child, and he recalls having a sore arm with associated fever following a previous diphtheria toxoid injection 15 years earlier.

3. Results

Several radioimmunodiffusion studies on the serum of the subject with the high YD who was given two diphtheria toxoid boosters were negative for YD antibodies except that one radioautograph which was allowed to develop for two weeks showed faint labelling of the YD after the second booster. Since there was no labelling of the preimmunization YD or of the myeloma YD on the same slide, the findings appeared to be significant. Therefore, the concentrations of each of the reactants was increased in an attempt to attain more clear cut results. This was done by precipitating the anti-YD serum and the subject's sera separately with 45% ammonium sulfate and concentrating each 10 times in the process. The concentrated fractions were then dialyzed and used in micro-Ouchterlony radioimmunodiffusion. The concentration of D-myeloma protein placed in the central reservoir was also increased to 1 mg/ml resulting in a precipitate line containing roughly 10 times more YD than on ordinary micro-Ouchterlony slides. Following these procedures, specific diphtheria toxoid binding was clearly evident in the post-immunization serum (Fig. 33). A lesser

degree of toxoid binding was evident in the pre-immunization YD but little if any was bound by the myeloma YD. Similar analyses were repeated a total of four times and the duration of washings was increased to 5 days to ensure thorough elution of all other immunoglobulins prior to application of the labelled toxoid. Each time the same results were obtained.

The five other antigens were then employed individually in a search for YD-binding in each sera. None was found even in the subjects who were recently immunized and in whom YG and YA antibody activity was readily demonstrable. Perhaps the lack of demonstrable YD antibodies in these instances was a reflection of a relatively mild local reaction to the immunization since in no instance was local swelling or a systemic reaction obtained approaching that which occurred following administration of diphtheria toxoid in the subject who developed YD antibody.

The fifty sera finally selected for study because they had previously been shown to have antibodies to one or more of the labelled antigens were then tested for YD antibodies by radioimmunodiffusion. A single unconcentrated serum had YD binding of bovine gamma globulin. This is shown in Fig. 34. This subject had chronic respiratory disease, clinical sensitivity to cow's milk, and multiple precipitins to cow's milk proteins in high titre, a syndrome previously reported (Heiner et al, 1962). Binding of BGG by the YD of this serum was repeatedly demonstrable.

4. Discussion

It appears that under some circumstances, YD may have antigen-binding capability. The observation that YD bound diphtheria toxoid only after a second booster injection which was associated with a moderate to marked local reaction and systemic symptoms suggests that a vigorous immune response is required to elicit certain YD antibodies. The same conclusion may be drawn from the YD binding of BGG by the subject who was immunologically hyper-reactive to a number of constituents in cow's milk. The latter subject also demonstrates that YD antibody activity occurs at least occasionally as a result of natural exposure to foreign antigens in hypersensitive subjects and that repeated parenteral injections are not necessarily required. Indeed, it is believed possible that YD may more frequently play a role in immune responses than these experiments would suggest. This might be so if YD antibodies ordinarily had an exceedingly low affinity for antigens so that procedures such as washing of precipitates in gel could lead to dissociation of the antigen-antibody complexes and hence prevent their recognition. If this occurred, one might also expect the antigen-binding avidity of YD antibodies to increase with repeated immunization as occurs with other immunoglobulins. Perhaps a similar increase in avidity occurs in certain spontaneous hyperimmune states. Although this possibility of a particularly low avidity of YD antibodies might permit a reasonable explanation for the difficulty usually encountered in

demonstrating YD antibody activity, no direct evidence has been brought to bear on this question during the current investigations.

H. Detection of YD Antinuclear Antibodies in Subjects with Disseminated Lupus Erythematosis (DLE)

1. Introduction

After the demonstration of antigen-binding activity in two sera as described above, a joint effort was made with Drs. I. Watson and A. Bootello in this laboratory to search for YD antinuclear antibodies by indirect immunofluorescence. This was considered a reasonable avenue for exploration since antibodies detected by this technique may be readily grouped according to immunoglobulin class, the technique is sensitive to small quantities of antibody, and a rather large number of human sera were available which had previously been shown to have high levels of YG and YM antinuclear antibodies.

2. Materials and methods

The indirect immunofluorescent technique described by Coons (1954) was used. Frozen sections were made of rat liver slices. These were fixed in absolute methanol, then flooded with a drop of serum from a subject with DLE. The non-reacting serum proteins were removed by four washes in buffered saline and each section was then flooded with specific rabbit anti-δ chain serum which had been absorbed with a

three-fold excess of a normal serum lacking in YD. The sections were then washed with four changes of buffer and fluoresceinated goat anti-rabbit YG was applied. The goat antiserum to rabbit YG had been absorbed with one-fourth volume of normal human serum to remove any possible cross-reacting antibodies which could have reacted with human YG attached to the liver cells to cause false positive reactions. This ensured that the fluoresceinated goat antibodies reacted only with rabbit YG which in turn was specific for human YD attached to the liver nuclei. Following application of the final layer, the sections were passed through another series of washes, were mounted in glycerin, and a coverslip was affixed prior to examination under the fluorescent microscope. The experiments and controls were arranged as follows:

	Antigen	First layer	Second layer	Third layer
Experimental	Rat liver nuclei	DLE serum	Rabbit anti-δ human chain	Fluoresceinated goat anti-rabbit YG
Control A	"	normal human serum	"	"
Control B	"	DLE serum	normal rabbit serum	"

3. Results

Forty sera from twenty-five patients with disseminated lupus erythematosus of varying disease activity were examined. Eighteen showed

some degree of YD anti-nuclear activity. In general the staining was weaker, was detected in lower titres and assumed a more homogeneous pattern than was the case for YG or YM antinuclear antibodies from the same serum. The same was true even when a higher titre of rabbit anti-YD serum than anti-YG or anti-YM was used. Normal human sera had no YD antinuclear activity and each of the above controls was consistently negative. An example of YD antinuclear antibodies is shown in Figure 35.

4. Discussion

The demonstration of YD antinuclear antibodies in DLE provides evidence of the relative frequency of YD antigen-binding activity. Again the findings suggest low antibody levels or perhaps weak binding to the nuclear antigens. High titres of YG or YM antinuclear antibodies do not appear to interfere with YD nuclear binding. They are in fact independent of each other but are frequently found in association one with another. It is possible that the YD antibodies are directed towards different nuclear antigenic determinants than are the YG antibodies. In fact, the homogeneous pattern of YD antibodies even when YG antibodies assumed a predominantly peripheral pattern suggests that this may be so.

I. Detection of YD Determinants on Lymphocytes

1. Introduction

The work of Sell and colleagues demonstrated that antisera to specific rabbit immunoglobulin classes caused blastogenic transformation of rabbit lymphocyte cultures (Sell and Asofsky, 1968). Since antiserum to specific rabbit YG allotypes would stimulate the transformation of lymphocytes from rabbits of the appropriate corresponding allotype but not from rabbits of other allotypes a high degree of specificity was evident. The proposal was made that there were probably immunoglobulin-like receptors on the lymphocyte surface which contained the allotypic determinants of the rabbits immunoglobulin heavy chain, and that perhaps the receptors consisted of immunoglobulin molecules themselves. This brought up the question of whether or not human lymphocytes had YD antigenic determinants on their surfaces, and if they did, were they present on only a small fraction of the lymphocytes or on a majority as Sell suggested was the case for each of the immunoglobulins in rabbits? With these questions in mind the following studies were undertaken in cooperation with Drs. Charles Naspitz and Maxwell Richter of this laboratory (1968a).

2. Materials and Methods

Peripheral white cells were harvested from healthy volunteers by collecting whole blood in heparin and allowing it to sediment by gravity for 45 minutes. The leucocyte-rich plasma was removed and the

lymphocytes counted in a hemocytometer. Four million lymphocytes were placed in each culture tube with 4 ml of Eagle's medium 199 containing 10% autologous serum. Penicillin 100 units per ml and streptomycin 10 ug per ml were added as were 0.1 ml of either rabbit antiserum to specific immunoglobulin or rabbit antiserum to human albumin as a control. Appropriate tubes were incubated without antiserum but instead received phytohemagglutinin (PHA) on day 4 of incubation.

Two microcuries of tritiated thymidine and 1 ugm carrier thymidine were added to each tube at day 6 of the incubation. Twenty-four hours later the tubes were centrifuged for 10 - 15 minutes at 1000 rpm, the supernatants discarded and the cell button suspended in 2 ml of 5% trichloracetic acid. A cell button was again obtained by centrifugation and was resuspended in trichloracetic acid. One-half ml of Hyamine (Packard Inst. Co.) was then added to each tube and allowed to digest for 24 hours in the dark. The contents of each tube were then transferred to counting vials, scintillation solution was added, and the vials were analyzed for radioactivity in a Packard Model 4000 Scintillation Counter. The results were expressed as counts per minute for each tube. All determinations were done in duplicate and the mean value recorded. Results were discarded if the duplicate determinations varied more than 20% from one another.

The antisera to human immunoglobulins were prepared as described previously but special absorptions were carried out in the case of the antiserum to YD. The aliquot of anti-YD which was unabsorbed contained

antibodies primarily to YD but also antibodies to YG as judged by immunoelectrophoresis and trace amounts of antibodies to two non-immunoglobulin serum proteins. Aliquots of this serum were mixed with increasing amounts of a normal human serum lacking in YD, the precipitates were removed by centrifugation, and the aliquots tested for antibody activity by both immunoelectrophoresis and Ouchterlony analysis and for the presence of YG and YD in antigen excess by Ouchterlony analyses.

Antisera were then selected so that one contained antiserum to YD only, the anti-YG having been absorbed out at equivalence (no free YG or anti-YG was detectable), and there were no antibodies to other serum proteins. This was called anti-YD abs. Eq. Another aliquot was absorbed with twice as much of the serum lacking YD and this was found to have trace amounts of free YG but a high titre of antibodies remained to YD heavy chain. It was called anti-YD abs 2X. A third aliquot was absorbed with an excess of D-myeloma serum in such a way that slight amounts of free YD and YG could be detected in antigen excess and no free anti-YD or anti-YG was present. This antiserum was completely inactive in regard to precipitating antibody and was called anti-YD inactive.

3. Results

A total of six separate experiments were performed in which peripheral leukocytes from 3 to 10 donors were used and a variety of antisera added to the culture tubes. The earlier experiments indicated

that anti-YD was stimulatory to the cultures in similar or greater degree than antisera to YG, YA, or YM. There was variation in the efficiency of antisera to a given immunoglobulin obtained from different rabbits. Thus one antiserum to YG consistently caused greater blastogenesis than a second anti-YG serum even though precipitating antibodies to YG were present in greater amounts in the second antiserum. Similar individual variations in antisera were noted for each of the four major immunoglobulins, and furthermore, antisera obtained one or two months apart from the same rabbit frequently varied in blastogenic capability. Therefore, antiserum pools were prepared and the most active used throughout these experiments. A typical experiment is shown in Table XIV. Since preliminary experiments demonstrated that antigen-antibody complexes in themselves could stimulate blastogenesis, antisera to YG, YA and YM myeloma proteins were selected which contained no antibodies to other immunoglobulin heavy chains without absorption. They contained small amounts of antibody to immunoglobulin light chains but to no other serum proteins. They were used unabsorbed to avoid the complexity which arises from the almost inevitable presence of antigen-antibody complexes in absorbed antisera.

TABLE XIV

Leucocyte response of five subjects to various rabbit antisera

(Uptake of tritiated thymidine in counts per minute per tube)

Additive	Five healthy leucocyte donors					Mean CPM
	M	B	Y	R	S	
none (control 1)	6,000	3,300	2,400	1,600	3,000	3,300
anti HSA (cont. 2)	6,200	2,000	1,500	1,500	3,100	2,900
PHA	160,000	138,000	95,000	145,000	180,000	148,000
anti YG	24,000	12,000	9,000	9,800	36,000	18,200
anti YA	6,000	3,500	4,000	2,400	8,000	4,800
anti YM	28,000	18,900	11,000	6,500	19,000	14,700
anti YD abs Eq	38,000	11,000	28,000	24,000	29,000	26,000
anti YD unabs	27,000	19,800	8,000	10,000	34,000	19,800
anti YD abs 2x Eq	11,000	12,400	12,000	21,000	16,000	14,500
anti YD inactive	13,000	7,600	12,000	10,000	11,000	10,700

It can be seen from the results that there was moderate stimulation from the antisera to YG and YM but less to YA. Aside from the response to PHA, the greatest stimulation in this experiment was produced by the antiserum to YD which was absorbed at equivalence. Since the anti-YD unabsorbed contained just as much anti-YD activity, it is possible that the greater effect of the absorbed antiserum represented the combined effect of anti-YD plus the effect of complexes still in solution but not detectable by immunodiffusion. Significant stimulatory activity persisted in the anti-YD which had been completely absorbed in antigen excess so that no free antibody to YD or other serum proteins was present. This is a further indication of the lymphocyte stimulatory effect of antigen-antibody complexes in antigen excess.

4. Discussion

The above results demonstrate that antisera to YD may stimulate blastogenesis of human leucocytes either when unabsorbed or when absorbed at equivalence for YG so that only free anti-YD (anti-δ chain) activity was present but minimal quantities of antigen-antibody complexes were also in solution. With further absorption producing YG anti-YG complexes in antigen excess, there was less stimulatory activity. Presumably the presence of certain complexes can decrease the stimulatory effect of anti-YD. On the other hand, when the antiserum was completely absorbed with D-myeloma serum in antigen excess for all proteins so that no precipitating antibody to any serum constituent remained, there still was persistent blastogenic activity. This indicated that antigen-antibody complexes in antigen excess may in themselves be blastogenic. The ratio of each antigen and antibody may be important, and perhaps the effects of some complexes are inhibitory and cancel out the effects of others. Therefore, one must be particularly cautious in interpreting the results of experiments of this nature when absorbed antisera are used to stimulate lymphocyte blastogenesis. The experiments did not answer the question of what proportion of the original lymphocytes carried YD-like receptors on their cell surface since the cultures were grown for seven days and there is no way of knowing how many cell divisions had taken place. There are no known allotypic variants of YD and no information supporting YD allotypy

was obtained during these studies. The fact that antiserum to a single D-myeloma protein was found to cause blastogenesis of the lymphocytes of a total of 10 different donors suggests that the antiserum was not specific to a particular allotype.

J. Physicochemical Studies

1. Introduction

Since there was very little information available in the literature concerning the physicochemical nature of γD it was felt that studies to elucidate this aspect of γD were necessary. These studies were done in collaboration with Dr. Anil Saha. Because there were relatively small quantities of purified D-myeloma available for study, every attempt was made to modify analytical procedures so they could be done with minimal material without sacrificing accuracy.

In all instances γD was purified by fractional ammonium sulfate precipitation, using the 35-45% fraction for subsequent DEAE-cellulose chromatography as described in section IV-A. Difficulty with loss of γD through fragmentation or unexplained denaturation precluded the additional use of G-200 for preparative purposes with the material at hand although this was attempted. The materials used for physicochemical studies were peak 4 of Fig. 14 (identical to a and c of Fig. 15), and peak 2 of Fig. 16. In each instance γD, γG, γA and γM were quantitated by the micro-Oudin

technique and γD was found to constitute 85-92% of the total immunoglobulins. In addition, a lack of other detectable proteins was demonstrated by immunoelectrophoresis.

In some of the later experiments, it was necessary to use γD which was largely fragmented and in all instances there was probably a degree of fragmentation of the molecule even though it was not always detectable by immunoelectrophoresis. In spite of this, it is believed that these physicochemical studies represent the most accurate picture of γD which could be attained at the time since some determinations were previously done on samples of the same D-myeloma protein fragmented to a lesser degree and a further check was available in the studies done on the second D-myeloma protein. In most instances, no information concerning a particular physicochemical property of γD was available in the literature to serve as a guide.

2. Materials and Methods

(a) Sedimentation velocity coefficient values were determined with a Beckman/Spinco Model E Analytical Ultracentrifuge with Schlieren optics, at 20°, and at a rotor speed of 56,000 rpm. Rotors were chilled to 20° before use. The sedimentation velocity coefficients were corrected for rotor temperature and stretching as well as for the viscosity and density of the solutions (Svedberg and Pedersen, 1940). Relative viscosity measurements were made on Cannon-Ubbelohde semi-micro dilution

viscometers (Kragh, 1961), 50 ml, at 20 $\pm$ 0.01°, with water flow time of 254 and 272 seconds. The solutions were filtered twice through Millipore filters of pore size 0.4 mμ before introduction into the viscometers. The concentration of protein used for the ultracentrifugal analysis and viscometry was determined by exhaustive dialysis of a concentrated protein solution against 0.2 M ammonium bicarbonate buffer, pH 8.6, followed by a determination of the weight obtained on drying an aliquot at 108° for 18 hr in a forced circulation oven. The density of solutions was determined with 5 ml capillary vent pycnometers. Solutions were kept dust-free by careful handling throughout and by repeated Millipore filtering.

Sedimentation velocity coefficient values were calculated according to the Svedberg equation,

$$s_{20,w} = \frac{1}{\omega^2} \frac{d \ln r}{dt} \frac{\eta_t}{\eta_{20,w}} \frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho)_{t,soln}}$$

where η is the viscosity of the solvent at the temperature of the experiment, ρ_{20} is the viscosity of water at 20°, $\bar{v}$ the partial specific volume of the solute, $\rho_{20,w}$ the density of the solution at 20° and the pressure of one atmosphere, r is the distance of the peak (the mid point) from the meniscus with respect to time, t, in seconds and ω the rotor speed in radians/sec. The term, $\frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho)_{t,sol'n}}$ was ignored because

of its insignificant contribution towards the S value of the globular proteins.

(b) Molecular weight determinations were made by sedimentation equilibrium using the Model E analytical ultracentrifuge fitted with an ultraviolet monochromator, an automatic double-beam photoelectric scanner, and multiple cell scanning accessories (Beckman Bull., 1967). Two rotors (AN-D, a 2-hole rotor, and AN-F, a 4-hole rotor) were used with double sector cells, 12 mm and 2.5° filled-Epon center pieces. In most instances the 4-hole rotor was used and three double sector cells were employed with one hole containing the special scanner counterbalance. The short liquid column method was used. A 0.115 ml volume of protein solution was layered over 0.05 ml of FC-43 fluorocarbon oil (Beckman Instruments Inc.) in each of two sectors and 0.2 ml of buffer in the fourth sector. Hamilton microliter syringes were used in each instance. Equilibrium was attained according to the method of Yphantis (1964) and the weight-average molecular weight calculated according to the equations of Svedberg and Pedersen (1940),

$$M_{w,app} = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \quad \frac{(C_b - C_m)}{C_o (r_b^2 - r_m^2)}$$

where R is the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume of the proteim, ρ the density of the solution, ω the angular velocity in radians/sec, and C the concentration of the protein solution evaluated at a radial distance, r.

The partial specific volume, $\bar{v}$, for 7G was determined to be 0.730. For IgA, IgM and IgD, $\bar{v}$ was assumed to be 0.72 - 0.71. The concentration of the protein solution, C_o, was determined at 280 mμ fifteen minutes after the rotor attained the speed of 9,000 rpm. An over-speed of 4,000 - 6,000 rpm above the actual operational rotor speed was occasionally used to facilitate early distribution of mass throughout the cell. The rotor speed was so chosen that the total time elapsing for the attainment of each equilibrium was approximately the same (15 hr). For each calibration step recorded by the scanner, a routine check was made to relate the step to the optical density of the protein solution as determined by a Cary double-beam Recording Spectrophotometer, Model 14. The operational parameters for equilibrium runs were as follows: operation - split beam; the optical slit width - 0.14 mm; mode- absorbance; optical density range - 1 O.D.; scanner recorder sensitivity - 5000 mv/cm; scan speed - medium (4.75 x 1); and scanner chart speed - 5 mm/sec.

(c) Amino acid analyses were performed on a Beckman Model 120 B Amino Acid Analyzer according to the method of Spackman, Stein and Moore (1958) using an accelerated procedure with a buffer flow rate of 70 ml/hr and a ninhydrin flow rate of 35 ml/hr at 55°. A short (0.9 x 5.0 cm) column for basic amino acids, and two long (0.9 x 58.0 cm) columns, for neutral and acidic amino acids, were used. These columns contained Beckman Custom Spherical Resin PA-35 and PA-28 (Beckman Instruments Inc.).

The analyzer was fitted with a high sensitivity cuvette, range expander and an automatic regeneration system. The recorder chart speed was 6 "/hr with dot printing every two seconds. Buffers contained n-propanol and benzyl alcohol as suggested by Hubbard (1965). Proteins were exhaustively dialyzed against 0.1 M ammonium bicarbonate buffer, pH 8.6, after which aliquots of 2-3 mg of protein were dried overnight at 108° in a forced circulation oven and weighed. One to two mg of dried protein was hydrolyzed in vacuo with glass distilled 6 M HCl at 108° for 22, 48 and 70 hours. Oxygen was removed from the tube by parching with special grade nitrogen previously flushed through pyrogallol solution. The hydrolysis loss was evaluated by extrapolating to 0 hours.

Total disulphide bonds were calculated according to Moore (1963) using performic acid oxidation.

Tryptophan was determined on a 0.9 x 16 cm column of Beckman resin PA-35 (7.5% cross-linked) after hydrolyzing 4-5 mg of γG or γA in 2 M $Ba(OH)_2$ for 70 hours at 108° according to Noltman et al (1962). Tryptophan and disulphide bond determinations were not done on γD due to a shortage of material

(d) Carbohydrate analyses

(ii) Total hexose (mannose plus galactose) was determined by the indole method of Dische and Popper (1926) with modifications proposed by Oettgen et al (1965) using preparations containing mannose to galactose

in a ratio of 5:3 as reference standards. This is the ratio of mannose: galactose found in γG by Clamp and Putnam (1964) and is similar to the ratios reported for γA and γM. When high mannose:galactose ratios (16:7 as has been reported for γA, or 2:1 as reported for γM) were used for the standards, the total hexose values became slightly greater but the relative hexose concentrations in different immunoglobulins remained unchanged, hence the 5:3 standard was used for all determinations. Carefully filtered 0.5 ml samples of purified immunoglobulins were thoroughly dialyzed against 100 volumes of 0.1 M tris-NaCl, pH 8.6 for 24 hours. Then 0.2 - 1.0 mg amounts of protein representing 10-80 ug of protein hexose were mixed with 4.5 ml of 75% sulphuric acid in an ice-water bath. To this was added 0.2 ml of a 1% solution of indole in absolute ethanol with thorough mixing. The mixtures were heated at 100° for 10 min, cooled in ice water, and optical densities read at 470 mμ. The optical densities were corrected for the color of blanks containing the protein and sulphuric acid but no indole. The hexose content of the protein was then calculated by reading from the standard curve obtained from different concentrations of the 5:3 mixture of pure mannose:galactose (Fig. 36). All glassware was washed with dust-free distilled water before use and all determinations were done in duplicate.

(ii) Fucose was measured by the cysteine hydrochloride method of Dische and Shettles (1958). One ml fucose standards containing from 2 to 20 ug/ml were prepared in dust-free distilled water. In one

experiment 1-4 mg quantities of filtered and dialyzed solutions of purified γG and γM immunoglobulins were used. In the other, known concentrations of purified γG, γA, γM and γD were added to 10.0 μg of fucose in amounts expected to increase the total fucose to 12-15 μg per ml according to published data for γG, γA and γM. The purpose of this was to minimize the loss of immunoglobulin fucose during handling and hence to maximize the reproducibility of the tests. The percent fucose in γD was assumed to be similar to that in γM (this was later confirmed experimentally). To these preparations were added 4.5 ml of ice-chilled concentrated H_2SO_4 mixed 6:1 with distilled water. The resulting mixtures were then warmed to room temperature before heating for precisely 3 minutes in a boiling water bath. They were then cooled and 0.1 M of 3% aqueous cysteine-HCl was added with thorough mixing. The samples were then stored in the dark for 1-2 hr and the optical densities read at 396 and 427 mμ. Both Tungsten and deuterium lamps were employed and the latter was found to give consistent results on repeated readings, hence if was used for all fucose determinations.

The corrected readings (O.D. 396 mμ - 427 mμ) of the pure fucose standards were used to construct a curve (Fig. 37) from which the fucose values of the protein or the fucose-protein solutions were read. In the analyses of the immunoglobulins to which 10.0 μg of pure fucose was added, this amount was subtracted from the final result to give the value of immunoglobulin fucose. There was good agreement between the values for γG done with and without added fucose as well as for γM. Thus added fucose was used for all γD determinations. All sets of

determinations were done in duplicate with close agreement between the duplicate samples.

(iii) Hexosamines (glucosamine and galactosamine) were determined after hydrolysis of protein samples in 4 M HCl for 4, 6 and 8 hr at 100° under nitrogen (Spiro, 1962). Amino sugars were separated and estimated on the amino acid analyzer using Beckman Custom Research Resin PA-35 (7.5% cross-linked) in a 0.9 x 16 cm column and a duplicate run on Beckman resin PA-28 in a 0.9 x 57 cm column using the usual amino acid analysis buffers containing n-propanol and benzyl alcohol. Extrapolated values to 0 hour took into account the loss of hexosamine due to hydrolysis.

(iv) Sialic acid was not determined for γD but the amount (18 residues per mole) recently reported by Spigelberg, Prahl and Gray (1969) has been assumed to be correct. This has been included with data on the other γD carbohydrates obtained in this laboratory in order to estimate the total carbohydrate content of γD. The method of Warren (1959) has been employed to determine the sialic acid content of a few myeloma proteins in this laboratory (as indicated in Table XVII) but at the time of writing of this thesis γD had not been analyzed.

3. Results

(a) The sedimentation velocity coefficients of the two D-myeloma proteins and the lambda Bence-Jones protein of L.P. were as follows:

J.M. D-myeloma protein: $S^{o}_{20,w} = 6.7$

L.P. D-myeloma protein: $S^{o}_{20,w} = 7.0$

L.P. Bence-Jones protein: $S^{o}_{20,w} = 4.1$

Schlieren patterns of whole L.P. D-myeloma serum (Fig. 38) were not abnormal. The ultracentrifugal analysis of 20-35% and 35-45% ammonium sulfate fractional precipitates (Fig. 39) were also unremarkable and failed to distinguish between γD and γG. However, the pattern of fraction 2 from the G-200 gel filtration of L.P. D-myeloma protein (Fig.12) showed a double peak corresponding to the γD and γG concentrations of the fraction (Fig. 40).

The sedimentation velocity coefficients of L.P. Bence-Jones protein at various protein concentrations are plotted in Fig. 41.

Sedimentation velocity coefficient values of γD proteins as reported by Fahey et al (1968) and as observed in our laboratory were higher than those of γG myeloma proteins which varied from 6.2 to 6.65. These observations prompted the use of comparative sedimentation equilibrium runs employing multiple cell scanning accessories.

(b). Sedimentation equilibrium analyses revealed that the cell meniscus clearance of γD was faster than that of γG for a given rotor speed and nearly equal initial protein concentrations. Fig. 42 illustrates the mass distribution of J.M. D-myeloma protein. The weight-average apparent

molecular weight of this γD was found to be 200,000 ± 2000. The partial specific volume, $\bar{v}$, was assumed to be 0.72, since the γD protein was found to contain a large amount of oligosaccharide moieties. Fig. 42 also demonstrates the plot ln c vs r^2, for γG, the theoretical mass distribution being obtained for γG on the basis of its mol. wt. as 150,000. The partial specific volume, $\bar{v}$, of γG myeloma proteins was determined as 0.73 in 0.1 M tris-HCl buffer, pH 8.6, at 20°. During sedimentation equilibrium runs, no molecular aggregation was observed at the cell bottom with or without FC-43 fluorocarbon oil. The theoretical slope difference between γD and γG reflected the ratio of their respective molecular weights.

During the characterization studies on γD, it was observed that storage at 3° often was accompanied by fragmentation of the native molecules. The presence of Fab and Fc-like fragments was reported by Fahey and Rowe (1965) and fragmentation during storage was reported by Skvaril and Radl (1966) and has also been observed in our laboratory (Fig. 21). It was considered of interest to assess the molecular weight of the fragments resulting from immunoelectrophoretically complete spontaneous degradation. The basic sedimentation equilibrium equation describes the mass distribution of a homogeneous component in the absence of self-association and interaction with others. This equation may be represented as follows:

$$\frac{d \ln c}{dr^2} = \frac{(1 - \bar{v}\rho)\omega^2}{2RT} \qquad M_{w,app} = A\,\omega^2 \; M_{w,app}$$

where $A = \frac{1 - \bar{v}\rho}{2RT}$. In a sectorial ultracentrifuge cell, where conservation of mass is assumed, the mass distribution of a component is related to $M_{w,app}$ for a suitable range of rotor speeds provided rigid experimental conditions (e.g. time for attaining equilibrium, temperature, density of the media, pH, etc.) are kept identical for a given $M_{w,app}$. The rotor speed has greater influence on the distribution of the heavier components than on the lighter ones and the greater the difference in the apparent molecular weights, the larger is the effect of rotor speed on the mass distribution of the heavier component. Comparative runs using different rotor speeds were carried out by using an An F rotor with multiplexer. Based on these considerations, we have tried to fit the theoretical plot of various assumed values of $M_{w,app}$ with the cell bottom part as the $\underline{\ln c \text{ vs } r^2}$ plot. The result of this determination has been represented in Fig. 43. The presence of intact 7D ($M_{w,app}$ = 200,000) could not be detected, although smaller fragments were present in the cell meniscus region. We have assumed $\bar{v}$ = 0.72 cc/g at 20° for our computation. The best fit of the theoretical slope was obtained with $M_{w,app}$ = 141,000 ± 2000 for fragmented J.M. 7D. A similar approach was also made for the evaluation of the average molecular weight of the smaller fragments. Fig. 44 shows the sedimentation equilibrium plot of the smaller fragments obtained from J.M. 7D. The best fit was obtained with an apparent molecular weight of 65,000 ± 2000. A similar study with L.P. 7D showed a best fit with apparent molecular weight of 60,000, again assuming $\bar{v}$ = 0.72 cc/g.

Thus the native molecules and split products from spontaneous degradation as identified by both immunoelectrophoresis and ultracentrifugation appear to be as follows:

Fragment	Molecular Weight
J.M. whole D-myeloma protein	200,000
L.P. whole D-myeloma protein	not determined
L.P. lambda light chain dimer	48,000
J.M. $F(ab)_2$ (?)	141,000
L.P. $F(ab)_2$ (?)	140,000
J.M. Fab and Fc	65,000
L.P. Fab and Fc	60,000

(c) The amino acid analyses of the two D-myeloma proteins are given in Table XV along with the representative amino acid analyses of other monoclonal immunoglobulins done by the same technique in this laboratory. The number of amino acid residues per mole of proteins were computed with consideration of their respective oligosaccharide content. Differences between γG and γA are most notable in regard to lysine, serine, valine, and leucine residues. γG contains more lysine, valine, and serine residues than γA but less leucine. γA molecules contain fewer total amino acid residues than γG since a larger part of the γA molecule represents oligosaccharide moieties. γD molecules contain fewer residues of lysine and more acidic amino acid residues (aspartic and glutamic acid). Leucine

content is quite high in γD and the proline content is unusually low in comparison with the other immunoglobulins. The two D-myeloma proteins differed considerably one from another in regard to several amino acid residues, i.e. aspartic and glutamic acids, serine, glycine, saline, valine and leucine.

TABLE XV

Amino Acid Composition of γG, γA, and γD

	γG		γA			γD	
	I	II	I	II	III	J.M.	L.P.
Lys	86	75	53	48	59	48	44
His	23	19	24	22	22	15	23
Arg	45	38	54	56	41	55	53
Asp	111	93	81	88	92	123	170
Thr	103	115	113	108	120	146	142
Ser	156	152	137	135	138	186	166
Glu	122	141	120	117	118	205	162
Pro	103	104	99	101	104	55	62
Gly	96	93	91	90	91	125	144
Ala	81	82	81	89	82	125	144
Cys/2	24	26	24	24	24	24	24
Val	128	127	87	94	84	123	133
Met	12	16	14	12	17	20	20
Ile	26	28	35	38	32	38	28
Leu	99	93	113	115	106	169	162
Tyr	63	49	50	41	40	67	75
Phe	43	52	43	49	41	73	65
Trp	20	22	22	18			
mol. wt.	150,000	150,000	150,000	150,000	150,000	200,000	200,000
carbohydrate mol. wt.	4,635	4,746	12,810	12,215	15,000	28,000	28,000

The amino acid analysis of L.P. Bence-Jones protein is compared with similar analyses done in this laboratory on five other lambda Bence-Jones proteins in the following table.

TABLE XVI

Amino Acid Composition of λ-Bence Jones Proteins

	L.P.	A	B	C	D	E
Lys	11	13	12	14	11	11
His	3	3	3	4	4	4
Arg	6	6	9	4	7	8
Asp	17	14	18	17	15	13
Thr	25	18	19	22	19	19
Ser	31	25	25	27	28	32
Glu	24	20	21	20	22	19
Pro	16	19	16	15	14	16
Gly	16	20	16	17	17	17
Ala	19	20	16	17	20	18
Cys/2	3	2	2	2	5	5
Val	17	17	17	17	15	17
Met	3	0	0	0	0	0
Ileu	5	5	5	6	5	4
Leu	15	15	15	14	15	15
Tyr	12	7	12	12	9	10
Phe	6	6	5	6	4	4

There is little which is unusual about the content of individual amino acids except for methionine which was not found in any of the other lambda proteins. The proline was not unusual, which contrasts with the low proline in the whole molecule.

(d) The carbohydrate analyses of the two D-myeloma proteins and other selected purified monoclonal immunoglobulins are given in Table XVII in order of decreasing oligosaccharide content. It may be seen that there is considerable variation between individual myeloma proteins, but γD must be classified as a high carbohydrate glycoprotein. The total hexose (mannose plus galactose) in γD was similar to that found in γE and γM as was the fucose content. However, the glucosamine plus galactoseamine content (or total hexoseamine) was considerably higher in the two D-myeloma proteins than in the other immunoglobulins.

Similar carbohydrate analyses were carried out on ten Bence-Jones proteins of the lambda variety and some carbohydrates were found in five. These are also summarized in the table. L.P. Bence-Jones protein was unique in its high carbohydrate content, and particularly in its hexose content. This amount of carbohydrate has not been found in any of the other Bence-Jones proteins analyzed in this laboratory.

TABLE XVII

Molar Ratio of Oligosaccharides to Immunoglobulins

		Mol. Wt.	Mannose + Galactose	Fucose	Glucose-amine	Galactose-amine	Sialic Acid
γD	J.M.	200,000	66.5	8.55	56	9.42	18 *
	L.P.	200,000	56.4	8.27	30	20	18 *
γE**		196,000	60.0	6.17	← 39.8 →		
$(\gamma M)_s$		180,000	63.4	7.89	30.5	2.43	-
		180,000	62.6	-	28.5	3.65	-
γA		150,000	26.6	2.56	12.6	12.0	10.36
		150,000	26.5	3.19	14.8	8.1	-
		150,000	25.8	2.20	15.3	7.8	11.15
γG		150,000	12.6	2.38	7.53	0.15	-
		150,000	12.4	2.36	7.5	0.0	2.23
		150,000	9.9	1.65	9.5	1.0	1.80
Bence-Jones Protein	L.P.	50,000	12.76	1.66	1.5	5.6	-
Others	I	50,000	0	0	0.34	0.34	-
	II	50,000	0	0.36	1.5	2.4	-
	III	50,000	2.5	0.91	1.0	1.0	-
	IV	50,000	0	0	1.0	1.0	-

* from Spiegelberger et al (1969)

** from Bennich & Johansson

4. Discussion

It is apparent that the monomer γD molecule is relatively large in comparison with other known four-chain immunoglobulin monomers. It appears to be approximately the same size as γE but to have an even higher carbohydrate content. Of particular interest is the close parallel which may be observed between the carbohydrate content and ease of denaturation of immunoglobulins under the stress of heat or variations of pH and molarity. γD is not only the most easily denatured of the known immunoglobulins, but is also the most susceptible to spontaneous fragmentation during storage and in association with mild separative procedures. Perhaps a large number of attached carbohydrate units make it difficult for an unfolded immunoglobulin molecule to refold to its native state once extended by thermal or electrostatic forces. If so, this could explain the ready loss of precipitating antigenicity of γD. It will not explain the ease of fragmentation of the γD molecule although perhaps there may be a reason for the association of these two features which is not readily apparent. The spontaneous fragmentation into Fc and Fab-like subunits suggests the existence of an area in the molecule which is particularly susceptible to the action of plasma enzymes such as plasmin, or of an area, linked by non-covalent forces which can be ruptured under conditions which do not ordinarily lead to breakage of immunoglobulin polypeptide chains.

A physicochemical feature of interest is the relatively low proline content of γD. Since proline causes an interruption of the α helix sequence in polypeptide chains it is possible that a low proline content might contribute to an altered molecular flexibility and to a reduced tendency to re-assume a globular shape after stress than is shared by the immunoglobulins, which have higher proline contents. It is also possible that the high carbohydrate content, the low proline content, and/or the marked lability of γD may contribute to the apparent low degree of antibody activity associated with this immunoglobulin, but more evidence is needed to demonstrate this in a convincing manner.

N.B. It should be added that during the April 14-18, 1969 American Association of Immunologists meetings in Atlantic City two papers were presented dealing with γD (Speigelberg, Prahl and Grey, 1969; Heiner, Saha and Rose, 1969). The authors of the first paper informed this writer that they had found that the addition of ε - amino caproic acid to serum and serum fractions containing γD, and to the buffers used in their separation will prevent much of the enzymatic splitting of γD which plagues workers in the field. This splitting is presumably due to plasminogen activation, and resultant action of plasmin on γD, a process specifically inhibited by ε-amino-caproic acid. Therefore, future efforts to isolate and work with γD should take this into account.

V. SUMMARY

The recently discovered and relatively unstudied immunoglobulin, γD, has been investigated to elucidate more of its biological, immunochemical and physicochemical nature. A method was developed for measuring concentrations of γD as low as 1 μg/ml. The mean level was found to be minimal (1.0 μg/ml) in umbilical cord sera wherein 7 of 20 healthy newborns examined had detectable γD (range 1-2 μg/ml). The level gradually increased during childhood until 6 years of age when that of healthy adults (80 μg/ml) was reached. The mean serum level of γD was markedly elevated (2-6 mg/ml or 25-75 times the normal mean) in 2 subjects with D-myeloma and was moderately elevated (3.6 times normal) in 18 subjects with cystic fibrosis of the pancreas, in 18 with Laennec's cirrhosis (2.5 times normal) and in 5 infants with the visceral larva migrans syndrome (3.7 times normal). It was also occasionally elevated in severe acute hepatitis where a level of 2.0 mg/ml was transiently present in one subject, and in occasional normals where levels of 350 μg/ml and 1.1 mg/ml were found in separate instances. The level was diminished in 9 of 10 subjects with hypoagammaglobulinemia.

Small quantities of γD were detected in colostrum, breast milk, mixed saliva, and occasionally in gastric or duodenal juice. Small amounts of γD were detected in both the urine and saliva of each of the two subjects with D-myeloma.

Evidence for 7D antibody activity was sought and antigen binding was demonstrated by radioimmunodiffusion in one subject who received several booster injections of diphtheria toxoid and in a child with spontaneous hypersensitivity to cow's milk. Twelve of 25 subjects with disseminated lupus had 7D antinuclear antibodies.

Specific rabbit antiserum to the δ-chain of 7D was shown to produce marked blastogenic stimulation of human lymphocyte cultures.

The protein was shown to be unusually labile to heat and to solutions of low pH or of high molarity. Spontaneous breakdown into Fc- and Fab-like fragments frequently occurred, presumably due to the action of plasmin.

Immunosorbents were prepared from complex-free antiserum to δ-chain and were found useful in purifying 7D from normal subjects.

Physicochemical studies of two D-myeloma proteins revealed $S^{o}_{20,w}$ values of 7.0 and 6.7. The molecular weight by sedimentation equilibrium was 200,000. Spontaneously occurring split products which retained δ-chain antigenic determinants had molecular weights of 140,000 and 60-65,000. The molecule was found to contain more carbohydrate residues than any other immunoglobulin and an unusual amount of carbohydrate was also found in the lambda Bence-Jones protein of one patient. The Bence-Jones protein also appeared to be unusually labile. Amino acid analyses revealed a particularly low concentration of proline in both D-myeloma proteins, relatively low concentrations of lysine and

histidine and high concentrations of serine, glutamic acid, lysine, alanine and leucine. γD was therefore found to be unique in many of its characteristics, and appears to possess functional activity. Its precise role in the immunologic responses of man remains to be elucidated. A number of important features of the five immunoglobulin classes are summarized in Table XVIII. The contributions of the current study are indicated with an asterisk.

TABLE XVIII

Human Immunoglobulins

	γG	γA	γM	γD	γE
Serum mg%	800-1600	100-400	50-200	0.1-40	.01-.09
Synthesis: mg/kg/d	20-40	3-55	3-17	.03-1.5	
Catabolism: % IV pool/d	4-7	14-34	14-25	18-60	16
% intravascular	50	40	65-95	65-85	50
Complement fixing	+	0	+		0
Placental transfer	+	0	0	0	0
Presence in CSF	+	+	0	0	
Exocrine Secretion	+	+++	+	±*	±
Skin sensitizing	PCA	0	0	0	PK
MW, basic unit	150,000	150,000	180,000	200,000*	196,000
Hexose %	1.2	3.3	5.4	6.1*	5.53
Hexosamine %	1.3	2.3	4.4	5.17*	3.64
Fucose %	0.20	0.22	0.7	0.74*	0.56
Sialic a %	0.30	1.8	1.3	2.78	1.0
Total CHO %	2.9	7.5	11.8	14.79*	10.7
antibody activity	+++	+++	+++	+*	+++
Heat Lability	-	+	++	++++*	+++

To convert mg% to μg/ml move the decimal one place to the right.

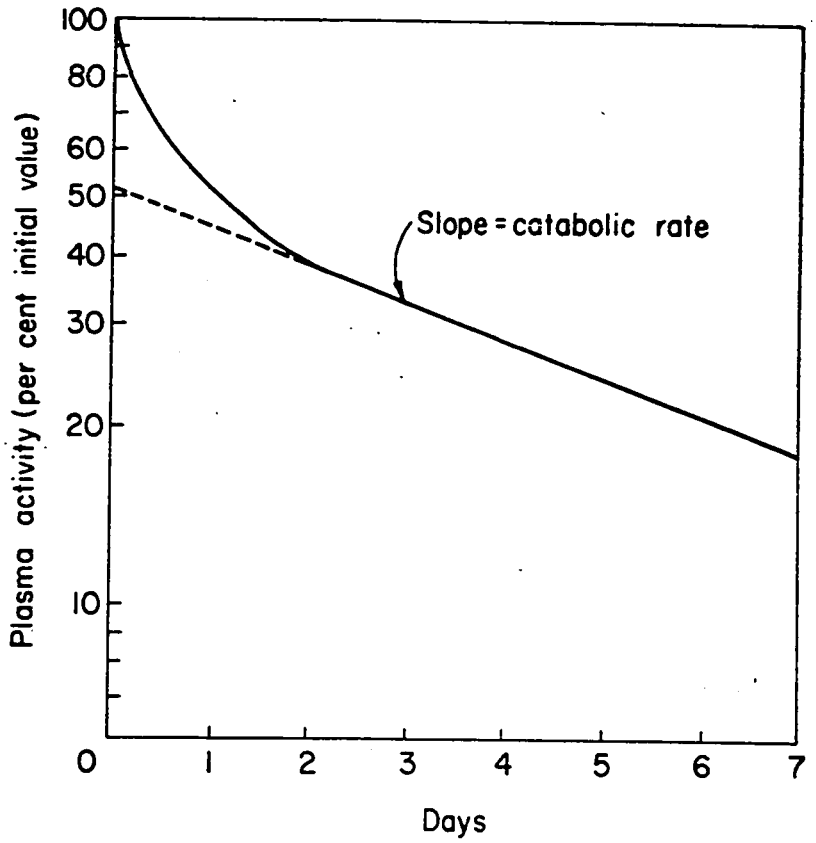
VI. CLAIMS TO ORIGINALITY

The following findings in regard to 7D are the original contributions of this study which have not previously been reported in the literature:

1. Elevated serum levels in cystic fibrosis of the pancreas and in the visceral larva migrans syndrome.
2. Its presence in breast milk and saliva of normals, in duodenal and gastric juice in iron deficiency anemia and in the saliva and urine of subjects with D-myeloma.
3. The appearance of 7D antibodies in response to immunization and in occasional subjects with spontaneous food sensitivity.
4. The induction of blastogenesis in human lymphocyte cultures by specific antiserum to the δ-chain of 7D.
5. An unusual lability upon exposure to heat or extremes of pH, or to solutions of high molarity.
6. A molecular weight of 200,000 for the whole molecule and of 140,000 and 60-65,000 for spontaneously occurring fragments.
7. An unusually high carbohydrate content (14.8%) of the whole molecule, part of which was probably represented in the light chain (Bence-Jones protein) of one D-myeloma subject.
8. An unusually low concentration of proline, which along with the high carbohydrate may be related to the lability of the molecule.

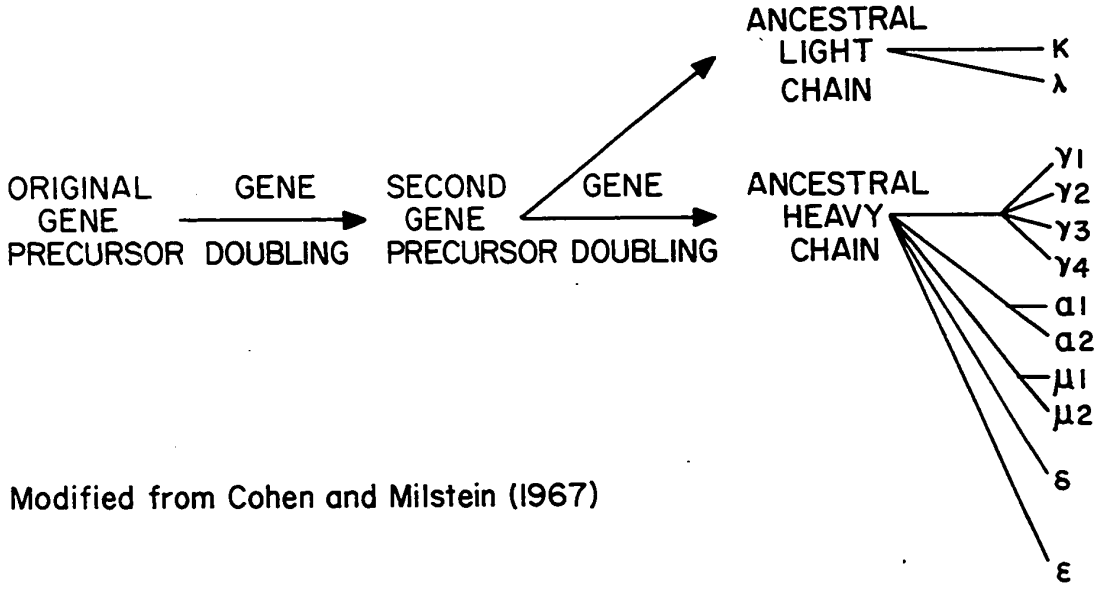
VII. ILLUSTRATIONS

Figure 1



Sterling model of disappearance of radiolabelled proteins from plasma. The slope represents the catabolic rate of the labelled protein and the intercept of the straight line with the ordinate represents the percent of protein in the intravascular space.

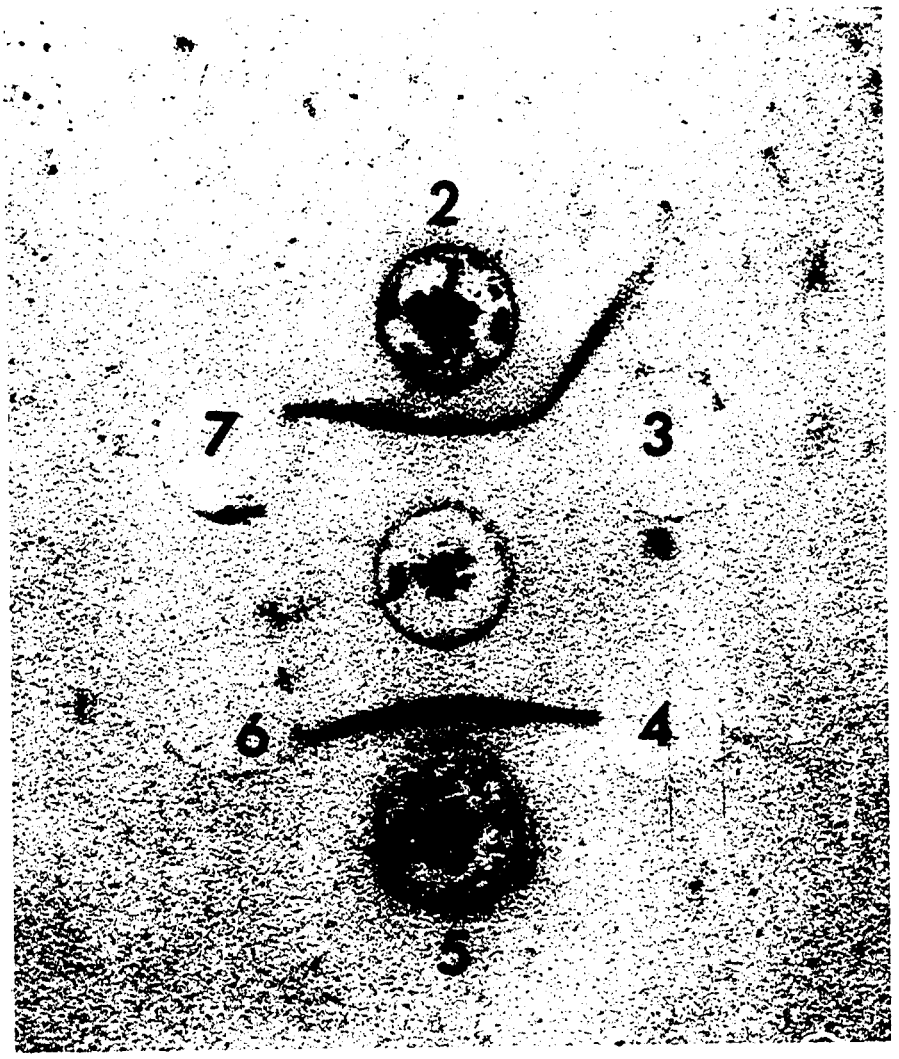
Figure 2



Modified from Cohen and Milstein (1967)

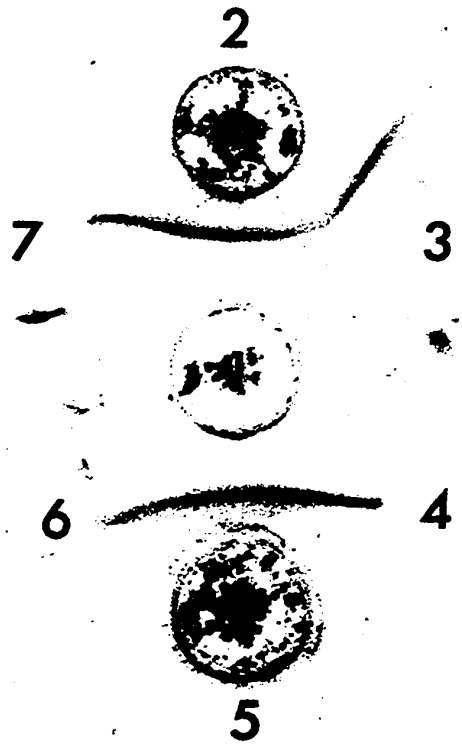
Possible evolution of immunoglobulin polypeptide chains. The ancestral immunoglobulin chain may have been half the size of immunoglobulin light chains and one quarter the size of heavy chains.

Figure 3



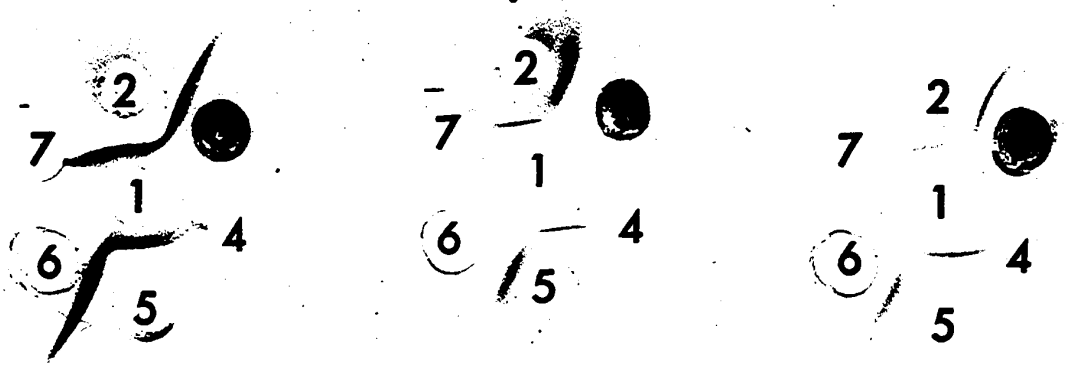
Precipitate lines formed by YD in micro-Ouchterlony. 1, myeloma YD 0.1 mg/ml; 2 and 5, antiserum to δ-chain; 3, normal serum containing 12 mg% YD (0.12 mg/ml); 6, normal serum with an absence of YD (under 1 µg/ml). Magnification x 9.

Figure 3



Precipitate lines formed by YD in micro-Ouchterlony. 1, myeloma YD 0
2 and 5, antiserum to δ-chain; 3, normal serum containing 12 mg% YD
mg/ml); 6, normal serum with an absence of YD (under 1 µg/ml).
Magnification x 9.

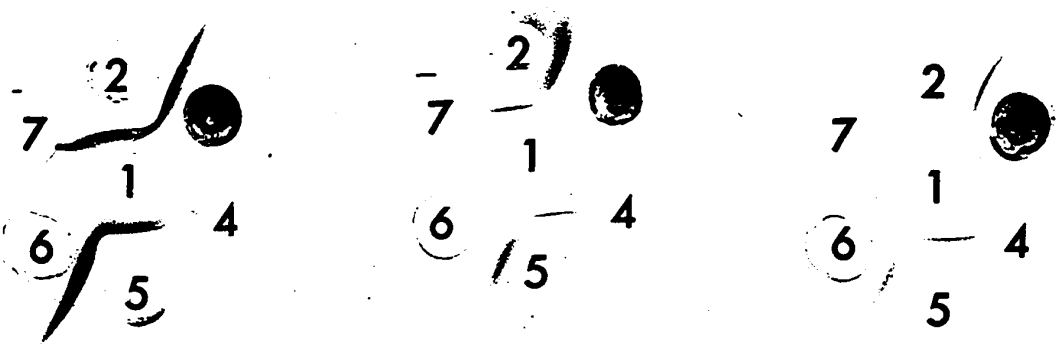
Figure 4



Proof of absorption of antiserum to δ-chain. Antisera (Rl65 and Rl34) to two D-myeloma proteins were absorbed with serum from a subject with an isolated absence of YD. There is an excess of YG, YA and YM in each of the anti δ-chain sera proving that each was absorbed in Ag excess for the three major immunoglobulins. Absence of antibodies to YE was proved by radioimmunodiffusion using specific antiserum to YE Fc fragment kindly supplied by Drs. Johansson and Bennich. Precipitating antibody activity to δ-chain remained intact as evidenced by identity on Ouchterlony analysis with antiserum to δ-chain obtained from Dr. Fahey. Antiserum Rl65 absorbed (anti-δchain) was used in Figure 3 and shows these antibodies to YD heavy chain.

A. 1, YG 0.1 mg/ml; 2,5, anti-γ chain; 3, anti-δ chain (Rl65); 6, anti-δ chain (Rl34).
B. 1, YA 0.1 mg/ml; 2,5, anti-α chain; 3, anti-δ chain (Rl65): 6, anti-δ chain (Rl34).
C. 1, YM 0.5 mg/ml; 2,5, anti-μ chain; 3, anti-δ chain (Rl65); 6, anti-δ chain (Rl34).

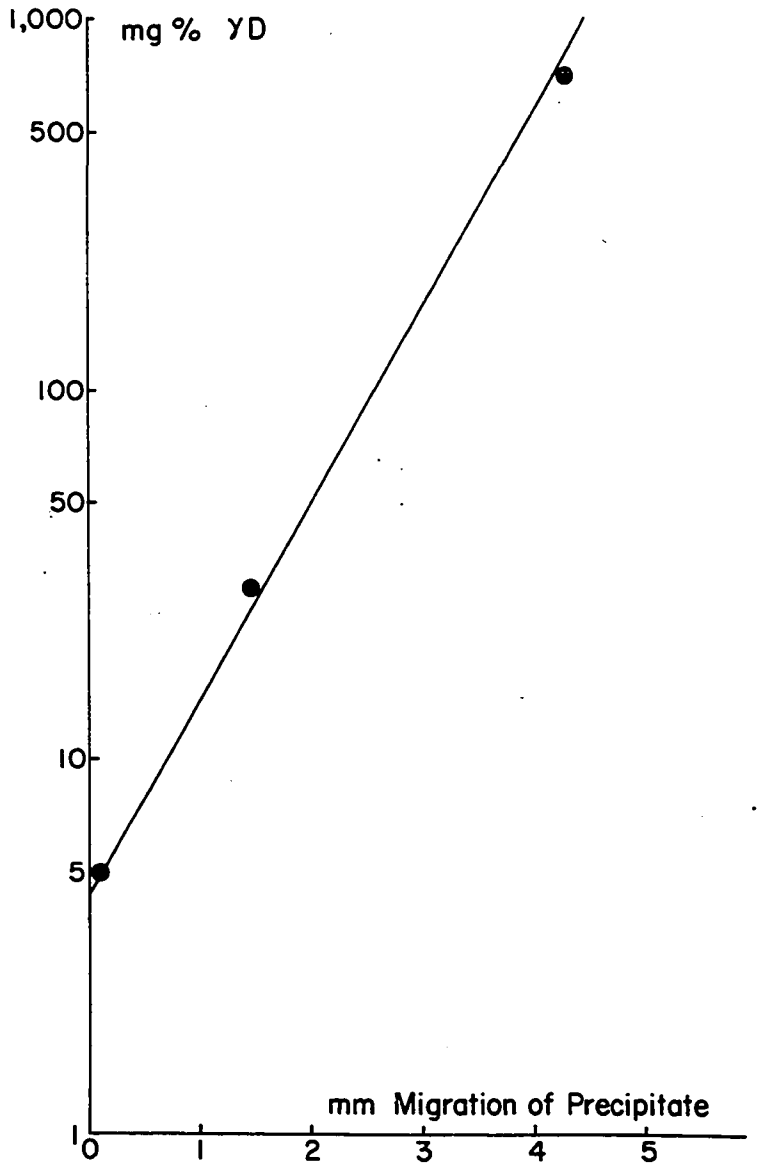
Figure 4



Proof of absorption of antiserum to δ-chain. Antisera (R165 and R134) to two D-myeloma proteins were absorbed with serum from a subject with an isolated absence of YD. There is an excess of YG, YA and YM in each of the anti δ-chain sera proving that each was absorbed in Ag excess for the three major immunoglobulins. Absence of antibodies to YE was proved by radioimmunodiffusion using specific antiserum to YE Fc fragment kindly supplied by Drs. Johansson and Bennich. Precipitating antibody activity to δ-chain remained intact as evidenced by identity on Ouchterlony analysis with antiserum to δ-chain obtained from Dr. Fahey. Antiserum R165 absorbed (anti-δchain) was used in Figure 3 and shows these antibodies to YD heavy chain.

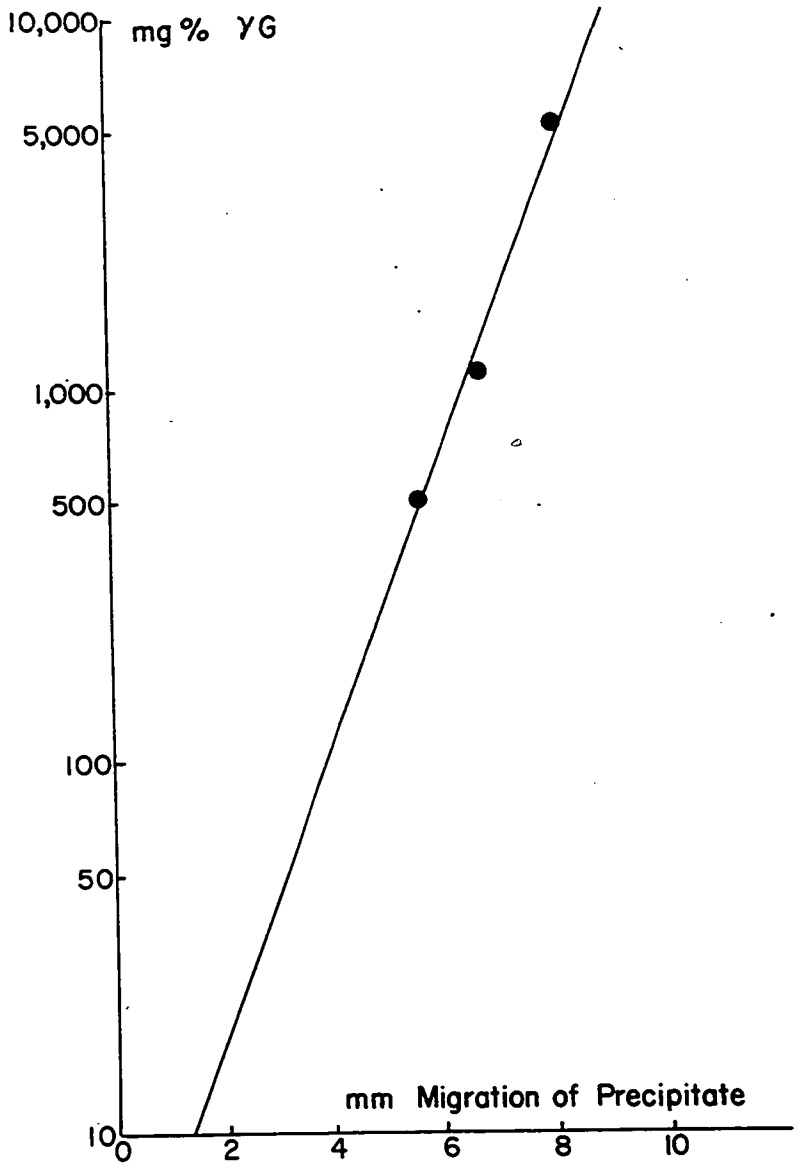
A. 1, YG 0.1 mg/ml; 2,5, anti-γ chain; 3, anti-δ chain (R165); 6, anti-δ chain (R134).
B. 1, YA 0.1 mg/ml; 2,5, anti-α chain; 3, anti-δ chain (R165): 6, anti-δ chain (R134).
C. 1, YM 0.5 mg/ml; 2,5, anti-μ chain; 3, anti-δ chain (R165); 6, anti-δ chain (R134).

Figure 5



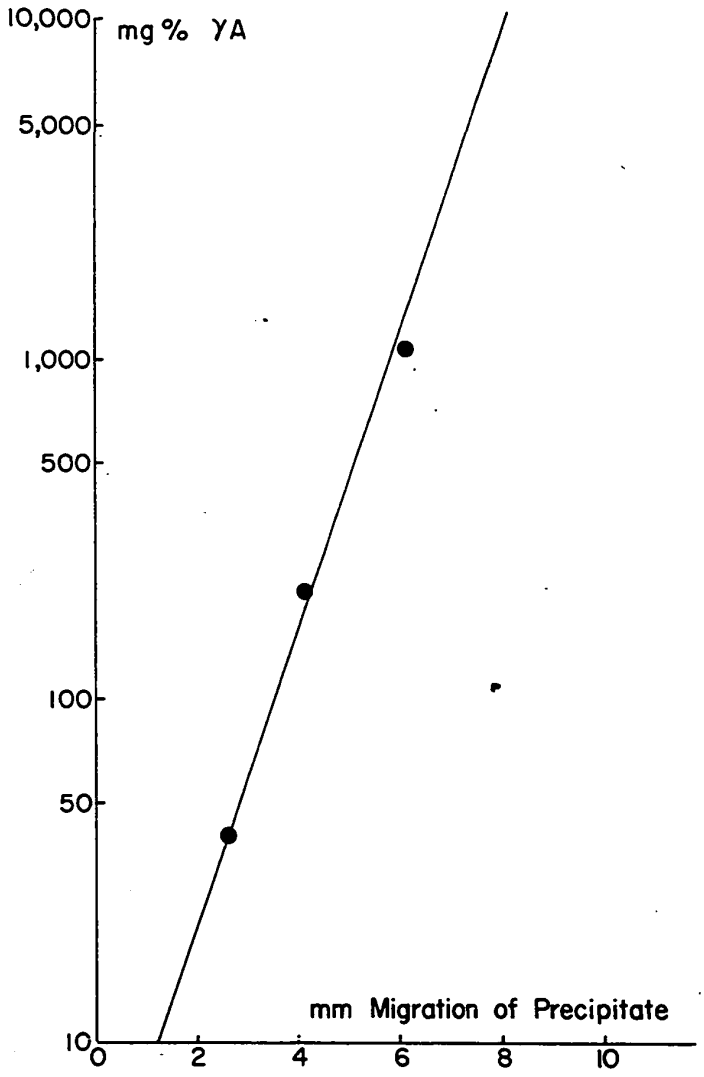
Standard curve for YD concentration (micro-Oudin technique). Standards consisted of D-myeloma serum containing 700 mg%, normal serum containing 30 mg% and pooled normal serum containing 5 mg% YD. The content of YD in unknown sera was determined by measuring the distance of migration of precipitate from the serum-gel interface and reading mg% YD directly from chart.

Figure 6



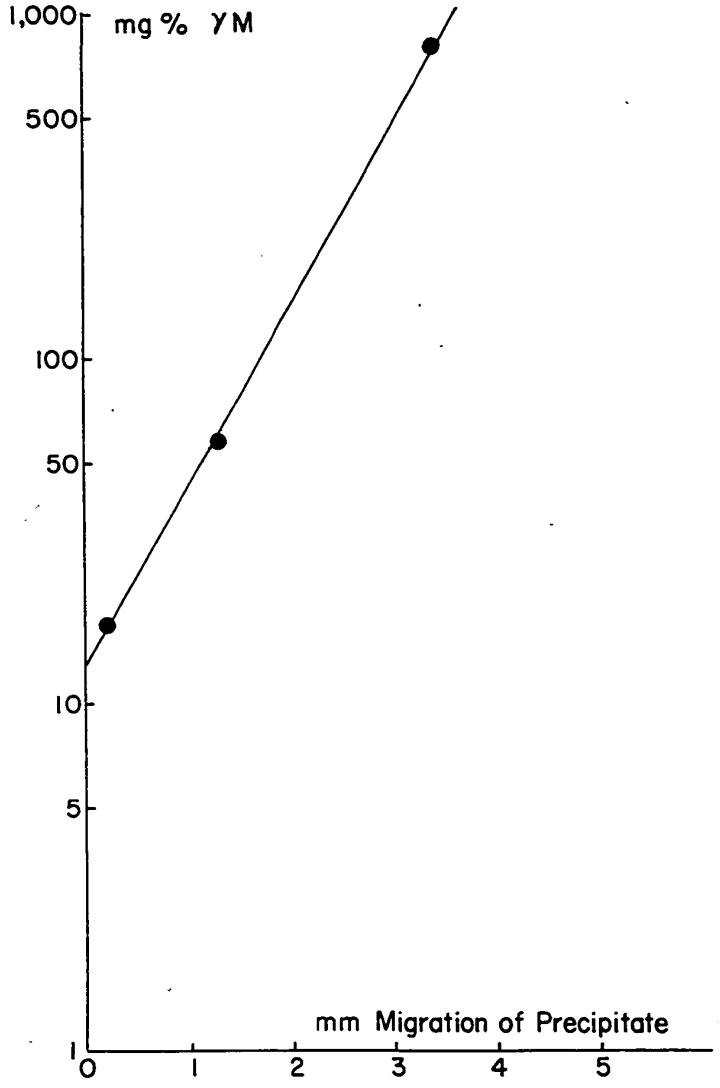
Standard curve for YG concentration. Standards were G-myeloma serum containing 5000 mg%, pooled normal serum containing 1200 mg% and D-myeloma serum containing 500 mg% YG.

Figure 7



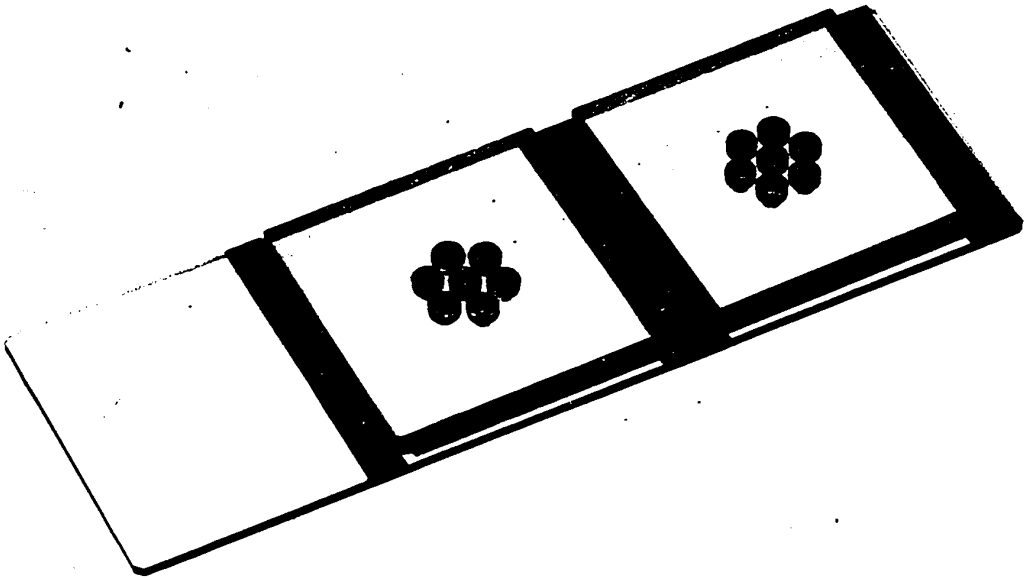
Standard curve for YA concentration. Standards were A-myeloma serum containing 1100 mg%, pooled normal serum containing 210 mg%, and D-myeloma serum containing 40 mg% YA.

Figure 8



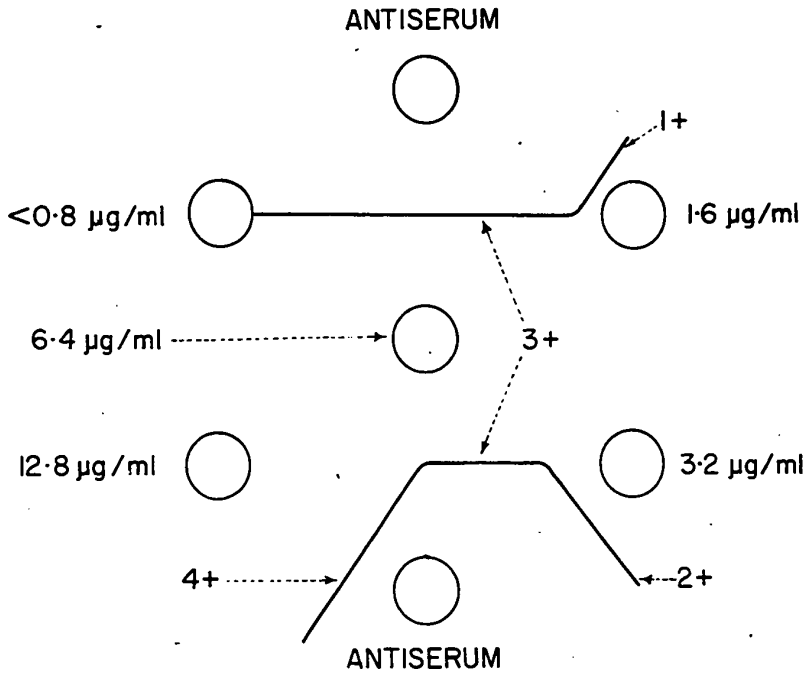
Standard curve for γM concentration. Standards were macroglobulinemic serum containing 800 mg%, pooled normal serum containing 60 mg%, and D-myeloma serum containing 17 mg% γM.

Figure 9



Plexiglas reservoirs for most sensitive micro-Ouchterlony. The plexiglas squares rest on double-thickness strips of water proof tape affixed to the microscopic slide.

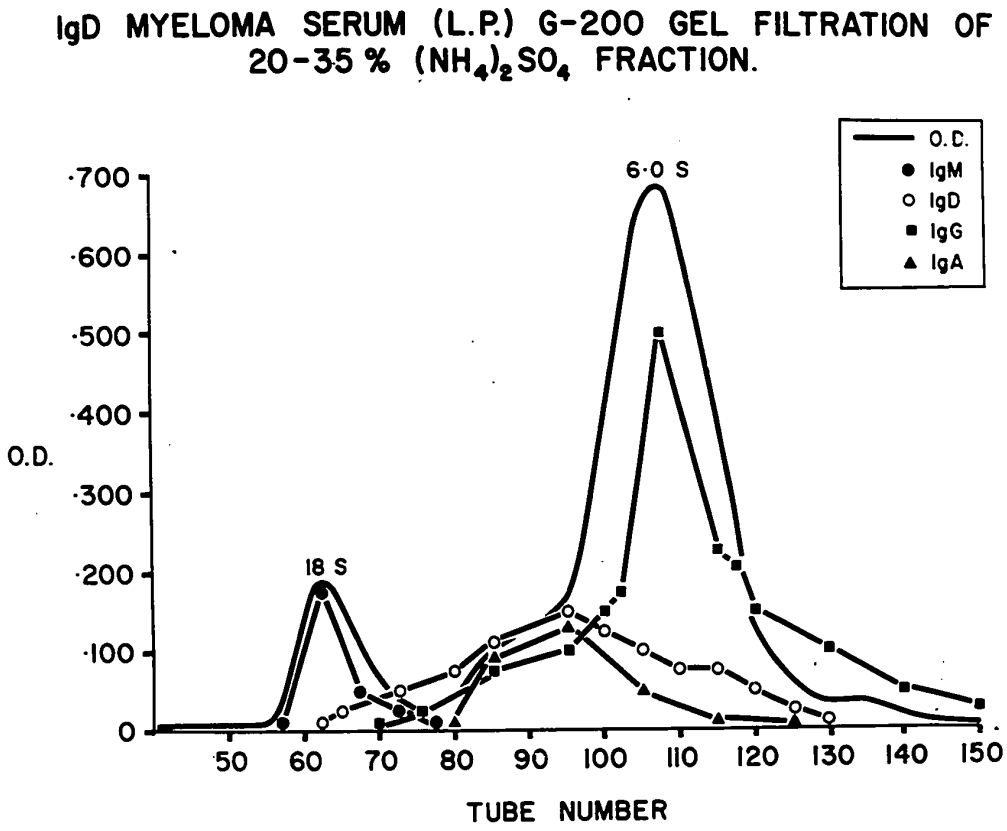
Figure 10



PLEXIGLAS MICRO-OUCHTERLONY

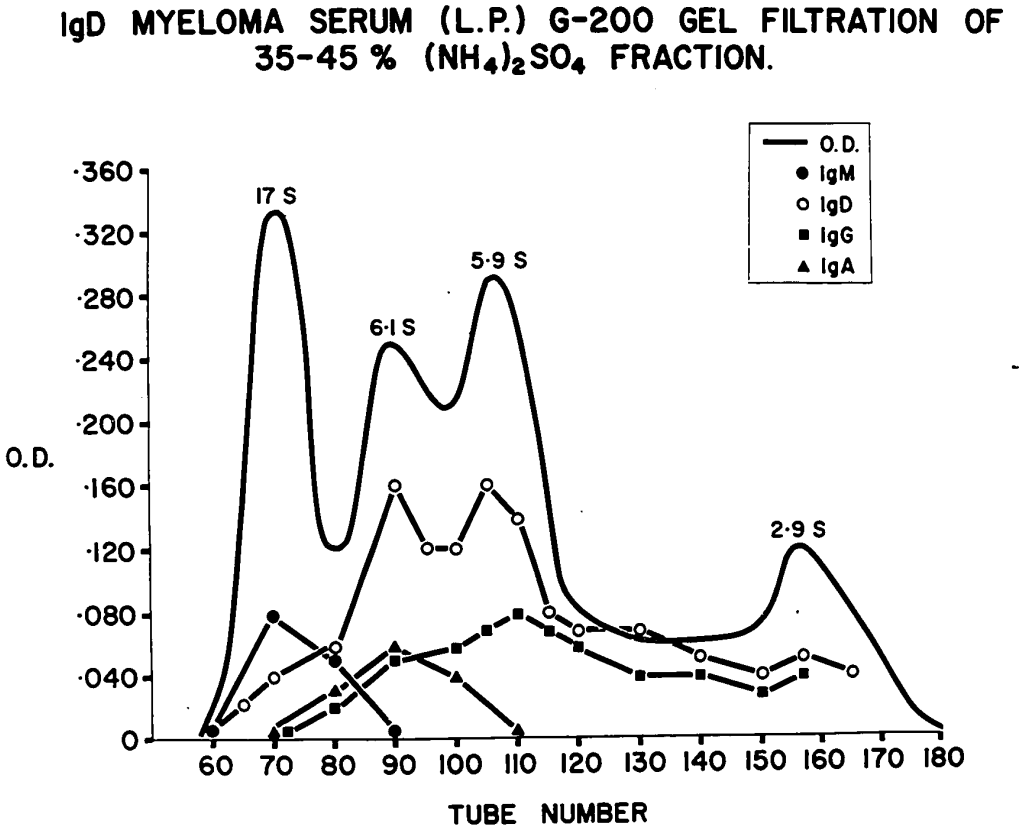
Schematic representation of sensitivity of plexiglas micro-Ouchterlony technique. With adjustment of antigen and antibody concentrations it is possible to attain the degree of sensitivity indicated for YD, YG or YA. Concentrations of YM 5 to 10 times as great are needed to attain comparable results. Plus signs represent method of indicating relative concentrations without assigning precise values.

Figure 11



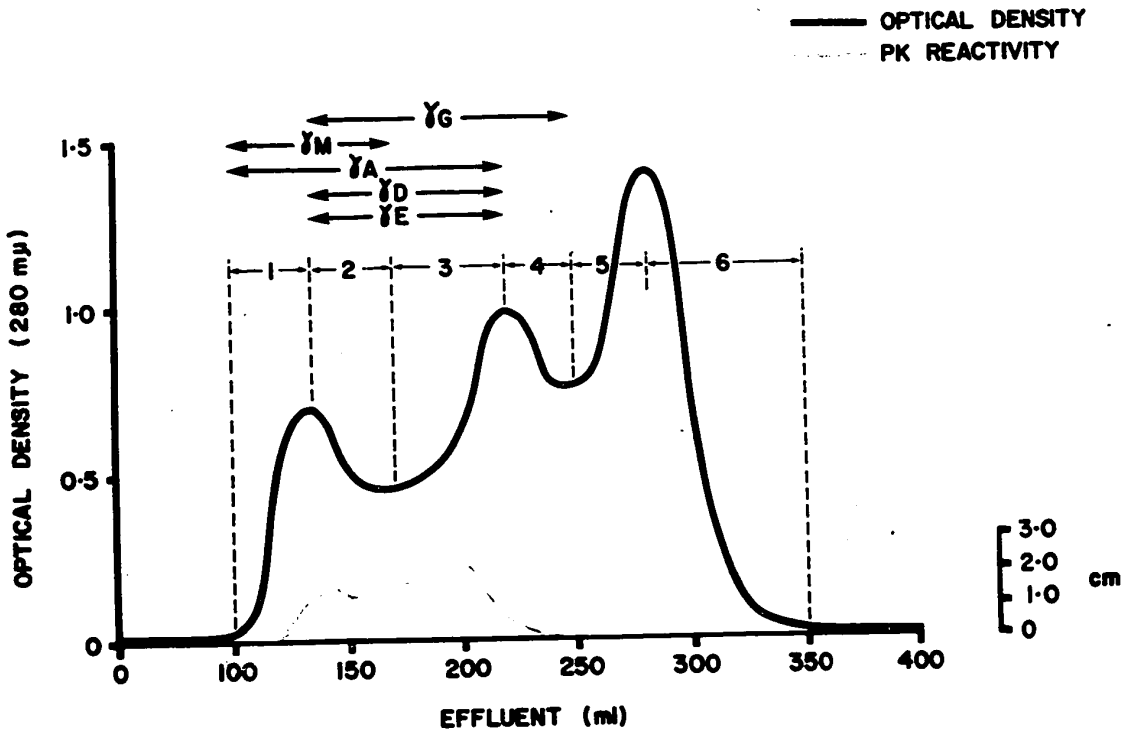
Sephadex G-200 gel filtration of 20-35% saturated ammonium sulfate fraction of L.P. D-myeloma serum. Relative concentrations of the four major immunoglobulins are given. Sedimentation velocity coefficients are indicated above each prominent peak.

Figure 12



Sephadex G-200 gel filtration of 35-45% saturated ammonium sulfate fraction of L.P. D-myeloma serum. The 2.9 S peak contained fragmented YD and YG molecules as well as albumin.

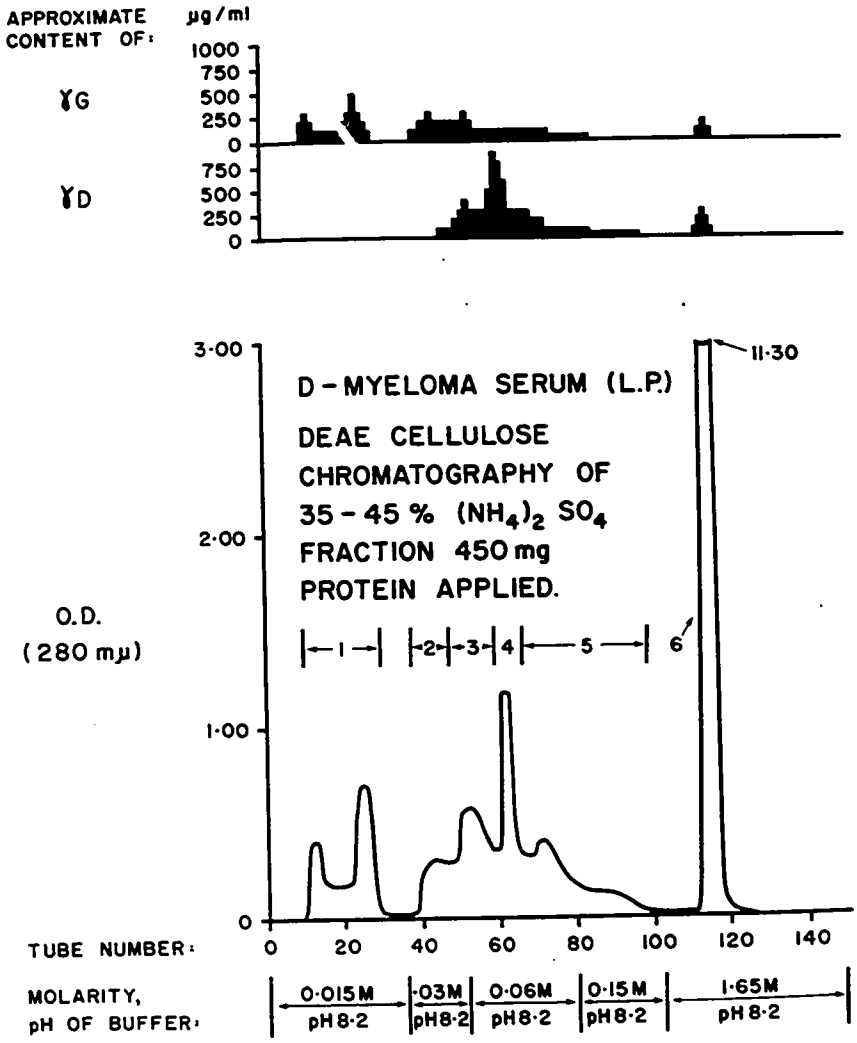
Figure 13



Sephadex G-200 gel filtration of reaginic serum showing distribution of all five immunoglobulins and Prausnitz-Kustner reactions of the fractions.

Figure 14

No. 157

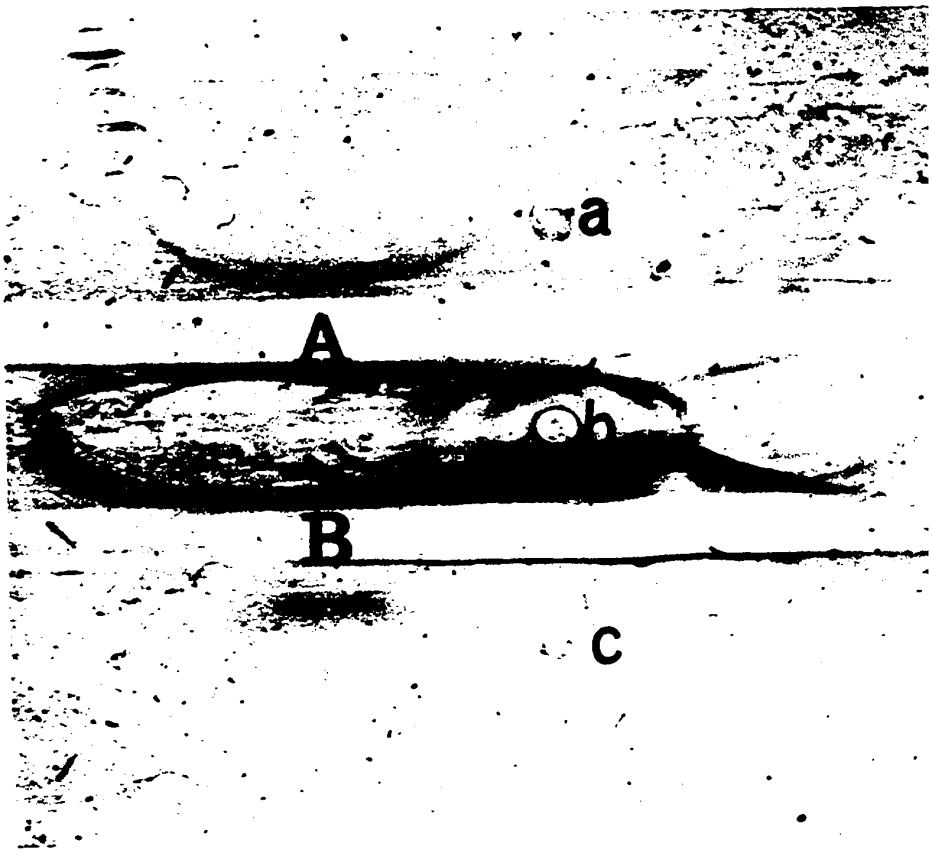


DEAE cellulose column chromatography of 35-45% saturated ammonium sulfate fraction of L.P. D-myeloma serum. Peak 4 contained YD in the highest degree of purity.

Figure 15

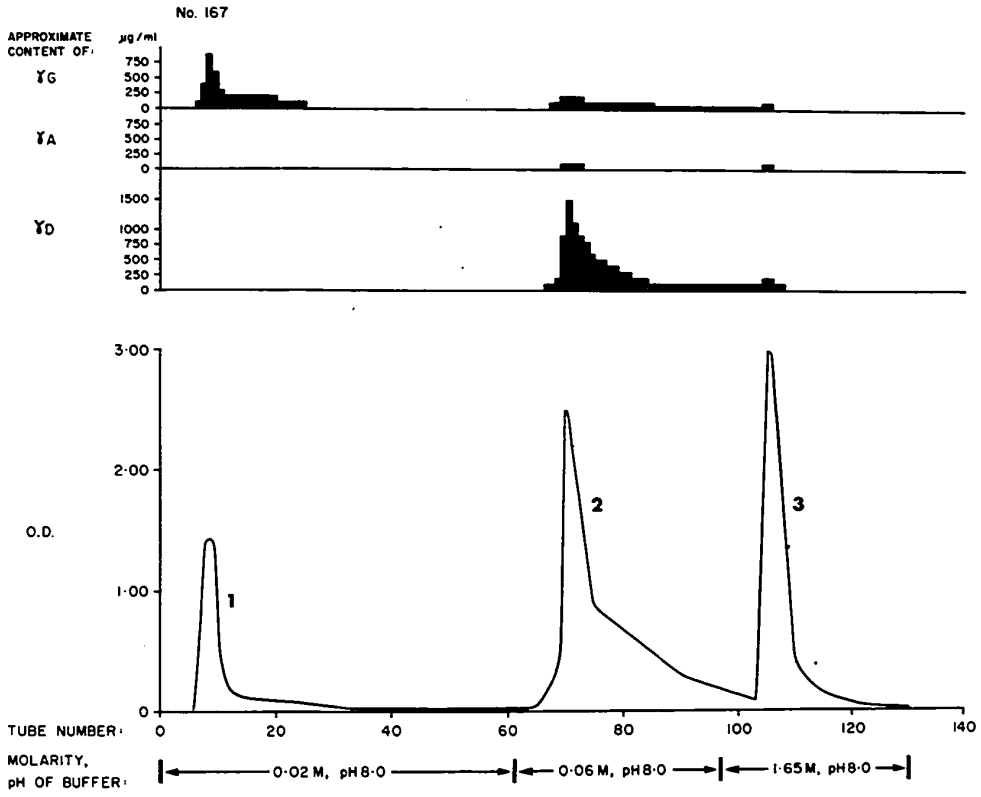
Immunoelectrophoresis of D-myeloma protein in peak 4 of Figure 14. None of the peaks contained significant amounts of γA or γM. A, unabsorbed antiserum to D-myeloma serum; B, antiserum to normal human serum lacking antibodies to γD. a and c, peak 4 of Figure 14; b, L.P. D-myeloma whole serum. The high degree of purity of γD in peak 4 is demonstrated. The anode is to the right.

Figure 15



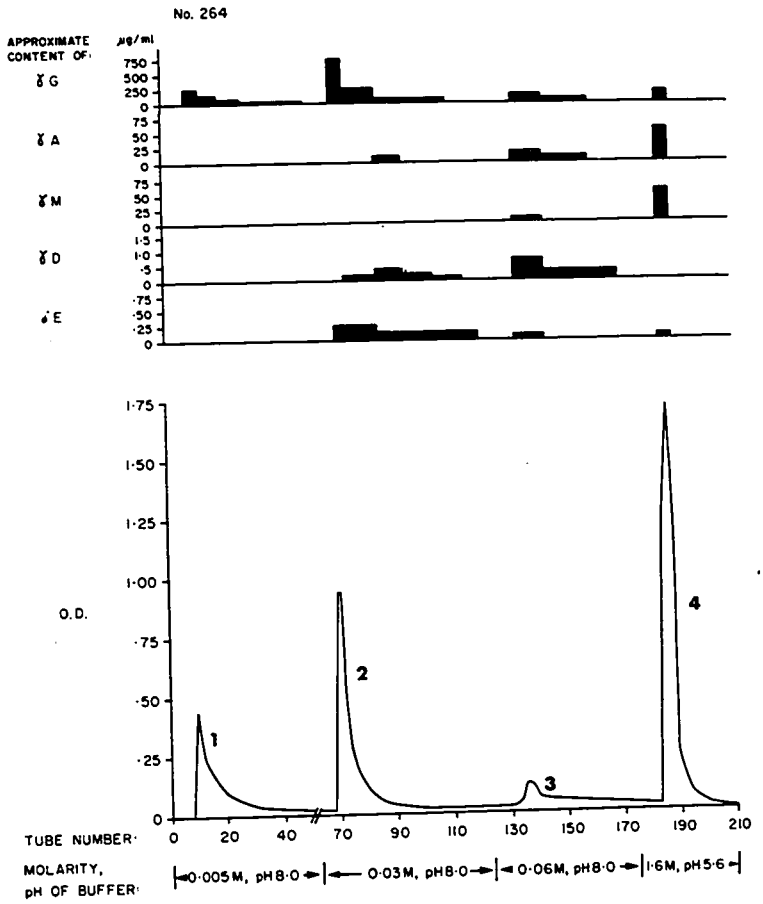
Immunoelectrophoresis of D-myeloma protein in peak 4 of Figure 14. No the peaks contained significant amounts of γA or γM. A, unabsorbed an to D-myeloma serum; B, antiserum to normal human serum lacking antibod γD. a and c, peak 4 of Figure 14; b, L.P. D-myeloma whole serum. The degree of purity of γD in peak 4 is demonstrated. The anode is to the

Figure 16



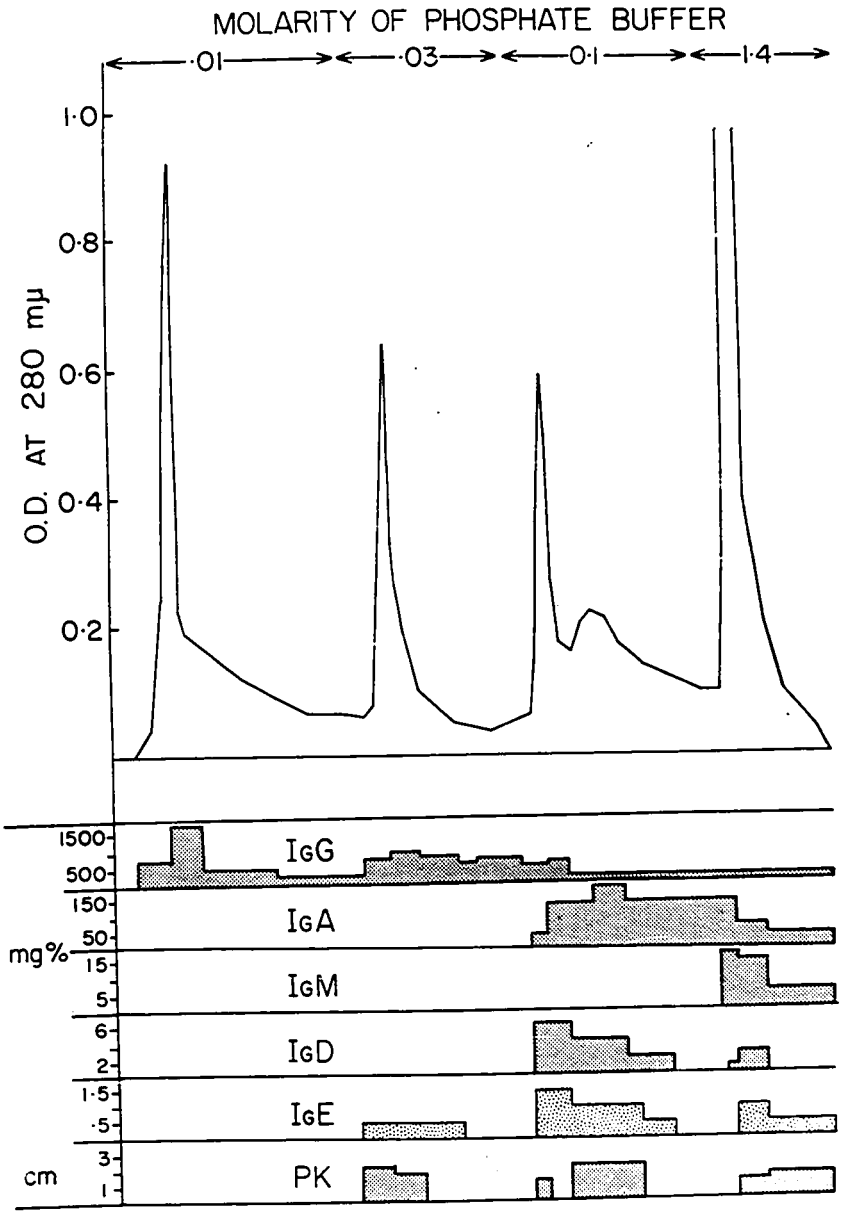
DEAE cellulose column chromatography of 35-45% ammonium sulfate fraction of J.M. D-myeloma serum.

Figure 17



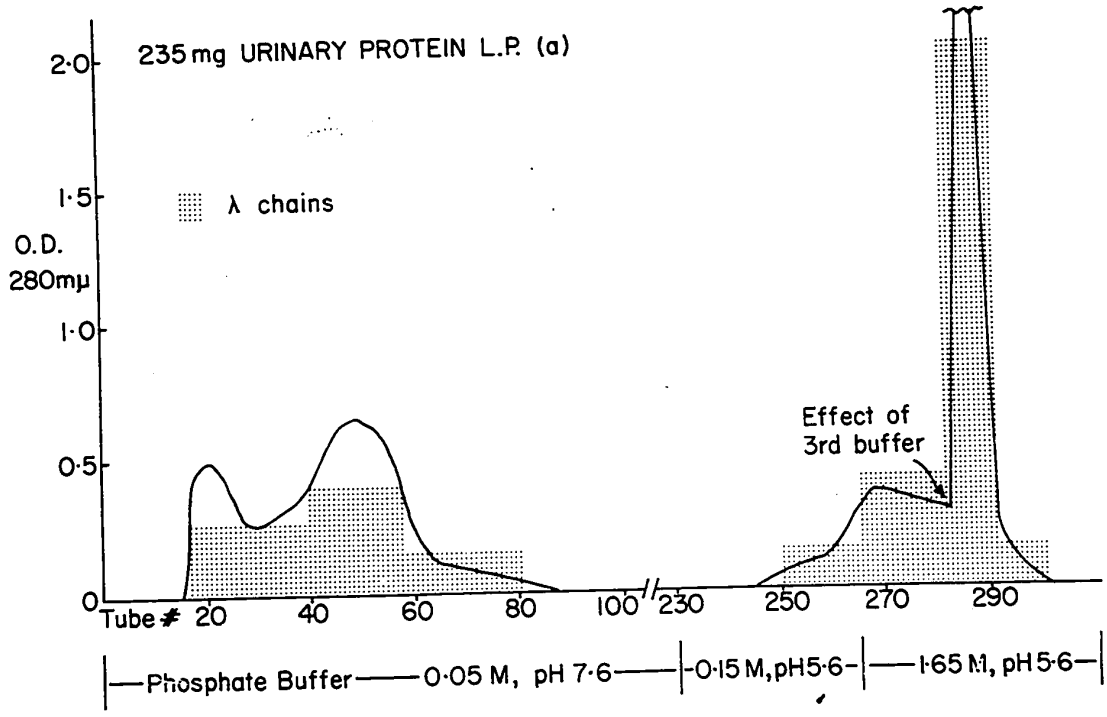
DEAE cellulose column chromatography of reaginic serum showing the distribution of five immunoglobulins.

Figure 18



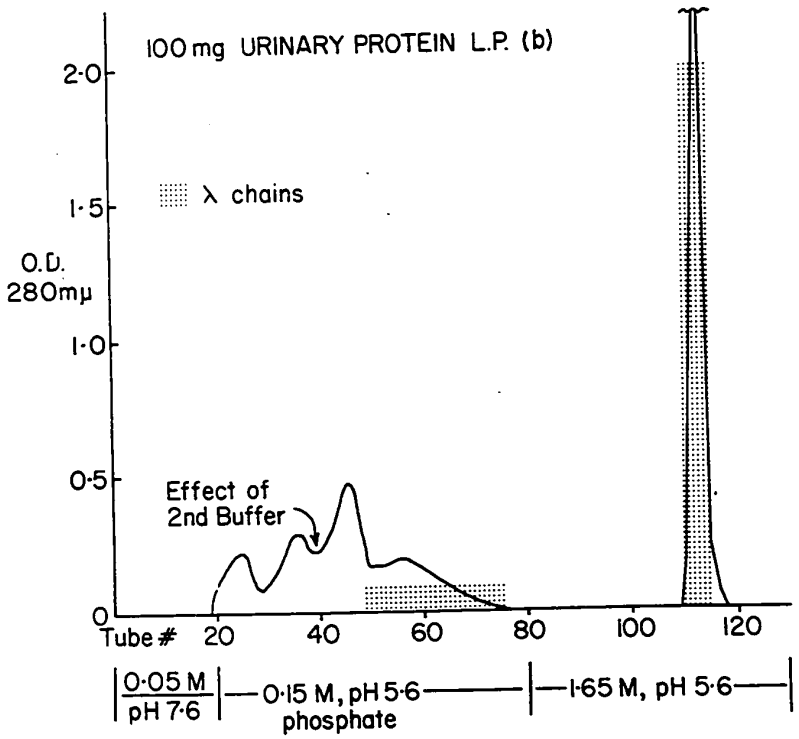
DEAE Sephadex column chromatography of reaginic serum showing the distribution of five immunoglobulins and Prausnitz-Kustner skin test activity.

Figure 19



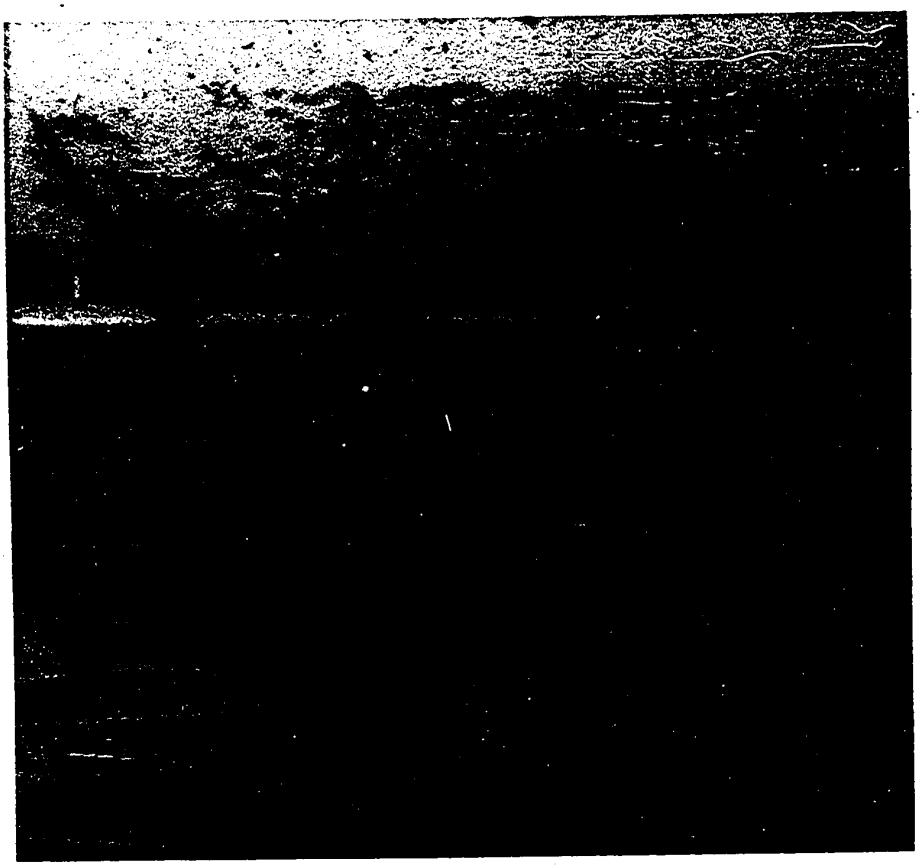
DEAE cellulose column chromatography of L.P. D-myeloma urine.

Figure 20



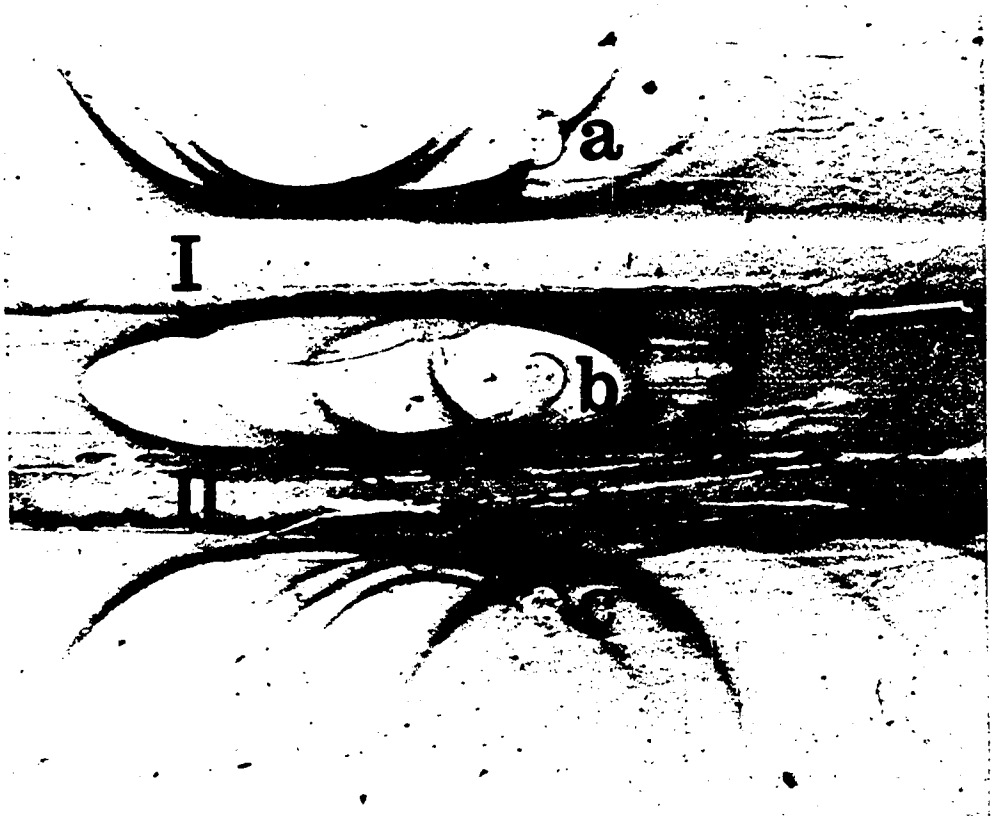
DEAE cellulose column chromatography of L.P. D-myeloma urine showing more restricted distribution of lambda Bence-Jones protein.

Figure 21



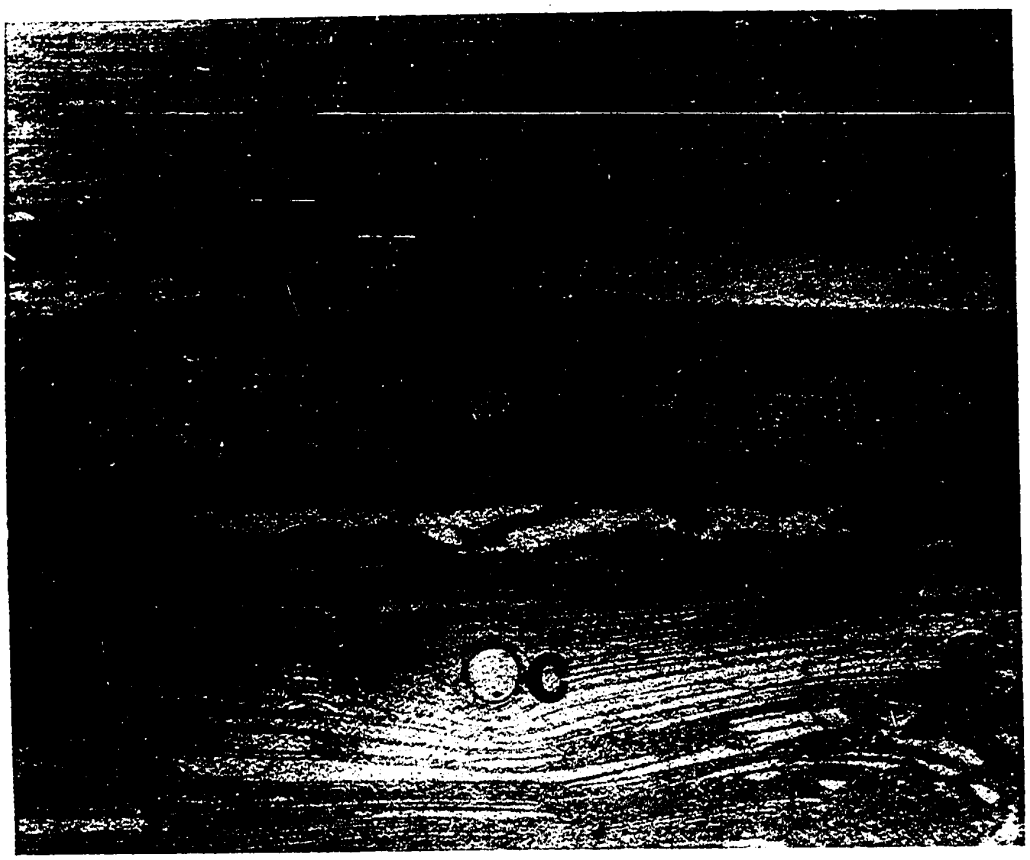
Immunoelectrophoresis of L.P. D-myeloma serum showing fragmentation of YD on freezing and thawing of serum. I, unabsorbed antiserum to D-myeloma serum fraction; II, antiserum to whole normal serum lacking antibodies to YD; a and c, D-myeloma serum after freezing and thawing; b, normal human serum. The YD precipitate arc is split. The anode is to the right.

Figure 21



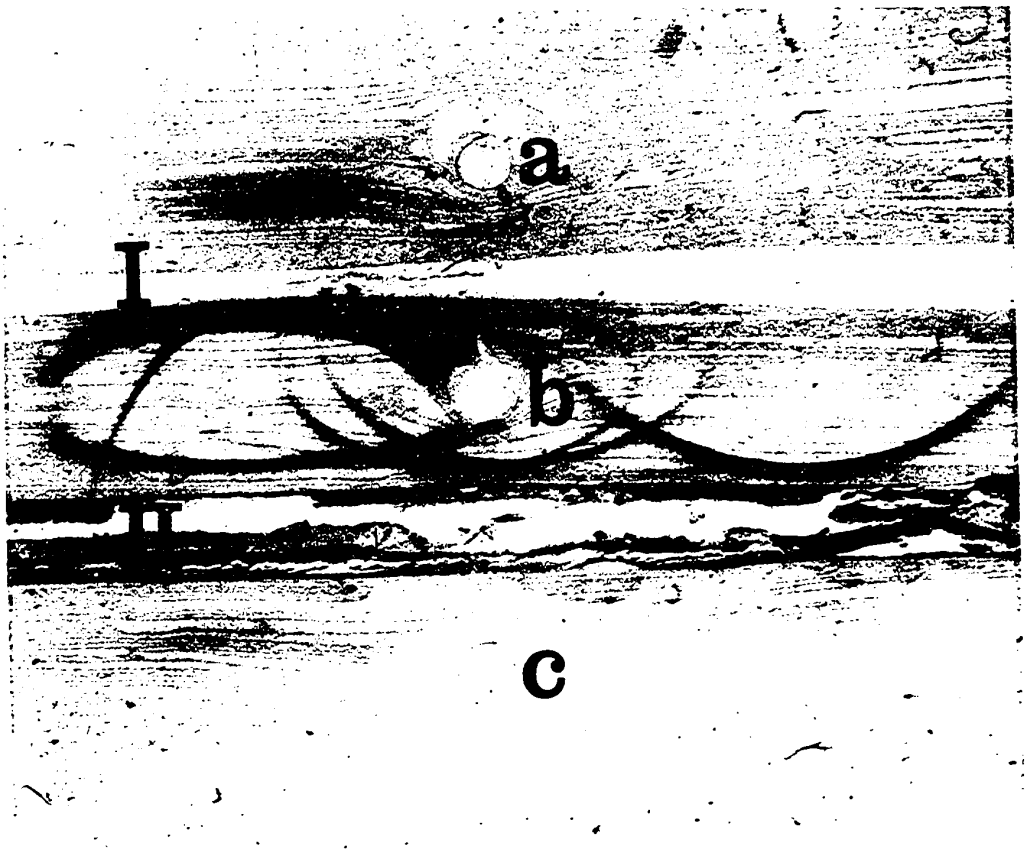
Immunoelectrophoresis of L.P. D-myeloma serum showing fragmentation of YD on freezing and thawing of serum. I, unabsorbed antiserum to D-myeloma serum fraction; II, antiserum to whole normal serum lacking antibodies to YD; a and c, D-myeloma serum after freezing and thawing; b, normal human serum. The YD precipitate arc is split. The anode is to the right.

Figure 22



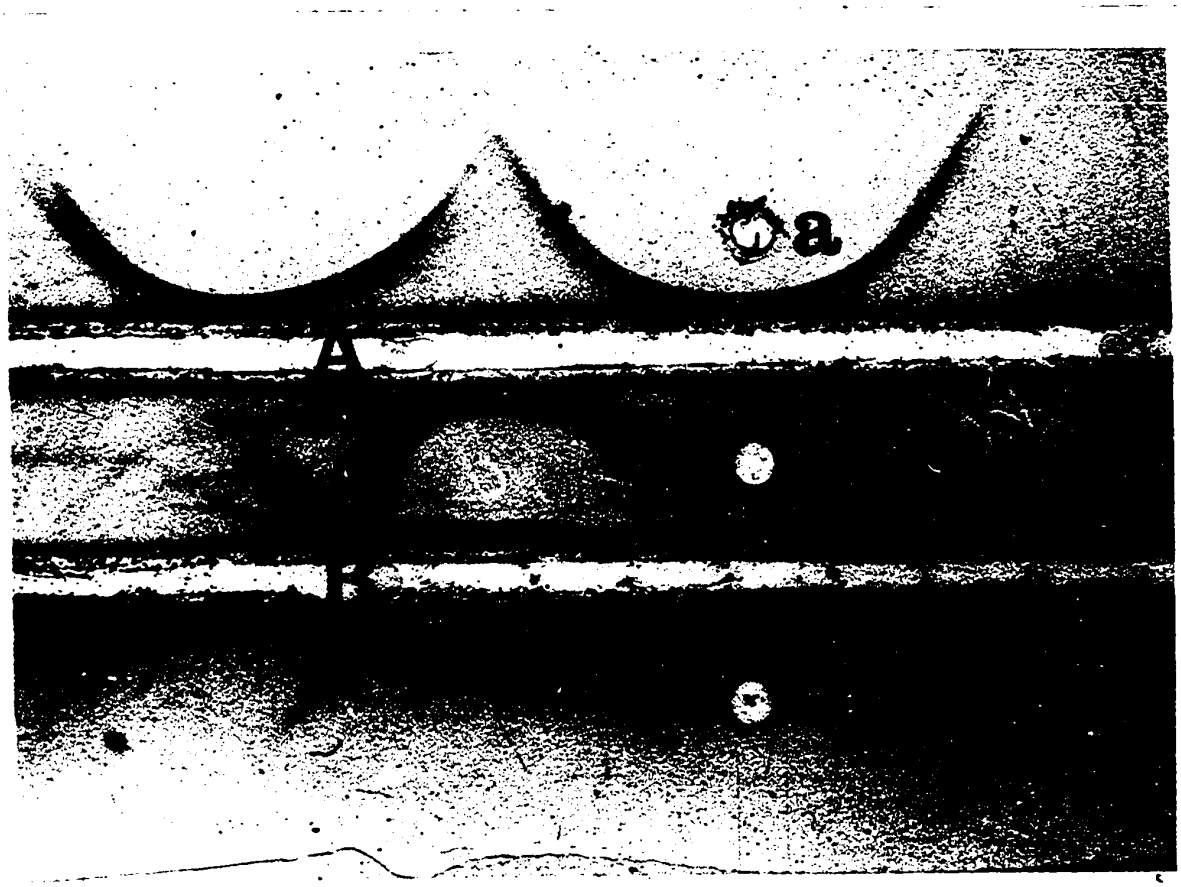
Immunoelectrophoresis of J.M. D-myeloma serum showing no evidence of splitting of the YD precipitate arc after freezing and thawing. I, unabsorbed antiserum to a YD-rich fraction of J.M. serum; II, antiserum to whole normal serum having no antibodies to YD; a and c, fraction containing YG and YD in concentrations too low to produce distinct precipitate lines; b, unfractionated J.M. D-myeloma serum. The sharp line pointing near the Roman numeral II is D-myeloma protein, it crosses the YG line near trough II. Anode to the right.

Figure 22



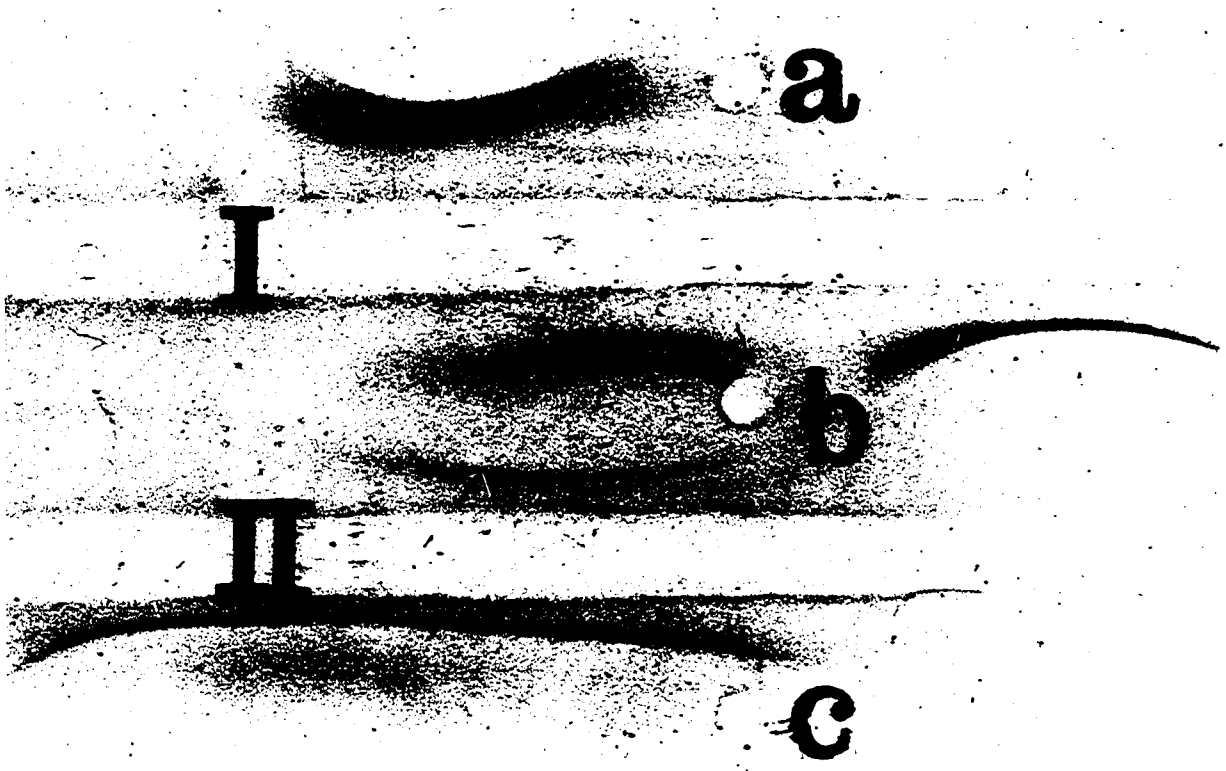
Immunoelectrophoresis of J.M. D-myeloma serum showing no evidence of splitting of the YD precipitate arc after freezing and thawing. I, unabsorbed antiserum to a YD-rich fraction of J.M. serum; II, antiserum to whole normal serum having no antibodies to YD; a and c, fraction containing YG and YD in concentrations too low to produce distinct precipitate lines; b, unfractionated J.M. D-myeloma serum. The sharp line pointing near the Roman numeral II is D-myeloma protein, it crosses the YG line near trough II. Anode to the right.

Figure 23



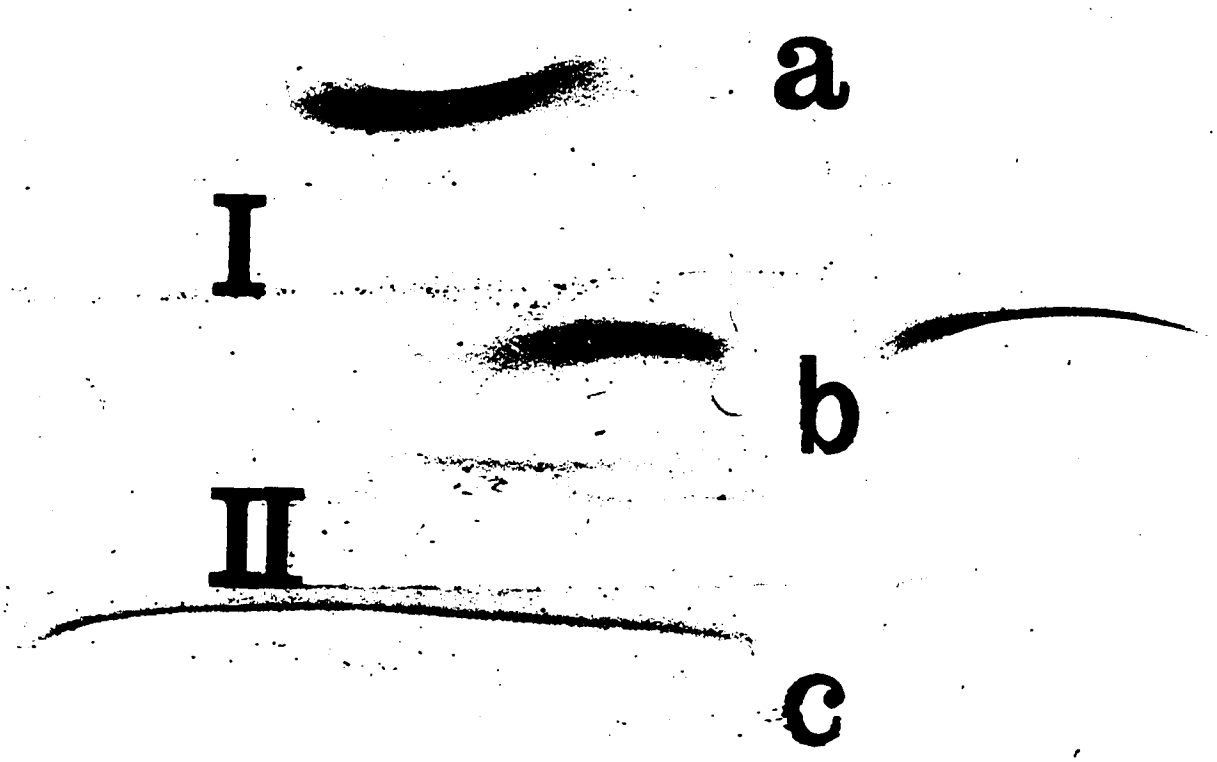
Immunoelectrophoresis of L.P. D-myeloma serum and a fraction containing spontaneously fragmented γD, Lambda Bence-Jones proteinemia is present. Only the slow moving fragment contains λ-chain. A, antiserum to δ-chain; B, antiserum to λ-chain; a and c, fraction of L.P. serum corresponding to peak 4 of Figure 14 but from different chromatogram in which γD had broken down to 3 S fragments; b, L.P. serum.

Figure 24



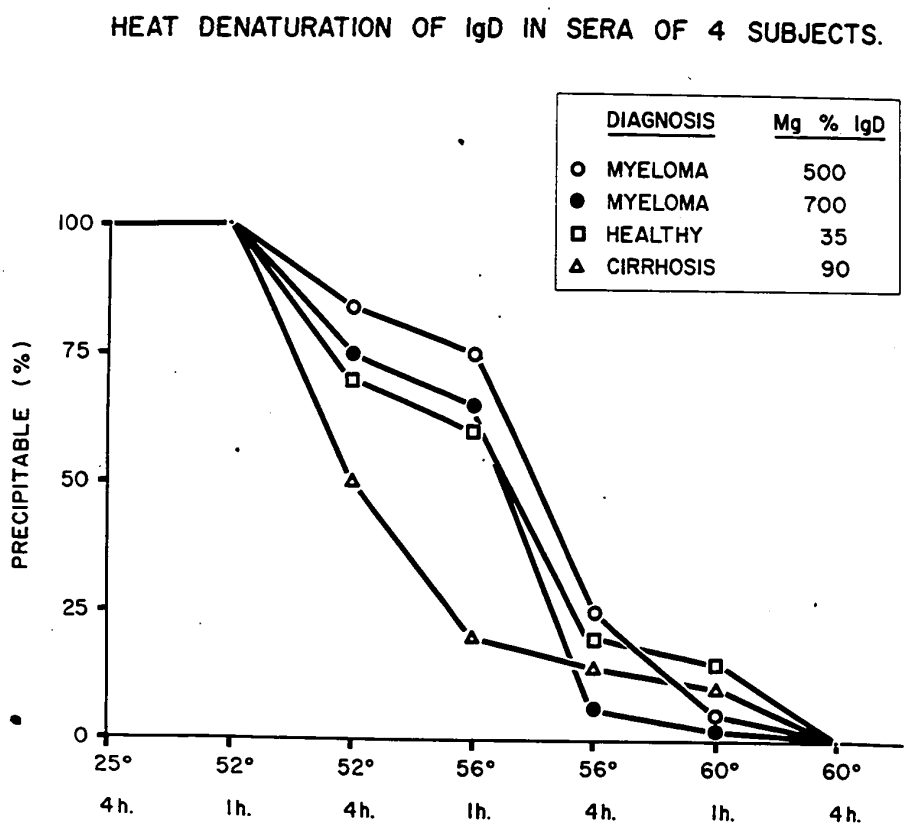
Immunoelectrophoresis of J.M. D-myeloma serum and a fraction of it containing spontaneously fragmented YD. Bence-Jones proteinemia is absent. Both fragments migrate more anodally than the native YD or the corresponding fragments of L.P. YD. I, antiserum to δ-chain; II, antiserum to λ-chain; a and c, whole J.M. serum, b, fraction of J.M. serum corresponding to peak 2 of Figure 16 but from a chromatogram in which the YD had fragmented.

Figure 24



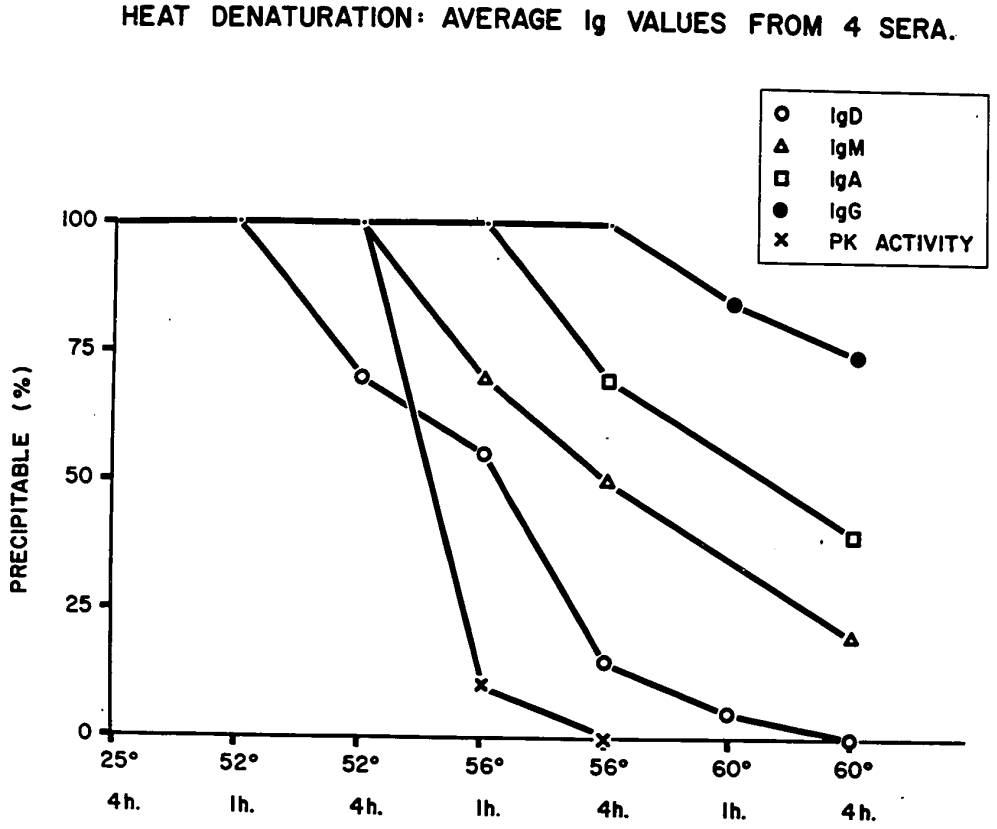
Immunoelectrophoresis of J.M. D-myeloma serum and a fraction of it containing spontaneously fragmented YD. Bence-Jones proteinemia is absent. Both fragments migrate more anodally than the native YD or the corresponding fragments of L.P. YD. I, antiserum to δ-chain; II, antiserum to λ-chain; a and c, whole J.M. serum, b, fraction of J.M. serum corresponding to peak 2 of Figure 16 but from a chromatogram in which the YD had fragmented.

Figure 25



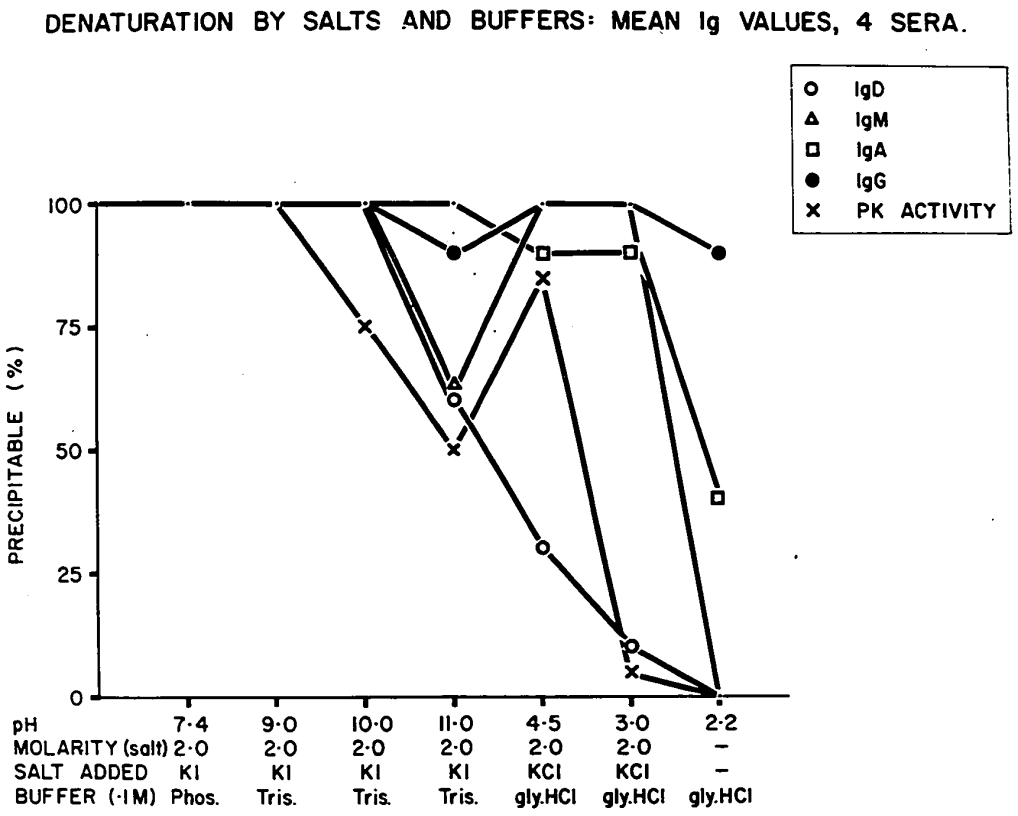
The amount of YD precipitated by specific antiserum is decreased after heating to 52° for 4 hours and none is precipitated after heating to 60° for 4 hours.

Figure 26



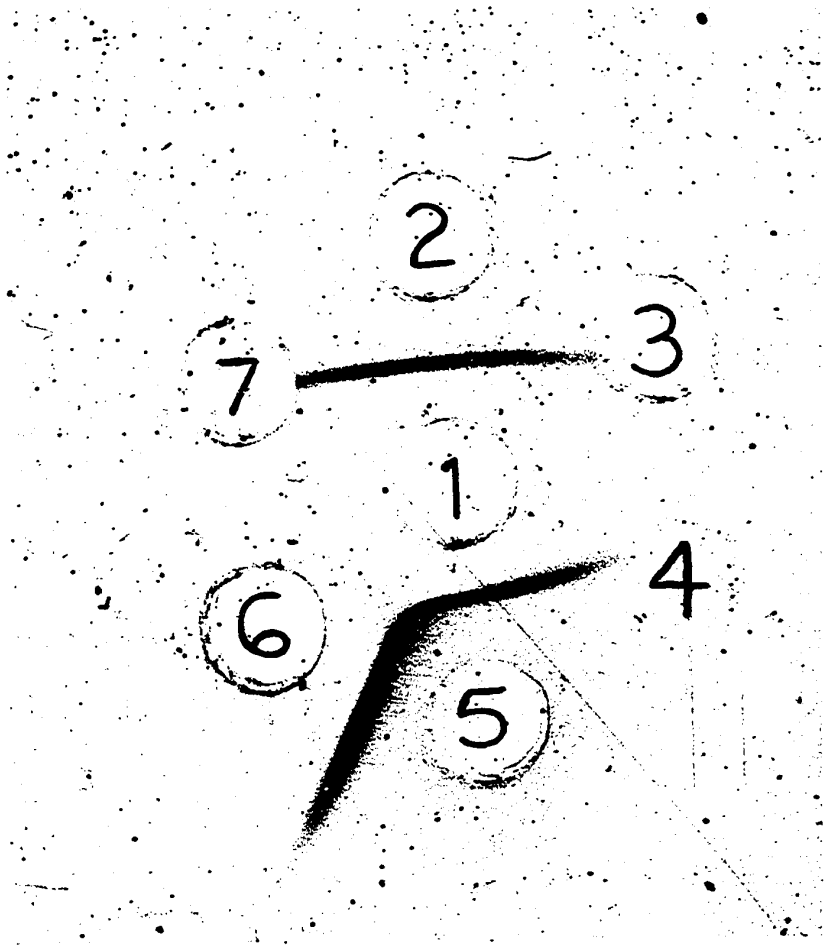
Heat denaturation of immunoglobulins varies from YD, the most labile, to YG, the least labile. Reaginic PK activity most resembles YD precipitability in its heat lability although the two are not strictly comparable.

Figure 27



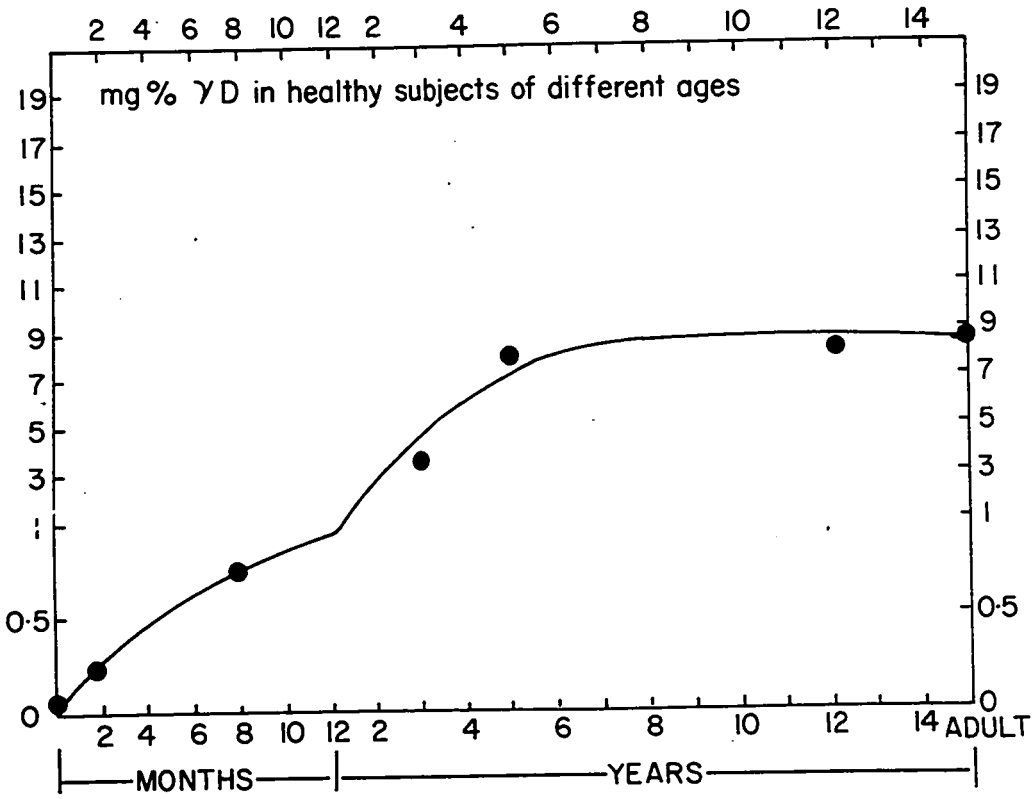
The buffers are arranged according to increasing irreversible denaturation of YD which is the most labile of the four major immunoglobulins when stressed in this manner. PK reactivity is similar to YD precipitability in its lability except that it is less affected by 2 M KCl at pH 4.5.

Figure 28



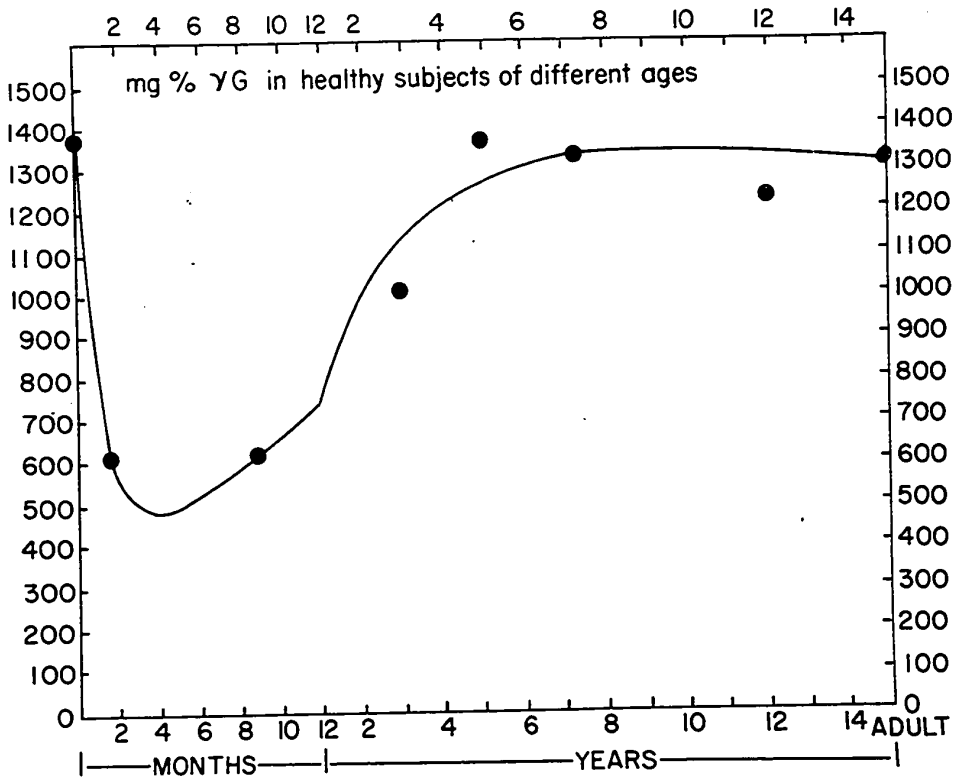
Removal of YG from a mixture of YG and YD using an anti-YG immunosorbent. 1, purified YG 0.1 mg/ml; 2 and 5, antiserum to γ chain; 3, serum fraction originally containing 0.1 mg/ml YG and 0.15 mg/ml YD but absorbed with anti-YG immunosorbent; 6, same fraction as in 3 but not absorbed with anti-YG immunosorbent; 7, saline. The YG is removed by the immunosorbent. Parallel tests showed no removal of YD.

Figure 29



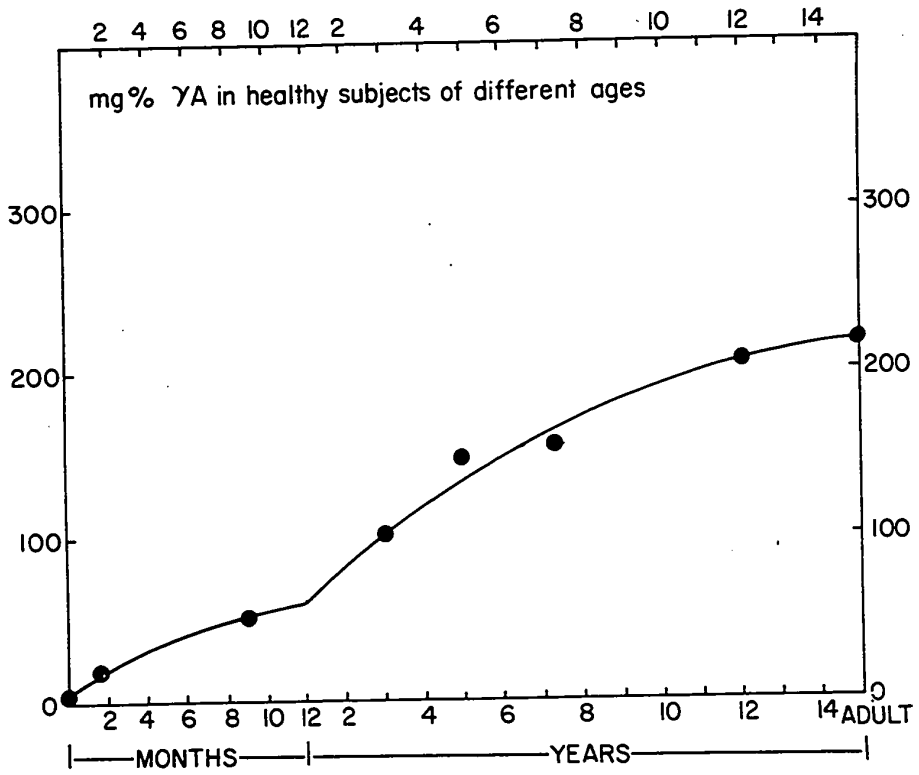
Mean YD levels in healthy subjects with assumed normal mean for all ages.

Figure 30



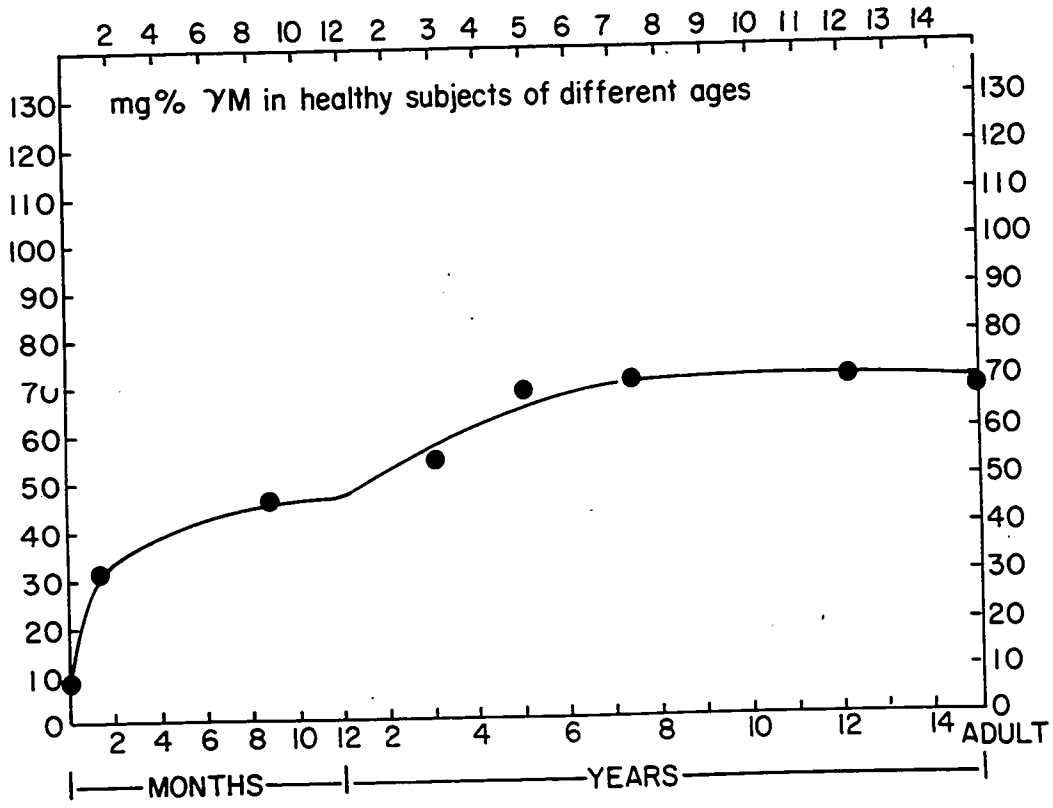
Mean γG levels in healthy subjects with assumed normal mean for all ages.

Figure 31



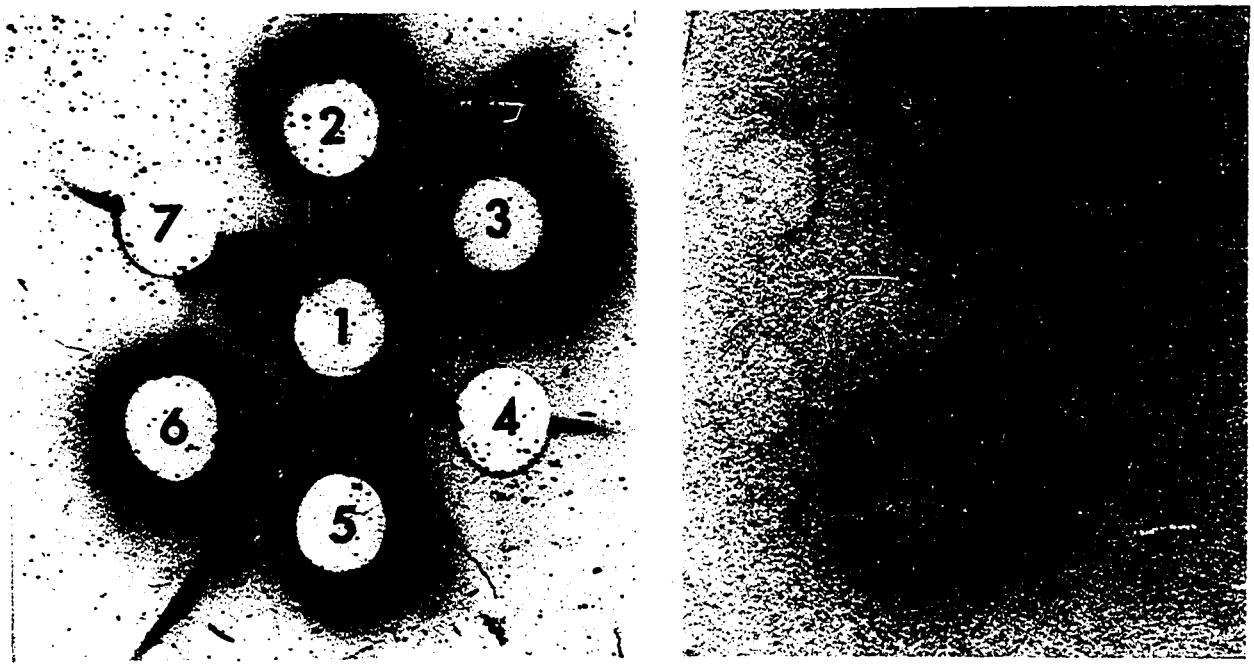
Mean YA levels in healthy subjects with assumed normal mean for all ages.

Figure 32



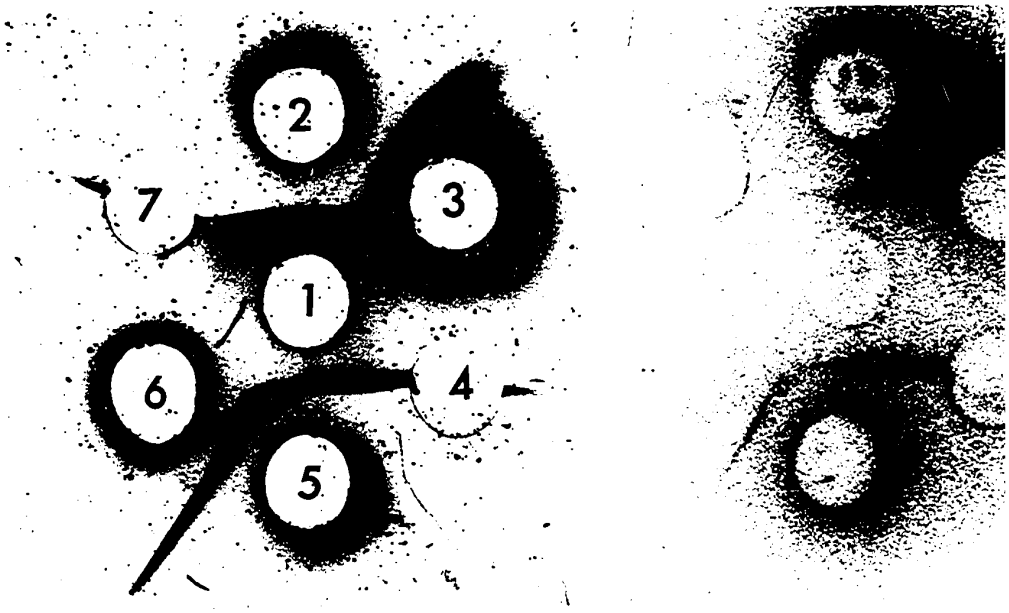
Mean YM levels in healthy subjects with assumed normal mean for all ages.

Figure 33



Radioimmunodiffusion showing YD binding of diphtheria toxoid in the serum of a healthy hyperimmunized subject having .12 mg/ml serum YD level. 1, myeloma YD 1 mg/ml; 2 and 5, concentrated antiserum to δ-chain; 3, concentrated hyperimmune serum containing .80 mg/ml YD; 6, concentrated serum containing 1 mg/ml YD, obtained before first booster immunization; 4 and 7, saline. Radiolabelled diphtheria toxoid was placed in reservoirs 2 and 5 after all nonprecipitated protein had been eluted for 4 days. The increase in YD antigen-binding after immunization is evident as is the lack of antigen binding by the myeloma YD line near reservoir 7. The labelling of YD near reservoir 4 is due to co-precipitation of YD from reservoirs 1 and 3.

Figure 33

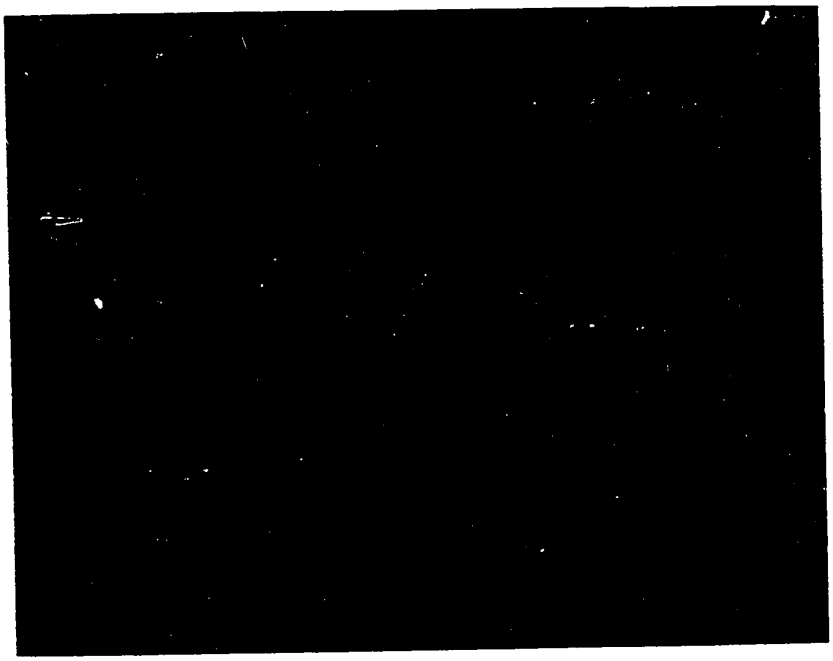


Radioimmunodiffusion showing YD binding of diphtheria toxoid in the healthy hyperimmunized subject having .12 mg/ml serum YD level. 1, YD 1 mg/ml; 2 and 5, concentrated antiserum to δ-chain; 3, concentr immune serum containing .80 mg/ml YD; 6, concentrated serum contain YD, obtained before first booster immunization; 4 and 7, saline. R diphtheria toxoid was placed in reservoirs 2 and 5 after all nonpre protein had been eluted for 4 days. The increase in YD antigen-bin immunization is evident as is the lack of antigen binding by the my line near reservoir 7. The labelling of YD near reservoir 4 is due precipitation of YD from reservoirs 1 and 3.

Figure 34

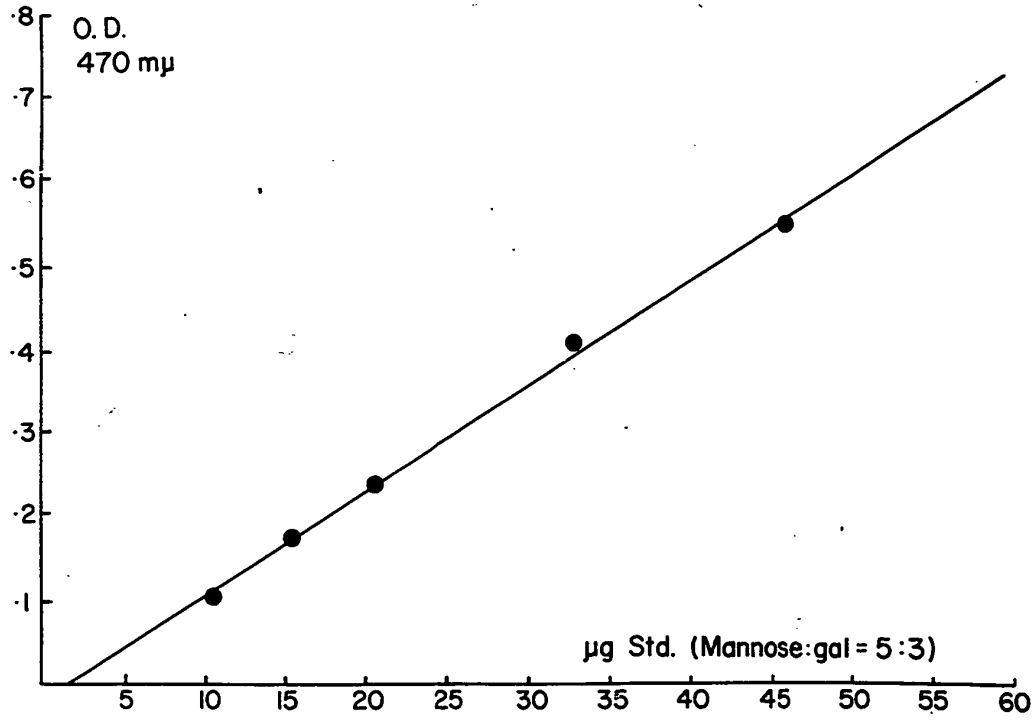
Plexiglas micro-Ouchterlony radioimmunodiffusion showing YD binding of bovine gamma globulin (BGG) in a milk sensitive subject who had YG and YA antibodies to BGG in high titre. 1, myeloma YD 0.12 mg/ml; 2 and 5, anti δ-chain; 3, serum of milk sensitive subject containing 0.15 mg/ml YD; 4, serum of a second milk sensitive subject containing less than 0.01 mg/ml YD; 6, serum of the same milk sensitive subject as in 3 but absorbed with one third volume of normal rabbit serum to remove any YG antibodies to BGG which might have cross-reacted with the rabbit gamma globulin in the YD precipitate line and subsequently bound the labelled bovine gamma globulin. Radiolabelled BGG was placed in reservoirs 2 and 5 after unprecipitated proteins had been eluted off. Specific binding of bovine gamma globulin by the serum in reservoirs 3 and 6 is evident in the radioautograph.

Figure 35



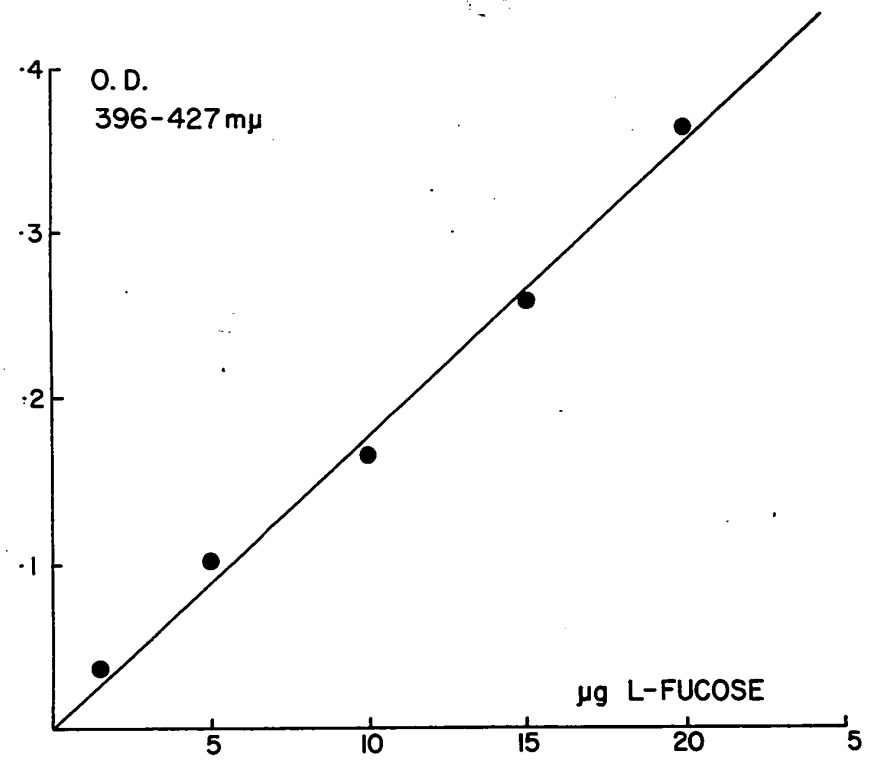
Antinuclear antibodies of the 7D variety.

Figure 36



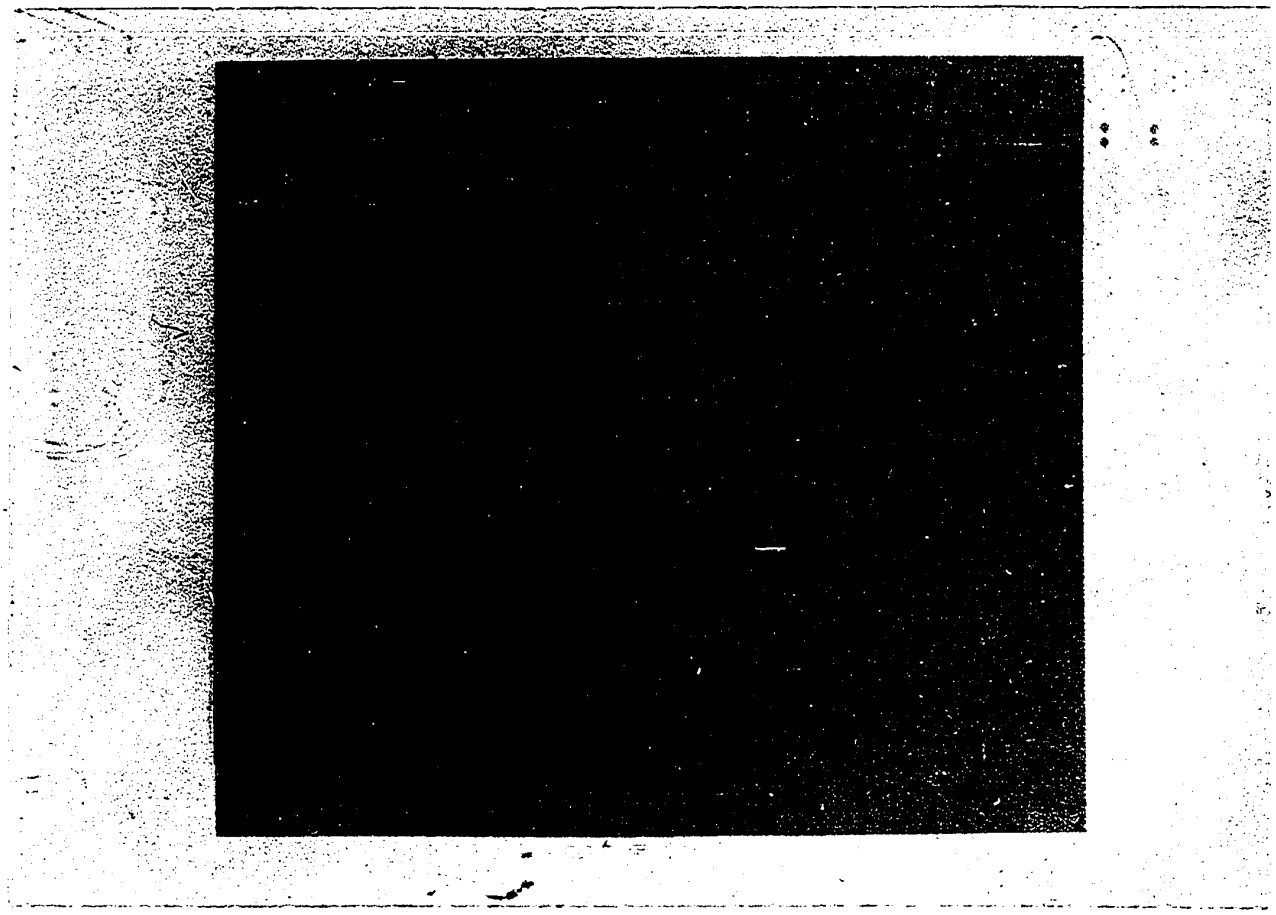
Standard curve for total hexose determinations.

Figure 37



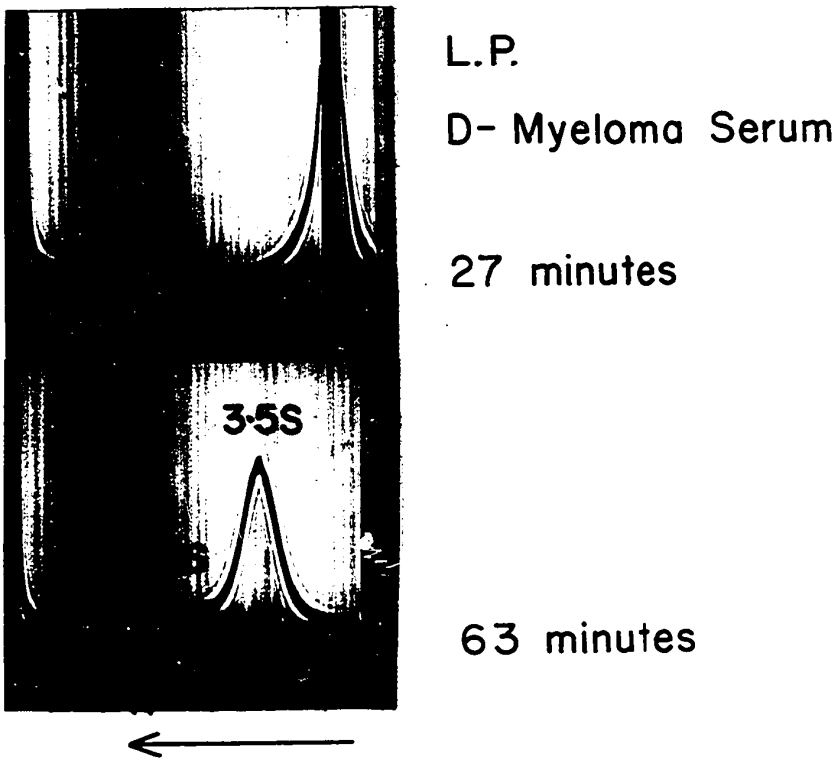
Standard curve for fucose determinations.

Figure 38



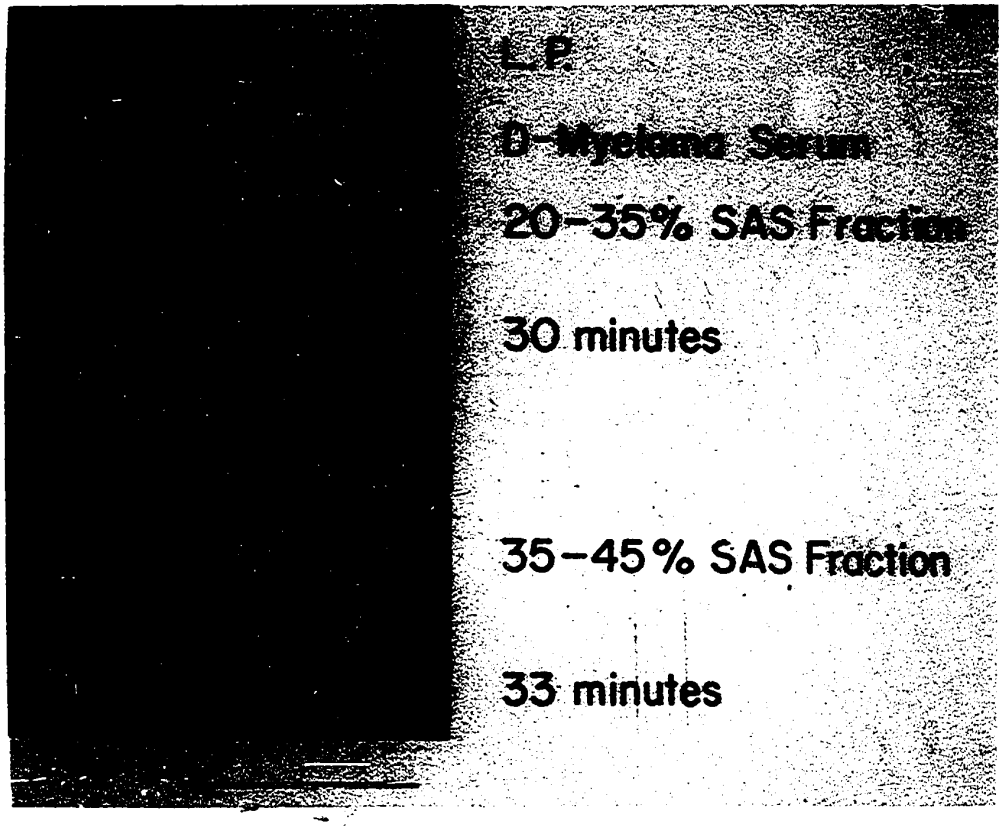
Analytical ultracentrifugation of D-myeloma serum. The Schlieren pattern is normal. 56,000 rpm.

Figure 38



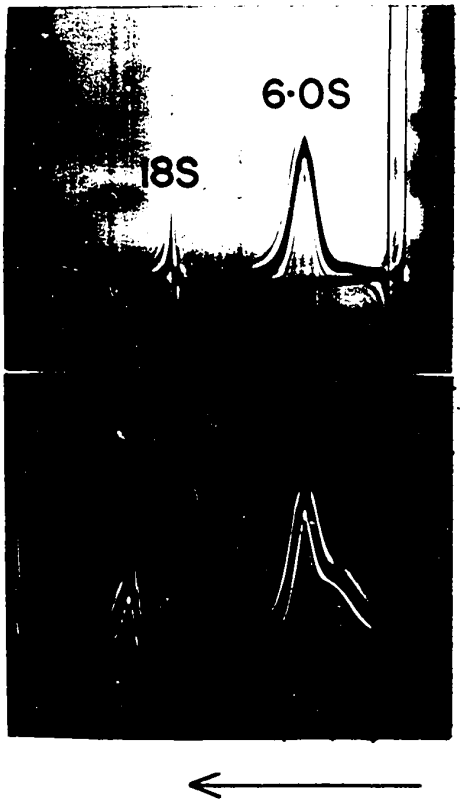
Analytical ultracentrifugation of D-myeloma serum. The Schlieren pattern is normal. 56,000 rpm.

Figure 39



Analytical ultracentrifugation of ammonium sulfate fractions of D-myeloma serum. The top pattern represents the fraction in Figure 11, the bottom pattern the fraction in Figure 12. 56,000 rpm.

Figure 39



L. P.

D-Myeloma Serum

20–35% SAS Fraction

30 minutes

35–45% SAS Fraction

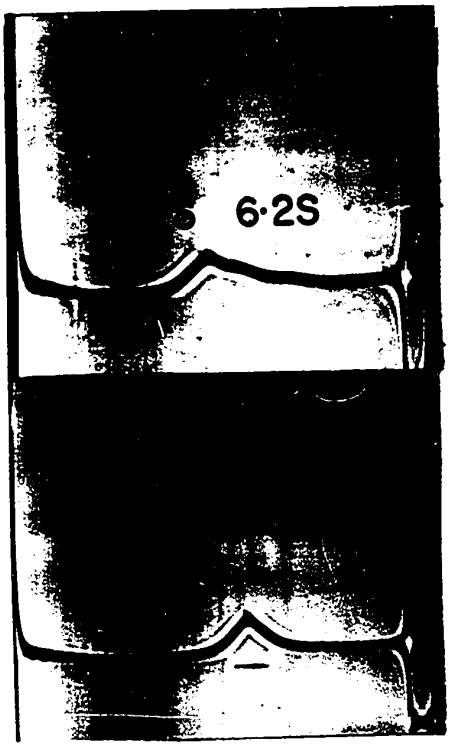
33 minutes

Analytical ultracentrifugation of ammonium sulfate fractions of D-myeloma serum. The top pattern represents the fraction in Figure 11, the bottom pattern the fraction in Figure 12. 52,000 rpm.

Figure 40

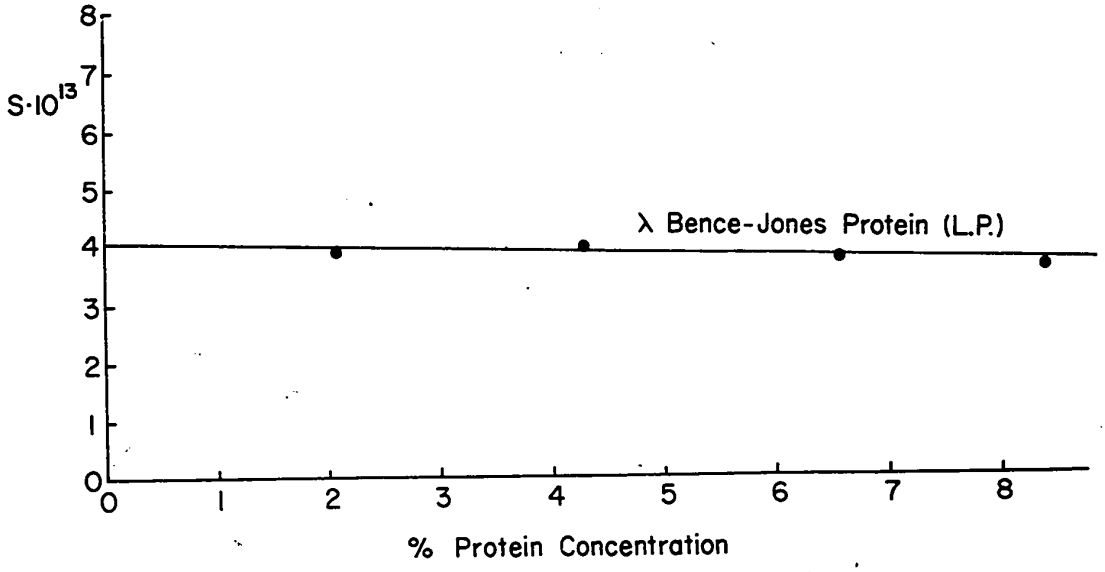
Analytical ultracentrifugation of peak 2 of Figure 12 demonstrates the 7 S 7D and the 6.2 S 7G components. Only a 6 S peak was evident in peak 3. 60,000 rpm.

Figure 40



Analytical ultracentrifugation of peak 2 of Figure 12 demonstrates the 7 S γD and the 6.2 S γG components. Only a 6 S peak was evident in peak 3. 60,000 rpm.

Figure 41



Sedimentation coefficient values of L.P. Bence-Jones protein at various solute concentrations.

Figure 42

Sedimentation equilibrium plots of 7D and 7G. The equilibrium run was carried out at 14,000 rpm for 7D and 16,000 rpm for 7G at 20°. ○—○ the initial protein concentration C_o ($A_{280\ m\mu}$) - 0.25 for 7D; □---□ C_o ($A_{280\ m\mu}$) - 0.25 for 7G. The theoretical slope was obtained for 7D with $\bar{v}$ = 0.72 cc/g and for 7G with $\bar{v}$ = 0.73 cc/g.

Figure 42

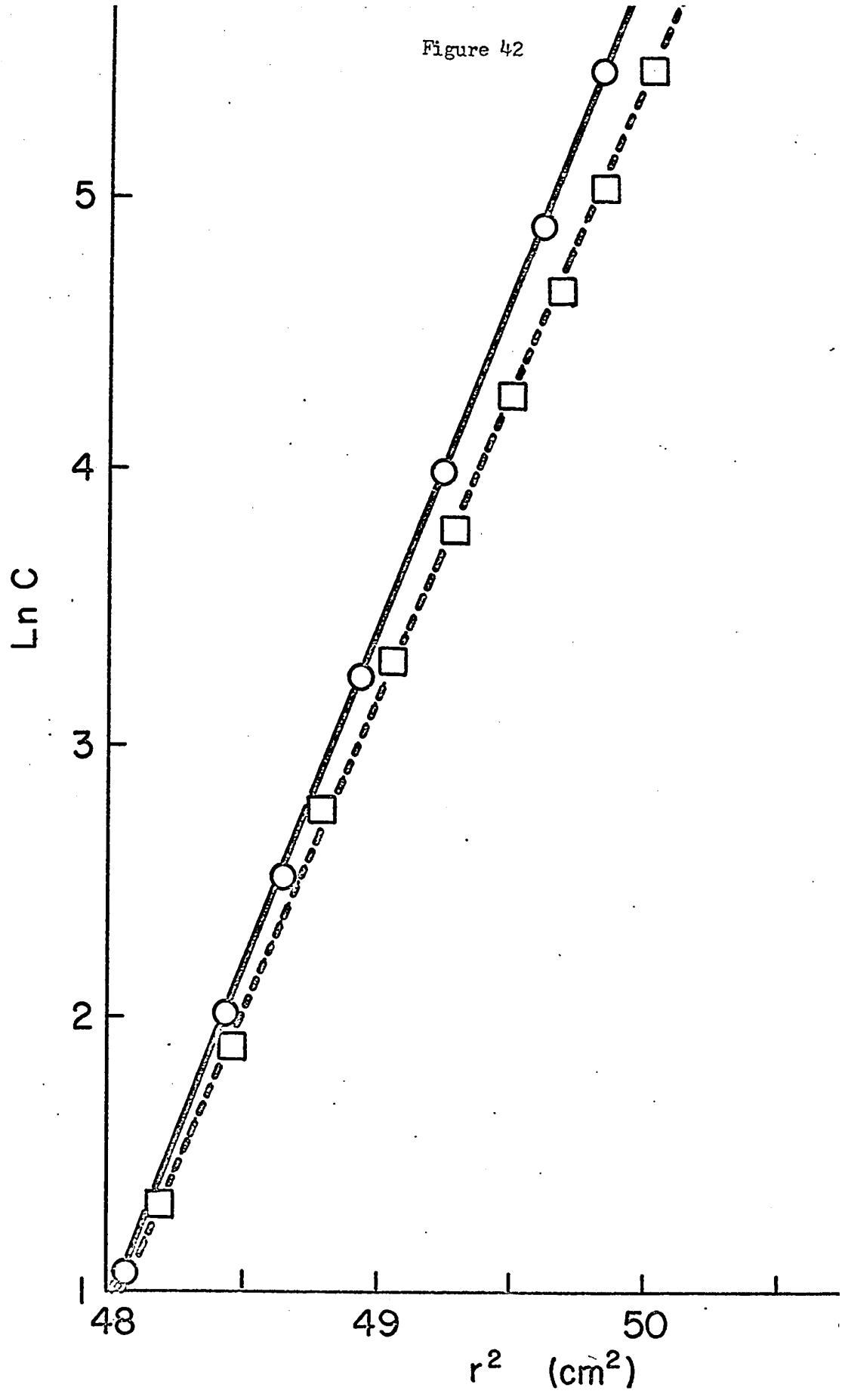


Figure 43

Sedimentation equilibrium plot of the larger fragment contained in a sample in which there was spontaneously degraded 7D. The equilibrium plot was obtained at 20°, with a rotor speed of 20,000 rpm and C_o - 0.254 ($A_{280\ m\mu}$). The partial specific volume, $\bar{v}$, was considered to be 0.72 cc/g.

Figure 43

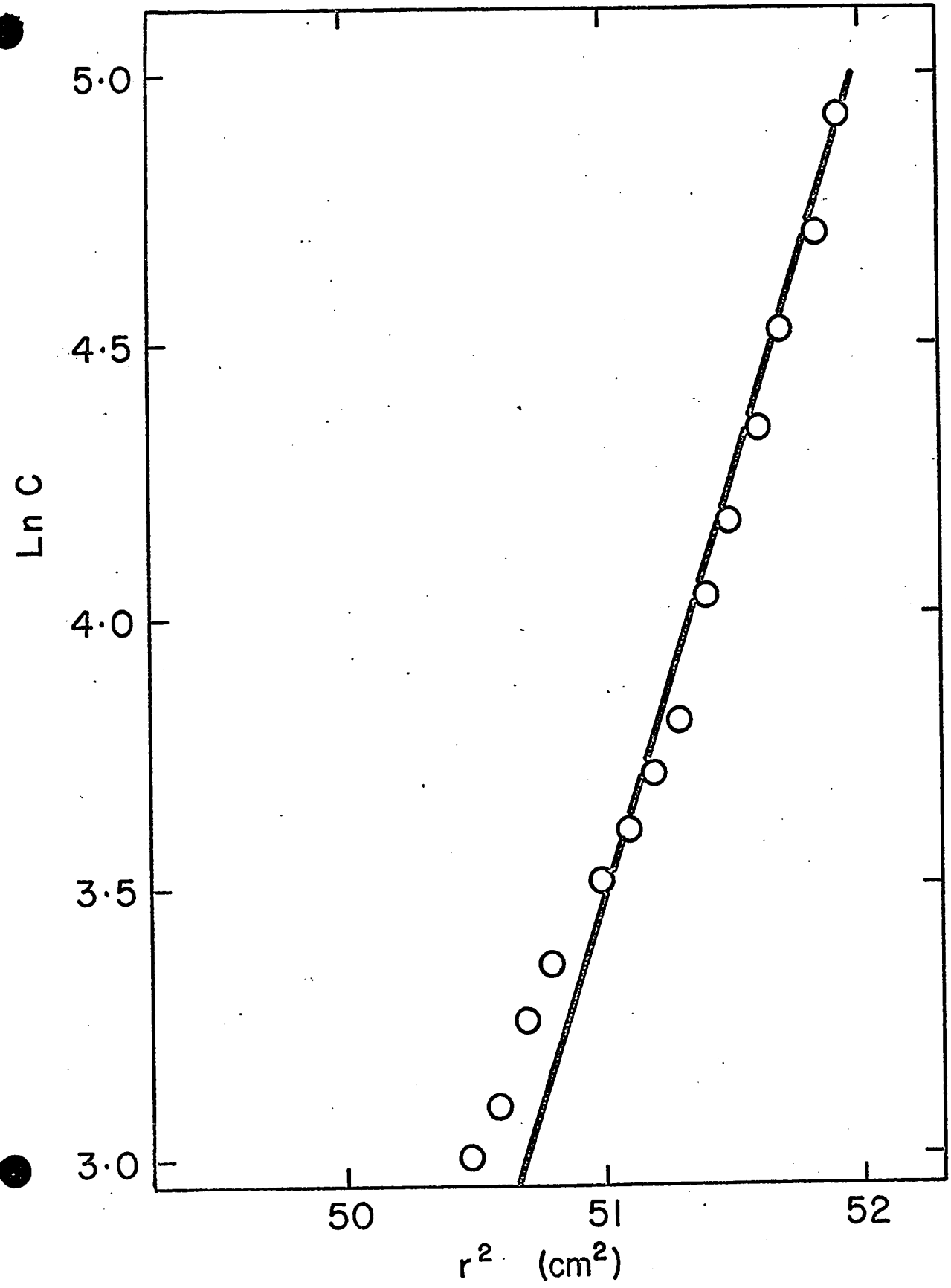
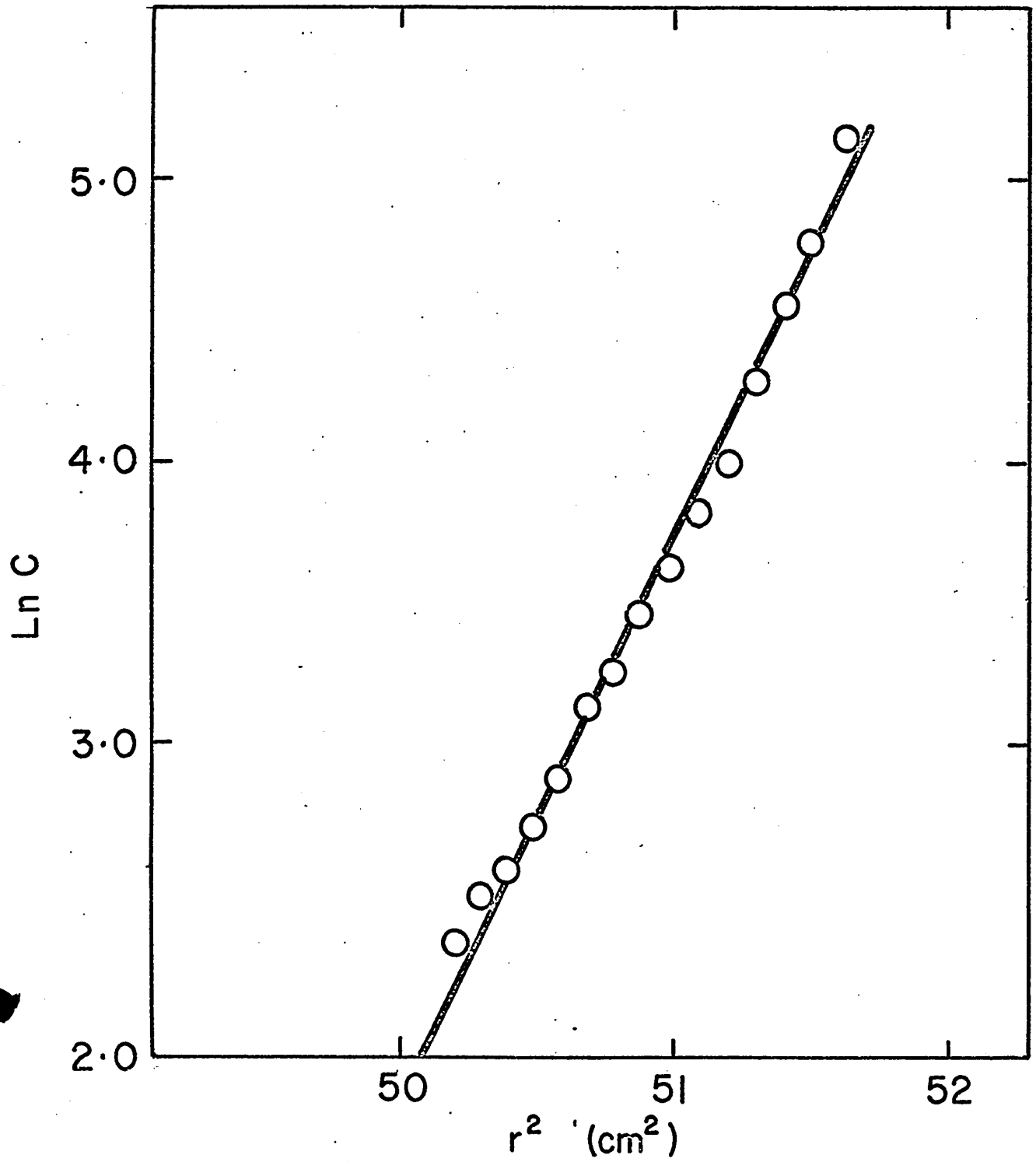


Figure 44

The mass distribution of the smaller fragments contained in spontaneously degraded 7D. The equilibrium plot was obtained at 20°, with a rotor speed of 24,000 rpm and C_o ($A_{280\ m\mu}$) - 0.50. The partial specific volume, $\bar{v}$, was assumed to be 0.72 cc/g.

Figure 44



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