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STUDIES OF THE INTERACTION BETWEEN RHEUMATOID FACTOR AND HUMAN γG IMMUNOGLOBULIN

by

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To my Wife without whose patience, and to my Children without whose impatience this work would not have been completed.

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INTRODUCTION

The existence of a class of human antibodies directed against autologous and isologous human gamma G immunoglobulin (γG)* has been recognized for some time. These substances were first found in patients with rheumatoid arthritis and were consequently termed "Rheumatoid Factor" (RF). It is now clear that these antibodies occur in individuals with a wide variety of other diseases characterized by inflammation, infection and/or granulomatous formation. They have in addition been detected in high incidence among the aged (1), and in mentally ill populations (2). They are also known to occur occasionally in healthy individuals. Indeed, there is evidence that more than half of individuals possessing RF do not have rheumatoid arthritis (3, 4). Since rheumatoid factor derived from all these various sources share in common the ability to react with some form of human γG, they have been also sometimes termed "anti γG factors" (AγG). In this report the two terms will be used interchangeably. Several useful general reviews have appeared on the rheumatoid factors (5, 6).

For the most part, the AγG factors belong to the class of serum proteins known as macroglobulins (γM)*. These are proteins of about 1×10^6 molecular wieght, of which about 10 per cent is carbohydrate in nature. Treatment of γM immunoglobulin with mercaptoethanol or other agents capable of reducing disulfide bonds results in splitting of the protein molecule to five subunits of about 1.6×10^5 MW (8). The subunits of γM are similar to but not identical with naturally occurring whole γG. One difference is

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* Both γG and γM globulin are classified as members of the immunoglobulin group of serum proteins. This group also includes the γA and γD globulins. Alternate nomenclature for these proteins are respectively IgG, IgM, IgA, and IgD (7).

that γG contains less carbohydrate (only about 2 per cent) than γM. Subunits of γM rheumatoid factor formed by reduction appear to lose their immunological reactivity. They will not agglutinate γG coated red cells, nor will they precipitate γG from solution, whereas the intact molecule does have these properties.

The location of the antibody active site on γM antibody molecules exhibiting anti γG activity is at present unknown. The reactivity and functional capacity of anti γG antibodies is apparently dependent on the integrity of the whole molecule as evidenced by the fact that dissociation of the γM molecule into subunits destroys anti γG activity. The subunits of γM as well as intact γG molecules are composed of four polypeptide chains; two "heavy" (H) chains, of MW = 5×10^4; and two "light" (L) chains, of MW = 2×10^4. The L chains are chemically similar to the Bence-Jones proteins frequently found in the urine of patients with multiple myeloma. Both H and L chains may be further subclassified according to antigenic types (for general reviews concerning the structure and antigenic characteristics of the immunoglobulins, see References 9 and 10).

In rare cases γG globulin possessing anti γG reactivity have been found (12). These constitute a very remarkable and possibly unique example of both antigen and antibody apparently consisting of the same type of protein.

By many criteria the rheumatoid factors are heterogeneous. For example gradient elution chromatography of rheumatoid factor sera (RFS) on DEAE cellulose has yielded five different active fractions (13). While the rheumatoid factors as a claas are all directed against γG, they appear to exhibit varying affinity for γG based on specific antigenic and/or physical characteristics of γG.

One aspect of the present work is concerned with the immunochromatographic fractionation of RFS and of anti γ sera raised in rabbits immunized with human γG, on columns of resins coupled to γG. It was reasoned that if the different RF subfractions do vary in their affinity for γG, then their chromatographic fractionation on columns containing γG should be possible.

The immunological heterogeneity of RF is a complex problem. For example, some of the rheumatoid factors are directed against various antigenic determinants on human γG. The controlling genes have been termed the Gm and InV alleles. There are some 14 Gm and 3 InV determinants, and no one individual posseses them all (11). The particular anti γG factors reactive with these determinants are isospecific rather than autospecific, that is, the factor in a particular individual is not directed against his own γG, but will react with γG from some other individual, notably his mother (14). The converse finding has also been made; for example, a mother may develop during the course of her pregnancy anti γG factors directed against antigenic sites on fetal γG (15). It appears that one cause of anti γG formation may be due to passage of γG across the placental barrier during the prenatal period. That γG, but not γM molecules can pass through the placental barrier, has been shown both in the anti γG system (15) as well as the RH antibody system (16).

Some rheumatoid factors have been shown to react with γG only when the latter is complexed to some other antibody (17). Still other RF fail to react with intact γG or with antigen-antibody complexes, but will react with γG when the latter has been degraded by partial enzymatic digestion (18). In these two cases the factors are directed against antigenic determinants "buried" in the intact γG molecule. When the γG molecule is split by

proteolytic digestion, or when its conformation is altered* by complexing with antibody, then specific antigenic sites are made available for reaction. It is alteration of the γG molecule which appears to be necessary for demonstration of reactivity with these rheumatoid factors.

The location of the various antigenic sites on γG against which the different anti γG factors are directed has only partly been elucidated. It has been established that the Gm locus is on the H chain, whereas the InV locus is on the L chain (11). RF appears to be directed primarily against sites on the H chain which are different from the Gm loci (20). The location of the "buried" antigenic sites with which certain anti-γG factors react, is unknown.

Anti γG factors found in patients with rheumatoid arthritis are characterized by still another pattern of specificity. For one thing, these factors crossreact with rabbit γG, and to a lesser extent with γG derived from other nonhuman species, whereas other AγG factors from patients without rheumatoid arthritis in general do not (5).

It is clear from these various observations that the rheumatoid factors obtained from different individuals, and even from the same individual, exhibit considerable immunological heterogeneity. This heterogeneity has posed great difficulty to the immunochemical study of RF. It became, therefore, the <u>first</u> purpose of this work to study the purification of RF and to devise a method for the subfractionation of the different RF found in a single individual.

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* Ishizaka and Campbell (19) have shown that the optical rotation of antigen-antibody complexes is greater than can be accounted for by the sum of the two components. These findings have been interpreted to mean that antigen and/or antibody are altered or denatured by their mutual interaction.

An important feature of RF reactivity is the fact that some RF react with human γG only when the latter is in an "aggregated" or denatured state (18). When pure monodispersed γG is added to an RF serum, no precipitin reaction occurs. If, however, the γG solution is heated to turbidity and added to RF, then copious precipitation results. The apparently nonreactive γG has an S_{20} value of about 7, but the reactive turbid thermal aggregates have S_{20} values ranging from 20 to 40, depending on the heating time and temperature employed (21).

In view of these findings and in view of the fact that naturally occurring in vivo aggregates of γG are extremely rare (22), and occur with apparently much less frequency than the incidence of RF in the population, it is difficult to see how most RF could be immunologically directed against naturally aggregated human γG globulin.

Another purpose of this work was to examine the possibility that reactivity of RF with thermally aggregated γG is primarily a consequence of denaturation which must precede the aggregation step. It is suggested here that RF is actually directed against antigenic sites on human γG which have been made available by molecular unfolding due to denaturation. It would appear, also, that aggregation does further enhance the reactivity of γG with RF, possibly because of the decreased solubility of RF-aggregated γG complexes.

If in fact denatured γG is the antigen for RF, one might question how denatured γG arises in vivo. This is a biological problem that lies outside the scope of this study. However, the denaturation phenomena that appears to accompany antigen-antibody reactions may suggest one possibility (19).

While the view advanced here regarding the reactivity of RF with aggregated γG as compared to denatured γG are not entirely new, evidence for support is scanty. It was a second purpose of this investigation to study the interaction between human anti γglobulin (rheumatoid factors), and human γglobulin in terms of the physical state of γG. To this end the reactivity of various anti γG preparations with purified γG, denatured γG, and aggregated γG have been compared and contrasted.

There is evidence to indicate that some RF, at least from patients with rheumatoid arthritis, is autospecific rather than isospecific, that is, it is directed toward a component of the patient's own γG. For example, in certain high titered RF sera, the RF is not found in the 19 S macroglobulin peak; rather it is found as a 22 S peak which consists of a complex between the 19 S RF and autologous 7 S G (23).

A third objective of this work, therefore, was to find additional experimental means to detect and study autospecific interactions between the RF in a given individual and his own γG globulin. The interactions between RF and various forms of human γG has been studied both here and in other work by various standard immunochemical techniques such as precipitin and agglutination reactions. Such techniques depend on secondary phenomena to demonstrate the primary antigen-antibody reaction. It was, therefore, deemed of interest to study techniques which directly relate to the primary reaction.

One such technique is that of fluorescence polarization (P). This technique is based in great measure on the work of G. Weber (24, 25, 26). Fluorescence polarization is based on the following general considerations:

The Brownian motion of large colloidal molecules such as proteins is affected by their size and shape. If a protein molecule is fluorescent,

either naturally or by virtue of chemically attached fluorescent groups, then the polarization of its fluorescence is related to its rotational and translational motion. Thus measurement of P can yield relative data about size and shape of macromolecules. In addition, interaction between different species, aggregation between identical species, or conformational changes can also be followed by changes in P.

Among the applications of P which have appeared in the literature are studies of micellar formation of soaps (27, 28, 29), protein conformation (30), monomerdimer transformation of actin (31), denaturation of rabbit γG (32), thyroglobulin (33), and growth hormone (34). Of particular relevance to this work is the study of Haber and Bennet (35) of various antibody-antigen interactions. They were able to show enhancement of P values in such reactions, with maximum values found at the equivalence point of the reaction. Dandliker and co-workers also employed P to demonstrate and quantitate antialbumin (36), and antipenicillin (37) antibodies.

Based on these considerations, it seemed that P measurements could be used to study the primary event in antigen-antibody reactions. Thus the fourth purpose of this study was to examine the use of P as a technique for studying interactions between RF and γG in its various forms.

To sum up, this study is concerned with four major problems:

1. Purification and fractionation of rheumatoid factors.
2. The role of denaturation and aggregation in enhancing reactivity of γG immunoglobulin with rheumatoid factor.
3. Detection of autospecific reactivity between individual rheumatoid factors and autologous γG.
4. Application of the technique of fluorescence polarization to these studies.

MATERIALS AND METHODS

RHEUMATOID FACTOR

The source of rheumatoid factor (RF) in this study was sera obtained from persons with definite rheumatoid arthritis.* Both pooled and individual RF sera were employed. Purification of RF was in general done by gel filtration of RF sera through Sephadex G-200 (see "Gel Filtration"). In some cases a preliminary globulin fractionation of the RF serum was performed with half saturated ammonium sulphate, or by 15fold dilution of the serum with cold distilled water. In either case the resulting globulin precipitate included all RF activity.

ASSAY OF RHEUMATOID FACTOR ACTIVITY

In the present work serological titrations were usually employed to assay relative RF activity. Such procedures employ as reagent an antigen (γG) coated suspension of particulate material such as latex particles, erythrocytes, etc., which is mixed with amounts of a twofold serial dilution of the test serum. The highest serum dilution capable of clumping or agglutinating the sensitized particles is referred to as the serum titer.

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* Rheumatoid arthritis is a systemic disease affecting primarily connective tissue. Joint inflammation is the dominant clinical feature. Inflammatory changes occur in the joint synovial membrane leading to thickening and later to erosion of this tissue. In advanced disease erosion affects articular cartilage and bone, and may result in total destruction of the original joint surfaces. The joint spaces may be infiltrated by fibrous tissue and ultimately by bone, completely obliterating the original joint. During active disease, systemic evidence of inflammatory process includes elevated erythrocyte sedimentation rate, C-reactive protein, and leukocytosis. Serum protein analysis shows decreased albumin and increased globulin, primarily in the α and γglobulin fractions. Rheumatoid factor is found in serum of 70 per cent to 80 per cent of patients with "definite" or "classical" disease. The relation of RF to either the etiology of pathogenesis of rheumatoid arthritis is unclear (38).

SLIDE LATEX TEST

Qualitative detection of RF in human sera was performed by means of the slide latex test, employing a commercial latex reagent, in most cases that made by the Hyland Co. ("RA-Test," latex globulin reagent, Hyland Division, Travanol Labs., Los Angeles, California). This reagent consists of a suspension of polystyrene latex particles to which human γG immunoglobulin is adsorbed. Analysis of the Hyland reagent revealed the following composition: latex solids 4.0 mg/ml, nonadsorbed protein 0.58 mg/ml, and adsorbed protein 0.2 mg/ml. The use of γG coated latex particles for detection of RF was first suggested by Singer and Plotz (39). These workers used a monodispersed latex suspension with a particle size of 0.8 micron diameter. Electron microscopy* of the Hyland reagent revealed that it contained a heterogeneous dispersion of latex particles, with a mean diameter of about 3×10^{-1} microns.

The test is performed by mixing one drop of a 1:20 dilution of serum in glycine buffer pH 8.2 and one drop of latex reagent on a clean glass slide. The drops are mixed with a clean wooden splint or toothpick, and gently rotated by hand for one minute. A positive test is indicated by clumping of the latex particles, whereas a negative test is indicated by a smooth homogeneous dispersion of the particles.

TANNED SHEEP CELL TEST

The tanned sheep cell test (TSC) is based on the method of Boyden (40) as modified by Heller et al (41), except that a glycine buffer pH 8.2 containing 0.15 M NaCl was used for all washing and dilution. A solution of

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* Courtesy Dr. S.K. Song, Department of Neuropathology, The Mount Sinai Hospital.

buffered tannic acid (1:20,000 w/v) was added dropwise with stirring to an equal volume of 4X washed 33.3 volume per cent suspension of sheep erythrocytes and incubated at 37°C for 10 minutes. The tanned cells, after one more washing, were resuspended to 33.3 volume per cent concentration in buffer and were sensitized at 37°C for 30 minutes with two volumes of a 2.5 g/100 ml solution of human γG (Squibb, Fraction II, #1812). The sensitized cells were then centrifuged and washed with three volumes of buffer. The washed cells were finally suspended in 400 ml of buffer per 1 ml packed cells (0.25 volume per cent) for use.

All test sera, unless otherwise stated, were heated at 56°C for 30 minutes to inactivate complement and then adsorbed overnight in the cold with an equal volume of 4X washed, nonsensitized tanned sheep cells. This procedure served to remove heterophil and related antibodies frequently present in human serum, and which react with antigenic sites on red blood cells. Adsorption did not affect RF activity.

To 0.5 ml amounts of a twofold serial dilution of the adsorbed and inactivated test serum in 13 x 100 mm test tubes was added 0.5 ml of the 0.25 per cent sensitized tanned sheep cell suspension. After mixing, the tubes were kept overnight in the cold. The test was then read according to the pattern of the sedimented cells (42).

As control a serial dilution of the test serum was also tested with 0.25 per cent washed uncoated tanned sheep cells. This was done to demonstrate complete removal of heterophil antibodies.

SENSITIZED SHEEP CELL TEST

The sensitized sheep cell test (SSC) is based on the method of Rose, Ragan, Pearce and Lipman (43). In this procedure, washed sheep cells

are sensitized, not with adsorbed human γG as in the TSC procedure, but with rabbit γG in the form of rabbit anti-sheep red cell antibodies. Used for this purpose was a rabbit anti-sheep cell gycerinated antiserum ("amboceptor, lot 61"), obtained from Mayer and Myles Labs., Allentown, Pa. The agglutination titer of this antiserum, with 2 per cent washed uncoated sheep red cells, was 1:1000. A subagglutinating dilution (1:2000) of the antiserum was prepared and added to an equal volume of 2 per cent 4X washed untanned sheep red cells. The mixture was kept at 4°C for 30 minutes prior to use. This procedure served to fix rabbit γG to the red cell surface without causing agglutination of the cells. However, hemolysis did occur about 48 hours after preparation. Therefore, sensitized cells were freshly prepared prior to use.

Test sera were adsorbed, inactivated and diluted as in the TSC procedure. Equal volumes of the now 1 per cent sensitized sheep cell suspension and test serum dilutions were mixed and kept overnight in the cold. Next day the test was read by gently tapping the tubes, and noting clumping or agglutination of the cells.

As control, a serial dilution of the test serum was also tested with 1 per cent washed uncoated sheep cells, in order to demonstrate removal of heterophil antibodies.

BLOCK TITRATION PROCEDURE

For each serum to be tested, six twofold serial dilutions (0.5 ml volume) were prepared, starting at a dilution of 1:10. Most consistent results were obtained by preparing one serial dilution of the serum under study, in a volume of 3 ml, and from this, preparing six rows with each tube containing 0.5 ml of diluted serum. To each of the six rows was added

0.5 ml of several tanned cell suspensions. The mixtures were incubated at 37°C for 30 minutes and refrigerated overnight. The next day, rows with 0.5 per cent, 0.25 per cent, and 0.125 per cent cells were read by pattern (42). The rows with 2.0 per cent, 1.5 per cent, and 1.0 per cent cells were read visually for agglutination after gently shaking. Both weak (plus-minus) and distinct agglutinations were recorded.

All values reported as TSC titers are those obtained with 0.25 per cent cell suspension. Titers are recorded as the highest dilution yielding complete agglutination. In some instances the next tube of dilution exhibited weak or partial (±) agglutination. Such a plus-minus tube is denoted in the tables by a plus sign (+) following the titer value.

GRAPHICAL ANALYSIS OF DATA

The six titers obtained for a given serum were plotted on double logarithmic paper as a function of cell concentration. In most cases, a straight line could be drawn throughout the plotted points. It was sometimes observed that some titration end points when followed by a plus-minus tube did not fall on a straight line. If, however, the mean of the logarithm of the apparent end point titer and the plus-minus titer was plotted instead of the apparent end point titer, then the fit was nearly always much better. Even with this correction, two sera did not exhibit linearity and these were not further considered in this study. The straight line was constructed visually from the six titration values, and the slope of this line was calculated.

Slope values are reported in the present study to one decimal place. In general, slope values varied on duplicate determination no more than ± 0.1. Significant differences between slope values are therefore taken as those that differ by more than 0.2 slope units.

Sera to be studied were examined for evidence of heterophil antibody by titration with 0.25 per cent tanned uncoated sheep cells (USC). Differential titers TSC/USC ranged from 8 to 512. It was found that removal of heterophil antibody by adsorption of the serum with packed sheep cells was without effect on the slope value obtained. Similarly, inactivation at 56°C for 30 minutes was also without effect on slope values, so that sera examined by the block procedure were studied in general without prior inactivation or adsorption.

INHIBITION OF AGGLUTINATION

This procedure is designed to measure the relative reactivity of a particular γG preparation in terms of its ability to inhibit agglutination by a standard rheumatoid factor preparation in the TSC test. To 0.2 ml of a dilution of a standard RF serum was added 0.2 ml in serial dilution of the γG preparation to be studied. The mixture was allowed to stand for one hour at room temperature, then 0.4 ml of 0.25 per cent γG coated tanned sheep cells was added. Tubes were kept overnight in the refrigerator. The next day results were read by pattern (46), and expressed as the minimum concentration of γG sufficient to inhibit agglutination.

Dilution of the standard RF serum was determined by preliminary titration and generally chosen at 1/10 of the serum agglutination titer.

PURIFICATION AND FRACTIONATION OF HUMAN γG IMMUNOGLOBULIN (γG)

GAMMA G IMMUNOGLOBULIN

Human γG (Squibb, Cohn Fraction II Lot 1979, obtained through the courtesy of the American National Red Cross), was employed in these studies. This preparation contained about 1 per cent of non γG protein impurities, and about 7 per cent of the γG was in an aggregated form.

SALT FRACTIONATION

To five volumes of a 2.0 g/100 ml γG solution in 0.15 M NaCl at room temperature, two volumes of 2.18 M Na_2SO_4 was added to yield a final concentration of 0.62 M Na_2SO_4. This served to precipitate aggregated γG which was removed by centrifugation. Aggregated γG, when used, was purified by 2x reprecipitation. Three volumes of 2.18 M Na_2SO_4 were added to the supernatant after aggregate removal, to yield a concentration of 1.55 M Na_2SO_4. The precipitate was recovered by centrifugation, dissolved in 0.15 M NaCl and exhaustively dialyzed against 0.15 M NaCl to remove added Na_2SO_4. This preparation was pure 7 SγG free of aggregates and non γG proteins. The 7 SγG was stored frozen in 10 ml aliquots until used.

DEAE CHROMATOGRAPHY

For preparation of smaller amounts of purified 7 SγG, chiefly from individual sera, chromatography on diethylaminothethylcellulose (DEAE) columns (20 x 2 cm) was employed. 2.0 ml of whole serum was applied to the column which was then developed with an 0.02 M pH 6.3 phosphate buffer. Under these conditions γG was preferentially eluted. Subsequent elution with 1 M NaCl yielded other serum protein components (44).

GEL PERMEATION CHROMATOGRAPHY (GEL FILTRATION)

This technique was found very useful for preparing both γG and RF, especially from the same serum. Columns of Sephadex G-200 (5 x 100 cm) were employed for this purpose.

The dry Sephadex G-200 powder was suspended in 40 volumes of phosphate pH 7.0 buffer containing 0.5 M NaCl. This mixture was heated to about 80°C

for one hour and then allowed to stand in the cold room overnight. This procedure hastened swelling of the Sephadex granules and insured removal of trapped air. The hydrated slurry was then poured into a 100 x 5 cm column and allowed to slowly settle. Additional slurry was added after settling to fill the column. This was done with slight stirring to eliminate boundaries in the packed gel.

In early experiments, serum samples were placed at the top of the column, which was then eluted downward with M/15 phosphate buffer at pH 7.0, containing 0.5 M NaCl. This proved unsatisfactory as the gel tended to compress, causing the buffer flow rates to decrease.

Better results were subsequently obtained by an upward flow technique. The column was equipped with upward flow adapters, a three-way valve, and a LKB Model 4912A peristaltic pump. Serum to be fractionated was pumped into the bottom of the column, followed by eluting buffer. A pumping rate of 12 ml/hr was used. Effluents were collected in 5 ml aliquots on a timed fraction collector (Buchler). Protein content was determined at first by manual ultraviolet spectrophotometry (Beckman DU spectrophotometer), and later by an automatic monitor (LKB Uvicord II, Model 8300, with LKB Recorder Model 6520A) (see "Results").

IMMUNOCHROMATOGRAPHY

POLYAMINOSTYRENE (PAS)

This material was obtained from Norsk HydroElektrisk, Oslo, Norway. According to the manufacturer the polymer has an average molecular weight of 200,000-300,000 and consists of about 70 per cent of poly-p-aminostyrene and about 30 per cent of poly-p-amino-o-nitrostyrene, poly-o-p-dinitrostyrene,

and poly-o-p-diaminostyrene. Elemental analysis (performed by M. Manzer, Postfach Basel-2, Switzerland), of the PAS yielded 11.6 per cent nitrogen, corresponding approximately to one amino group per monomer residue.

PREPARATION OF POLYAMINOSTYRENE COUPLED TO HUMAN γ-GLOBULIN (PAS-γG)

The conjugates between PAS and γG were prepared essentially by the method of Gyenes and Sehon (44). Ten grams of PAS were suspended in about 80 ml of cold 3 N HCl. To this chilled mixture, 40 ml of cold 20 g/100 ml $NaNO_2$ was added dropwise with constant stirring. The mixture was kept overnight at 5°C. The following day the diazotized resin was filtered on a Buchner funnel and washed sequentially with 200 ml each of: 5 per cent sodium acetate, 5 per cent urea, 0.15 M sodium chloride, and pH 8.0 borate buffer. The washed diazotized resin was suspended in 200 ml of borate buffer, and to this suspension 1.0 of γG dissolved in 50 ml of borate buffer was added dropwise with stirring. The pH was adjusted from time to time to 8.0 with a few drops of 2 M K_2CO_3, and the mixture stirred for 24 hours. The PAS-γG resin conjugate was then filtered on a Buchner funnel, and the filter cake was washed with 400 ml of 0.15 M NaCl; then with sufficient pH 3.2 citrate buffer until washes were protein free, and finally with 400 ml of pH 8.2 glycine buffer. The washed resin was then suspended in 200 ml of glycine buffer and heated at 55°C for about eight hours to decompose unreacted diazonium groups.

For chromatography, the PAS-γG was suspended into 20 x 2 cm columns. Details of the immunochromatographic fractionation of RF is described under "Results."

FLUORESCENCE AND OTHER PHYSICAL TECHNIQUES

PREPARATION OF γG TAGGED WITH DNS

The procedure employed was similar to that of Steiner and Edelhoch (46). However, during initial phases of this work, a number of problems were encountered in preparing γG labelled with dimethylamino-naphthalene sulfonate (DNS). The technique which finally yielded adequate tagging of DNS to γG, and removal of unreacted DNS as well as of aggregated γG, is outlined as follows: 10 grams of human γG (Squibb Fraction II Lot 1979) was dissolved in 500 ml 0.15 M NaCl. As γG is slowly soluble, this was done by sprinkling the dried protein on the surface of the liquid and slowly stirring the mixture overnight in the cold. Next day insolubles were removed by centrifugation and the γG solution was adjusted to about pH 7.5-8 by adding 2 M K_2CO_3 dropwise with stirring. A cold solution of 300 mg of dimethylamino-naphthalene sulfonyl chloride in 20 ml of acetone was then added dropwise with stirring in the cold room to the γG solution. This mixture was slowly stirred for 48 hours in the cold, with occasional adjustment of pH to about 8. After 48 hours, insoluble material was removed by centrifugation.

At this stage, the solution contained in addition to DNS- 7 S γG, a number of impurities and undesired side products, Eg : DNS- aggregated γG; free hydrolyzed DNS, and hydrolyzed DNS adsorbed to aggregated γG. Purification was effected by adding at room temperature two volumes of 2.18 M Na_2SO_4 to 5 volumes of crude DNS- γG solution to a final concentration of 0.64 M Na_2SO_4. This served to precipitate aggregated γG. The supernatant was reduced to about one tenth of original volume by dialysis against dry

sucrose, and then filtered through a 6 x 50 cm Sephadex G-75 column. The free DNS was retained near the top of the column as a green fluorescent band, whereas the tagged protein was eluted with 0.15 M CaCl, which could be seen passing through the column as a yellow fluorescent zone. The yellow column eluate was then dialyzed with stirring against several portions of 0.15 NaCl to remove sodium sulphate, sucrose and traces of untagged DNS. The purified DNS- γG was stored frozen in 10 ml aliquots until used. Freshly purified DNS- γG was devoid of aggregates as judged by the Tyndall effect and ultracentrifugation, and yielded one line on immunoelectrophoresis against an antihuman serum antiserum.

Quantitation was done by ultraviolet spectrophotometry (Beckman DU) with γG measured at 280 mμ (E, γG, 1 mg/ml = 13.3), and DNS at 330 mμ (E, DNS, mM/ml = 4.3×10^3) (45) (see "Results").

FLUORESCENCE MEASUREMENTS

Fluorescence measurements were made with an Aminco-Bowman Spectrophotofluorometer, Model 4-8202, equipped with a Grace microphotometer, Model 380. The Grace photometer was employed in place of Aminco photometer, because the former has a damping circuit useful in suppressing "noise" and meter flickering at high gain setting. For most measurements, "arrangement #5" (46) of the eight collimating slits was employed.

FLUORESCENCE POLARIZATION

For fluorescence polarization (P) measurements, a jacketed cell compartment equipped with two Glan-Thompson polarizing prisms, one in the incident light path, and one in the emitted light path was employed. In order to obtain temperature control, thermostatted water was circulated

through the cell compartment as well as through specially designed quartz sample cells. These cells were jacketed in such a way that neither incident nor emitted light passed through the double wall or the circulating water (Fig. 2). Sample temperature in the jacketed cell was determined with a thermistor resistance probe thermometer (Tele-Thermometer, Model 42-SC).

The Glan-Thompson prisms are used in one of two settings, e or b. Fluorescence measurements were made at the four possible combinations of the two prisms; ee, eb, bb, and be. The polarization (P) value was calculated from the following relationship: (where I is fluorescent intensity)

$$P = \frac{Iee - Ieb\ (Ibe/Ibb)}{Iee + Ieb\ (Ibe/Ibb)} \qquad \text{Eq. (1}$$

The arithmetic solution of this equation for large numbers of measurements was found to be very tedious. Accordingly a simple computer program* was devised and calculations performed with it using an Olivetti Programma Desk Computer, Model 101.

Fluorescence measurements of systems containing human γG coupled to DNS were done at excitation and emission wavelengths of 350 mμ and 510 mμ, respectively.

VISCOSITY

Viscosity measurements were made with a Cannon-Manning Semi-Micro Viscosimeter, Model 100, A75 (flow time for water about 60 seconds), in a constant temperature water bath at 30.0°C ± 0.05°C. Before use, the

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* The program in the symbolism of the computer is AV, MS, M↓ MS, M÷, B↕, MS, b↑, MS, b↑, MS, b↑, MS, c↑, c↓, BX, B↕, B↓, b↙ +, C↕, b↓, B−, C÷, A◇, MV, V. Data are entered in the following order: Ibe, Ibb, Iee, and Ieb.

viscosimeter was cleaned with sulfuric-chromate cleaning solution, then rinsed with tap water and finally with distilled water to a constant water outflow time. All samples prior to measurement were centrifuged to remove particulate matter. Flow times were determined in triplicate to 0.1 second. Under optimum conditions, flow times varied less than $\pm$ 0.2 seconds.

TURBIDITY

Nephelometric measurements were made with a Beckman DU spectrophotometer. Turbidity was measured as the apparent absorbance (optical density) of the particle suspensions, over a wavelength range where true absorbance was negligible. Latex particles and aggregates of human γG were measured between 400 and 700 mμ. Turbidity (T) data were plotted as an inversefunction of wavelength (ζ) according to the relation:

$$T = K/\zeta^n \qquad \text{---- Eq. (2}$$

where n is an integer between 0 and 4. Data were tested for graphic linearity for different values of n. For such plots, T was converted to extinction values (E) where E = optical density/mg/ml.

LIGHT SCATTERING PHOTOMETRY

Absolute turbidity measurements for γG were made at 25°C in a precalibrated Brice Phoenix Model 2000 Universal Light Scattering Photometer using incident unpolarized light, at a wavelength of 435.8 mμ. Temperature control was achieved by circulating thermostated water through a special cored light scattering cell base plate and coiled jacket surrounding the cell. Refractive index increments (dn/dc) of samples were determined with a Brice Phoenix Differential Refractometer, Model BP-200-V.

OPTICAL ROTATION

Optical rotation measurements were made with a Schmidt and Haench polarimeter using sodium D light. Results are reported as specific rotation, i.e., observed rotation X 100/path length (2dm) X concentration (g per 100 ml).

BUFFERS

Various buffers were employed in these studies. Their composition is tabulated below, and are based on dilution with distilled water to a final volume of 1 L, and an ionic strength of 0.1. Unless otherwise stated higher ionic strengths were obtained by adding NaCl.

Nominal pH	Measured pH	Buffer Type	Composition
1	1.0	HCl	50 ml 2N HCl
2	2.0	HCl	5.0 ml 2N HCl + 5.26 g NaCl
3	3.2	citrate	25.48 g citric acid + 50 ml 2N NaOH
4	3.96	acetate	37.9 ml glacial HAc + 50 ml 2N NaOH
4.5	4.50	acetate	16.0 ml glacial HAc + 50 ml 2N NaOH
5	4.91	acetate	9.3 ml glacial HAc + 50 ml 2N NaOH
5.25	5.25	acetate	7.54 ml glacial HAc + 50 ml 2N NaOH
5.5	5.50	acetate	6.74 ml glacial HAc + 50 Ml 2N NaOH
6	6.00	phosphate	9.00 g $NaH_2PO_4.H_2O$ + 1.63 g Na_2HPO_4
6.3	6.30	phosphate	2.15 g NaH_2PO_4 + 0.625 g Na_2HPO_4(0.02M)
7.5	7.50	phosphate	1.20 g KH_2PO_4 + 10.32 g $Na_2HPO_4.2H_2O$ + 5.85 g

Nominal pH	Measured pH	Buffer Type	Composition
6	6.04	borate-phosphate	123 ml A + 877 ml B (0.094M)
7	7.01	borate-phosphate	390 ml A + 610 ml B (0.081M)
8	8.13	borate-phosphate	550 ml A + 450 ml B (0.072M)
9	8.97	borate-phosphate	868 ml A + 132 ml B (0.067M)
8.2	8.2	glycine	7.507 g glycine + 10.0 g NaCl + 2.5 ml 1N NaO
7	7.0	"Tris"	13.34 g "Tris" + 50 ml 2N HCl
8	8.0	"Tris"	25.1 g "Tris" + 50 ml 2N HCl
9	9.0	"Tris"	33.2 g "Tris" + 50 ml 2N HCl
9	8.93	carbonate	20 ml C + 230 ml D (0.05M)
9.6	9.63	carbonate	80 ml C + 170 ml D (0.05M)
10	9.99	carbonate	138 ml C + 112 ml D (0.05M)
10.6	10.89	carbonate	212 ml C + 38 ml D (0.05M)
12	12	NaOH	5.0 ml 2N NaOH + 5.26 g NaCl
13	13	NaOH	5.0 ml 2N NaOH

A: Sodium Borate, 19.1 g/L (0.05M)

B: KH_2PO_4 13.6 g/L (0.1M)

C: Na_2CO_3 21.2 g/L (0.2M)

D: $NaHCO_3$ 16.8 g/L (0.2M)

RESULTS AND DISCUSSION

I. FRACTIONATION AND PURIFICATION OF RHEUMATOID FACTOR

GEL PERMEATION CHROMATOGRAPHY (GPC)

The general technique employed has been described under "Methods." Figure 3 represents the results obtained when 5.0 ml of whole rheumatoid serum (R-OJ) was eluted with a M/15 phosphate buffer pH 7.0 containing 0.5 M NaCl. As can be seen, three peaks were obtained on the chromatogram. Peak 1, which presumably consists of macroglobulin and other high weight protein components, also contained the bulk of the RF agglutinating activity. Peak 2 contained the bulk of the protein components in the sample and a small amount of RF apparently due to "tailing" (from 144-162 ml effluent). Finally, peak 3, considered to be principally albumin, was free of all RF activity.

Serum R-OJ exhibited a TSC titer of 1:40,960 prior to chromatography. After chromatography all tubes exhibiting RF activity were pooled, reduced in volume to 10.0 ml by dialysis against 20 per cent polyvinylpyrrolidone (PVP). Concentrated peak 1 so obtained yielded a TSC titer of 1:1280. Multiplying titer values by sample volume it can be estimated that recovery of RF activity was 6 per cent. This value must be qualified by the fact that titers have an inherent uncertainty of up to $\pm$ 1 tube. Considering maximum possible titer variation in either direction, then recovery of RF could vary from 2 per cent to 25 per cent. Taking the most favorable situation it was clear that even so mild a technique as GPC resulted in considerable loss in RF activity.

Figure 4 is a representation of another GPC experiment, this time on 4.0 ml of serum R-W. Again three major peaks could be discerned. In addition this time a fourth peak following albumin was obtained. As before, RF activity was associated with peak 1, but with considerable "tailing" into peak 2.

In this experiment rechromatography of the RF fractions by GPC was

performed in an effort to eliminate nonmacroglobulin components. Accordingly all fractions exhibiting RF activity were pooled, reduced in volume by dialysis against PVP and rechromatographed. Results are shown in Figure 5. It may be seen that this procedure resulted in a complete removal of peak 4, and virtually complete removal of peak 3. Considerable material from peak 2 was obtained, but now peak 1 was predominant. All RF activity observed originally under peak 2 was in fact due to "tailing" and not to the presence of a γG component with RF-like properties.

Material from peak 1, exhibiting RF activity, was again pooled, reduced in volume and rechromatographed by GPC. This experiment is shown in Figure 6. It may be seen that as a result of three cycles of GPC, a virtually pure peak 1 (macroglobulin) fraction was obtained which contained all recovered RF activity. A small amount of peak 2 "tailing" was noted. RF activity in these experiments could be completely destroyed by treatment with 0.05 M mercaptoethanol, thus confirming the macroglobulin nature of RF.

This sequence of experiments on serum R-W is summarized in Table 1, together with titer data on the original serum and the various fractions. Again, as in the case of serum R-OJ, purification of RF was attended by considerable losses in activity. This difficulty was to persist throughout the course of these investigations, and to date no completely satisfactory solution to the problem has been obtained. These results possibly reflect the greater sensitivity to denaturation of the macroglobulins, than that of other serum proteins. As will be demonstrated later, rabbit γG antibodies (belonging to the 7 S γG class) directed against human γG, proved to be far more stable during the course of purification.

A number of other human sera were subjected to fractionation by GPC. As the results were similar to those shown in Figures 3 and 4, they are not presented here. The optical density data given in Figures 3 through 6 were obtained by ultraviolet spectrophotometry on individual 4.0 ml fractions' collection. In later work, an automatic ultraviolet monitor and recorder was used to measure protein content in GPC eluates. A typical example of such an experiment is shown in Figure 7.

LOW IONIC STRENGTH PRECIPITATION

It was originally elected to use GPC in order to obtain a highly purified RF preparation, free of non γM components, for use in other phases of this work. The results, however, demonstrated that purity was obtained at the expense of yield. It was therefore necessary to employ whole RF serum, or partially fractionated RF serum for most other studies.

For many purposes a low ionic strength (l.i.s.) precipitate of whole serum, containing the RF activity , was found satisfactory. These precipitates are somewhat variable in their composition, but generally appear to contain the γM, part of the γG, and some other globular components of serum. They are substantially free of albumin.

In the present study, precipitates obtained at low ionic strength, were prepared as follows:

To one volume of a rheumatoid factor serum was added 15 volumes of cold distilled water. The diluted serum was kept in a cold room at 4°C for two days, and the precipitate thus formed was harvested by centrifugation. The precipitate was then dissolved in a minimal volume (as little as 1/20 the volume of original serum) of 0.15 M NaCl or appropriate buffer.

Table 2 is a summary of results obtained when four RF sera were fractionated in this way. In these sera, virtually all RF activity was found in the l.i.s. precipitate.* Judging from the TSC titers obtained on the redissolved precipitates, substantially all RF activity was recovered. Of particular interest was serum R (P 7), which exhibited an unusually high TSC titer of 1:818,200 in plasma. After clotting the sample (with a few drops of 2 per cent $CaCl_2$, the TSC titer dropped one tube to 1:409,600. A subsequent l.i.s. precipitate solution yielded the same titer value.

In other experiments a rheumatoid factor serum was subjected to precipitation with half saturated ammonium sulphate. Here too, RF was found in the precipitated globulin fraction with no loss of activity. A second ammonium sulphate precipitation followed by l.i.s. precipitation again did not result in any loss of RF activity. However, when the precipitate solution was subjected to GPC fractionation on Sephadex G-200, then about 50 per cent of RF agglutinating activity was not recovered.

These experiments demonstrated, from the point of view of recovery of RF activity, that l.i.s. precipitation was an acceptable first step in the fractionation and purification of RF.

IMMUNOCHROMATOGRAPHY

Preparation of PAS γG Conjugates. Table 3 is a summary of the effect of varying experimental conditions on the coupling reaction between diazotized PAS and human γG. The amount of protein coupled to the resin was strongly dependent on the reaction time. Thus conjugates containing 1 per cent γG (as measured by ultraviolet absorbance of supernatant before

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* Several RF sera, where some or all RF activity did not appear in the l.i.s. precipitate fraction but was instead found in the supernatant fraction, are reported in Table 17.

and after reaction could be prepared by allowing the reaction to proceed for six days. However, the final product was very fine and could not be conveniently used for chromatography without employing high external pressure. For this reason, a one-day reaction time which yielded a product containing 10 per cent γG was chosen as optimum.

Treatment of PAS with insufficient nitrous acid resulted in a sharp decrease in protein coupling; this in spite of the fact that only about 0.1 per cent of the estimated available diazotized amino groups were apparently utilized for coupling to the protein. Based on this experiment, the procedure finally adopted and described under "Methods" continued the use of excess nitrous acid in the diazotization step.

Varying the amount protein added to the diazotized resin had only a moderate affect on the amount coupled to the PAS. Thus when 100mg γG/gram of resin was added, protein coupling was virtually complete. However when 250 mg γG/gram was added, the amount of protein coupled increased by only 11 per cent in spite of the large number of excess diazotized sites on the resin.

Adsorption Experiments: The capacity of PAS-γG to adsorb RF activity from a RAS, and anti-γG antibodies from a rabbit anti-γG antiserum* was tested and the relative adsorbtive capacity of γG coupled to a resin in contrast to polymerized γG not coupled to a resin was determined.

Polymerized γG was prepared by coupling the protein to diaminophenol-HCl (DAP-HCl) as follows:

To 160 mg (1.0 m mole of DAP-HCl dissolved in cold water was added 0.35 ml of cold 20 gram per cent $NaNO_2$ (1.01 m mole) dropwise with stirring.

*Kindly supplied by Mrs. Bella Shore, Department of Microbiology, The Mount Sinai Hospital, New York, N.Y., 10029. Antigen used for immunization was Ag-7S human γG (ICC grade lot R-58, Immunology Inc., Chicago Ill.), in Freund's complete adjuvant.

After the solution had turned black, 1.0 ml of cold concentrated HCl was added, followed by the dropwise addition of 0.50 ml of cold 20 gram per cent $NaNO_2$ (1.45 m mole). After one hour, 0.6 ml of 5 per cent urea was added, followed by 1,600 mg of human γG dissolved in 16 ml of 0.05 N NaOH. The pH was adjusted to about eight with 2 M K_2CO_3 and the mixture was stirred for 24 hours in the cold room. A coarse gelatinous insoluble precipitate was obtained which was washed three times with pH 3.2 citrate buffer, and three times with pH 8.2 glycine buffer. The yield of polymerized protein (DAP-γG) was 1,429 mg (89 per cent). The insoluble protein was homogenized and kept as a suspension in glycine buffer.

Table 4 is a summary of adsorption experiments. A RF positive human rheumatoid arthritis serum, and a rabbit anti-γG serum were both repeatedly adsorbed with PAS-γG and DAP-γG. RF agglutinating activity was not easily removed from RF serum. Nine adsorptions with PAS-γG or DAP-γG were required to do so. On the other hand, the anti-γG antiserum was completely depleted of antibody after five adsorptions with PAS-γG and only one adsorption with DAP-γG. It appeared from these experiments that the rabbit anti-γG antibodies possessed greater affinity for γG than did RF and further, that the immunoadsorptive efficiency of γG was decreased by coupling to diazotized PAS, possibly due to blockage of antigenic sites on γG. In preliminary work it was found that preparations of PAS coupled to heat aggregated or alkali denatured γG were not more effective in adsorbing RF than PAS coupled to untreated γG.

Immunochromatography on PAS-γG. The rheumatoid factor serum studied came from a patient with classical rheumatoid arthritis. Twenty ml of this serum was fractionated by precipitation with half saturated ammonium

sulfate and low ionic strength dialysis. The resulting precipitate was dissolved in 5.0 ml of phosphate buffer pH 6.3 (0.02 M), and applied to the top of a 20 x 20 cm column of PAS-γG. The column was developed under 5-8 pound pressure by stepwise elution, with buffers of decreasing pH from 0.02 M phosphate pH 6.3 to 0.1 M citrate pH 3.2. Five ml fractions were collected and adjusted to pH 7 with 2 M K_2CO_3. The optical absorbance at 280 mμ of the fractions was measured, and RF agglutination titers were determined. These data are graphically presented in Figure 8.

At pH 6.3 much of the protein content was eluted but no RF activity was recovered. Between pH 5.25 and pH 4.0, seven regions of RF agglutinating activity were discerned. Some overlap of activity was noted and absolute separations were not obtained.

The several fractions (II-VIII) obtained were concentrated in volume, and separately rechromatographed on the same column.

Table 5 is a summary of these rechromatography experiments. Fractions II and III which appeared at pH 5.25, reappeared at the same pH. Fractions IV and V, which originally appeared at pH 5.0, also reappeared at the same pH. Fraction VI originally was obtained at 0.5 M, pH 5.0. Upon rechromatography, this fraction proved to be heterogeneous but with most of the activity appearing at the original pH. Fraction VII also proved to be heterogeneous with about one third of the activity appearing at the original pH of 4.5. Finally, fraction VIII obtained originally at pH 4.0 now appeared at pH 4.5.

Table 6 is a summary of various data obtained upon the seven pooled RF subfractions. The percentage of total agglutinating activity is calculated from the titration values of individual eluate tubes. Treatment

of the pooled subfractions with mercaptoethanol destroyed agglutinating activity in all cases, indicating that the subfractions were all macroglobulin in nature.

Only fractions IV and V exhibited cross reactivity with rabbit γG, that is, a positive reaction in the Waaler-Rose sensitized sheep cell test. It appeared from these results that resolution of RF fractions, reactive with human γG only, from those reactive with both rabbit γG and human γG, was obtained.

Inhibition experiments were done in which amounts of the subfractions were mixed with twofold serial dilution of heat aggregated γG and then tested for agglutinating activity. The highest dilution of aggregated γG sufficient to inhibit agglutination was determined. The values in Table 6 are the ratio of the test sample dilutions to the inhibition titers. As such they are only of relative significance. The higher the ratio the more γG was required to inhibit the test sample. Thus a higher ratio suggests that the sample in question has less affinity for aggregated human γG. The initial subfractions (II, III, and IV) all yielded higher inhibition ratios than later subfractions (V to VIII). Thus the later fractions apparently had more affinity for γG than did the earlier ones. This conclusion is consistent with their chromatographic order, since presumably those fractions with greater affinity for γG would be held more strongly to the column, and therefore be eluted at lower pH values. It must be noted that only a fourfold difference in inhibition ratios was observed. The interpretation given of these data must therefore be qualified by the fact that the uncertainty of serial dilution techniques if of the same order. However, the trend of the values was in the expected direction.

The fractions were examined by agar gel diffusion against several antisera. In most cases multiple lines were seen with anti-whole human serum antiserum, indicating that not all nonspecific protein was eluted at pH 6.3 prior to elution of the RF subfractions. Fractions II to V yielded precipitin lines with an anti-γM antiserum but VI and VII did not, even though their agglutinating activity was destroyed by mercaptoethanol. The failure to obtain precipitin lines was probably due to the diluteness of the test samples. Unfortunately, fraction VIII was accidentally lost prior to this experiment and therefore no data are reported.

Chromatographic experiments on other rheumatoid factor preparations also yielded complex elution patterns indicative of RF heterogeneity. In general, this procedure resulted in considerable inactivation of RF as indicated by the fact that the sum of RF agglutinating activity in subfractions was always considerably less than that present in the original unfractionated serum. Calculated in this way, recovery of RF activity was only about 10 per cent.

IMMUNOCHROMATOGRAPHY OF RABBIT ANTI-HUMAN γG ANTISERUM

Five ml of whole rabbit antiserum directed against human γG were chromatographed on PAS-γG. In this experiment the sequence of eluting buffers was altered. The first buffer employed was 0.1 M "Tris" pH 7.0 followed by 0.2 M phosphate pH 6.3, 0.1 M acetate pH 4.0, and 0.1 M citrate pH 3.2. The other buffers used in chromatography of RF were omitted because it was previously determined that they failed to elute any antibody activity from this antiserum.

As before, 5 ml fractions were collected and pH of the eluates was adjusted to about pH 7 with 2 M K_2CO_3. Antibody agglutinating activity was

determined with the TSC test. Results of this experiment are diagrammed in Figure 9.

A small amount of antibody acitvity was recovered early in the experiment at pH 7.0. The major portion of the antibody activity, however, was obtained in two fractions, one at pH 4.0, the other at pH 3.2. Further elution with a pH 2.5 citrate buffer did not yield any more antibody. This was in marked contrast to the elution pattern obtained with the rheumatoid factor serum, where most of the RF agglutinating activity was obtained above pH 4.0.

The pH 4.0 and pH 3.2 fractions were both unaffected by mercaptoethanol treatment, suggesting that neither were macroglobulins. When these antibody fractions were rechromatographed under the same conditions, they were recovered at the same pH at which they were originally obtained.

Recovery of rabbit anti-γG activity was far better than recovery of RF after immunochromatography. It was estimated that about 80 per cent of the antibody activity of the rabbit antiserum was recovered after fractionation. This enhanced recovery, as compared to RF, presumably reflects the fact that the rabbit antibodies were probably γG rather than γM immunoglobulin. It would seem that the former are less susceptible to denaturation and inactivation than the latter.

I. DISCUSSION

Immunoadsorptive agents, i.e., insoluble complexes of resins or polymers with antigens of various types, have been utilized by many investigators for the purification and fractionation of a wide variety of antibodies (48). It is now evident that many antibodies are heterogeneous with respect to their affinity for, and their elutability from, specific immunoadsorbents (49, 50). Thus, Webb and Lapresle (51), found that rabbit antihuman serum albumin antibodies adsorbed to conjugates of polyaminostyrene and albumin could be desorbed in three distinct fractions: the first was eluted at pH 3.0 and the other two were obtained by further elution with strong acid (0.1 N HCl).

Similarly, Perlmutter, Freedman and Sehon (52), found that erythrocyte-ragweed-pollen-extract conjugates preferentially adsorbed skin sensitizing antibodies over hemagglutinating antibodies from sera of ragweed sensitive individuals. To cite one more example, Kitagawa et al (49), have shown that rabbit anti-p-azobenzoate antibodies were heterogeneous with respect to their adsorption to a specific immunoadsorbant and their elution by specific hapten.

Various studies have shown that RF found in many individuals is also heterogeneous. At least three different rheumatoid factors have been distinguished based on their differential reactivity with human and rabbit γG (53, 54). Seifert (55) has employed adsorption and chemical fractionation techniques to demonstrate four RF fractions in a rheumatoid arthritis serum. Heimer, Schwartz and Freyberg (13), have chromatographically separated on DEAE-cellulose five different rheumatoid factors reacting with human γG, two of which also exhibited reactivity with rabbit γG. From

another point of view, the existence of different anti-γG-agglutinating substances directed against the various Gm and Inv allotypic varieties of human γG have also been demonstrated (56, 57).

The immunochromatographic results in the present work again demonstrate the heterogeneous nature of RF. Seven different regions of agglutinating activity were discerned in the chromatogram. Judging from the rechromatography data, it appears that there were some five or six "different" rheumatoid factors in this sample. Two of these proved to be also reactive with sheep cells sensitized with rabbit γG, a finding similar to that of Heimer et al (13).

Williams and Kunkel (58), have also used a chromatographic technique, employing PAS-γG conjugates to fractionate RF. Their technique was to pass whole serum through the column, followed by a two-step elution procedure using 0.15 M NaCl, followed by 6 M urea or an acid buffer. Under these conditions there was incomplete adsorption of RF activity. Of interest was that the nonadsorbed RF which appeared in the first few tubes yielded high titers with SSC test, but were negative with the latex fixation test. In parallel experiments with PAS coupled to rabbit γG, early tubes with nonadsorbed RF yielded significant latex titers but were virtually devoid of SSC activity. The authors conclude from these data that one of the rheumatoid factors present reacts primarily with rabbit γG and not with human γG.

The existence of this type of rheumatoid factor has been previously suggested by Milgrom et al (54). However, the use of negative latex data as evidence for their presence, must be qualified by the fact that the latex fixation test is very sensitive to nonspecific inhibition by serum protein, whereas agglutination tests employing sheep cells are not (59).

In the sera studied in the present work, no RF was found in any fraction that reacted in SSC test (rabbit γG), but failed to react in the TSC test (human γG).

The immunochromatographic experiments in the present study revealed that the rabbit antihuman γG antibodies were also heterogeneous, with two major fractions, one eluted at pH 4.0 and the other at pH 3.2. The heterogeneity observed in the sample studied was apparently not of the 7 S-19 S type, as both fractions were resistant to inactivation by mercaptoethanol. Of interest was the finding that virtually all RF was eluted from PAS-γG column with buffers of pH greater than 4.0; this in marked contrast to the findings with anti-γG antibodies. This suggests that the rabbit antihuman γG antibodies studied here have greater affinity for human γG than do the rheumatoid factors. This view is supported by the adsorption experiments (Table 4), in which both PAS-γG and DAP-γG removed anti-γG antibodies from antiserum more efficiently than they removed RF from a rheumatoid arthritis serum.

What the stimulus is for RF production in the human remains obscure. Even more obscure is the biological mechanism underlying RF heterogeneity and antibody heterogeneity in general. The various rheumatoid factors appear to represent a family of anti-γG directed against various antigentic sites on native and/or "altered" γG. In the present study, evidence will be presented that in most (if not all) rheumatoid factor sera, at least one of the rheumatoid factors present is directed against some portion of the individual's own γG. Consequent autoinhibitory phenomena may introduce uncertainties in the serological approach to definition of all RF specificities in an individual serum. For these reasons techniques of immunochromatography can be of value in characterizing the rheumatoid factors in

individual sera. Further work is required, however, to improve the yield of recovered active RF subfractions. Then the immunological and physico-chemical characterization of these subfractions can be attempted.

II. REACTIVITY OF RF WITH AGGREGATED γG

THERMAL AGGREGATION OF γG

Constant volumes of a human γG (Squibb, Fraction II, Lot 1812) solution in 0.15 M NaCl, containing 21.9 mg/ml protein were heated at 60°C for varying times from 0 to 120 minutes. Aggregates formed by this procedure were precipitated with 0.62 M Na_2SO_4 and purified by two reprecipitations. This served to remove all unaggregated 7S γG, as demonstrated by ultracentrifugal analysis. The yields of aggregated γG with increasing heating time are listed in Table 7, columns 2 and 3 and plotted as a function of time in Figure 10. It should be emphasized that this experiment measured only the total formation of aggregates insoluble in 0.62 M Na_2SO_4 and did not permit distinction as to particle size.

Figure 10 shows that the formation of aggregates appeared to be characterized by an initial lag period. The kinetics of aggregate formation appeared to be complex as judged by the changes in slope between 10 min and 120 min. Data beyond 120 min was not obtained. Kinetic analysis of the data was attempted by plotting $\log [a/(a - x)]$ and $1/(a - x)$ as a function of time (where a is the initial γG concentration and x is the aggregated γG concentration at any time). In both cases complex non-linear plots were obtained. It appeared that under the conditions of this experiment, the formation of γG aggregates could not be characterized by simple first or second order kinetics.

Two aliquots of the purified aggregates were dissolved, one in 0.15 M NaCl and the other in 0.05 M NaOH. These preparations were then

tested for their ability to inhibit agglutination of tanned sheep cells by a RF serum.

In appearance the solutions of aggregates in 0.05 M NaOH were perfectly clear and exhibited no Tyndall effect, suggesting that the aggregates were now completely dispersed in the alkaline solution. However optical rotation measurements as shown in Table 8 indicated they were extensively denatured.

Even though the γG aggregates appeared to be completely dispersed at high pH they were still very reactive with RF as judged by the fact that a mean concentration of 18.75 µg/ml was sufficient to inhibit RF agglutination. By comparison, 200 µg/ml of undenatured purified 7SγG was required to inhibit the same test system. In this experiment little or no correlation between heating time and inhibitory potency of the alkali dissolved aggregates was noted.

In performing the inhibition experiments the alkali dissolved γG was carefully back titrated to pH 8.6. Under these conditions aggregation did not reoccur and the denaturation was apparently not reversed (Table 8.). In view of the denaturing effect of alkali on proteins these results suggested that γG when denatured, but not aggregated, could still react with RF.

Other aliquots of the purified aggregates were dissolved in 0.15 M NaCl. These solutions were markedly turbid and exhibited a strong Tyndall effect. When these aggregated γG solutions were tested in the agglutination-inhibition procedure it was found that their inhibitory capacity (per unit weight) increased with the time of heating employed in their

preparation. Since these preparations were virtually free of unaggregated 7SγG this effect could not be ascribed to variation in aggregate concentration but reflected the increased reactivity of the aggregates themselves.

These results are shown graphically in Figures 11 and 12. Log concentration of aggregated γG required to inhibit RF agglutination decreased linearly with heating time, suggesting that the enhancement of reactivity was kinetically first order.

In relative terms, these data showed that the reactivity for RF of denatured γG was about 100 times greater than that of undenatured γG. Aggregated γG however proved to be from 250 to 2500 times as reactive as undenatured γG, or 2.5 to 25 times as reactive as denatured γG.

The term "denatured" has been applied to γG preparations exhibiting enhanced optical rotation but no enhanced turbidity or marked Tyndall effect. Denatured γG is considered to be monomeric as evidenced by sedimentation constants of about 7, determined with the ultracentrifuge. The term "aggregated" has been applied to γG preparations exhibiting strong Tyndall effects and ehnanced turbidity at non-absorbing wavelengths. Aggregated γG, purified as previously described, did not contain monomeric 7SγG when studied in the ultracentrifuge, but did consist of a heterogeneous mixture of components ranging in S value from about 10 to 40 (21).

It would be expected that heating of γG solutions first denatured the protein, and then that the denatured γG polymerized to form aggregates. Initial fractionation with 0.62 M Na_2SO_4 precipitated aggregates as well as monomeric γG. The latter component may have been denatured but its properties were not further studied as it was subsequently removed by

reprecipitation of the aggregates.

TURBIDITY STUDIES

Latex Particles: In light of the findings that the reactivity of γG aggregates with RF increased with the heating time used to prepare them, it seemed possible that this effect was related in some way to changes in particle size of the aggregates themselves, associated with difference in heating time. To examine this question turbidity (T) measurements of γG aggregates were undertaken.

For purposes of comparison the turbidity of polystyrene latex particle size was determined as described under "Methods". The various LP suspensions employed are listed in Table 3. Turbidity (optical density) measurements were made as a function of wavelength and latex solids concentration.

In general it was found that turbidity at 600mμ was linearly proportional to latex solids concentration for a given particle size. A plot of this type for latex particles of 0.365 microns diameter is shown in Figure 13. Similar results (not shown) were obtained for LP of other sizes employed in this work.

Figure 14 is a plot of LP turbidity, expressed as extinction (E = OD/mg. solids/ml) as a function of particle diameter. These data were obtained at three different wavelengths, 400, 500 and 600mμ. For the smaller particles from 0.088 to 0.365 microns diameter, turbidity was linearly proportional to particle size.

At larger particle sizes* a turbidity maximum was noted approximately in the region where $\lambda/r = 1.3\text{-}1.6$. This turbidity maximum was wavelength dependent. At still larger particle sizes, i.e. $\lambda/r = 0.6\text{-}0.8$ a drop in turbidity was noted. Light scattering theory predicts for large particles, $\lambda/r \leq 0.5$, turbidity is essentially a function of the number of particles in a system and is unrelated to particle size. Thus at constant weight concentration it follows that:

$$T_{r_1}/T_{r_2} = r^3_2/r^3_1 \qquad \text{---- Eq. (3}$$

Inserting turbidity and radius data (at d=1.0 and 1.4), interpolated from downward portion of the 400mμ turbidity curve, into equation 3) yields: $T_{r_1}/T_{r_2} = 2.67$, and $r^3_2/r^3_1 = 2.74$. This fairly close agreement indicates that at 400mμ the 0.796 and 1.30 micron LP behave as typical "large" particles.

The turbidity of various LP suspensions was also examined as a function of wavelength of incident light. The data was tested by plotting turbidity vs. $1/\lambda^n$ for various values of n, to find the best linear fit. This approach was based on the theoretical consideration that the scatter of light by "small" particles (whose particle diameter are less than $\lambda/20$ of the wavelength of the incident light) is proportional to $1/\lambda^4$ whereas the scatter of light by "large" particles (whose particle diameter are more than 0.05 times the wavelength of the incident light) is independent of λ. The best linear plots of the data as a function of λ are shown graphically in Figures 15 through 18 and the results are summarized in Table 9.

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* As will be shown later the diameter of these LP are in a transition range. Below these sizes turbidity was proportioned to $1/\lambda^4$; above this wavelength, turbidity was independent of λ.

It can be seen that "small" particle behavior (T : $1/\lambda^4$) was exhibited by LP suspensions of 0.088 and 0.126 microns diameter. For larger particles, that is, 0.264 and 0.365 microns diameter, turbidity was still related to $1/\lambda^n$, where however n=3. For still larger particles, i.e. 0.557μ and 0.796μ the best linear fit was obtained at n=1 and n=0.5 respectively. Finally for the largest LP (1.305μ) n approached 0, i.e. turbidity was almost independent of λ.

In general it appeared that particles exhibiting a turbidity dependence with wavelength of $1/\lambda^4$ also exhibited a turbidity particle size dependence proprotional to r. These results suggested that at least in an approximate way, the range of mean particle size of γG aggregates could be deduced from turbidity data.

Aggregates of γG Immunoglobulin: Three 10.0 ml aliquots of a 1.0 g/100 ml solution of γG in 0.15 M NaCl was heated at 63.0°C for 30, 60, and 120 min. respectively. From a portion of the heated γG solutions, aggregates of γG were precipitated and purified as described under "Methods". The purified aggregates were redissolved in 0.15 M NaCl to original volume. Turbidity measurements were made on the heated γG solutions as well as on the purified aggregate solutions prepared from them. Some of the data is summarized in Table 10.

It can be seen that turbidity values obtained immediately after heating were somewhat lower than those obtained 24 hours later, indicating that some further aggregation occured after cessation of heating. However turbidity of purified aggregates was virtually the same as

turbidity of the same aggregates when not separated from monomeric γG. Trubidity values of aggregates measured 24 hours after purification were not significantly different from values obtained immediately after purification indicating that no further change in aggregation occured after 24 hours.

In Figure 19 are plotted optical density values for aggregates (from sample 2) as a function of wavelength, $f(\lambda)$, of the incident light. Conversion data for $f(\lambda)$ are given in Table 11. It can be seen that a linear relationship was obtained when $f(\lambda)=1/\lambda^4$, but not when $f(\lambda)=1/\lambda^2$ or $1/\lambda$ was used. Extinction ,E, (O.D. / mg/ml) for all three aggregate preparations is plotted vs $1/\lambda^4$ in Figure 20. In all three cases a substantially linear fit was obtained. Some slight positive deviation from linearity at low values of λ were noted for sample 1, and a slight negative deviation from linearity at high λ values was seen for sample 3. Extrapolations of the linear plots to zero values of $1/\lambda^4$ did not pass through the origin. Only in the case of sample 1 did such an extrapolation pass near the origin.. Additionally, plots of OD for all three samples as a function of $1/\lambda^2$ or $1/\lambda$ (shown only for sample 2 in Fig. 19) were distinctly non-linear.

Figure 21 is a plot of E values obtained at 400mμ for the three aggregate preparations, as a function of heating time. It can be seen that E increased regularly with heating time used to form the aggregates. Based on the observation that the turbidity of small spherical latex particles (i.e. where T : $1/\lambda^4$) was proprotional to particle radius it

appeared, by analogy, that increased heating time in thermal aggregation of γG resulted in not only the formation of more aggregates, but also in the formation of larger aggregates.

VISCOSITY MEASUREMENTS

As part of the study of physical properties of aggregates of γG immunoglobulin, viscosity measurements were performed on γG. The protein was freed of aggregates, and viscosity measurements made as described under "Methods".

Flow times (t) were determined in quadruplicate for a series of γG solutions in glycine buffer pH 8.2 varying in concentration from 0 to 2.0g protein/100 ml buffer. This data was used to calculate specific viscosity ($n_{sp}=(t_s/t_o)-1$) and reduced viscosity ($n_{red}=n_{sp}/C$). Density corrections were not employed as it was estimated that the error thereby introduced was less than 0.5%. (t_s = sample flow time, sec; t_o = solvent flow time, sec.; C = sample concentration, gm/100 ml).

Both measured and calculated data are given in Table 12, and reduced viscosities are plotted as a function of protein concentration in Fig. 22.

Linear extrapolation of reduced viscosity to zero concentration yielded a value for the intrinsic viscosity $[n]$ (where $[n]=n_{\underset{c\to o}{red}}$) for γG of 0.048. This value is somewhat lower than most reported in the literature for the intrinsic viscosity of γG. Edsall (61) and Kochwa et al (22) obtained values of 0.060. Jergensons (62, 63) has reported values varying from 0.048 to 0.083. Effects of buffer salts, ionic

strength and pH as well as the tendency of γG to dimerize may all be related to these differences in results.

Viscosity data for aggregates of γG have not yet been obtained. It is reasonable to believe that aggregated γG should yield higher intrinsic viscosity values than monomeric γG. One report gives a $[\eta]$ value for a natural, in vivo, aggregate of γG as 0.095 (22).

Many studies have sought to relate viscosity to molecular weight. For example, Kushner and Frankel (64) found that Log $[\eta]$=K Log M for four proteins of different molecular weights (M). Other investigators have sought to relate viscosity (65) or viscosity and sedimentation data (66, 67) to size and shape of macromolecules. It would be of great interest therefore to compare viscosity data for different sized γG aggregates, especially when other data on particle size becomes available.

II. DISCUSSION

The fact that aggregated γG will react in an inhibition or precipitin reaction with RF is well established (17, 21, 68). The usual laboratory procedure for the formation of γG aggregates is to heat γG solutions for various periods of time at temperatures ranging from 56° to 70° C. Under these conditions the γG solutions soon become turbid and reactivity with RF is markedly enhanced. Although it has been customary to refer to the reactive species in whole γG as "aggregated", this term does not take into account the fact that aggregation is a secondary phenomenon, resulting from the thermal denaturation of γG (69). Aggregation is not an inevitable consequence of denaturation since proteins exposed to high pH or urea or spread as surface films exhibit numerous criteria of denaturation yet are not aggregated (69). Thus the reactivity ascribed to aggregated γG could in principle be a reflection of the denatured condition of the protein.

Aggregation or coagulation may be viewed as analogous to a condensation polymerization reaction resulting from thermal denaturation (70). Huggins (71) has pointed out that for such reactions the number average polymer molecular weight increases with the extent of reaction. Further it has been shown experimentally (71) that aggregates formed as a result of heating bovine serum albumin increase in molecular weight with heating time. Therefore, it is reasonable to assume that γG aggregates, which are the result of increased heating times, should also exhibit increased molecular weights, and that increased reactivity is therefore associated with larger particle size. It may be argued that the solubility of

aggregated γG-RF complexes would decrease with increasing particle size. This would favor increased precipitation and also increased inhibition of agglutination since the γG on the particulate carrier surface would be less able to capture the RF already tightly complexed by the aggregated γG in solution.

The molecular shape and size of monomeric γG has been given (72) as that of a prolate elipsoid, 44A by 235A, in diameter andlength respectively. Letting λ=400mu and taking r= 11.8mμ then in the longest dimension $\lambda/r = 34$, thus putting the γG monometer in the "small" particle class. Thermal aggregates of γG were characterized by a linear dependence of turbidity when plotted as a function of $1/\lambda^4$. However since these plots did not pass through the origin, the aggregates could not be unequivocally characterized as "small" Rayleigh particles.

The actual size and shape of γG aggregates is unknown. The experiments reproted here do not directly yield information on this subject. Yet the data permit certain speculative observations. If thermal aggregation resulted in "end to end" linkages of γG, then λ/r would be 17 for the dimer: 11.3 for the trimer, and so on. Clearly beyond the dimer or possibly trimer stage turbidity of such aggregates would no longer be proportional to $1/\lambda^4$. If on the other hand the thermal aggregation proceeded primarily by "side to side" linkages then λ/r in the longest direction up to the 9-mer would still be > 20, and therefore $T : 1/\lambda^4$ would still hold.

Thus the data obtained is at least consistent with the idea that

thermal aggregates of γG, up to N=18-20, consist of an end to end dimer and a side to side decamer of monomeric γG. For smaller aggregates of γG it is of course not possible from the data, to picture which of these processes occur first.

As regards actual particle size of γG aggregates no direct information is available. We can however suggest, by analogy with the LP turbidity data, that the average particle radius for the 120 min. aggregates was no more than 0.05 micron. If turbidity of γG aggregates is proportional to particle radius as in the case of LP, then the average value for r in the longest direction for the 60 and 30 min. aggregates was about 0.04 and 0.02 microns respectively. The foregoing argument is admittedly simplified and speculative. In addition it must be recognized that thermal aggregates are almost certainly heterogeneous. A more rigorous approach to this problem could be developed as follows:

(1) Fractionate γG aggregates into more homogeneous fractions by gel permeation chromatography or density gradient centrifugation.

(2) Measure aggregate particle size by one or more of the following methods:

a) Light Scattering.

b) Gel permeation chromatography in calibrated columns.

c) Analytical ultracentrifugation.

d) High resolution electron microscopy.

In this regard light scattering measurement on monomeric γG were made during the course of this work. These data yielded a MW=208,000, which

is considerably higher than the more generally accepted value of 160,000 for γG. The high value probably reflected experimental defects, i.e. dust in solvents, or presence of trace amount of aggregates in the γG solution. Light scattering studies of the (presumably) heterogeneous γG aggregates were not attempted since it was felt that Zimm plots of such data would be too skewed to be useful.

The observation that aggregates of varying reactivity when dissolved in alkali lost only part of their reactivity and were now all equally reactive suggests that aggregate particle size does not completely account for reactivity. Aggregates dissolved in alkali are completely dispersed but they are also denatured as a result either of previous heating or the effect of alkali itself.

In other studies (73) γG devoid of aggregates, and subjected to the action of denaturing agents such as NaOH or urea, became reactive with RF even though apparently no aggregates were formed. This induced reactivity of 7SγG appeared to be associated with protein denaturation as evidenced by increases in optical rotation. Jergensons has previously demonstrated that optical rotation measurements may be used to characterize alkaline denaturation of γG (24) and that guanidine salts will denature γG without aggregation (62). Still other studies have also shown that NaOH or urea treatment of γG resulted in increased reactivity which in these cases however was associated with the presence of aggregates (17, 68). Presence of aggregates in these studies appeared to be due to exposure of the altered γG to pH below 8. Under these conditions denatured γG spontaneously and rapidly aggregates.

Evidently therefore, γG, when denatured but not aggregated, can react with the rheumatoid factors. This is consistent with the fact that 7SγG can sensitize polystyrene latex for agglutination with RF (75) presumably either due to the denaturing effect of the emulsifying agents present in commercial latex suspensions or to surface denaturation of the protein upon adsorption. Christian has also shown that large excesses of nonaggregated γG can inhibit the precipitin reaction between aggregated γG and RF (21) and that the reaction of aggregated γG with the 22S rheumatoid factor complex results in the dissociation of 7SγG from the complex (76). Whether these effects are due to the presence of denatured material in the 7SγG preparations or whether they represent intrinsic reactivity of "native" 7SγG for RF is not known.

It appears that denaturation is an essential prerequisite for converting γG into a reactant for RF. The subsequent aggregation of denatured γG serves to enhance reactivity possibly because of decreased solubility of the RF-γG complex (21). The reactivity of denatured 7SγG with RF supports the views of some investigators that the rheumatoid factors may represent autoantibodies to the host's own altered γG (77, 78). An immune response to altered γG of this type has already been demonstrated in the rabbit (79, 80) and guinea pig (80). How such alteration of "own" γG could occur is suggested by the observations of Ishizaka and Cambell (19). They demonstrated that the specific optical rotation of certain soluble antigen-antibody complexes was greater than could be accounted for by the contributions of the two components. They concluded that this could be due to denaturation

of either antigen or antibody as a result of complex formation. These results are consistent with the view that the rheumatoid factors might arise in response to antibodies elicited by environmental antigens (81). It may be reasoned that when such antigens are complexed with corresponding circulating antibody γG they serve to denature the globulin and cause it to become autoantigenic.

Many studies with a wide variety of antigen-antibody systems have revealed (82) that denaturation of protein antigens <u>decreases</u> their reactivity with antibody directed against the undenatured antigen. Another pertinent observation (83) is that the "valence" of antigen per mole tends to increase with increasing antigen molecular weight. However antigen "valence" per unit weight of antigen tends to <u>decrease</u> with increasing molecular weight.

The RF-γG System appears to be distinctive if not unique with regard to the above two observations. Firstly, denaturation tends to <u>increase</u> γG reactivity for RF. Secondly, antigen "valence" (per unit weight of antigen" appears to <u>increase</u> with increasing antigen molecular weight (when caused by thermal aggregation) as judged by the inhibition studies presented here or by the precipitin experiments in other studies.

This second point remains equivocal. The present findings, with the inhibition technique, of increased reactivity with increasing γG aggregate size suggest an increase in antigen "valence". Other studies, (73) of the reaction between RF and aggregated γG, using the precipitin technique, show enhanced precipitation with larger aggregates. It is

however not clear if this finding reflects a true increase in antigen "valence" or simply greater co-precipitation of aggregated γG.

This problem could be resolved experimentally if tagged (either with a fluorescent or radio-active label) homogeneous fractions of aggregated γG were employed in such precipitin experiments. Then it would be possible to determine both total precipitation as well as γG precipitation as well as γG precipitation and by difference, RF precipitation. Appropriate immunochemical analysis of such data, would help clarify the relation between antigen "valence" and molecular size of γG aggregates.

III. INTERACTION OF RHEUMATOID FACTOR WITH AUTOLOGOUS γG

BLOCK TITRATION STUDIES

Relation of titer to cell concentration: It was observed that agglutination titers of RF sera were dependent on the concentration of γG coated tanned sheep cells used. In all cases, titers decreased with increasing cell concentration. The manner of titer reduction, however, varied among the sera tested, and three different types of sera could be distinguished on this basis. These three types are illustrated in Figure 23. In type (a) sera, there was a 1-tube decrease for each doubling of cell concentration; in type (c) sera, the corresponding titer decrease was 2 tubes. In addition, an intermediate type (b) was recognized where the corresponding titer reduction was greater than 1 but less than 2 tubes.

The titers obtained were plotted on double logarithmic paper. Since such plots were nearly always linear, the various serum types could be characterized by the slope values of the plots. Of 63 rheumatoid factor sera studied, 27 were type (a) and had a mean slope value of -1.0 ± 0.04. These have been termed "low slope." Another 8 sera which were type (c) yielded a mean slope value of -2.0 ± 0.13 and were termed "high slope type". The remaining 28 sera of type (b) were found to have intermediate slope values. The mean slope value for this group was -1.4 ± 0.12. Examples of the three slope types are shown in Figure 24.

Reproducibility: The classification of RF sera into three slopes types clearly depended on the reproducibility of the titration and calculation procedure. In order to determine whether the variability

inherent in the use of different lots of tanned sheep red cells was of any consequence, the experiments listed in Table 13 were performed. Three sera (one of each type) were titrated by the block method at weekly intervals using different batches of γG coated tanned sheep red cells. The standard TSC titer (that obtained with 0.25% cells) showed the often encountered 1-tube variation. However, the slopes were far more reproducible. This is illustrated by the data for serum R2, where the variation from the slope mean offour determinations was $\pm$0.07. Similar results were obtained in other replicate determinations. Based on these results, differences in slopes of 0.2 or more were considered to be significant.

It was found that the heterophil activity frequently encountered in many human sera was without influence on slopes. Several undiluted sera were adsorbed overnight at 4°C with equal volumes of packed washed sheep red cells. This procedure served to remove heterophil activity but did not significantly affect the slope values. Heat inactivation of complement at 56°C for 30 min. also did not affect either high or low slope values. Consequently, all sera were subsequently studied without adsorption of inactivation.

Effect of added γG: It is generally accepted that the prozone* phenomena occasionally observed in agglutination titrations of RF sera are due to the presence of autoinhibitory substances in these sera. The

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* In the serological titration of some RFS it is sometimes observed that the first few tubes of serial dilution are negative, then subsequent tubes turn positive until an end point is reached. This region of initial negativity in an otherwise positive serum is referred to as a "prozone."

fact that prozones are usually obtained only at low serum dilution suggested that the effect of autoinhibitors is variable and dependent on the serum dilution (84, 85). It was, therefore considered possible that the variable interaction of autoinhibitors with the patient's own RF could be responsible for observed slopes greater than -1.0. In order to determine if the presence of inhibitors could cause an increased slope, the experiments listed in Table 14 were carried out.

Amounts of heated (63°C, 15 min) human γG from 0 to 10 mg/ml were added to a low slope serum, incubated for 60 min at 37°C and analyzed by the block titration method. It was found that the slope of the test serum, R5 in the absence of added γG was -0.97. With increasing amounts of added γG to 10 mg, the slope value increased to a value of -2.15.

This effect was also demonstrated in another way (Table 15). Aliquots of a 1.0% solution of γG were heated at 63°C for varying times from 0 to 60 min. Then 0.01 ml (0.1 mg of γG) was added per ml of serum. After incubation and block titration, the slopes were found to increase from -1.4 to -2.0 over the range of heating times employed. These experiments showed that the addition of aggregated γG correlated with an increase in slope. Since aggregated γG is known to be an inhibitor of RF agglutination, these experiments offered support for the view that the high slopes were due to autoinhibitory substances in some RF sera. The effect of added γG on titer and therefore on slope may be seen by comparing titers obtained with cell concentration of 0.125% and 2.0%. At a cell concentration of 0.125% a titer reduction of less than one tube was obtained; in contrast, at 2.0% cell concentration, the titer reduction was

four tubes i.e., 16-fold. In effect, these data show that the inhibitory effect of added γG decreased with increasing dilution of the mixture, in spite of the fact that the ratio of added γG to RF was the same at all serial dilutions.

Effect of heating serum: Aliquots of serum were heated at 63°C for varying times, from 0 to 60 min. Results are summarized in Table 16. Heating of the serum for 60 min. destroyed all agglutinating activity. Heating for 30 min. reduced the titer 64-fold and raised the slope -1.0 to -1.8. Heating 1:20 diluted serum for 30 min. resulted in only a two-fold titer decrease and a slope rise from -1.0 to -1.3

In other experiments, RF serum was also heated at 60°C and 56°C. At 60°C, heating resulted in titer decrease andslope increase. However, heating at 56°C for 30 min. resulted in a four-fold titer decrease but no change in slope. This observation suggested that the titer decrease was due to heat destruction of RF whereas the increase in slope was due to formation of denatured or aggregated γ-globulin in the sample, and that the two processes proceeded at different rates.

Fractionation experiments. In an attempt to locate the autoinhibitors presumed responsible for the high slope effect, several RF sera were fractionated by 10-fold dilution with cold distilled water. Typical results are presented in Table 17. Where RF activity appeared in the precipitate fraction (R6) slopes were reduced, but not to -1.0. Where some (R7) or all (R2) RF activity appeared in the supernatant, these titers were associated with an increase in slope. Thus, it appeared that under

the conditions of this experiment the autoinhibitors appeared principally but not exclusively in the supernatent (pseudoglobulin) fraction regardless of where the RF activity was found. Serum R2 is of the type reported by Nosenzo, Primack and Heimer (86) where RF activity is located in the "pseudoglobulin" fraction.

Several RF sera were separated into 7SγG and RF fractions by column chromatography on DEAE cellulose. Removal of the γG component by this procedure resulted in a drop in slopeof the RF fraction (Table 18) Serum R7 (intermediate slope type) was reduced from -1.6 to -1.2 in this way. Similarly R10 (high slope) was reduced from -1.8 to -1.0. Serum R8 which was a low slope type showed no change in slope. Addition of a low slope γG to intermediate or high slope RF was without effect on the slope. On the other hand, addition of a high slope γG to low, intermediate, or high slope RF served to raise the slope in all cases.

A similar experiment was carried out employing the more conventional TSC inhibition procedure (87). Varying quantities of low, intermediate, or high slope 7SγG were added to three RF preparations obtained by DEAE cellulose column chromatography (Table 19), in order to determine the relative potency of these three types of γG in inhibiting RF agglutination. In the cases tested, high slope γG was more inhibitory than intermediate or low slope γG. This was the case with both autologous and isologous RF preparations. Both intermediate slope and low slope γG exhibited less inhibitory potency with all RF preparations, and no distinction could be discerned between the two.

Intermediate complexes: It was considered a possibility that high slope phenomena was related in some way to the presence of RF complexes of the 22S or the intermediate type. In order to investigate this possibility, the rheumatoid arthritis serum Do* was examined by the block titration method. This serum has been investigation by Kunkel et al. (88) who have shown that it contains large amounts of intermediate complexes.

Titration of whole serum Do yielded a slope value of -1.5 (Table 20). Intermediate complexes have been shown (88) to be dispersed to lower weight substances by the addition of excess γG. If such complexes were the principal cause of the high slope effect, then addition of excess γG to such a serum should result in a lowered slope. When 10 mg of pooled 7SγG was added to a 1:40 and 1:80 dilution of serum Do, slope values of -1.5 and -1.6 were obtained. No significant lowering of slope was observed. Addition of heated 7SγG resulted in an expected increase in slope to -2.0 in this case.

Serum Do was fractionated on DEAE-cellulose as previously described. The RF containing fraction (Peak II) yielded a slope of -1.0. Addition of unheated 7SγG caused a non-significant slope rise; heated 7SγG

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* Kindly supplied by Dr. H. F. Kunkel, The Rockefeller University, New York, N.Y.

caused a sharp slope rise.

Two intermediate slope sera were examined in the analytical ultracentrifuge. Serum R21 (slope -1.6, TSC titer, 20,480) contained 9.6 S (3.3%) 17.0 S (0.4%) and 20 S (0.2%) components. However, serum R7 (slope -1.4, TSC titer, 2560) had only a 17.1 (1.2%) component in addition to the usual 4S and 7S fractions. All S values are uncorrected. Only in the higher titered serum were intermediate complexes or "22S" complexes observed. It appeared that increased slopes could be found in both the presence and absence of RF complexes.

Adsorption experiments: It was considered possible that low slope sera also contained autoinhibitors but in concentrations too low to be detected by the block technique. Partial removal of RF should, therefore, increase the relative autoinhibitor concentration. Accordingly, several low slope RF sera were repeatedly adsorbed with a 10% γG conjugate to polyaminopolystyrene. This technique served slowly and gradually to remove RF activity from the sample. After each adsorption, the slope value of the remaining supernatant was determined. Of the four samples studied in this way (Table 21) all showed an increase in slope with removal of the RF content. Ultimately, with continuing adsorption, the effect was reversed and the slope values decreased under conditions where most of the agglutinating activity had been removed. The slope rise observed in these experiments suggested that autoinhibitory substances were in fact present in the four low slope samples studied. The subsequent slope decrease observed after repeated adsorptions was possibly due to removal of the RF most specifically directed against

the autoinhibitors and also, in part, to the relative increase in heterophil antibody concentration. In a control adsorption experiment 0.15 M NaCl was substituted for serum (Table 21, footnote a). After each adsorption, the supernatant was tested for the presence of γG by gel diffusion against rabbit antiserum to human γG. Even after eight adsorptions no γG could be detected in this way. Thus, the slope rise was not due to desorption of inhibiting γG from the polyaminostyrene-γG conjugate.

Results with cells coated with animal γG: Four RF sera were studied with two sets of tanned sheep cells, one coated with human γG, and the other coated with rabbit γG (Table 22). Except for the different proteins coats employed, identical experiments were carried out with the two sets of cells.

Higher titers were obtained with human γG coated cells as compared to rabbit γG coated cells. The ratio of titers obtained with these two cell preparations ranged from 4 to 128.

Two sera yielded identical slope values with both test systems whereas two sera did not. Thus, R19 was of the low slope type and R18 was of the intermediate slope type with both human and rabbit γG coats. On the other hand, R17 which was high slope with human γG coated cells, was low slope with rabbit γG coated cells. A similar but less pronounced difference was seen with R20.

Tonder and Milgrom (89) have compared titer values of four rheumatoid arthritis sera using the sensitized Sheep Cell Test and two different anti sheep cell antisera, one from rabbit and the other from guinea

pig. In the cases studied, higher titers were obtained with rabbit antiserum than with guinea pig antiserum at the same sensitizing concentration. Slope values have been calculated by us from their titration data at the different antisera concentrations (Table 22).

Three of the four sera tested with rabbit γG coats were low slope, and the fourth serum was high slope. All four sera when tested with guinea pig γG coats yielded high slopes. In all cases, sera tested with guinea pig γG coated cells yielded higher slope values than when tested with rabbit γG coated cells. In fact, two sera yielded slope values of -2.5 with guinea pig cells. This value exceeds the highest slope value of -2.1 previously obtained in this study with a rheumatoid arthritis serum when tested with cells coated with human γG.

Studies on rabbit anti-human γG antiserum: Four rabbits were immunized with 7.5 mg of human γG (dissolved either in saline or in Fruend's complete adjuvant), followed by a second injection of 10 mg, 4 days later. Initial bleedings were taken on the 11th day and approximately weekly thereafter. These bleedings all exhibited high agglutination titers when tested with human γG coated tanned sheep cells (Table 23). In all cases the initial bleedings were of the low slope type. In three of four animals, slope increases were observed in sera obtained on day 18 or 25. Rabbit 942 exhibited a steady increase in slope from -1.0 to -1.9 between days 11 and 38. The slope increase was paralleled by a decrease in antibody titer during the same period of time. A similar pattern was seen with rabbit 216. In the case of rabbit 945, the slope

rise on day 25 was accompanied by a rise in agglutinating antibody titer.

Rabbit 942 was given a booster injection of 10 mg of human γG on day 48 which resulted in a second increase in antibody titer. However, slope values obtained from bleedings from days 56 to 80 remained low (-1.0 to -1.2) and did not rise to the previous high value of -1.9 obtained on day 38 with this animal.

Portions of all antisera obtained were subjected to treatment with 0.1 M mercaptoethanol, which is known to depolymerize γM antibodies. In no instance wasa significant difference in antibody titer value or in slope obtained as a result of this treatment.

III. DISCUSSION

In the present study, various RF sera were serologically titrated with six different concentrations of γG coated tanned sheep cells. It was demonstrated that double logarithmic plots of titers vs. cell concentration were linear. It might be expected that decrease in titer should be proportional to the increase in cell concentration and, therefore, that the slopes of such plots should be -1.0. This is based on the simple assumption that the minimum number of RF molecules required for agglutination is proportional to the number of cells to be agglutinated. As the cell concentration increases, the required number of RF molecules also increases or, conversely, the maximum serum dilution (titer) at which agglutination is still observed decreases. Algebraically, the relation between cell concentrations (C) and titer (T) would be:

$$TC=K \qquad \text{---- Eq. (4}$$

rearranging and taking logarithms:

$$\text{Log } T = \text{Log } K - \text{Log } C \qquad \text{---- Eq. (5}$$

Thus it follows that a plot of Log T vs. Log C should be linear with a slope of -1.0.

In fact it was observed that only 43% of the sera examined yielded slopes of -1.0. Another 44% gave a mean slope value of -1.4 and 13% gave a mean slope value of -2.0. Thus, more than half (57%) of the sera studied exhibited slope values greater than the theoretical value of -1.0.

Of direct pertinence to the present results were the findings of Tonder in his detailed investigation of the Waaler-Rose (sensitized sheep cell) test (90). In this study, 40 RF sera were titrated with several batches of sheep cells sensitized with progressive dilutions of rabbit anti-sheep cell antiserum (amboceptor). In effect, he found that in most cases double logarithmic plots of titers vs. amboceptor dilution were linear. Further, 42% were of the low slope type (Type P in Tonder's notation); 35% yielded intermediate slopes (Type M) and 22% were high slope (Type S). In short, 57% of the sera tested yielded slopes greater than -1.0. The data in the present study are in general agreement with these findings. They suggest that the high slope phenomenon is, in fact, a characteristic property of many RF sera. It appears that the use of constant cell concentration sensitized with different dilutions of amboceptor is the equivalent of the use of different tanned cell concentrations all coated with the same quantity of γG per cell. In both cases, the parameter which is varied is the number of potential reactive sites available for combination with RF.

What then, is the cause of the high slope effect. The fact that it is revealed by two different test systems which employ different reactants suggests that the slope effect is not a consequence of the test procedure. The fractionation experiments listed in Tables 17 and 18 are indicative of the reason for this effect. Removal of 7S γG by DEAE cellulose column chromatography, from a high slope serum converted

that serum to a low slope one. Recombination of the two separated components restored the high slope effect. These experiments demonstrate that a component of the patient's own 7S γG is responsible for the slope effect. Similarly, addition of aggregated γG to a low slope serum caused an increase in slope (Table 14, and 15). Where constant amounts of aggregated γG heated for varying times were added, the slope increased with heating time. Commercial pooled γG heated or not, is known to act as an inhibitor of RF agglutination reactions. The effect on slope of addition of pooled γG to a low slope serum appears to be analogous to the process which occurs naturally in many RF sera. Therefore, it seems probably that the high slope effect is a consequence of autoinhibitory components present in the patients' own 7S γG fractions.

Data in Table 16 shows that inhibition varied with serum dilution. In this experiment, addition of γG of increasing inhibitory potency decreased titers by 4 tubes at 2% cell concentration (e.g., low serum dilutions). On the other hand, titer reduction was at most 1 tube at 0.125% cell concentration (high serum dilution). In these experiments both serum and inhibitor were of necessity serially diluted together; hence, the ratio of RF to inhibitor was always constant. In spite of this, the added inhibitor was more effective at low dilutions than at high dilutions.

Thus, the effective concentration$^{(c)}$ of RF available for agglutination varied with serum dilution. If we employ an exponential expression of the type:

$$C = K\,(RF)^{a} \qquad \text{---- Eq. (6}$$

where (RF) is the concentration of rheumatoid factors, and K and a are constants. Since (RF) is inversely proportional to serum dilution (titer),(RF): (1/T) it follows that:

$$\text{Log } C = \text{Log } K - a\text{Log } T \qquad \text{---- Eq. (7}$$

A plot of Log C vs. Log T should be linear with a slope of -a.

It may be inferred from these results that the association between RF and inhibitory γG is labile, and reversible upon dilution. This is in agreement with the results and views of Grubb (85), Vaughan and Waller (91) and Kaplan and Jandl (84).

The literature is replete with numerous investigations that have demonstrated by various fractionation techniques the presence of inhibitory substances in rheumatoid arthritis sera. The prozone phenomena observed in the titration of some sera have been ascribed to the effect of inhibitors competing with the γ globulin coating of the sensitized sheep cells for the RF in the serum (92, 93, 94). Vaughan and Waller have shown (91, 94) that the γ globulins obtained from rheumatoid sera exhibiting prozones were more inhibitory in a standard test system than γglobulins obtained from nonprozone sera. Some RF sera have been shown to undergo spontaneous precipitation (95, 96), presumably as a result of reaction between the patient's own RF and his autologus altered γ globulin (76). Allotypic 7S γglobulins have been described which can inhibit RF agglutination of erythrocytes sensitized with selected anti-Rh sera. This has been the basis for the Gm system of γglobulin classification (97). Various investigators (98, 99, 100) have also reported

the existence of thermolabile inhibitors which interfere with RF agglutination, notably in procedures employing γG coated latex particles.

It seems reasonable to conclude that many of the above mentioned phenomena are due to the simultaneous presence of RF and its specific inhibitor in the same serum. Harboe (101), for example, has reported that, of 25 RF sera tested which exhibited prozones, and were Gm a+, 17 contained anti Gm (a) agglutinating substances. However, Harboe (101) and Schoenfield and Epstein (102) have also reported prozones or autoinhibition in RF sera of Gm (a-b+) and antiGm (a) specificity. These findings indicate that inhibitory activity cannot always be accounted for by the presence of type specific γ globulins coexisiting in the same serum with the corresponding anti-γglobulin agglutinating substances. Allen and Kunkel (103) have shown that 8 out of 12 individual isolated RF preparations agglutinated certain Rh sensitized red cells while the parent sera did not. This finding is also evidently due to autoinhibition by autologus γ globulin.

It is not possible in the present investigation to identify the nature of the inhibitor responsible for the high slope effect. One may, however, with reasonable assurance exclude thermolabile inhibitors from any role in the process since heating a serum at 56^{o}C for 30 min. caused no change in slope. Under these conditions, the thermolabile inhibitors, such as described in the literature, are destroyed.

The block technique here is essentially demonstrative rather than quantitative in its identification of the autoinhibitors. A meritof

this procedure is that no fractionation or treatment of the test serum is required nor is the addition of extraneous γglobulin necessary. According to the present data as well as that of Tonder (90) most RF sera contain autoinhibitory 7SγG. It may well be that the remaining sera also contain autoinhibitors at levels below that detectable by the block technique. Evidence for this view is the observation by Kaplan and Jandl (84) that, of 35 randomly selected RF sera, all showed prozones at very low dilutions in the Rh sensitized red cell agglutination test. Further, Tonder (90) has calculated from Vaughan's data (94) that adsorption of an individual RF serum with immune complexes converted that serum from a -1.0 to -2.0 slope. Similar studies in the present report (Table 21) also showed a slope raising effect after adsorption. Apparently, by removing some of the RF by adsorption, the inhibitor concentration increased relative to the remaining RF concentration, to a level sufficient for the high slope effect to be demonstrable. This suggests that autoinhibitors may actually be present in all or nearly all RF sera including sera of -1.0 slope. It is possible, therefore, that RF sera exhibit slopes greater than -1.0 only when the ratio of inhibitor concentration to RF concentration exceeds some minimum and as yet undetermined value. It migh be expected that a serum such as Do (Table 20) with high inhibitor concentration (as evidence by the presence of RF complexes) should yield a high slope approaching -2.0. However, this serum also had a high RF titer and consequently the inhibitor to RF ratio was presumably diminished. This was reflected in the intermediate slope value of -1.52 that was found. This view is consistent with the present findings that a high titer serum R21 (slope -1.55,

TSC 20,480) exhibited RF complexes, whereas a lower titered serum R22 (slope -1.44, TSC 2,560) did not. Thus, it appears tha autoinhibition can be related to the presence of RF complexes only in sera containing high concentration of RF.

The autoinhibitory effect of autologous γG did show a measure of specificity in that it did depend on the species of γG cell coating employed. Thus of four sera tested with both human and rabbit γG coated cells, two yielded significantly different slope values with two test systems. In both cases, higher slopes were obtained with human γG coated cells than with rabbit γG coated cells.

Comparison of slope values (Table 22) derived from data of Tonder and Milgrom (89) showed that guinea pig γG coats yielded higher slopes and lower titers than did rabbit γG coats. One may presume that RF agglutination of guinea pig γG coated cells is due to a "cross-reaction" which, because of decreased specificity, is relatively more easily inhibitable by autologous human γG.

The situation with rabbit γG coats is more complex. Reactivity with such coats may in part be due to rheumatoid factors directed against antigenic determinants on rabbit γG which are not normally available on unaltered human γG. In sera with such RF, the autoinhibitory effect of autologous γG would presumably be minimized when using rabbit γG coated cells for testing.

These findings support the view that the rheumatoid factor are autoantibodies. However, the view that the rheumatoid factors are isoantibodies is supported by findings of Fudenberg and Kunkel (104) that

RF possessing anti - Gm specificity exhibit in the main isospecificity rather than autospecificity. Further, Fudenberg and Franklin (105) have demonstrated that several individuals with high RF titers consistently failed to agglutinate Rh positive red cells coated with their own anti Rh incomplete antibody.

The question of autospecificity vs. isospecificity of the rheumatoid factors must sill be considered open at the present time. In view of the heterogeneity of RF from various individuals (13, 104) it is quite possible that both types of specificity may exist in the same individual (106). The adsorption experiments of Matte et al (107) where RF with anti Gm(a) specificity could be removed from sera, leaving behind RF lacking any Gm specificity, are consistent with this view.

Landy et. al (108) and Shiebel (109) have employed passive hemagglutination with varying concentrations of tanned cells coated with diphtheria toxoid to assay diphtheria antitoxic sera. Double logarithmic plots of cell concentration vs. serum titers were substantially linear. Scheibel (109) found that guinea pig antisera obtained after one or two stimulations with toxoid yielded slopes not significantly different from -1.0. However, sera of high avidity obtained after three stimulations yielded slopes of -1.3. In addition, the International Reference Serum, obtained in a horse after multiple stimulation, gave a slope of -1.35.

Present studies of rabbit anti human γG antisera showed that early bleedings also yielded slopes of -1.0. Later bleedings, however, exhibited significantly elevated slopes as high as -1.9 in one instance.

Autoinhibition of agglutination of manifested by the elevated slope effect has now been recognized in three immunosystems: diphtheria antitoxin, anti human γG, and rheumatoid factors. Only in the last case has another serum component been implicated in this autoinhibitory process. It is difficult to see how autologous protein could inhibit the diphtheria toxin-antitoxin system since the antigen is not closely related to host serum components. One may speculate that the phenomenon might be explained in another way. After miltiple antigenic stimulation and extended antibody production, there may arise "anti-antibodies" (110) directed against the original antibodies. The original antibodies could have acquired autoantigenicity due to denaturation caused by in vivo reaction with the original immunizing antigen as suggested by Milgrom et al (111). Such anti-antibody could interfere with the agglutination reaction between antibody and original antigen adsorbed to tanned sheep cells. If the association of antibody and anti-antibody is "weak", then dissociation upon dilution could occur. Autoinhibition would, therefore, be manifest only at low serum dilutions, and elevated slopes would result.

Aho and Wager (112) and Abruzzo and Christian (81) have succeeded inducing anti-antibody formation in rabbits after hyperimmunization with ovalbumin or killed bacteria respectively. It is noteworthy that in rabbits immunized with Bacillus subtilis, appearance of rheumatoid factor like anti-antibody occurred consistently later than did anti B subtilis agglutinins (113).

In the case of RF, the possibility that the autoinhibitor is "antibody" rather than "antigen" cannot be excluded. Thus Swierzynska

and Woznicsko-Orlowska (114) have reported the existence of an "incomplete" rheumatoid factor that can bind to sensitized sheep cells but without causing their agglutination. It is possible that such an "incomplete" RF could exert a blocking effect in agglutination tests which, if dilution dependent, would result in enhanced slopes.

Of interest in this respect are the findings of Heimer and Levin (115) that there exists in sera from patients with rheumatoid arthritis a γM protein component with the property of inhibiting complement fixation by various antigen rabbit antibody complexes. This macroglobulin is distinct from rheumatoid factor and does not agglutinate particles coated with γG. Its inhibitory properties are destroyed by treatment with mercaptoethanol and it is apparently not adsorbed to heat aggregated γG or to antigen antibody complexes. Present evidence indicates that the complement fixation inhibitor and the RF agglutination autoinhibitor are different proteins. What relation, if any, they bear to each other is as yet unclear.

IV. FLUORESCENCE STUDIES

PREPARATION OF DNS COUPLED TO γG GLOBULIN

Application of fluorescence and fluorescence polarization techniques to the study of RF-γG interactions requires that one of those two components have distinctive fluorescence properties. In principle the intrinsic fluorescence of either protein could be employed but in practice this was not deemed suitable for several reasons: a) protein fluorescence intensity due to tyrosine or tryptophan residues was rather low, b) extensive purification of RF serum to remove non protein fluorescence would be required and c) separation of the fluorescent contribution from γG and from RF would not be possible.

For these reasons it was elected to use a chemically attached fluorescent tag in this work. One such material that has been widely used for this purpose is dimethylamino naphthalene sulphonyl chloride (DNS-Cl). This material when dissolved in acetone or dioxane and added to cold protein solution at pH 7 to 8 readily reacts with the protein to yield the intensly fluorescent dimethylamino naphthalene sulphonate (DNS) derivative.

Theoretically the fluorescent tag could be applied to either antibody or antigen. However, in this work tagging of antibody (rheumatoid factor) proved to be unsuitable, since the tagging procedure caused considerable inactivation of RF activity as well as non specific tagging of other serum components. As a result it was found expedient to tag the antigen (γG immunoglobulin) instead.

In the course of this work various problems were encountered in preparing pure γG coupled to DNS, which was free of γG aggregates, or hydrolyzed DNS-γG either free or adsorbed to aggregated γG. The method finally adopted is detailed under Methods and generally follows the procedure of Steiner and Edelhoch (32). The complete sequence of experiments performed in establishing this procedure will not be detailed here. Instead selected data will be presented to illustrate the solution of the purification and other problems encountered.

In early experiments removal of free unreacted or hydrolyzed DNS-Cl was attempted by exhaustive dialysis against frequent changes of 0.15M NaCl. In general the volume ratio outside and inside the dialysing bag ranged from about 5:1 to 10:1. After each change the dialysate was examined with an ultraviolet lamp for evidence of fluorescence. In one instance considerable fluorescence was found even after more than a dozen changes. It appeared that free DNS was slowly being released with each dialysis step. This suggested that considerable DNS was adsorbed to γG rather than being covalently bound to it. In view of the tendency of γG to aggregate and the well known observation that denatured or aggregated proteins tend to bind dyes better than native proteins, it seemed possible that free DNS was adsorbed to aggregates of γG. Accordingly the DNS, γG mixture, after tagging, was subjected to precipitation with 2.18M sodium sulphate (5:2v/v) as described in Methods. This served to precipitate aggregates of γG. When the γG aggregates were redissolved in 0.15M NaCl and subjected to exhaustive dialysis slow release of fluorescent material was again

observed. On the other hand exhaustive dialysis of tagged 7SγG quickly removed unbound DNS and fluorescent free dialysates were soon obtained.

As indicated above dialyzable fluorescence was present in the 7SγG-DNS fraction. In an attempt to remove the free fluorescence, the 7SγG-DNS fraction was subjected to gel permeation chromatography (GPC) in a 6 X 50cm column of Sephadex G-25. This experiment is shown in Figure 25. Essentially all fluorescent actively passed through the column in association with theprotein band which was largely collected in tubes 5-8. Under UV light one diffuse yellow band, characteristic of the γG-DNS conjugate, could be seen moving through the column during the experiment. Dialysis of eluted protein fractions again yielded considerable dialyzable fluorescence. It was apparent from these results that GPC with Sephadex G-25 did not resolve free DNS from the γG-DNS conjugate.

In another experiment GPC was performed, this time with a 6 X 50cm column of Sephadex G-75. Results are shown in Figure 26. As in the previous experiment the major peaks of fluorescence and protein content coincided and were collected in tubes 4 & 10. This material when pooled and dialysed did not yield any discernable free fluorescence in the dialysate. In contrast to the experiment shown in Fig. 25 a second peak of fluorescent activity was obtained (tubes 16-17). This material was substantially protein free* and the fluorescence was readily dialyzable.

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*As can be seen from the spectral data for free DNS (Fig 29) the observed absorbance at 280mμ in Fig. 25, tubes 16 and 17 can be accounted for by free DNS alone.

The resolution of fluorescence into two components was strickingly visualized by looking at the column under a UV lamp. Fig. 27 shows the column appearance just before collection of tube 4. Two clearly distinct fluorescent bands could easily be seen. One, a blue-green band near the top of the column was due to free DNS. Its movement down the column was strongly retarded. The other thicker yellow band near the bottom of the column was due to DNS-γG and its passage down the column was little retarded.

It was clear from these experiments that a good separation of free DNS from conjugated DNS was obtained by GPC with Sephadex G-75 but not with G-25. In this connection Cuatrecasas, Edelhoch and Anfinsen (116) have recently reported separation of free DNS from DNS conjugated by means of GPC with Sephadex G-25 (fine). To insure complete removal of free DNS they however resorted to multiple filtration through Sephadex as well as repeated precipitation with saturated ammonium sulphate. In the present work, sodium sulphate precipitation and filtration through G-75 followed by brief dialysis proved sufficient to purify DNS-γG. Alternately a second filtration through G-75 could replace the dialysis step.

Table 24. is a summary of three conjugation experiments for preparation of DNS-γG. Batch A was prepared with twice as much DNS- Cl as B, and was allowed to react 4 times as long. Yet the degree of tagging was virtually the same. The tagging ratios of 0.4 and 0.5 respectively, were clearly non integer values, suggesting that tagging was not homogeneous. In B a small amount (3.5%) of tagged protein was obtained with a much higher tagging ratio (Table 24. footnote) than the major fraction. This finding again indicated non-homogeneous

tagging. Batch C which was done at a lower protein concentration and a higher ratio of added DNS/γG yielded a much higher tagging ratio than did A or B. Aliquots of this preparation were purified by GPC or dialysis. In either case the degree of tagging was about the same.

In these and other experiments, the degree of tagging varied considerably from batch to batch. This variation could not be clearly correlated with such variables as pH, reaction time, or protein concentration. Increase in tagging did seem to relate to increased added DNS- Cl, in spite of the fact that over 99% of added DNS was not found coupled to γG. Cold acetone or dioxane were both found useful as solvents for DNS-Cl. However, dioxane froze at cold room temperature, hence it was found desirable to either dilute the dioxane-DNS-Cl solution with 0.15M NaCl or to adjust its temperature to about 12° to 15°C before adding it dropwise to the γG solution.

Finally the recovery of non-aggregated γG coupled to DNS varied greatly among different batches, from 7 to 32% of starting γG. Again the reason for this variation was unclear. It did appear that better recoveries occurred at lower starting γG concentrations, i.e. aggregation of γG during the tagging reaction was promoted by higher γG concentration.

SPECTRAL DATA

Dimethylaminonaphthalene sulfonyl chloride (DNS-Cl): Fig. 28. is a plot of the light absorbance of this compound, in the visible and ultraviolet wavelength ranges, employing 95% ethanol as the solvent. In this solvent, DNS-Cl exhibited absorbance maxima at 360 and 255mμ with an isosbestic point at about 350mμ. The reason for this spectral shift was thought to be possibly due to reaction between the acid

chloride solute and the ethanol solvent yielding the ethyl sulfonate derivative.

Dimethylaminonaphthalene sulfonic acid (DNSA): The light absorbance of the free sulfonic acid in the visible and ultraviolet wavelength ranges is shown in Fig. 29. As might be expected for a compound of this type, absorbance exhibited a marked pH dependence. Thus DNS dissolved in 0.01N HCl and therefore unionized yielded a λ max. at 282.5 mμ. When dissolved in 0.01N NaOH however, λmax. of the unionized form of DNSA was observed at a higher wavelength, 315mμ. The isosbestic point for this transition was 298mμ.

A similar effect was seen in the DNSA fluorescence excitation spectrum (Fig. 30). In this experiment λ max. values were at 295mμ and 330mμ in acid and base respectively. These values were about 10-15 mμ higher than those obtained in the absorption experiment. Such peaks do not always coincide for theoretical or instrumental reasons.

The fluorescence emission of DNSA is shown in Fig. 31. A major fluorescence peak occurred at 505mμ in acid and 500mμ in alkali indicating little or no effect of pH on λmax. of fluorescence emission. Intensity of emission was however strongly pH dependent. Emission intensity in alkali was four times stronger than in acid and fluorescence excitation in alkali (at λ max) was about 3.5 times higher than in acid (Fig. 30.).

It can be seen in Fig. 31 that a secondary emission peak was obtained at lower wavelength. The one in alkali at 330mμ coincided with the excitation wavelength employed and can therefore be ascribed to light scattering rather than fluorescence. However, λ max in acid was obtained at a wavelength of 340mμ; a value distinctly higher than the excitation wavelength of 295mμ. It would thus seem to be authentic fluorescence emission.

Fig. 32 is a double logarithmic plot of fluorescence intensity (I) vs DNSA concentration. It may be seen that in the range studied Log I was linearly proprotional to Log C. In this experiment, fluorescence was about 40 fold greater in alkali than in acid solution. This additional enhancement of alkaline fluorescence was largely due to the fact that a single excitation wavelength at 350mμ was employed and that this value is closer to the maximal excitation wavelength of DNSA in alkali than in acid.

DNS-γG: Figures 33 and 35 summarize fluorescence behavior of the tagged protein preparation, DNS-γG. Fig. 33 shows the fluorescence excitation spectrum of the tagged protein, with emission measured at 510mμ. At alkaline or neutral pH values, excitation was observed at about 340mμ. In acid excitation was bimodal with one peak at 370mμ and another at 300mμ. It will be recalled (see Fig. 30) that the free DNSA in acid exhibited a peak of excitation at 295mμ. Why such an apparently similar peak should be observed for the conjugated compound was not clear.

A higher order excitation peak was observed at 510mμ. Neither

the intensity nor wavelength of this peak was strongly influenced by pH.

The differential absorption spectrum for DNS-γG is shown in Fig 34. Three DNS tagged preparations were compared to equal concentrations of untagged γG. Maximum UV light absorption, due to the DNS label was in the wavelength range, 335 to 340mμ. This compared favorably with the results obtained in the fluorescence excitation experiment (Fig. 33).

The emission spectrum of DNS-γG is given in Fig. 35. An emission peak was seen at about 340mμ, close to the excitation wavelength of 350mμ evidentally also due to light scattering. The intensity of this peak was somewhat diminished in alkali possibly due to the solution of strongly scattering γG aggregates at this pH.

Major fluorescence emission was observed at 510-520mμ. In this instance fluorescence intensity was strongly affected by pH, with intensity increasing with pH.

A very small peak was observed at 385mμ and a somewhat larger one at 675mμ. The nature of these peaks was not investigated but it seems probably that these were due to Raman or higher order Rayleigh scatter (118).

Figures 36 and 42 show the fluorescence intensity of DNS-γG as a function of protein concentration. As is generally observed in fluorescence work (e.g. see Fig. 31) a linear relation was obtained between log intensity and log concentration in the concentration range studied. This was true both in the absence of polarizing elements (Fig 36) and in their presence (Fig 42). Under conditions employed

in this experiment, fluorescence intensity was about twice as great in 0.01N NaOH as in phosphate buffer, pH 7.5.

Effect of temperature: Fig. 37 is a plot of fluorescence intensity at various temperatures relative to fluorescence intensity at 25°C (I/I_{25}) for DNS-γG. A regular decrease in I/I_{25} was seen with increasing temperature. Thus for a 30°C temperature increase, fluorescence emission of DNS-γG, dissolved in 0.15M NaCl, decreased about 29%. The same protein when dissolved in 1.0M NaCl exhibited a similar but less pronounced (14%) diminution of fluorescence over approximately the same temperature range.

Effect of viscosity: Fig. 38 is a plot of relative fluorescence intensity (I/I_o) of DNS-γG dissolved in 0.15M NaCl containing various proportions of added glycerol from 0% to 80% (V/V). It can be seen that addition of glycerol resulted in a marked enhancement of fluorescence. Thus at 80% glycerol I/I_o increased some 3.5 times. Control experiments without DNS-γG showed that the enhanced fluorescence was not due to glycerol itself. The increase in I/I_o was related to increased medium viscosity as a result of the added glycerol.

Effect of pH: Experiments previously cited (Figures 35 and 36) indicated that the fluorescence intensity of DNS-γG was affected by pH and was greater in alkali and in acid. This effect is shown in somewhat more detail in Figure 46, which includes a plot of fluorescence (EE) vs pH. Minimal intensity was obtained at pH 1. With increasing pH to about pH 7, intensity tended to increase. Between pH 7-12, intensity remained relatively constant. However, a sharp intensity increase occurred at pH 13. Comparing data at pH 1 and pH 13,

the transition from acid to base resulted in an enhancement of fluorescence intensity of about 15 fold. The various pH values studied were obtained with appropriate buffers. Small fluorescence variations differing from the generalized description above may have been due to specific buffer composition effects.

Other data: Figure 39 is a log-log plot of fluorescence intensity vs concentration of quinine sulphate. This compound is widely used in fluorescence work, and the data presented here illustrate the three possible relationships between concentration and fluorescence.

At low concentrations of quinine (less than 0.05μg/ml) fluorescence intensity was only slightly above blank values and a non-linear relation between log I and log C was obtained. At higher concentrations (from 0.05 to 5.0μg/ml) log I vs log C was linear. Finally at still higher concentration (greater than 5.0μg/ml) fluorescence intensity decreased with increasing concentration. This effect has been termed fluorescence quenching and has been attributed to collisional deactivation of molecular electronic excitation energy (117, page 22).

Fluorescence data has been presented in terms of "relative fluorescence intensity". While fluorescence yields depend on the actual quantum efficiency of the process, the instrument meter readings are also a function of electronic arrangement of the detector. Figure 40 is a plot of relative fluorescence intensity (meter reading) as a function of the detector phototube voltage. It may be seen that at 400 V or less, the detector was inoperative. Between 500 and 700 V sensitivity increased 17 fold. In the present work, phototube voltage was

normally fixed at 600 V.

FLUORESCENCE POLARIZATION (P)

Reproducibility: The use of P data as an index of physical or conformational change is based on the relative comparison to other P values obtained for some standard or previous state. As such the significance of P values can not be assessed without some consideration of the reproducibility of the absolute value of P.

As previously shown Eq. [(1, Methods)] P is calculated from fluorescence intensity values obtained with two Glan-Thompson polarizing prisms oriented in their four possible positions. These 4 positions have been termed EE, EB, BB, BE. Actual readings were taken in a photometer with 100 divisions each about 1.2 mm wide. It was therefore possible to interpolate readings to 0.1 unit (0.12 mm). Under these circumstances it is not unreasonable to assume that the variation in meter readability is about $\pm$ 0.2 units. The question arises what would be the variation in P be in consequence of a $\pm$0.2 unit variation in the four fluorescence readings. Some hypothetical fluorescence readings, exhibiting $\pm$ 0.2 unit variation, and their associated P values are tabulated below.

#	EE	EB	BB	BE	P	ΔP
1	50.0	25.0	20.0	25.0	0.2308	--
2	50.2	24.8	19.8	25.2	0.2248	-2.6%
3	50.2	24.8	20.2	24.8	0.2449	+6.1%
4	49.8	25.2	20.2	24.8	0.2334	+1.1%
5	49.8	25.2	19.8	25.2	0.2164	-6.2%

It can be seen that if the maximum readability error occur in the proper directions (lines 3 and 5) for the four fluorescence values, then the variation in P is about ±6%. Considering that no other source of error is included in this calculation this value seems very large.

Table 25 tabulates repetitive measurements of EE and P on DNS-γG under conditions of stable electronics (Exp 1) and unstable electronics(Exp. 2 and 3). Under the most favorable conditions the standard deviation for the replicate determination of P was 2.8%. Considering all three experiments together, as better reflecting day to day experimental conditions, a mean S.D. of 3.8% was obtained. Based on the oft expressed idea that a significant difference is equal to two or more standard deviations, then for a P value of say 0.20 a significant change in P would equal ± 0.015 or more.

The above data were obtained under closely controlled conditions using one protein preparation and one cell. When several preparations were measured in different cells (table 26) the S.D. was sharply increased, to at least 6.6%. Thus when comparing different proteins in different cells, then for a hypothetical P of 0.20, a significant change in P would equal at least ± 0.03. In evaluating P data presented here, this value has been used in assessing the significance of changes in polarization.

Excitation Wavelength: As indicated elsewhere, most fluorescence measurements in the present work were made at an excitation wavelength (λ ex) of 350mμ and an emission wavelength (λ em) of 510mμ.

To determine how variation in λ ex affected both fluorescence (EE) and fluorescence polarization (P), spectra were determined at different pH values. These spectra are given in Figures 42, 43 and 44.

In general the spectrum of EE proved to be a complex function of both wavelength and pH. At pH 1.0 (Fig 42) three intensity maxima were seen between 200 and 400mμ, and at pH 8.13 four such maxima were seen (Fig 43). In both cases one of them was at or near 350mμ. In alkali (pH 13.0, Fig 44) a single but more intense λ ex maxima at 330mμ was obtained.

It will be recalled that similar fluorescence data was also presented in Fig 33. This earlier data was characterized by a less complex excitation spectrum than those shown in Figures 42 and 43. The reason for this was not clear but may relate to technical differences. The earlier data (Fig 33) was obtained in an Aminco instrument without polarizers but equipped with automatic scanning and recording. In contrast the later data (Fig 42, 43, 44) were obtained with a different Aminco instrument equipped with polarizers but not equipped for automatic scanning and recording.

The spectrum of P also exhibited various complexities in these experiments. At pH 1.0 four maxima were also obtained. The general shape of the P spectrum roughly corresponded to the EE spectrum. This was also true at pH 8.13. The spectrum of P in alkali, pH 13.0 also exhibited considerable variation but was generally less complex than at lower pH values. These results indicated the complex variability of P, and the importance of comparing P values under identical experimental conditions.

Emission wavelength: One spectral experiment was carried out at constant λ_{ex} (350mμ) and variable λ_{em}. This is shown in Figure 45. Maximum fluorescence emission was obtained at 510mμ in agreement with previous work. The spectrum of P exhibited considerable variation that did not seem to be related to the EE spectrum. At 435-440mμ a maximum in P seemed to correspond to a minimum at EE. At 510mμ P appeared comparatively stable and less subject to variation than at other wavelengths.

Effect of pH: Previous experiments have alluded to the effect of pH on both fluorescence intensity and polarization. This question was examined in greater detail in various experiments. The results of these are shown in Figure 46. At pH 1.0, P tended to be low, increasing to pH 2.0. Between pH 2-4, P was substantially constant. Between pH 4-10 there was a regular decrease in P with increase in pH. Finally at alkaline pH values, P again increased between 10 to 12, and sharply decreased at pH 13. The above data were obtained by using different pH buffers. Since some of the effects might be due to buffer composition effects rather than pH effects the experiment was repeated, this time, by titrating aliquots of DNS-γG to various pH values (see footnote, Fig 46). In general P values obtained this way were similar to the buffer experiments. A high P value was obtained at pH 2.5 and values at higher pH's tended to decrease to pH 10.5. However the plateau in P values observed between pH 2 and 4 when using buffers was not observed in the titration experiment.

Effects of DNS-γG concentration: Fig 47 is a plot of P as a function of increasing DNS-γG concentration. In the range from 0.1 to 10 mg/ml (0.01% to 1.0%) P tended to increase with protein concentration. At higher concentration a tendency toward decreased P values was noted. Similar results were obtained with 0.15M NaCl and with 1.0M NaCl as protein solvents.

Ultracentrifugal sedimentation analysis* of a non tagged γG preparation that had not been purified, revealed that the relative concentration of dimers and higher aggregates increased with concentration. Fluorescence polarization analysis of the same protein (using the intrinsic fluorescence of the native untagged γG) revealed a substantial increase in P with increasing concentration. These results are shown in Table 27.

These findings suggest that the increase in P noted with increasing concentrations of DNS-γG may also have been related to the formation of dimers or higher polymers at higher concentrations. The DNS-γG preparations had been freed of aggregates during preparation and then stored frozen until used. Ultracentrifugal analysis* done soon after tagging and purification failed to reveal any aggregates of γG. However in view of the above results the possibility that aggregates were formed during freezing or thawing, is not excluded.

Calculation of Relaxation Time (ρ_H) : Equation (8 has been derived from the theory of fluorescence polarization by Perrin (119)

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*Kindly performed by Dr. S. Kochwa, Dept. of Hematology, Mount Sinai Hospital, N.Y., N.Y.

Weber (24, 25, 26) and others (121). For incident polarized light:

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{RT\tau}{\eta V}\right) \qquad \text{------Eq (8}$$

Where P_o is the fluorescence polarization in the absence of molecular rotation, R is the gas constant (8.314×10^7 ergs/degree/mole); T is the absolute temperature, τ is the lifetime of the excited state of the fluorescent group (13×10^{-9} sec for DNS) (121), η is the solution viscosity (poises) and V is the molecular volume of the fluorescent molecule.

For spherical macromolecules:

$$\rho_H = 3\eta V/RT \qquad \text{-----Eq. (9}$$

Where ρ_H is the mean relaxation time of the fundamental rotating unit

Substituting (9 into (8 one obtains equation (10:

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_H}\right) \qquad \text{-----Eq. (10}$$

As ρ_H has been defined in eq.(9 this expression is applicable only to spherical molecules. Since γG is ellipsoidal in shape, ρ_H in eq. (10 becomes equal to $1/3\left[\frac{1}{\rho_x}\ \frac{1}{\rho_y}\ \frac{1}{\rho_z}\right]$ and ρ_H obtained by means of eq. (10 represents the harmonic mean of the three rotational relaxation times (ρ_x, ρ_y, ρ_z) for each axis of the ellipsoid, x, y, and z.

Following from eq. (10 if one plot $1/p$ vs T/η then the intercept is $1/P_o$ and the slope (5) is defined by:

$$S = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(\frac{3\tau}{\rho_H}\right) \qquad \text{-----Eq. (11}$$

It is thus possible to calculate in this way ρ_H, the relaxation time for DNS-γG.

Figure 48 is a plot according to eq. (10 for two DNS-γG preparations under varying conditions of temperature and viscosity. These two plots as well as others (not shown) tended to be linear, in conformity with the modified Perrin equation (eq. 10). Extrapolation of data to $1/P_o$ yielded a value for ρ_H of 135 nsec (nsec $= 10^{-9}$ sec) when viscosity was varied at constant temperature, and 240 nsec when temperature was varied. In these and other experiments not presented here considerable variation in ρ_H was obtained (60 to 248 nsec).

It has been supposed that the relaxation time of a macromolecule is characteristic of its molecular size and shape and should be a fairly distinctive value. This appeared to be the case in various previous fluorescence polarization studies of γG. Thus Steiner and Edelhoch (32) reported ρ_H values ranging from 140-160 nsec for bovine and rabbit DNS-γG. Chowdhury and Johnson (120) reported distinctly lower ρ_H values ranging from 76 to 93 ηsec. for bovine DNS-γG. Weltman and Edelman (121) have carefully studied this problem for human DNS-γG. Their ρ_H values varied from 43 to 288 nsec depending on conditions of temperature, viscosity, and wavelength of fluorescence excitation. Although they were unable to theoretically account for the wide variation in ρ_H they did demonstrate the influence of experimental conditions on the determination of this perameter. They also pointed out that both protein aggregation as well as contamination by free DNS could affect ρ_H values. In the present work free dye contamination was rigidly excluded, but as previously pointed out,

dimerization or aggregation after purification might have occured in our DNS-γG samples.

Experiments with pooled RF serum: The ultimate objective of this work was to explore the use of the fluorescence polarization technique to study the interaction between RF and human γG. Figure 49 is a plot of P values obtained from a mixture of constant amounts of DNS-γG and a serial dilution of pooled RF serum. At low serum dilutions (high RF concentration) rather high P values were obtained. These values decreased with increasing serum dilution. The mixtures were kept for 5 days in the refrigerator andprecipitation qualitatively noted (Fig 49, top). The region between serum dilutions 1:16 and 1:32, where the sharpest decrease in P occured, more or less corresponded to the end of the precipitin region.

For control purposes this experiment was carried out with a normal human serum devoid of RF agglutinating or precipitating activity. With this RF negative serum significantly lower P values were obtained at equivalent dilutions (Both test sera were selected for the same protein concentration and approximately the same electrophoretic composition). However the trend toward decreasing P values with increasing serum dilution was again noted. In Fig. 49, the ratio of polarization obtained with RF serum to that obtained with normal serum (P_{RF}/P_{NS}) is also plotted.

Between serum dilution 1:1024 to 1:32 this ratio remained relatively constant between 1.0 and 1.1. However at 1:32 and lesser serum dilutions a distinct increase in P_{RF}/P_{NS} to about 1.4 was seen. This ratio increase occurred at serum dilutions where a distinct precipitin reaction between RF and DNS-γG occurred later. The

polarization and ratio data are listed in Table 28.

The rise in P value for both RF and normal serum, with decreasing serum dilution presumably reflected in part the effect of increasing sample viscosity due to increasing serum protein concentration. However the increment in P for RF serum over normal serum, as reflected both by $P_{RF}-P_{NS}$ and P_{RF}/P_{NS} data in Table 28 must be ascribed to the additional effect of the reaction between RF and DNS-γG. This conclusion is confirmed by the precipitin reaction data. It is clear that the fluorescence polarization technique was able to detect the reaction between RF and γG almost immediately whereas agglutination or precipitation methods required hours or days to do so.

The interaction of RF with γG was manifested in the above experiment in another manner as well as by changes in fluorescence polarization. Fig. 50 is a plot of the ratio of fluorescence intensity of DNS-γG in the presence of serum to the fluorescence intensity of DNS-γG in the absence of serum ($EE_S/EE_C = R$). With both normal serum and rheumatoid serum this ratio decreased with decreasing serum dilution (increasing serum concentration). At serum dilutions of 1:128 or less this quenching of DNS-γG fluorescence was greater with RF serum than with normal serum.

The increment of fluorescence quenching by RF serum as compared to normal serum has been measured by plotting the ratio R_{RF}/R_{NS} as a function of serum dilution. This plot is also shown in Fig. 50. In general at high serum dilution where no RF-γG precipitin reaction occurred this ratio was at or near 1 indicating no enhancement of fluorescence quenching by RF serum as compared to normal serum.

However at lower serum dilutions (1:64 or less) this ratio rose markedly to a maximum value of 1:47. This enhancement of fluorescence quenching with RF serum occurred at dilutions where the RF-γG precipitin reaction was manifested. These results indicated that the reaction between RF and γG could be detected not only by increases in fluorescence polarization but also by increases in fluorescence quenching.

Fig. 51 is a plot of P values obtained with mixtures of a constant amount of pooled RF serum and increasing amounts of unheated or heated (aggregated) DNS-γG. In both cases increases in P were noted with increasing γG concentration that appeared to be distinctly greater than that previously seen in the absence of RF serum (Fig. 47). P values obtained with heated DNS-γG were in all cases greater than P values obtained with native unheated DNS-γG. A plot of P_h/P_u over the DNS-γG concentration range studied was maximal at 0.1 mg/ml and its shape was similar to that of classical precipitin curves which exhibit maximum precipitation at or near the equivalence point. This comparison with a precipitin curve may be fortuitous and without significance. However it appears reasonable to suggest that the increase in P noted here reflects the reaction between RF and DNS-γG, and that the higher values for P obtained with heated as compared to unheated DNS-γG are a consequence of the greater reactivity of denatured, as compared to native, γG with RF.

IV. DISCUSSION

The propagation of light is associated with both electric and magnetic fields oriented at right angles to each other and to the direction of propagation. When polarized light is used as a source of excitation in fluorescence it preferentially excites those molecules that have oscillators of absorption with an appreciable component in the plane of the electric field of the exciting beam. Although molecules in solution are randomly oriented, the excited molecules represent a selected group with a preferred orientation. If the mean lifetime of the excited state (τ) is long compared to the time required for disorientation by Brownian Motion (rotational diffusion) then the emitted fluorescence will exhibit little polarization. Consequently small molecules, at high temperatures and in solutions of low viscosity yield low values for P.

Conversely when τ is short compared to the time required for Brownian Motion randomization, then the emitted fluorescence occurs before randomization and shows a higher degree of polarization. This is the case for larger molecules at lower temperatures and in solution of high viscosity.

The degree of polarization especially for macromolecules such as proteins is sensitive to various structural and environmental factors. For this reason many physical processes such as denaturation, molecular expansion (or contraction), association and dissociation are reflected by changes in fluorescence polarization.

Some of the applications of the fluorescence polarization technique

to the study of physical changes in macromolecules have been briefly reviewed in the Introduction. About 50 reports have appeared in the scientific literature dealing with such applications.

Of particular relevance to the present work has been the use of fluorescence polarization for the study of antigen-antibody reactions. The first such application was that of Dandliker and Feigen (36) who demonstrated a decrease in polarization associated with the reaction between fluorescein labelled ovalbumin and rabbit anti-F-ovalbumin antibody This reaction also resulted in pronounced fluorescence quenching. Haber and Bennet (35) studied a variety of antigen-antibody systems by mean of the fluorescence polarization technique. Amoung the antigens studied were: the B chain of insulin coupled to para amino-hippuric acid, bovine pancreatic ribonuclease, and bovine serum albumin, all coupled to fluorescein. In general, when antisera were titrated with increasing amounts of corresponding antigen, a rise in P was noted. A maximum value of P was obtained at antigen-antibody ratios which in the precipitin reaction yielded maximum precipitation. With further addition of antigen, P values decreased. It may be noted that in the present work titration of RF with DNS-γG also yielded increasing values of P to a maximum, and decreasing values of P beyond (Fig. 51). These findings perhaps account for the early observation (36) of a fall in P with increasing antigen. Apparently the direction of ΔP is a function of the ratio of antigen to antibody, (Ag/Ab).

In later work Dandliker et al (37) used the fluorescence polarization technique to study the reaction between penicillin and rabbit and human anti-penicillin antibodies. Using purified antibody as well as antigen these workers were able to calculate both equilibrium and hetero-

geneity constants for these immune reactions. Titration of constant antigen with increasing antibody resulted in increased P values, as was also found in the present work (Fig. 49). However titration of constant antibody with increasing antigen resulted in decreasing P values. This corresponds to the findings of Haber and Bennet (35) and to the present work (Fig. 51) in the (presumed) region of antigen excess.

Tengerdy (122) in a study of conalbumin-bovine anti conalbumin antiserum also obtained decreased P val ues in the region of antigen excess. With increasing antibody P values rose and achieved a maximum value at Ag/Ab ratios more or less corresponding to the equivalence point in parallel precipitin tests.

These findings again illustrate the necessity of relating P values to antigen-antibody ratios and to the equivalence points as determined by other means. Additionally both the size of the antibody (eg. "7S" or"19S") and the size (valence) of the antibody must be factors in determining changes in P values for immune reactions.

Further studies by Dandliker et al (123) and Dandliker and Levison (124) have detailed the theoretical and experimental basis for application of the fluorescence polarization method to obtain both thermodynamic and kinetic information regarding immune reactions.

Other fluorescence-based techniques have proved useful for the study of antigen-antibody reactions. Velick and coworkers (125) have used fluorescence quenching as a measure of the interaction between anti-ϵ-N-dinitrophenyl-lysine antibody and specific hapten. They were able to determine that the dissociation constant for this reaction was

very small, about 10^{-9}. Of interest is that according to these workers, the determination of such small values of K is not possible by the more widely used equilibrium dialysis technique. Eisen and Siskind (126) have employed fluorescence quenching to study hapten-antibody interactions. Their most striking finding from an immunological point of view was that the association constants of sequentially drawn antisera increased progressively with time after immunization. The rise in Ka was delayed by an initially large dose of immunizing antigen and on the other hand hastened by a small dose of antigen.

Other studies of various hapten-antibody reactions by fluorescence quenching include those of Saha, Karush and Marks (127) and Green, Paul and Benacerraf (128). Of particular interest are the studies of Parker and coworkers (129, 130) who showed that the interaction of rabbit antibody specific for the 5-dimethyl aminonaphthalene-1-sulfonamido (DNS) group with the homologous hapten ϵ-DNS-lysine is accompanied by marked fluorescence enhancement. Based on DNS spectral data obtained in both polar and non polar solvents they concluded that the specific combining region of anti-DNS-antibody was essentially hydrocarbon-like in nature.

The fluorescence polarization results presented here concerning the reaction between rheumatoid factor and DNS labelled human γG immunoglobulin are essentially qualitative andpreliminary in nature. It has been seen that addition of increasing amounts of antibody (RF) to constant antigen (DNS-γG) resulted in increased polarization of the DNS fluorescence (Fig. 49) as well as increase in the quenching of the fluorescence (Fig.50). Both fluorescence effects occurred parallel to increased yield in the precipitin test.

When the polarization experiment was done in a more conventional way, following usual immunological techniques, that is by addition of increasing antigen to constant antibody, then the P curve followed the shape of a precipitin curve with an initial rise followed by a later fall (Fig. 51). The maximum in the precipitation curve represents the equivalence point where all antibody and antigen are complexed, form large aggregates, and ultimately precipitate, leaving little or no free antibody or antigen in the supernatent (131, 132). Clearly the maximum amount of complexed antigen occurs at the equivalence point. Since in the present work it is the polarization of antigen fluorescence that has been measured it follows that a maximum in P should occur and that such a maximum should correspond to the equivalence point. This would be in consequence of both complete complexing of antigen by antibody up to the equivalence point, as well as formation of larger antigen-antibody aggregates in the region of the equivalence point. It would be of interest to correlate a maximum in P such as seen in Fig. 50 with light scattering data, to establish this view.

It became clear during the course of this work, than meaningful interpretations of fluorescence data required close attention to the experimental perameters affecting these data. Temperature, viscosity, pH, concentration, and specific buffer effects, all appeared to be variables. Instrumental stability, particularly that of the exciting light source, was crucial in obtaining data of good precision. To deal with various defects of currently available commercial instruments

Dandliker et al. (134) have described a modified light scattering photometer, of their own design, adapted for fluorescence polarization work.

The variation described here, of P, with both excitation and emission wavelength appears to be a general phenomena in fluorescence polarization. Winkler (135) has described this effect for excitation, using DNS labelled rabbit γG. That this effect is not confined to macromolecules is shown by the work of Chen (136) who found for quinine that P was also function of both excitation and emission wavelength. The theoretical basis for these findings remain unclear.

The dependence of P on DNS-γG concentration reported here is of more practical concern. A similar effect has been reported by Winkler (136). On the other hand several other investigators studying DNS-γG have reported that P is independent of concentration (32, 121). The reasons for these discrepancies is not clear. All investigations cited were presumably done on aggregate free preparations of DNS-γG. However it is known that γG can, under certain conditions, dimerize to form a "10S" species (137, 138). It seems possible that this dimerization of γG is related to the various results cited above.

Since γG is the major reservoir of antibodies, and can also serve as an antigen, the application of fluorescence polarization to immune studies requires clarification of the monomer-dimer transition and its effect on polarization.

To sum up at this point, the use of fluorescence polarization and fluorescence quenching to study the reaction of γG with RF has proved useful for a number of reasons:

1). It has permitted the study of the primary reaction between γG

and RF without reference to secondary phenomena such as agglutination or precipitation.

2). It has permitted the study of γG under conditions where a secondary reaction was not obtained, as in the case when γG is undenatured or "native".

3). It offers a possible technique for obtaining kinetic and thermodynamic information (123, 124). Other techniques have not been so applicable.

The present fluorescence data offers support for the view that the interaction of γG with RF is analogous to other antigen-antibody reactions. It has been shown that undenatured γG (unchanged except for DNS labelling) will react with RF but that aggregated γG is more reactive. While this conclusion is suggested by the results of earlier serological investigations it has not been previously demonstrated by a purely physical technique. The results permit one to speculate that RF (or at least some of them) do react with undenatured γG, but that denaturation and subsequent aggregation serve to potentiate this reaction.

It may be argued that the use of impure RF (i.e., whole serum) in the present experiments nullifies the above results. It is true that changes in polarization as well as quenching were also found with normal sera, although always in lesser degree, than with RF sera. Similar effects have been noted by others (37). The possibility exists that DNS-γG, in addition to reacting with RF, complexes or interacts with serum components other than RF, as for example, complement or albumin. Such interactions while devoid of immunological specificity would nevertheless serve to increase the polarization of DNS fluorescence.

Farah and Kurban (133) have demonstrated the formation of albumin-γG complexes as the result of heating serum. In any event it is clear that further work seeking to determine, for example, association constants, will probably require the use of purified RF preparations. However, the present qualitative conclusions, based on distinct differences observed between RF and normal sera, appear to be valid.

SUMMARY AND CONCLUSIONS

These studies have generally been concerned with the reaction of the macroglobulin antibody, known as the rheumatoid factor (RF) with human γG immunoglobulin (γG). Four points have been considered: I. the purification and fractionation of RF; II. the role of denaturation and aggregation of γG in enhancing its reactivity with RF; III. detection of autospecific reactivity between an individual's RF and his autologous γG; and IV. application of fluorescence polarization to study RF-γG interactions.

Purified RF was prepared by three filtrations through Sephadex G-200 columns, but this purification was attended by considerable loss of antibody activity. In other experiments conjugates of diazotized polyaminostyrene and human γG (PAS-γG) were employed in adsorption and immunochromatographic studies of rheumatoid factor serum (RFS) and antihuman γG antiserum. Euglobulin fractions of RFS were chromatographed on PAS-γG columns using stepwise elution with buffers of decreasing pH. At pH 6.3 much of the protein content was eluted but no RF. Between pH 5.25 and pH 4.0 seven fractions of RF agglutinating activity were obtained. In most cases, upon re-chromatography the RF fractions were recovered at the same pH at which they were originally obtained. Treatment of RF fractions with mercaptoethanol resulted in complete loss of agglutinating activity. The capacity of aggregated γG to inhibit agglutinating activity was determined. The earlier fractions obtained from the column required more aggregated γG to inhibit their agglutination in the tanned sheep cell test than did the later fractions.

These results are consistent with previous reports of RF heterogeneity. The variations observed in elution of RF subfractions and inhibition of their agglutination reaction suggest that they differ in their affinity for pooled γG.

The immunochromatographic pattern of an anti-γG antiserum differed from that of the RFS in that no antibody was eluted until pH 4.0 and pH 3.2 where two fractions were obtained. These results reflect the greater affinity of anti-γG antibody, as compared to RF, for pooled γG.

The thermal aggregation of human γG has been studied turbidometrically. For purposes of standardization a series of polystyrene latex suspensions of known particle size were examined. Turbidity was measured as the absorbance of the suspensions as a function of wavelength between 250 and 450 millimicrons. Latex particles of 0.088 microns diameter exhibited the expected relation between absorbance (turbidity=T) and wavelength: $T = K/\lambda^4$ where λ = wavelength. On the other hand latex particles of 1.3 microns diameter followed the relation $T = K/\lambda^0$, that is, turbidity was approximately independent of wavelength. Intermediate sized particles showed an intermediate type of dependence. These data are in qualitative accord with light scattering theory which predicts that the turbidity of "large" particles is independent of wavelength and that the turbidity of "small" particles is inversely proportioned to the fourth power of wavelength.

Human γG was aggregated by heating at 63°C for ½, 1, and 2 hours. In all three cases $T = K/\lambda^4$ in the range of λ from 400-600 millimicrons suggesting that even after 2 hours of heating the largest aggregates were less than 0.1 microns in diameter. For a given sample at a given wavelength turbidity was proportional to concentration. However, the ratio of turbi-

dity to aggregate concentration increased with increasing heating time. Purified aggregates yielded identical turbidity values as did the same concentration of aggregates when not separated from unaggregated γG.

Various RF sera were t itrated with human γG coated tanned sheep cells at six cell concentrations ranging from 0.125% to 2.0%. It was found that in 36 of 63 sera tested there was an abnormally large decrease in titer with increasing cell concentration. Double logarithmic plots of titers vs. cell concentrations were linear. While linearity with a slope of -1.0 was expected, it was found that these 36 sera tested yielded slopes greater than -1.0.

Addition of aggregated γG to a -1.0 slope serum caused a rise in slope. Removal of autologous 7SγG, by diethylaminoethyl cellulose column chromatography, fron a high slope serum lowered the slope. Addition of 7SγG, prepared either from a high slope serum or from some but not all normal sera, to a -1.0 slope serum served to raise the slope. Partial removal of RF by adsorption of a low slope serum also caused an increased slope. Heating at 55°C for 30 min. did not affect slope values.

Block titration of rabbit antisera directed against human γG yielded normal -1.0 slopes with the initial bleedings. However, anti sera obtained 3 to 4 weeks after initial immunization showed elevated slopes. This was true when agglutination titers were increasing, as well as when such titers were decreasing.

It is suggested that in RF sera at least, elevated slopes are due to a dilution dependent autoinhibition of agglutination which arises from the interaction between at least part of the patient's rheumatoid factor with his autologous γG.

The techniques of fluorescence polarization and fluorescence quenching have been applied to the study of the RF-γG reaction. For this purpose γG has been coupled to the fluorescent compound dimethylamino naphthalene sulphonyl chloride (DNS). Removal of excess DNS (as the sulfonic acid) was complicated by the fact that denatured or aggregated γG strongly adsorbed unconjugated DNS. Therefore aggregated γG was removed following conjugation with DNS by sodium sulphate precipitation. Filtration through Sephadex G-25 followed by dialysis yielded preparations containing 2-3 moles DNS bound per mole of γG and substantially free of unbound DNS.

Constant amounts of DNS-γG were mixed with serial dilutions of normal serum (NS) and RFS and polarization (P) of fluorescence measurements were made. In both cases P increased with increasing serum concentration, in part due to viscosity effects. At a serum dilution of 1:16 P was 43% higher with RFS than with NS. At this dilution an appreciable precipitin reaction was later obtained. This increment in P was evidently due to the reaction of DNS-γG with RF. The reaction was also characterized by a considerable degree of fluorescence quenching.

In other experiments RF serum was titrated with increasing amounts of DNS-γG. P values rose to a maximum and then declined. The shape of the P curve was similar to typical immunological precipitin curves.

These results show that the reaction between RF and γG was immediately detectable by fluorescence polarization or quenching whereas agglutination or precipitation techniques required hours or days for completion. Undenatured γG was shown to react with RF as did also aggregated γG.

Summing up the experimental results, the heterogeneity of RF is confirmed and techniques for sub-fractionation have been devised. Evidence has been presented that the reactivity of RF with aggregated γG is related to the particle size of the aggregates. The view that RF is an autoantibody is supported by evidence of interaction between an individual's RF and his own γG. Finally the RF-γG reaction has been studied by fluorescence polarization and quenching. Their reaction appears to be similar to other antigen-antibody reactions.

The discovery of rheumatoid factor in patients with rheumatoid arthritis was received at the time with great interest and excitement by rheumatologists. The rheumatic diseases, of which rheumatoid arthritis is one, as a class have offered clinicians many difficulties in regards both diagnosis and therapy. It was felt by many that RF must be related in some manner to both the etiology and pathogenesis of RA. The research road seeking to establish these correlations has however proved to be a difficult one. More than 1,000 publications have appeared in the last decade on the subject of RF, yet its connection to rheumatoid arthritis or any other disease for that matter, remains unclear.

The most striking feature of RF is its reactivity with γG immunoglobulin, but a curious aspect of the reaction was that γG had to be denatured or "aggregated" before the reaction was manifested. RF would agglutinate particulate matter coated with γG or, when added to a γG solution, precipitate as an RF-γG complex. In either case the reaction would not take place when γG was undenatured and purified of aggregates.

While it has been widely held that RF is in fact an antibody to γG, serological investigations revealed a wide and sometimes confusing spectrum of reactivity between various RF preparations and different types of γG.

The present work as well as that of other investigators (13, 53, 56) clearly shows that RF is indeed heterogeneous. Of the seven RF subfractions found, five reacted specifically with human γG whereas two cross reacted with rabbit γG. Most of the seven fractions could be differentiated by their difference in affinity for aggregated human γG. Further, all of them appeared to have less affinity for γG than did anti γG antibodies prepared by immunizing a rabbit with γG. It would appear that the varying patterns of serological reactivity of RF observed by others are a consequence of this heterogeneity.

Another problem is whether RF is an autoantibody (to ones own γG) or an isoantibody (to someone elses γG). In light of various heterogeneity studies we now recognize that both may be true. In the present work autospecific interaction between RF and γG from the same individual has been demonstrated. This offers strong support for the autoantibody theory. Yet other investigators have demonstrated isospecific RF selected individuals. In the cases studies isospecific RF appears to be the consequence of γG "immunization" in the human either by repeated blood transufions (139) or by placental transfer of γG from mother to child (14,140) or vice versa (141). It appears possible that in some individuals both auto-and iso-specific RF coexist but how this comes about remains unclear.

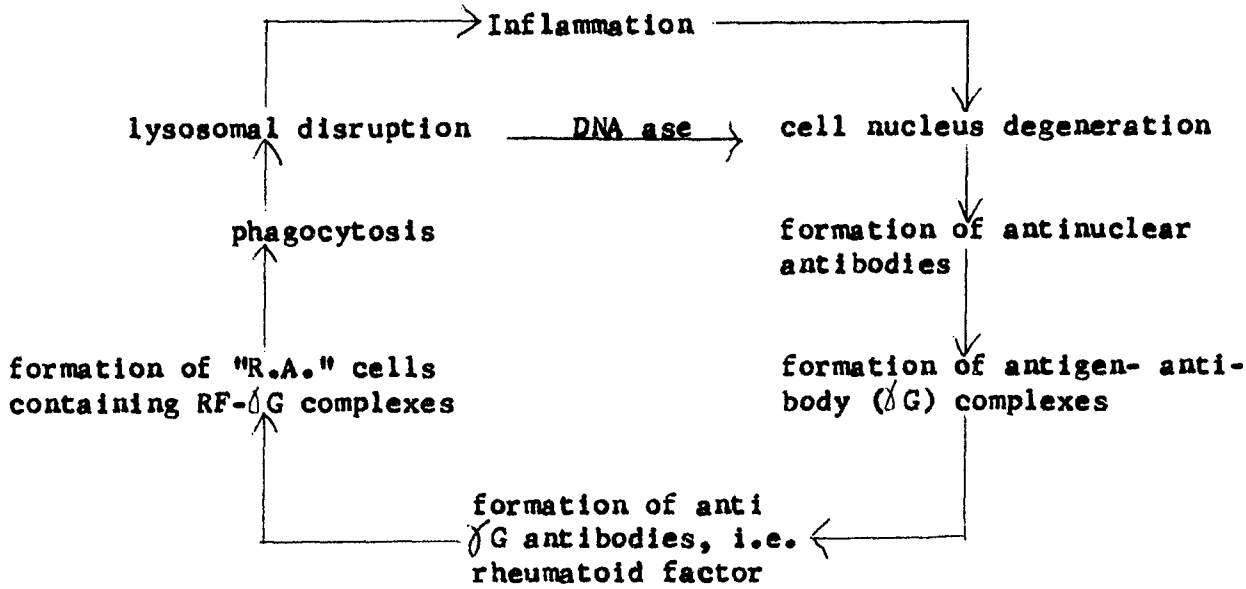
The nature of γG antigecity vis-avis RF remains as a major question. The present study demonstrates that both turbidity as well as reactivity of

thermally aggregated γG with RF increased with heating time. This would suggest that reactivity was directly related to particle size. Unfortunately for the view, studies of other antigen-antibody systems indicate the opposite (142). Alternate possibilities remain. One is that increased heating time serves to enhance γG reactivity by promoting further denaturation and thereby uncovering "hidden" antigenic determinants in the γG molecule to which RF is directed. It is also possible that larger thermal aggregates of γG appear more reactive merely due to decreased solubility as a result of larger size and/or greater denaturation.

While much immunochemical information has been obtained about antigen-antibody reactions by the "classical" techniques of agglutination and precipitation it is also true that deeper and more sophisticated knowledge has come from the application of various physical techniques to these problems. This study has endeavored to apply such techniques, in particular, fluorescence polarization and fluorescence quenching to the study of RF-γG reaction. It has been demonstrated that the reaction between RF and γG is accompanied both by an increase in fluorescence polarization and a decrease in fluorescence intensity. The pattern of these changes resembles those reported for other antigen-antibody reactions. While the results reported here are qualitative they indicate the possibility of using fluorescence techniques in a quantitative manner to obtain equilibrium and thermodynamic data.

The difficulty in relating RF to etiology or pathology of human disease has been previously mentioned. This question lies outside of the limits of this study. Yet it is of interest insofar as it sheds light on the biological processes that make for γG autoantigenicity. A cyclic

mechanism has been described seeking to relate RF to other clinical and immunological phenomena (143). With some adaptation this theory is schematically presented below.



This theory focuses on the primary clinical sympton in rheumatoid arthritis, that of synovial inflammation. This leads to cellular degeneration, release of antigenic substances and formation of so called "anti nuclear" antibodies. The existence of such antibodies and their association with RF is fairly well documented. It is postulated that the antinuclear antibodies circulate as Ag-Ab complexes with nuclear antigens. In the complex γG is "antibody" which as a result of complex formation suffers conformational alteration (19) and so becomes "antigen" leading to formation of anti γG antibodies, that is, RF.

RF now complexes with altered γG and is taken up in leukocytic cells to form so called "R.A." cells. These cells are then phagocytized which leads to lysosomal disruption and release of hydrolytic enzymes and inflammatory substances. In this manner the cycle is completed.

According to this view RF is a product rather than a cause of inflammatory disease, but once arising these antibodies serve to exacerbate and continue the disease process. To the extent that high titers of RF appear to correlate with more severe rheumatoid disease (144) this is a consistent and plausible picture. Whatever the outcome is of these pathogenetic speculations, rheumatoid factor remains a subject of considerable immunochemical interest

TABLE 1.

GEL PERMEATION CHROMATOGRAPHY OF
RHEUMATOID FACTOR SERUM R-W

	Original Serum	AFTER GPC First	Second	Third
Area under peak % of total				
1	-	13.2	43.3	95.2
2	-	42.2	53.7	4.8
3	-	42.7	3.0	0.0
4	-	1.9	0.0	0.0
RF fraction				
initial volume(ml)	4.0	105.0	37.5	10.0
final volume (ml)	4.0	13.5	9.0	10.0
TSC titer				
after volume reduction	40,960	10,240	1,280	20
RF activity recovered				
% of original	100	85	7	1
specific activity*	-	46	18	1

* Titer/area under curve containing RF (in planimeter units)
Values corrected for differences in scale.

TABLE 2.

LOW IONIC STRENGTH FRACTIONATION OF RHEUMATOID FACTOR SERA

Serum	Original Volume	TSC titer	Final Volume*	TSC titer	TSC titer adjusted to original volume
R-(P2)	165 ml	2,560	20 ml	20,480	2,560
R-(P3)	20 ml	1,280	10 ml	1,280	2,560
R-(P4)	360 ml	1,280	20 ml	40,960	2,560
R-(P7)	250 ml	409,600	250 ml	409,600	409,600

*Volume of 0.15 M NaCl used to dissolve low ionic strength precipitate obtained from original volume of RF serum.

TABLE 3.

COUPLING OF DIAZOTIZED POLYAMINOSTYRENE TO HUMAN ɣ G IMMUNOGLOBULIN

Nitrous Acid Added: (m mole/m mole PAS-NH_2)	ɣG Added: (mg/gm. PAS)	Reaction time (Days)	ɣG Coupled (mg/gm. PAS)
1.5	100	1	98
1.5	150	1	99
1.5	250	1	109
1.5	250	3	131
1.5	250	6	174
0.15	250	6	77

TABLE 4.

AGGLUTINATION TITERS (TSC TEST) OF RHEUMATOID ARTHRITIS SERUM AND RABBIT ANTIHUMAN GAMMA GLOBULIN ANTISERUM AFTER REPEATED ADSORPTION WITH PAS-γG AND DAP-γG.

ADSORPTION	RAS		RABBIT ANTI γ G	
	PAS-γG	DAP-γG	PAS-γG	DAP-γG
Before	2560 (U.S.C. 20)		2560 (U.S.C. 160)	
1	2560	1280	1280	20
2	2560	640	1280	10
3	1280	320	320	
4	1280	640	320	
5	640	640	160	
6	640	320	40	
7	320	160		
8	80	40		
9	20	20		

Adsorptions were carried out on 1.0 ml. serum diluted with 1.0 ml. buffer, employing 200 mg. of PAS-γG (20 mg. γ G) or 20 mg. of DAP- γG. Adsorbent and serum were mixed by turning on a rotatory mixer (10rpm.) for three hours at 5°C. Then adsorbent was removed by centrifugation, and 0.1 ml. of supernatent was taken for serological titration. Balance of supernatent was reserved for next adsorption. Titer values are reciprocal of end point dilution. Adsorption step below dotted line was where RF activity was completely removed.

TABLE 5.

RECHROMATOGRAPHY EXPERIMENTS ON RHEUMATOID FACTOR SUBFRACTIONS

FRACTION	pH AT WHICH FRACTION WAS ORIGINALLY ELUTED	pH AT WHICH FRACTION WAS ELUTED UPON RECHROMATOGRAPHY
II	5.25	5.25
III	5.25	5.25
IV	5.0 (0.1M)	5.0 (0.1M)
V	5.0 (0.1M)	5.0 (0.1M)
VI	5.0 (0.5M)	5.25,13%; 5.0(0.1M)13%; 5.0(0.5M)74%
VII	4.5	5.0(0.1M)58%; 4.5,33%; 4.0,8%
VIII	4.0	4.5

TABLE 6.

SUMMARY OF DATA ON POOLED RHEUMATOID FACTOR SUBFRACTIONS

RACTION	pH	PERCENT OF AGGLUTINATING ACTIVITY	AGGLUTINATION TITERS			INHIBITION RATIO	LINES OBTAINED ON IMMUNODIFFUSION vs		
			TSC	TSC AFTER MERCAPTO-ETHANOL	SSC		ANTI HUMAN SERUM	ANTI γG	ANTI γM
II	5.25	23	320	---	---	5.0	1	1	1
III	5.25	21	640	---	---	2.5	2	1	1
IV	5.0 (0.1M)	24	1,280	---	160	2.5	2	1	2
V	5.0 (0.1M)	11	640	---	80	1.25	2	0	1
VI	5.0 (0.5M)	12	640	---	---	1.25	3	0	0
VII	4.5	6	160	---	---	1.25	2	0	0
VIII	4.0	3	160	---	---	1.25	---	---	---

TABLE 7.

REACTIVITY OF THERMALLY AGGREGATED HUMAN γG WITH RHEUMATOID FACTOR*

Heating Time (60°C) (min.)	Amount of Precipitable Aggregated γG (mg/ml)	Amount of Precipitable Aggregated γG (percent)	Amount Heated γG Required to Inhibit TSC Test (μg/ml)	Amount Purified Aggregated γG Required to Inhibit TSC Test (μg/ml) Dissolved in 0.15M NaCl	Amount Purified Aggregated γG Required to Inhibit TSC Test (μg/ml) Dissolved in 0.05M NaOH
0	0.131	0.6	273	----	----
5	0.192	0.9	68	----	----
10.0	0.400	1.8	68	6.25	16.9
16.5	0.644	4.7	34	7.94	24.4
21.5	1.406	6.4	34	5.50	18.1
31.0	2.256	10.3	17	4.37	23.8
40.0	3.112	14.2	17	3.06	14.8
50.5	3.450	15.8	17	3.37	31.3
60.0	3.700	16.9	17	1.81	16.3
75.0	3.800	17.4	17	1.88	20.0
92.0	4.681	21.4	17	1.13	10.6
120.0	6.019	27.6	17	0.75	11.3
					18.75 Mean
					± 6.03 SD

* Test serum R(5458A) TSC titer 1:12, 800, serum diluted 1:500 for inhibition tests. Human γG, original conc. 21.875 mg/ml.

TABLE 8.

OPTICAL ROTATION MEASUREMENTS
OF 7S γG IN VARIOUS SOLVENTS

Solvent	Specific Optical Rotation
0.15M NaCl	-54.6
pH 7.7 buffer	-51.5*
pH 9.4 buffer	-51.2*
0.05M NaOH	-88.0
0.05M NaOH for 10 min. then adjusted to pH 8.2	-86.0

* Data of Jirgensons (145)

TABLE 9.

RELATION OF TURBIDITY TO WAVELENGTH FOR LATEX PARTICLES* OF DIFFERENT DIAMETERS

LP Diameter (Microns)	Lot	N**	S. D.*** (Microns)	Turbidity Proportional to
0.088	LS-040-A	1164	0.0080	$1/\lambda^4$
0.126	LS-052-A	328	0.0043	$1/\lambda^4$
0.264	LS-057-A	577	0.0060	$1/\lambda^3$
0.365	LS-061-A	438	0.0079	$1/\lambda^3$
0.557	LS-063-A	373	0.0108	$1/\lambda^1$
0.796	LS-449-E	85	0.0083	$1/\lambda^{0.5}$
1.305	LS-464-E	142	0.0158	$1/\lambda^0$

* Obtained through the courtesy of Drs. J. Vanderhoff, and L.J. Lippie, Dow Chemical Co., Midland, Mich. Information in columns 1-4 supplied by mfg.

** N=No. of measurements of diameter (electron microscope)

*** S. D. = Std. deviation of mean diameter

TABLE 10.

TURBIDITY DATA OF γG AGGREGATES

Sample	#1	#2	#3
Heating time (min.)	30	60	120
Percent conversion to aggregates	45.0	47.0	56.5
	Optical Density (400 mμ)		
Whole sample, immediately after heating	0.140	0.270	0.457
Whole sample, 24 hrs. later*	0.153	0.296	0.508
Aggregates, after purification **	0.158	0.293	0.502
Aggregates, 24 hrs after purification*	0.150	0.287	0.502

---

* Samples kept in refrigerator at approx. 4°C

** Done 24 hrs after heating of samples

TABLE 11.

RECIPROCALS OF WAVELENGTHS RAISED TO VARIOUS POWERS

λ (μm)	$(1/\lambda^{0.5})\times10^{2}$	$(1/\lambda)\times10^{3}$	$(1/\lambda^{2})\times10^{5}$	$(1/\lambda^{3})\times10^{8}$	$(1/\lambda^{4})\times10^{10}$
400	5.00	2.50	0.625	1.56	0.391
425	4.85	2.35	0.554	1.30	0.306
450	4.71	2.23	0.494	1.10	0.244
475	4.59	2.11	0.443	0.933	0.197
500	4.47	2.00	0.400	0.800	0.160
525	4.36	1.90	0.363	0.691	0.132
550	4.26	1.81	0.331	0.601	0.109
575	4.17	1.74	0.302	0.526	0.091
600	4.08	1.67	0.278	0.463	0.077
625	4.00	1.60	0.256	0.410	0.066
650	3.92	1.54	0.237	0.364	0.056
675	3.85	1.48	0.219	0.325	0.048
700	3.78	1.43	0.204	0.292	0.042
725	3.71	1.38	0.190	0.262	0.036
750	3.65	1.33	0.178	0.237	0.032

TABLE 12.

VISCOSITY MEASUREMENTS OF γG IMMUNOGLOBULIN

γG Concentration (g/100ml)	Flow Time (sec)		Specific Viscosity $\left(\frac{t_s}{t_o}\right)-1$	Reduced Viscosity $\frac{\left(\frac{t_s}{t_o}\right)-1}{C}$
0.0	62.5 62.8 62.8 62.7	62.7	0.0	0.0
0.25	63.7 63.7 63.8 63.6	63.7	0.01595	0.06380
0.50	65.0 64.8 64.9 64.8	64.9	0.03509	0.07018
1.00	70.0 69.9 70.0 69.8	69.9	0.1115	0.1115
1.50	70.8 70.7 70.8 70.9	70.8	0.1292	0.08613
2.00	82.5 82.5 82.4 82.7	82.5	0.3158	0.1579

T=30.0°C, Viscosimeter: Cannon-Manning semi-microtype No. 100 A 462 constant (100°F)= 0.01402 centistokes/sec; (212°F)= 0.1399 centistokes/sec protein dissolved in pH 8.2 glycine-saline buffer.

TABLE 13.

REPRODUCIBILITY OF SLOPE MEASUREMENT WITH DIFFERENT BATCHES OF γG COATED TANNED SHEEP CELLS

SAMPLE	CELL BATCH	TSC TITER	USC TITER	-SLOPE
R1	1	2,560	40	0.98
(low)	2	5,120	40	1.01
	3	2,560	20+	0.98
R2	2	20,480	40	1.46
(inter.)	3	10,240	20	1.39
	4	5,120	20	1.54
	3 (a)	10,240	-	1.39
R3	1	2,560	40	1.99
(high)	2	5,120	40	1.98
R4	2	1,280	20+	1.38
(inter.)	2 (a)	1,280	-	1.37

(a) Serum adsorbed with washed packed sheep cells
USC test negative at 1:5

TABLE 14.

EFFECT OF ADDING HEATED HUMAN γG IMMUNOGLOBULIN TO RHEUMATOID FACTOR SERUM ON AGGLUTINATION TITER AND SLOPE

AMOUNT γG ADDED (mg/ml.serum)	TSC TITER	-SLOPE
0	5,120	1.0
0.005	2,560	1.0
0.05	2,560	1.4
0.10	1,280	1.3
0.50	2,560	1.3
1.00	640	1.4
5.00	640	2.0
10.00	160	2.2

Procedure: 1.0% γG heated at 63°C for 15 min. then added to R5 and incubated for 1 hour at 37°C prior to titration.

TABLE 15.

EFFECT OF ADDED HEATED HUMAN γG IMMUNOGLOBULIN ON AGGLUTINATION TITERS AND SLOPES OF A RHEUMATOID FACTOR SERUM (R5)

Tanned Cell Conc.	Serum alone	Serum + γG heated for varying times (Min.)					Titer decrease (Number of Tubes)
		0	10	20	30	60	
2.0 %	640	160+	80+	80	80	40	4
1.5	640+	320	160	80+	160	80	3 +
1.0	1,280	640	320	160+	160+	160	3
0.5	2,560	1,280+	640+	640+	640	640	2
0.25	5,120	5,120	2,560+	2,560	2,560	2,560	1
0.125	10,240+	10,240+	5,120	5,120	5,120	5,120+	<1
-Slope	1.0	1.4	1.5	1.5	1.6	2.0	

1.0 g.% solution of γG heated at 63°C for times shown, then 100 mg(0.1 ml) added to 1.0 ml. of test serum and incubated for one hour at 37°C prior to titration.

TABLE 16.

EFFECT OF HEATING RHEUMATOID FACTORS SERUM (RS) ON AGGLUTINATION TITERS AND SLOPE

HEATING (TIME) Min.	63°C SERUM DILUTED 1:20 TSC TITER	-SLOPE	63°C SERUM UNDILUTED TSC TITER	-SLOPE	60°C SERUM UNDILUTED TSC TITER	-SLOPE	56°C SERUM UNDILUTED TSC TITER	-SLOPE
0	5,120	1.0	5,120	1.0	2,560	1.0	2,560	1.0
10	2,560	1.2	640	1.3	640	1.3	-	-
20	2,560	1.1	160	1.6	320	1.5	-	-
30	2,560	1.3	80	1.8	160	2.0	640	1.0
60	2,560	1.4	0	-	0	-	640	1.0
120	-	-	-	-	0	-	320	1.1
240	-	-	-	-	0	-	80	1.5

TABLE 17.

COMPARISON OF AGGLUTINATION TITERS AND SLOPES OF WHOLE RHEUMATOID FACTOR SERUM AND SUBFRACTIONS*

SAMPLE	FRACTION	TSC TITER	- SLOPE
R6	Serum	2,560	1.9
	Precipitate	1,280	1.7
	Supernatant	80	-
R7	Serum	5,120	1.5
	Precipitate	1,280	1.3
	Supernatant	1,280	1.9
R2	Serum	40,960	1.6
	Precipitate	80	-
	Supernatant	40,960	1.9

* Prepared by 1:15 dilution of 1.0 ml. RFS with distilled H_2O and 24 hour refrigeration. L.i.s. precipitate dissolved in 1.0 ml. 0.15 N NaCl. Supernatant titers adjusted for original serum volume.

TABLE 18.

FRACTIONATION OF RHEUMATOID FACTOR SERA BY DEAE COLUMN CHROMATOGRAPHY INTO 7S γ G AND RF FRACTIONS

SAMPLE			7S γ G SLOPE TYPE	TSC TITER	- SLOPE
R8				2,560	1.0
R8-RF				5,120	1.0
R8-RF	+	R8-7S	low	2,560	1.0
R8-RF	+	R7-7S	high	2,560	1.4
R7				5,120	1.6
R7-RF				5,120	1.2
R7-RF	+	R7-7S	intermediate	5,120	1.5
R7-RF	+	R8-7S	low	2,560	1.2
R7-RF	+	R9-7S	high	1,280	1.5
R10				10,240	1.8
R10-RF				5,120	1.0
R10-RF	+	R10-7S	high	2,560	1.3
R10-RF	+	R11-7S	low	5,120	1.0
R12				20,480	1.6
R12-RF				20,480	1.2
R12-RF	+	R12-7S	intermediate	20,480	1.7
R12-RF	+	R9-7S	high	10,240	1.8
R12-RF	+	R8-7S	low	20,480	1.9

TABLE 19.

AMOUNT OF 7S γ G REQUIRED TO INHIBIT AGGLUTINATION OF THREE RHEUMATOID FACTOR PREPARATIONS*

	R9-7S (high slope)	R12-7S (intermediate slope)	R18-7S (low slope)
R9-RF (1:320)	0.2	0.9	0.9
R12-RF (1:320)	0.7	0.9	0.9
R8-RF (1:80)	0.1	0.9	0.6

* 0.25 ml. RF preparation (diluted to one fourth of TSC titer)
† 0.25 ml. 7S γG, incubated at 37° for 30 minutes, then 0.5 ml. of 0.25% TSC added. Mixture read by pattern, after overnight refrigeration. Value reported is minimum quantity of 7S γG required to inhibit agglutination.

TABLE 20.

AGGLUTINATION TITERS AND SLOPES OF A RHEUMATOID FACTOR SERUM (Do) CONTAINING INTERMEDIATE COMPLEXES

SAMPLE	TSC TITER	- SLOPE
1) Whole serum	81,920	1.5
2) 10 mg. 7S γG* heated 63°C 30' added to 6.0 ml. of 1:40 dilution of Do.	10,240	2.0
3) As in 2) except 7S γG was unheated.	40,960	1.5
4) As in 3) except 1:80 dilution of Do.	81,920	1.6
5) Rheumatoid factor fraction (Do-Rf)	40,960	1.0
6) 5 mg. 7S γG heated 63°C. 30' added to 6.0 ml. of 1:80 dilution of Do-Rf	20,480	1.9
7) As in 6) except 7S γG unheated	20,480	1.1

* Human 7S Gamma Globulin, Lot 8284, Immunology, Inc.

TABLE 21.

AGGLUTINATION TITERS AND SLOPE VALUES OF LOW SLOPE RHEUMATOID FACTOR SERA REPEATEDLY ADSORBED WITH PAS-γG*

NUMBER OF ADSORPTIONS	SAMPLE NO. TITER (-SLOPE) R12	R14	R15**	R16
0	20,480 (1.2)	5,120 (1.0)	640 (1.0)	2,560 (1.1)
1	10,240 (1.3)	5,120 (1.2)	160 (1.0)	1,280 (1.0)
2	5,120 (1.3)	5,120 (1.3)	320 (1.0)	1,280 (1.0)
3	5,120 (1.4)	2,560 (1.2)	320 (1.5)	640 (1.2)
4	5,120 (1.4)	2,560 (1.5)	***	1,280 (1.3)
5	5,120 (1.5)	2,560 (1.3)	160 (1.2)	1,280 (1.3)
6	2,560 (1.5)	2,560 (1.5)	160 (1.0)	320 (1.0)
7	640 (1.0)	1,280 (1.5)	80 (1.0)	320 (1.0)
8	640 (1.0)			80 (1.0)
USC TITER BEFORE ADSORPTION	20	20	40	10
USC TITER AFTER ADSORPTION	10	20	40	10

* 1.0 ml. of sample was diluted with 1.0 ml. 0.15 N NaCl and adsorbed with 200 mg. polyaminostyrene coupled to human globulin (10%) for three hours at 5°C. on a rotating drum (5 R.P.M.). The PAS-γG was removed by centrifugation; 0.10 ml. of the supernatent removed for titration and the balance transferred to a fresh pellet of PAS-γG.

** Synovial fluid

*** Sample lost.

TABLE 22.

AGGLUTINATION TITERS AND SLOPES OF RHEUMATOID FACTOR SERA TESTED WITH TANNED SHEEP CELLS COATED WITH HUMAN γG OR ANIMAL γ G

	Human γG		Rabbit γ G		Significant Slope Difference
Serum Sample	Titer	-Slope	Titer	-Slope	
R17	20,480	1.9	160	1.0	Yes
R18	1,280	1.5	320	1.4	No
R19	5,120	1.0	160	1.0	No
R20	163,840	1.5	40,960	1.2	Yes
	Guinea Pig γ G*		Rabbit γ G*		
2245	320	1.9	640	1.0	Yes
2300	160	1.9	1,280	1.0	Yes
2402	80	2.5	640	2.0	Yes
2439	160	2.5	1,280	1.0	Yes

* These data are derived from Tonder and Milgrom (89). Agglutination titers are their values, obtained with sheep cells sensitized with rabbit or guinea pig amboceptor at 1/4 B.A.T. Slopes were calculated by us from their agglutination data obtained with cells sensitized at 1/2, 1/4, 1/8, 1/16 and 1/32 B.A.T.

TABLE 23.

AGGLUTINATION TITERS AND SLOPES OF ANTISERA FROM RABBIT IMMUNIZED WITH HUMAN γG

Rabbit	942		216		934		945	
Immunization*	γG dissolved in saline				γG dissolved in Freund's complete adjuvant			
Day	Titer	-Slope	Titer	-Slope	Titer	-Slope	Titer	-Slope
11	2560	1.0	10240	1.0	5120	1.0	320	1.0
18	2560	1.0	5120	1.2	2560	1.0	320	1.0
25	2560	1.2	2560	1.5	5120	1.0	2560	1.5
32	640	1.2	1280	1.5			2560	1.5
38	640	1.9	Died day 33		Died day 27			
48	320	1.7						
48	10 mg. boster i.v.							
56	5120	1.1						
63	1280	1.1						
73	2560	1.2						
80	640	1.2						

* 7.5 mg. human γG injected I.V. followed by 10 mg. human γG four days later

TABLE 24.

PREPARATION OF DNS CONJUGATES TO HUMAN γG IMMUNOGLOBULIN AT pH 8.0 AND 5°C

Batch	A	B	C	
Concentration, γG, g%	5	5	2	
Volume, 0.05 NaCl, ml.	200	200	200	
Amount of DNS, mg.	200	100	100	
Solvent	Dioxane, 10 ml.	Dioxane, 10 ml.	Acetone, 20ml.	
Reaction time, days	4	1	2	
Purification	Na_2SO_4 ppt volume red. dialysis	Na_2SO_4 ppt volume red. GPC-G75	Na_2SO_4 ppt volume red. GPC-G75	Dialysis
Molar ratios DNS/γG	0.4	0.5*	6.7	6.5

*Principal eluate from column, which contained 96.5% of recovered protein. An initial eluate contained 3.5% of recovered γG had a DNS/γG ratio of 3.7.

TABLE 25.

REPRODUCIBILITY OF FLUORESCENCE POLARIZATION (P) MEASUREMENTS OF DNS-γG*

Time (Min.)	Exp. 1		Exp. 2		Exp. 3	
	EE	P	EE	P	EE	P
1	22.5	0.2075	24.0	0.2262	17.8	0.2886
4	22.5	0.2145	23.0	0.2289	19.0	0.2912
6	22.4	0.2125	23.2	0.2472	19.5	0.2789
10	22.5	0.2078	22.8	0.2459	19.8	0.2802
20	22.6	0.2021	22.5	0.2421	21.7	0.3064
30	22.8	0.2142	22.6	0.2321	22.5	0.3171
40	22.5	0.2075	22.7	0.2394	22.0	0.2821
Mean		0.2094		0.2374		0.2921
S.D.		0.00579		0.00837		0.0145
% S.D.		2.77		3.53		4.97
Mean % S.D.				3.76		

* DNS-γG, batch 5, concγG, 9.962 mg/ml. molar ratio DNS/γG 6.44. Exp. 1: pH 8.13 buffer. Exp. 2: pH 4.90 buffer, Exp. 3: pH 3.95 buffer.

TABLE 26.

REPRODUCIBILITY OF FLUORESCENCE POLARIZATION (P) MEASUREMENTS OF DNS-γG IN DIFFERENT CELLS*

Cell	P of DNS-γG			
	A	B-1	C-1	C-2
I	0.311	0.255	0.241	0.272
II	0.291	0.267	0.278	0.278
III	0.260	0.268	0.266	0.283
Mean	0.2873	0.2633	0.2617	0.2777
S.D.	0.08127	0.007246	0.02670	0.007811
% S.D.	28.29	2.75	10.20	2.81
Mean % S.D.		11.01		

* Cell I: unjacketed, 4 quartz walls, Cell II: jacketed all quartz, Cell III: jacketed with quartz windows at right angles. Cells II and III reduced fluorescence intensity approximately 25%, compared to Cell I. When data was pooled and analysed in one batch the following results were obtained, P=0.2725 ± 0.0179 (6.58%)SD

TABLE 27.

ULTRACENTRIFUGATION AND FLUORESCENCE POLARIZATION ANALYSIS OF HUMAN γG IMMUNOGLOBULIN (FRACTION II) NOT FREED OF AGGREGATES

γG Conc. (mg/ml)	No. of Components	Sedimentation Constants	Percent of Total	Percent Aggregates
5.0	3	12.25	3.8	21.9
		9.33	18.1	
		6.26	78.1	
10.0	3	11.59	5.3	24.9
		9.01	19.6	
		6.14	75.2	

γG Conc. (mg/ml)	EE*	P
5.0	0.500	0.493
2.0	0.625	0.316
1.0	0.645	0.255
0.5	0.435	0.211

* Eex, 295 mμ; Eem, 325 mμ T=25.0°C

TABLE 28.

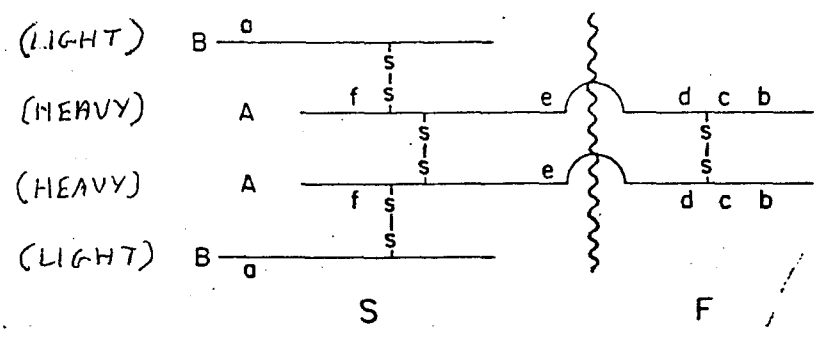
FLUORESCENCE POLARIZATION OF DNS-γG IN THE PRESENCE OF RHEUMATOID FACTOR SERUM AND NORMAL SERUM

Serum Dilution	Polarization of DNS-γG*					Precipitin Reaction
	Normal Serum	RF Serum	RF-NS	RF/NS	RF/NS / 1.18	
1:2	0.356	-	-	-	-	+++
1:4	0.244	0.419	0.175	1.72	1.46	++
1:8	0.243	0.393	0.150	1.62	1.37	+
1:16	0.224	0.377	0.153	1.68	1.43	+
1:32	0.220	0.280	0.060	1.27	1.08	±
1:64	0.204	0.263	0.057	1.29	1.09	±
1:128	0.192	0.251	0.059	1.31	1.11	-
1:256	0.187	0.241	0.054	1.29	1.09	-
1:512	0.185	0.220	0.035	1.19	1.01	-
1:1024	0.172	0.206	0.034	1.20	1.01	-
DNS-γG*	0.152	0.179	0.027	1.18	1.00	

* Batch la, γG conc. 1.94 mg/ml, DNS/γG molar ratio, 1.6, diluent pH 7.0 phosphate buffer. Polarization values: corrected for serum fluorescence. Precipitin reaction, samples taken up in capillaries and read after 5 days.

FIGURE 1.

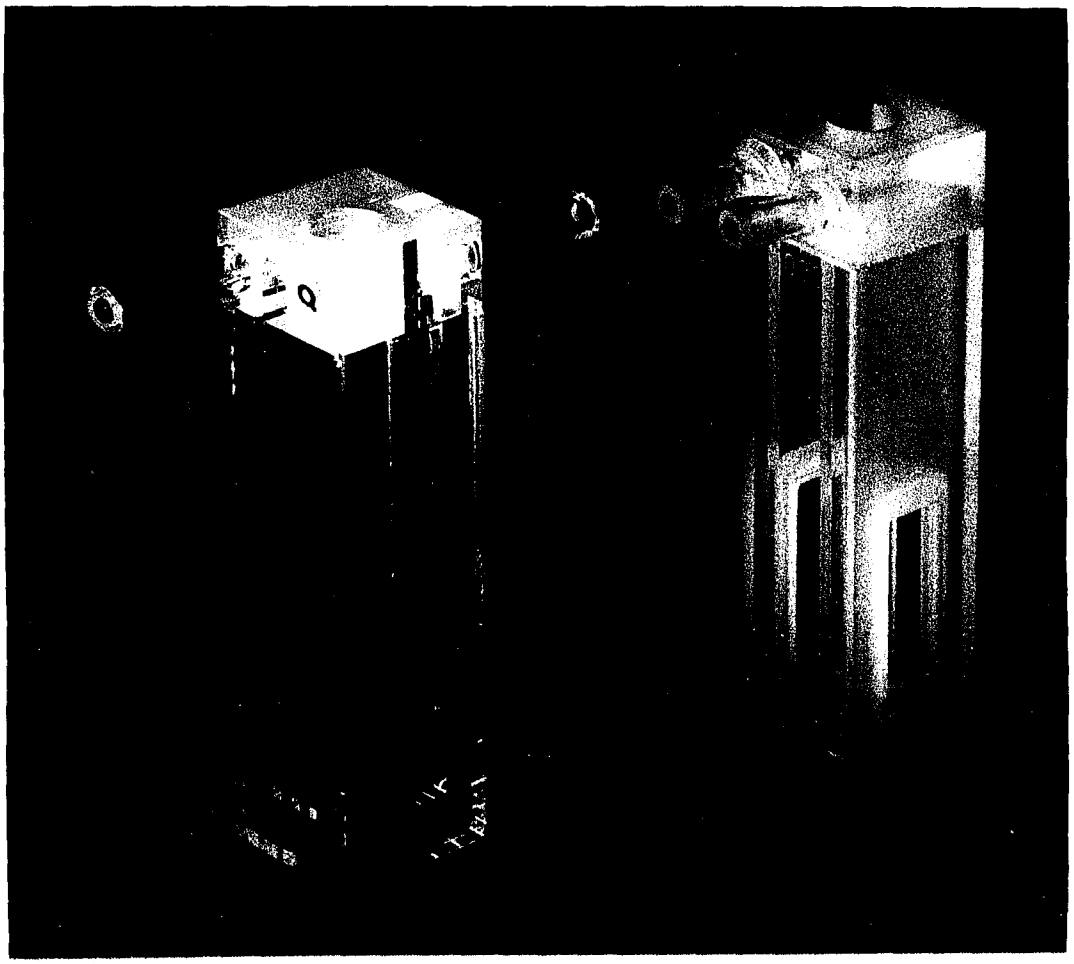
STRUCTURE OF HUMAN γG IMMUNOGLOBULIN



Tentative diagram of human γG immunoglobulin molecule, showing the heavy and light chains and indicating possible locations of sites on the molecule with which various anti-γ-globulin factors react. The S and F indicate the fragments produced by papain. Letters indicate sites as follows: a-Inv site; b-Gm site; c-rheumatoid factor site 1; d-Rheumatoid factor site 2 (rabbit-γ-globulin cross-reacting factor); e-"pepsin" site; f-Milgrom factor site. Illustration reproduced from Williams R.C. and Kunkel, H.G. Ann. N.Y. Acad. Sci. 124:860, 1965.

FIGURE 2.

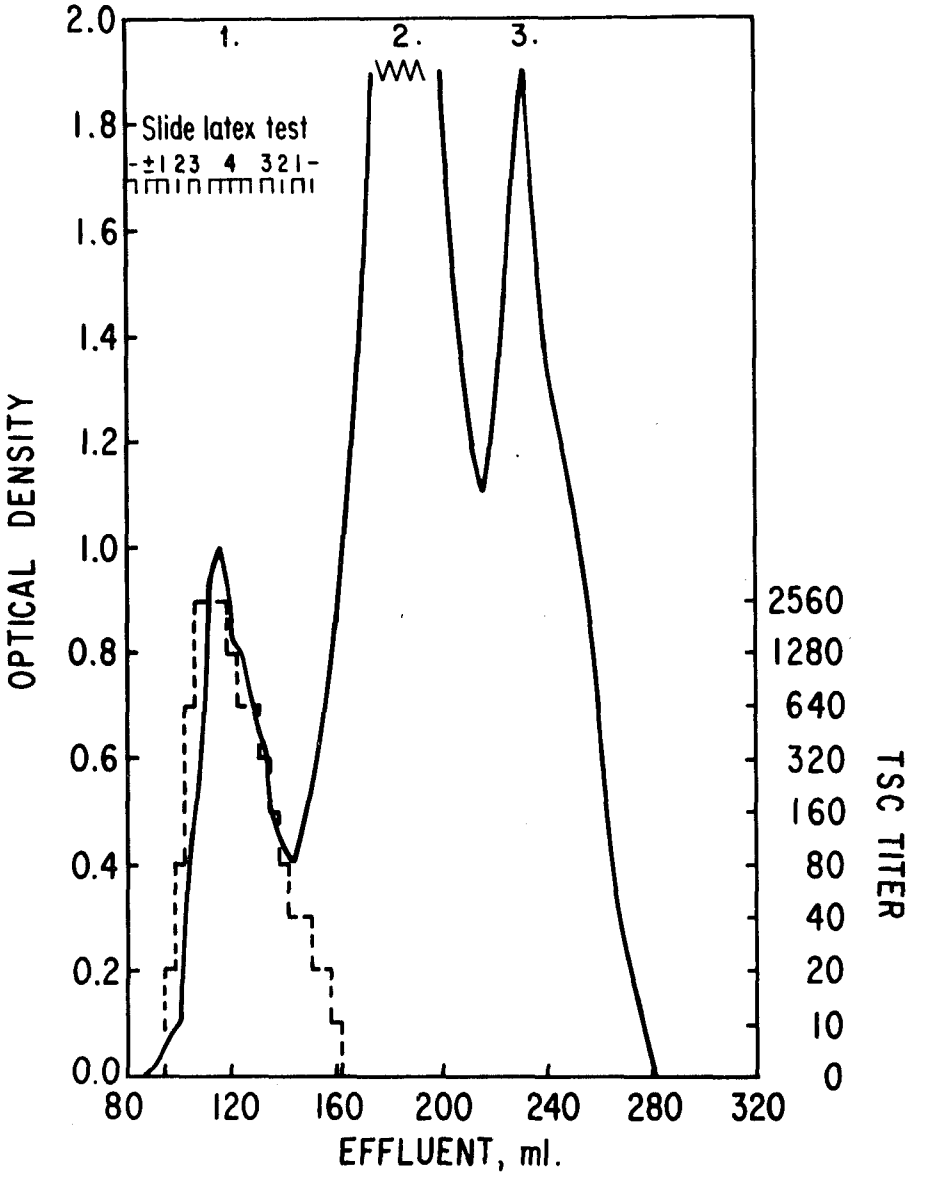
JACKETED QUARTZ FLUORESCENCE CELLS



Right: window type, made by Scientific Cell Co., 118-21 Queens Blvd, Forest Hills, N.Y. 11375.
Left: All quartz type, made by Precision Cell Co., 401 Broadway, N.Y., N.Y. 10013.

FIGURE 3.

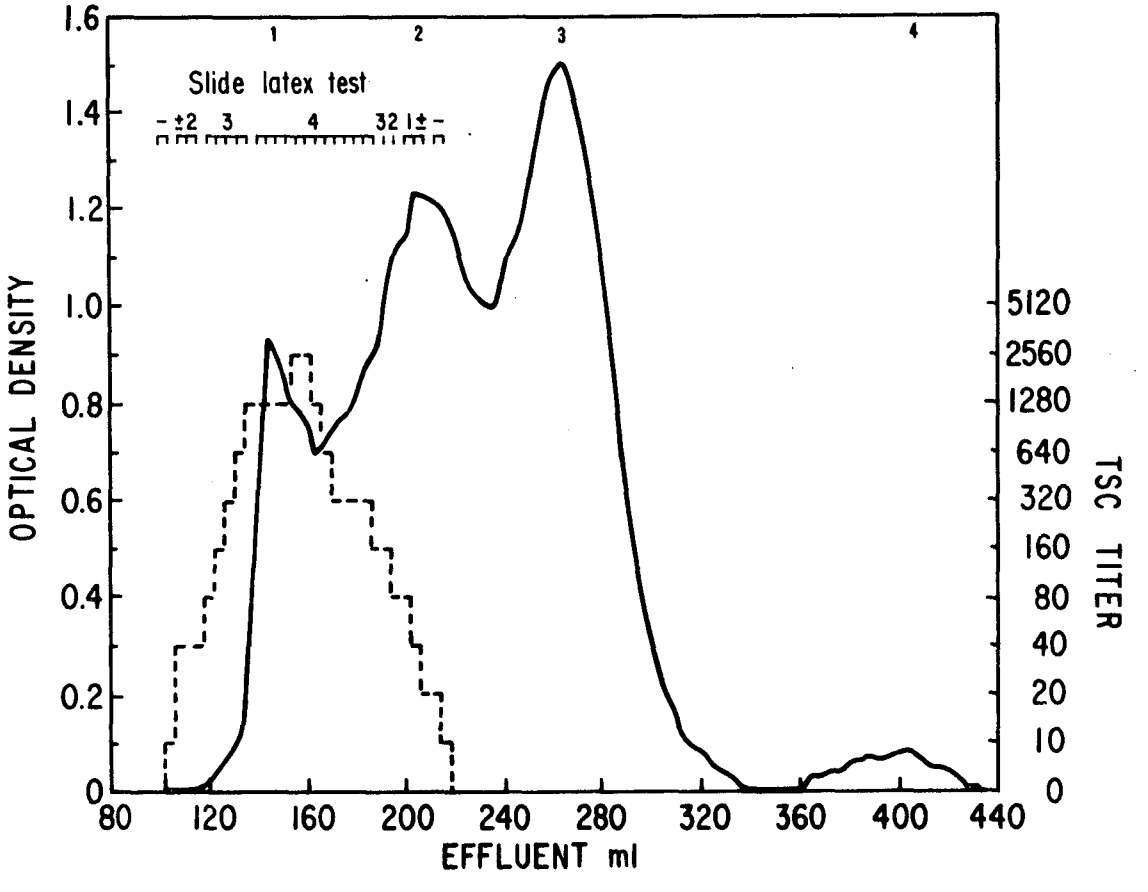
GEL PERMEATION CHROMATOGRAPHY



Serum R-OJ. Sephadex G-200. Optical density, 280mμ,--- tanned sheep cell titer.

FIGURE 4.

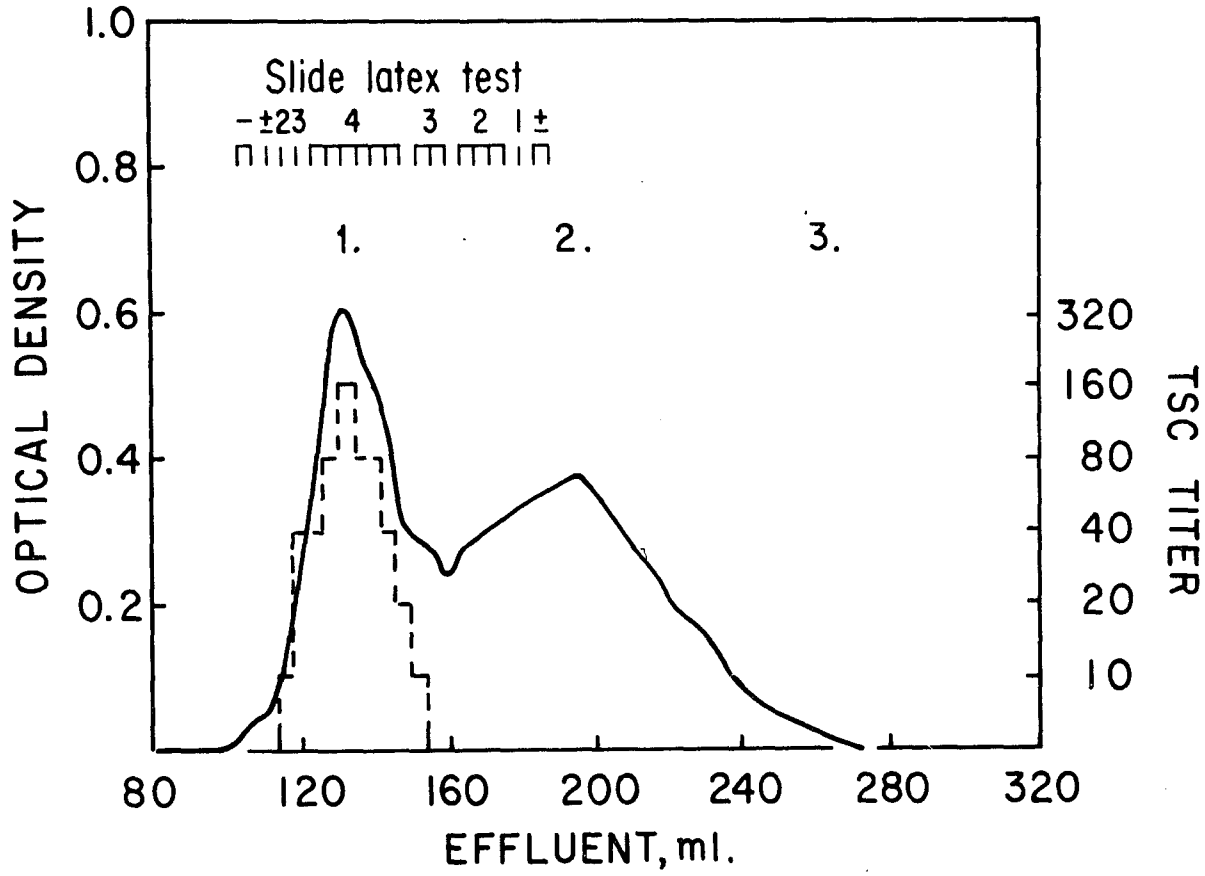
GEL PERMEATION CHROMATOGRAPHY, FIRST CYCLE



Serum R-W, Sephadex G-200, first pass._____, optical density, 280 mμ
----- Tanned sheep cell titer.

FIGURE 5.

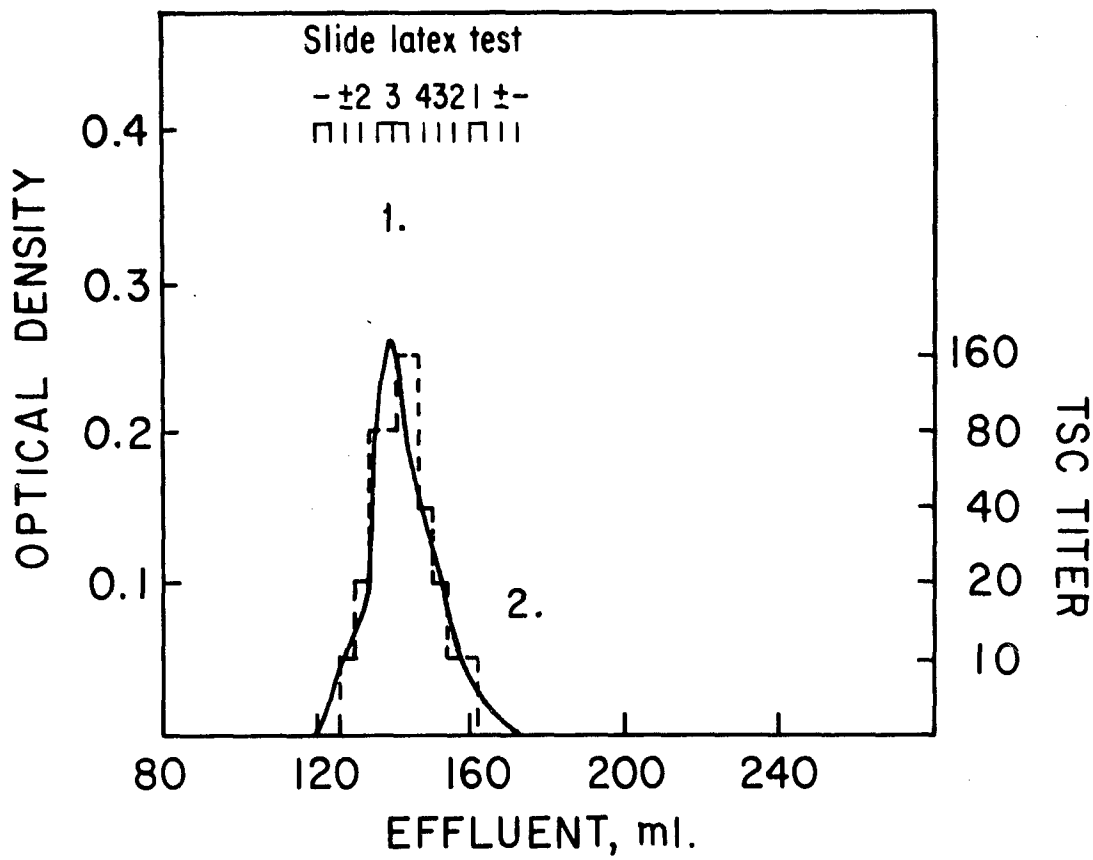
GEL PERMEATION CHROMATOGRAPHY, SECOND CYCLE



Serum R-W, Sephadex G-200, Second pass, of TSC positive pooled fraction from first pass. _____ optical density, 280mμ ----- tanned sheep cell titer.

FIGURE 6.

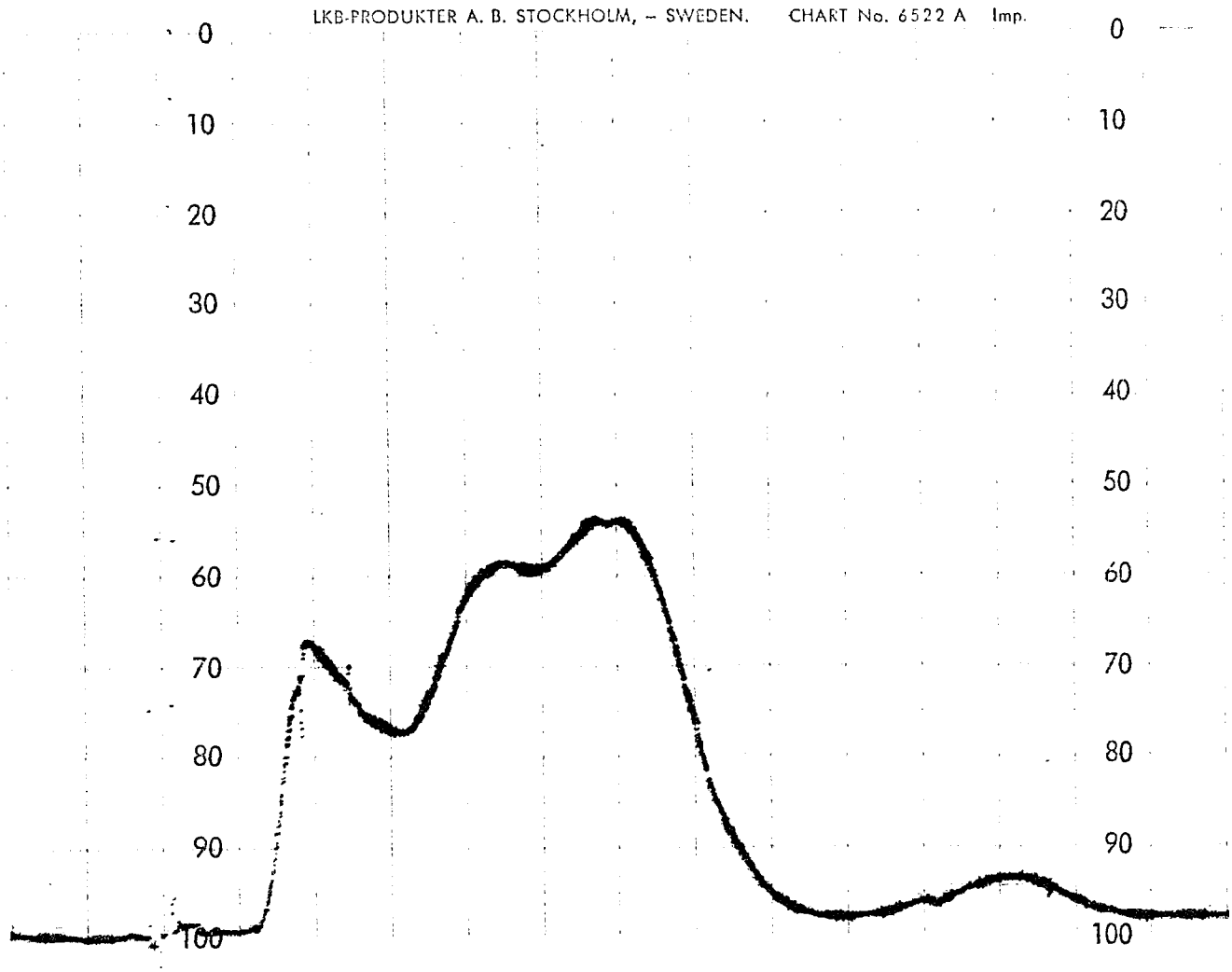
GEL PERMEATION CHROMATOGRAPHY, THIRD CYCLE



Serum R-W, Sephadex G-200, third pass, of TSC positive pooled fractions from second pass. _____ Optical density, 280mμ. ---- Tanned Sheep Cell Titer.

FIGURE 7.

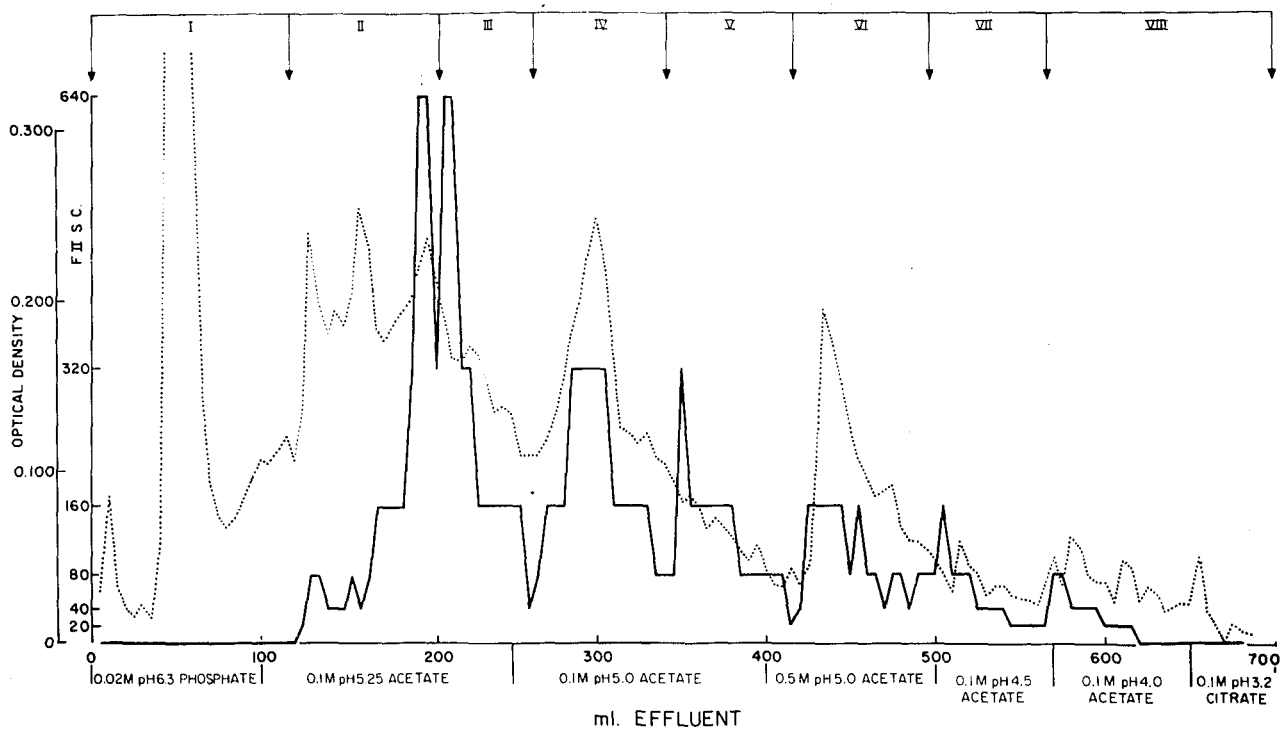
GEL PERMEATION CHROMATOGRAPHY WITH AUTOMATIC MONITOR



GPC chromatogram of normal human serum. Protein content monitored at 280mμ with a LKV Unicord II, Model 8300 and a LKB Recorder Model 6520A. Sephadex G-200; buffer flow rate 12.0ml/hr. Paper speed 10.0mm/hr. Alteration of color in original recorder trace at 20′ intervals marks the change of tube in the fraction collector.

FIGURE 8.

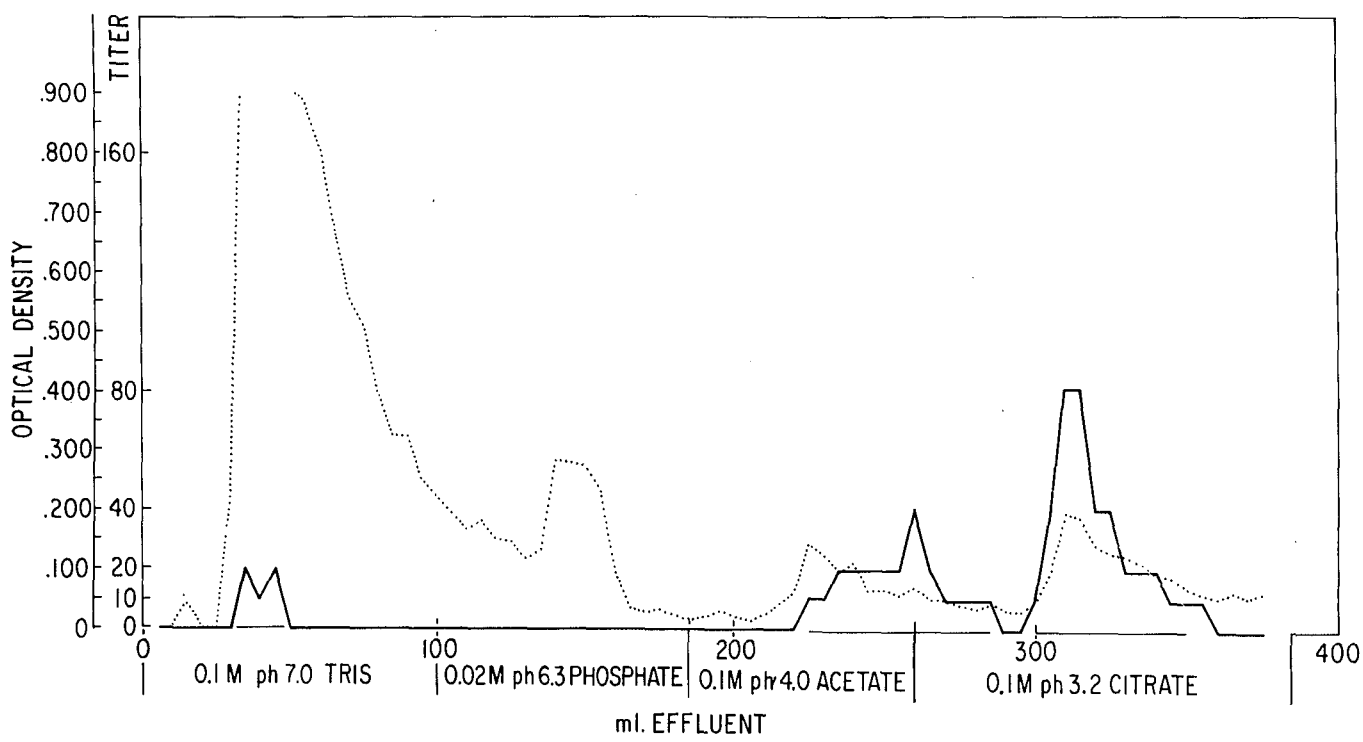
IMMUNOCHROMATOGRAPHY OF RHEUMATOID FACTOR



Immunochromatographic pattern obtained from euglobulin fraction from 20.0 ml. of rheumatoid arthritis serum #9005 developed on PAS-γG column. Original TSC titer 1:40,960, SSC titer 1:640.---optical density (280 mμ) _____ serum TSC agglutination titers.

FIGURE 9.

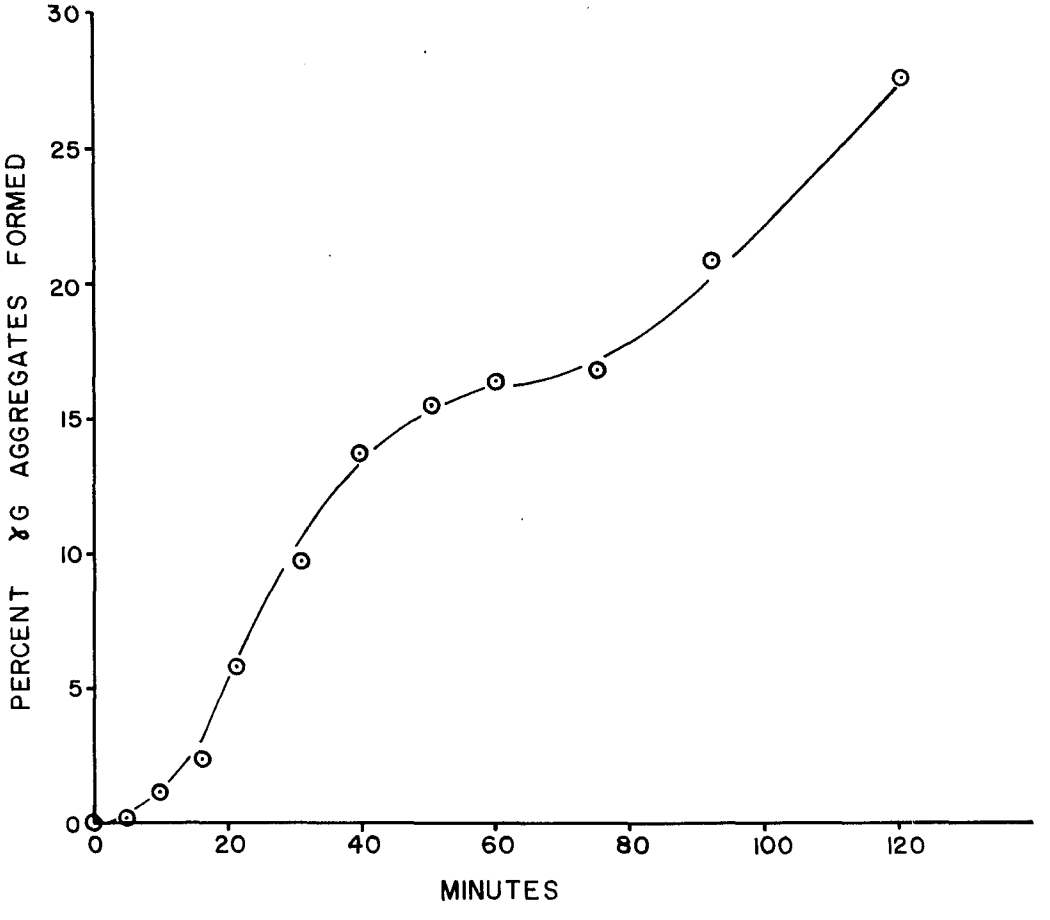
IMMUNOCHROMATOGRAPHY OF A RABBIT ANTI HUMAN γG ANTISERUM



Immunochromatographic pattern obtained from 5.0 ml. of rabbit anti human γ G antiserum developed on PAS- γG column. Serum TSC titer 1:5,120;---- optical density (280mμ)_____TSC agglutination titers.

FIGURE 10.

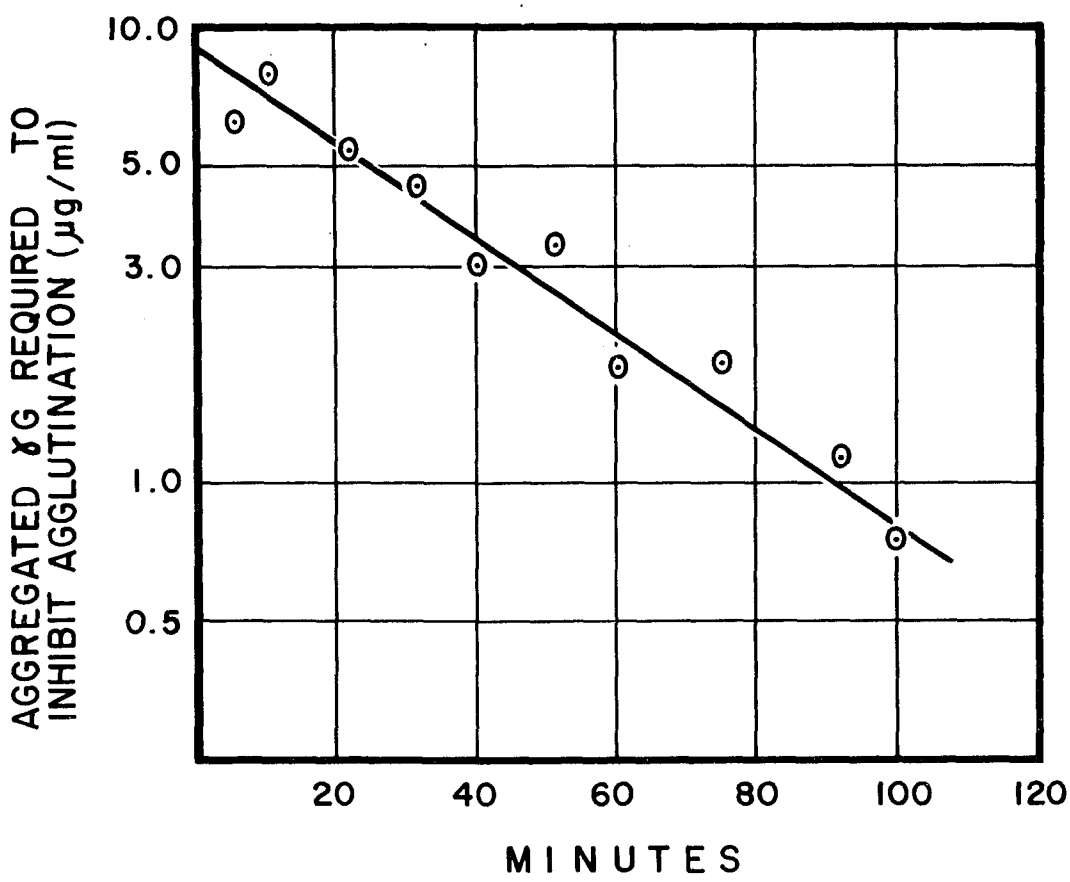
KINETICS OF THERMAL AGGREGATION OF γG



Formation of γG aggregates as a function of heating time at 60.0°C ⊡ percent γG precipitable as aggregates, corrected for aggregate concentration at T=0. Data from Table 7.

FIGURE 11.

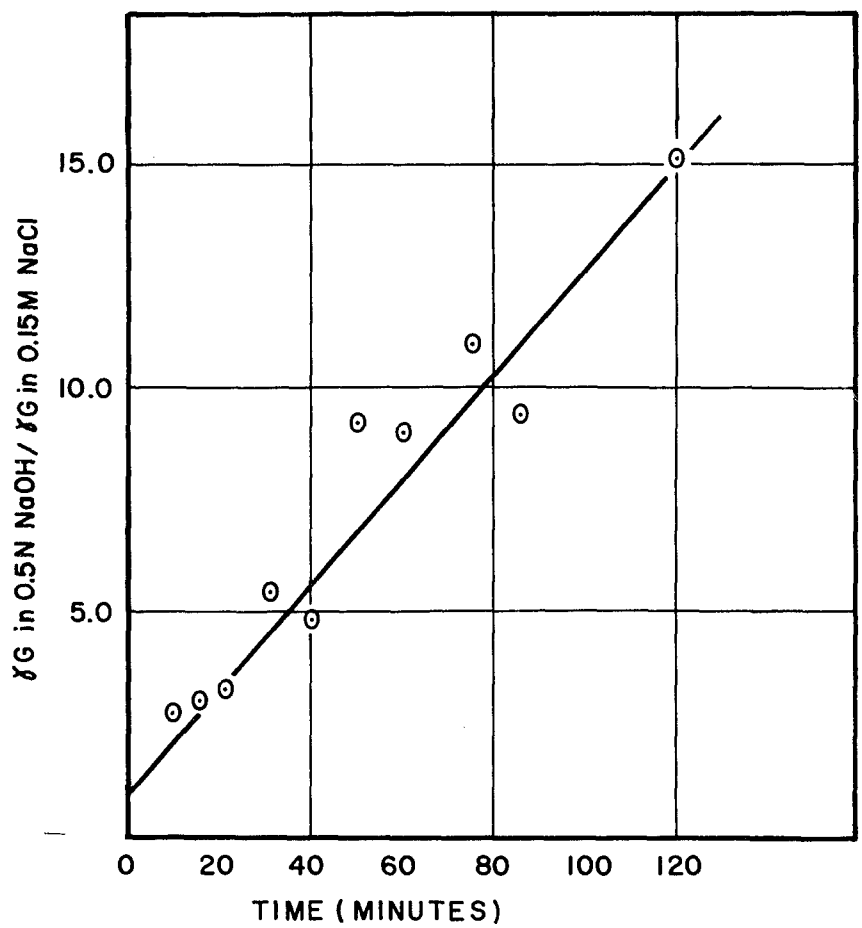
INHIBITION OF RHEUMATOID FACTOR AGGLUTINATION BY γG AGGREGATES



⊙ concentration of aggregated γG, (μg/ml), prepared with different heating times, required to inhibit tanned sheep cell agglutination of a 1:500 dilution of a RF serum (R23).

FIGURE 12.

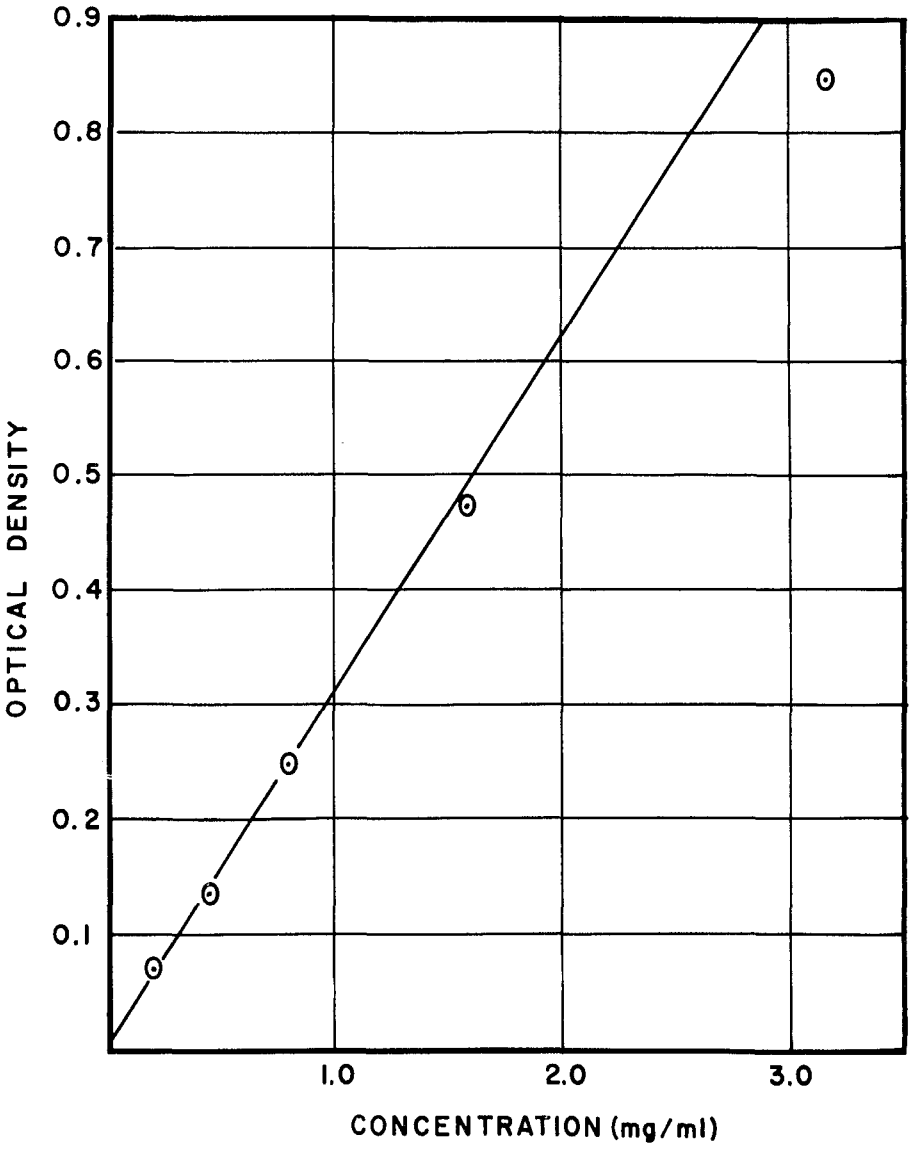
INHIBITION OF RHEUMATOID FACTOR
AGGLUTINATION BY γG AGGREGATES



Ratio of concentrations of aggregated γG dissolved in 0.15 M NaCl to that dissolved in 0.05 M NaOH, required to inhibit tanned sheep cell agglutination of a 1:500 dilution of a RF serum.

FIGURE 13.

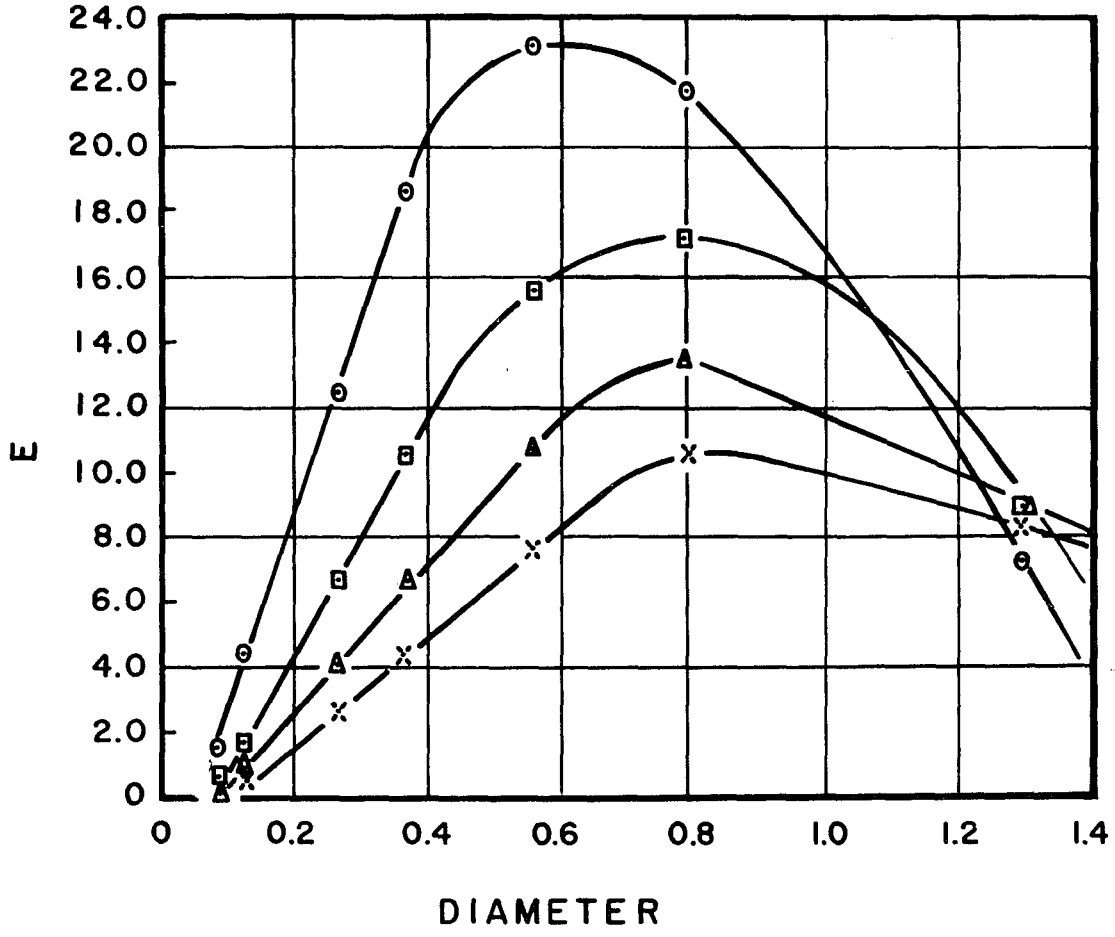
TURBIDITY OF LATEX PARTICLES



Optical density (turbidity) vs total solids concentration of polystyrene latex particles, 0.365 microns diameter.

FIGURE 14.

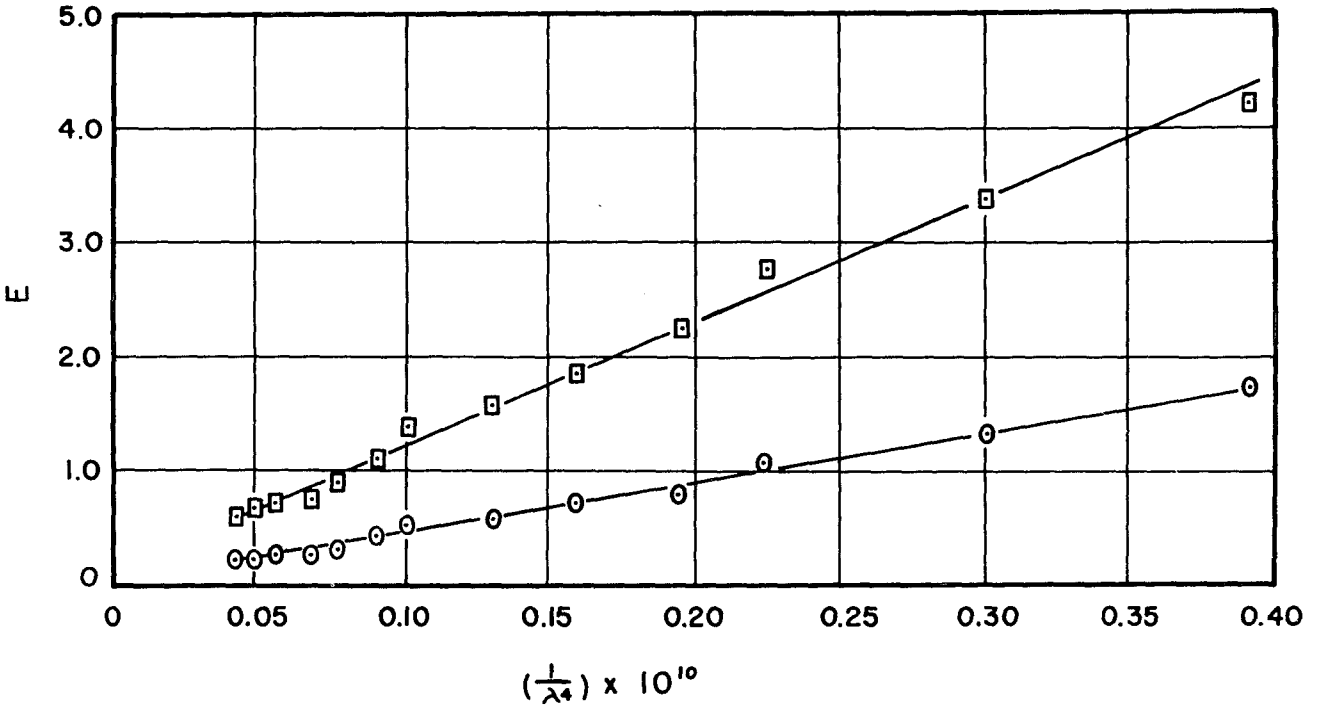
TURBIDITY OF LATEX PARTICLES RELATED TO DIAMETER AND WAVELENGTH



Extinction value (optical density/mg/ml) as a function of latex particle diameter at various wavelengtha: ⊙ 400mμ; ⊡ 500mμ; ▽ 600mμ; ✕ 700mμ.

FIGURE 15.

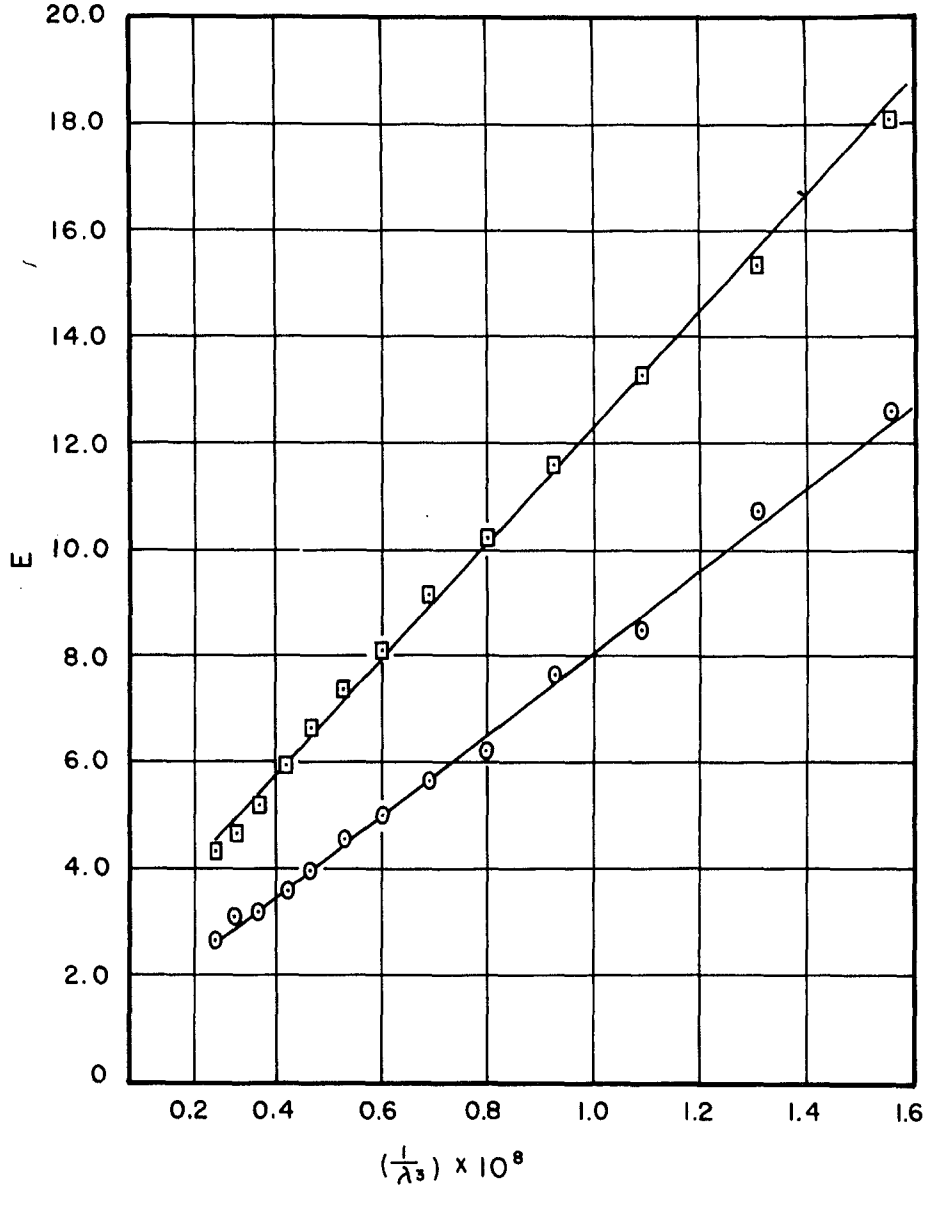
TURBIDITY OF LATEX PARTICLES AT
DIFFERENT WAVELENGTHS



Extinction of "small" LP vs $(1/\lambda^4)X10^{10}$. Diameters in microns; ⊙ ,0.088; ⊡ ,0.126.

FIGURE 16.

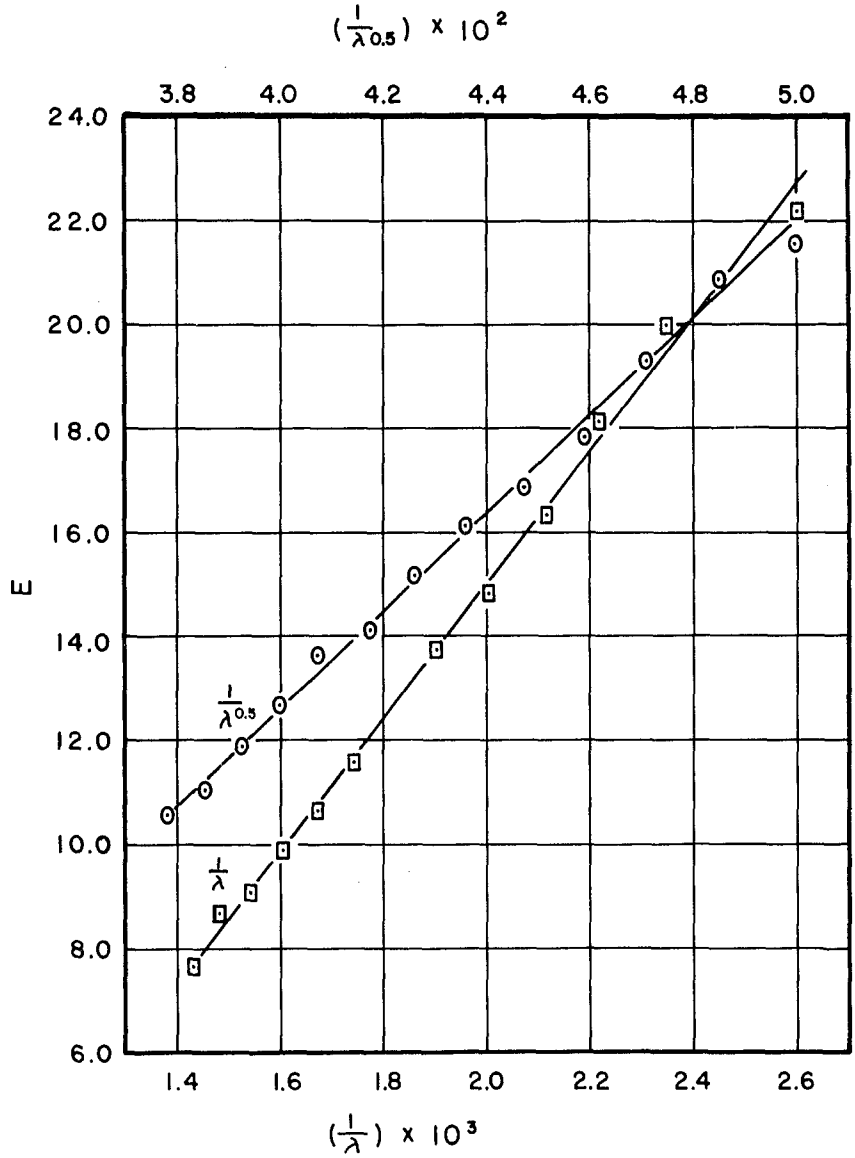
TURBIDITY OF LATEX PARTICLES AT
DIFFERENT WAVELENGTHS



Extinction of LP vs $(1/\lambda^3)X10^8$. Diameters in microns; ⊙ ,0.264; ⊡ ,0.365.

FIGURE 17.

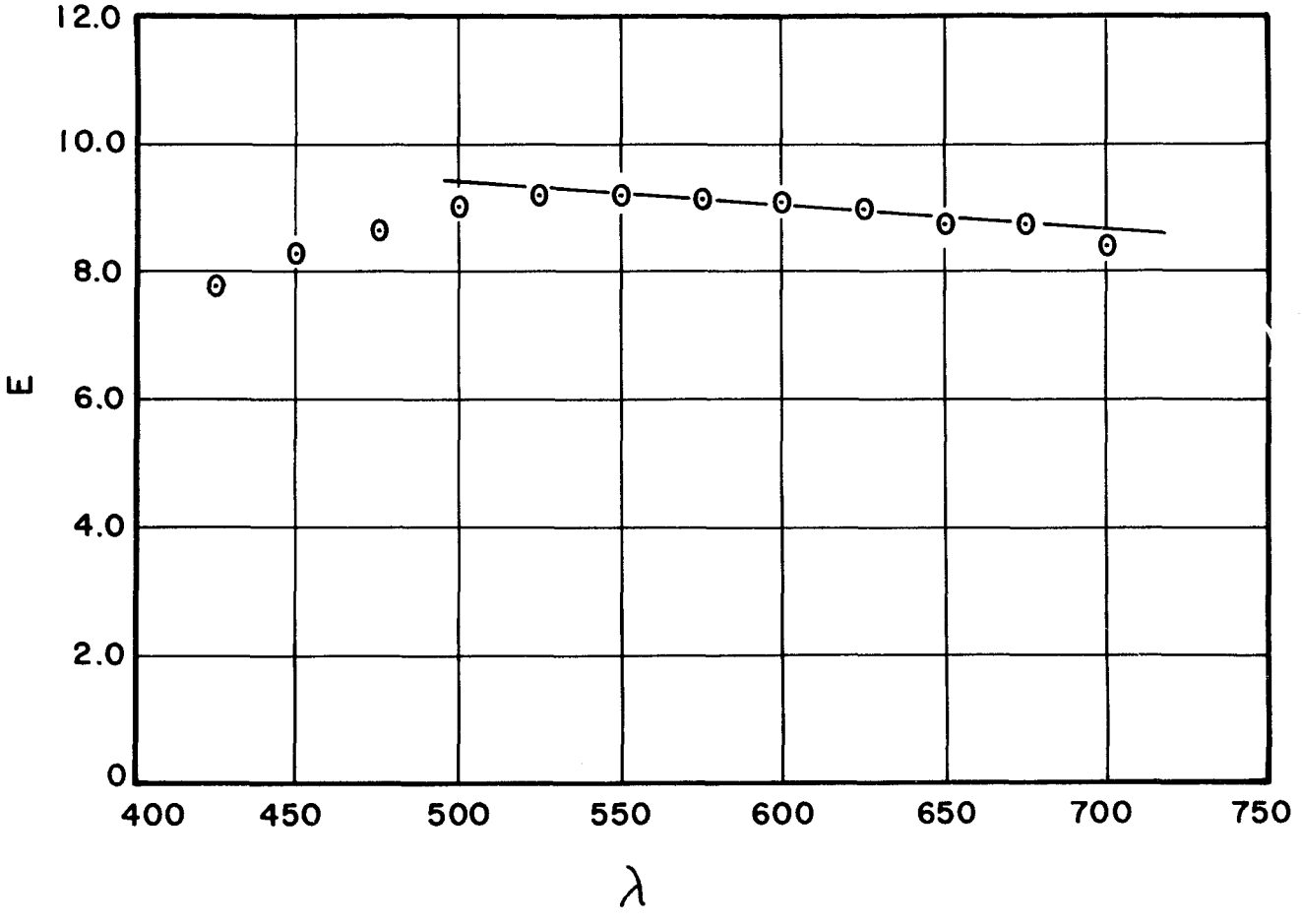
TURBIDITY OF LATEX PARTICLES AT DIFFERENT WAVELENGTHS



Extinction of 0.577 micron diameter LP vs $(1/\lambda^{1.0})X10^3$, ⊡ ; extinction of 0.796 micron diameter LP vs $(1/\lambda^{0.5})X10^2$, ⊙ .

FIGURE 18.

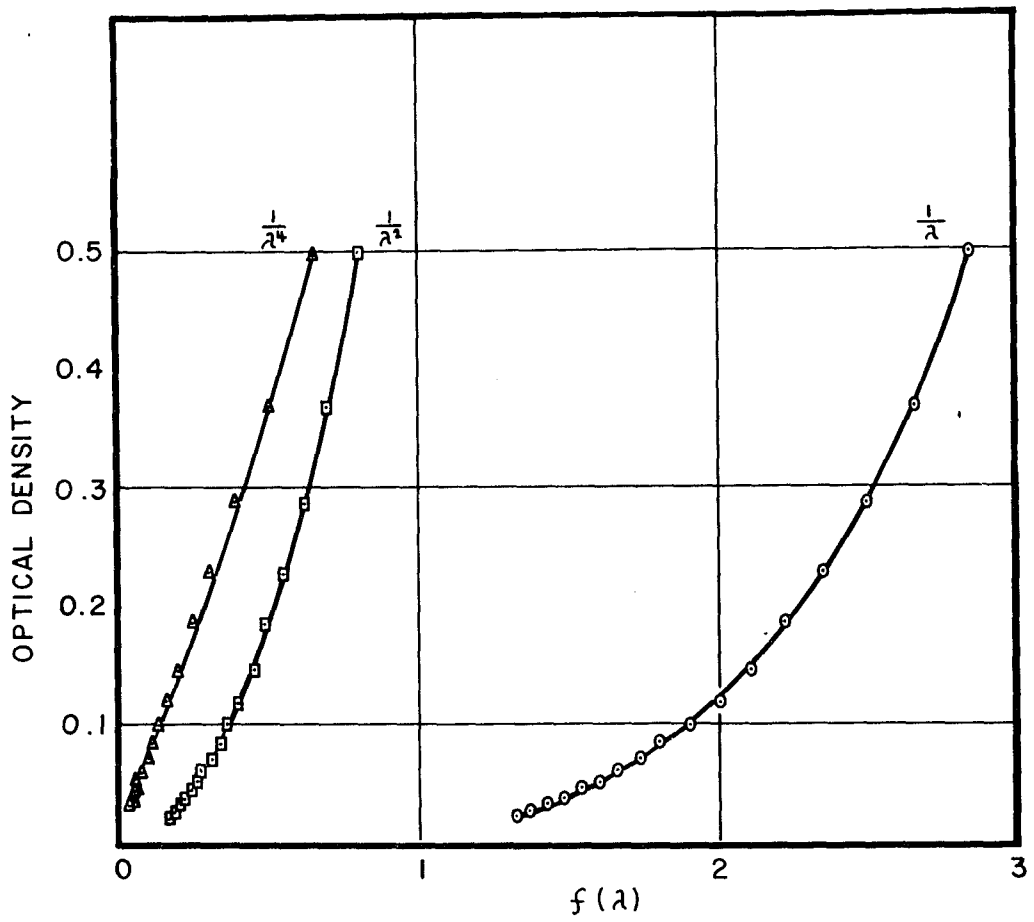
TURBIDITY OF LATEX PARTICLES AT
DIFFERENT WAVELENGTHS



Extinction of 1.305 micron diameter LP vs λ.

FIGURE 19.

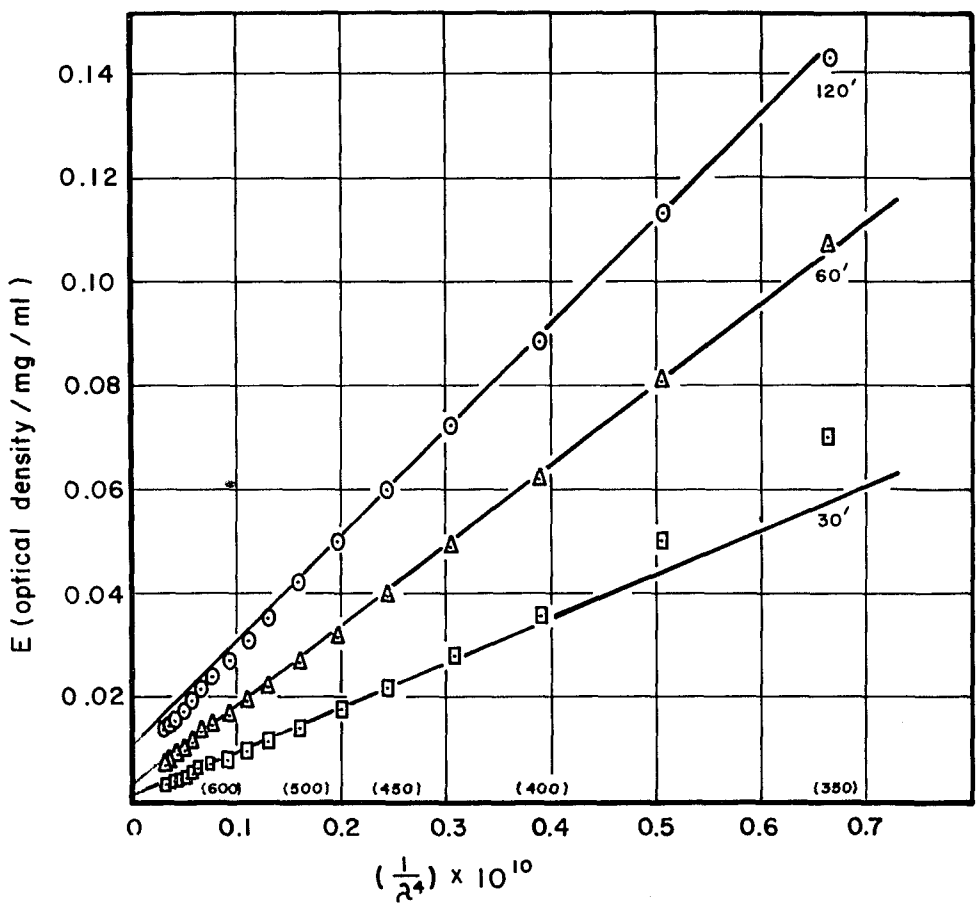
TURBIDITY OF AGGREGATED γG AT DIFFERENT WAVELENGTHS



Optical density of aggregated γG (prepared from γG heated for 60 min at 63°C) vs $f(\lambda)$, where $f(\lambda)=(1/\lambda)\times10^3$, ⊙ ; $f(\lambda)=(1/\lambda^2)\times10^5$, ⊡ ; $f(\lambda)=(1/\lambda^4)\times10^{10}$, △ . Values of λ for each $f(\lambda)$ are listed in Table 11.

FIGURE 20.

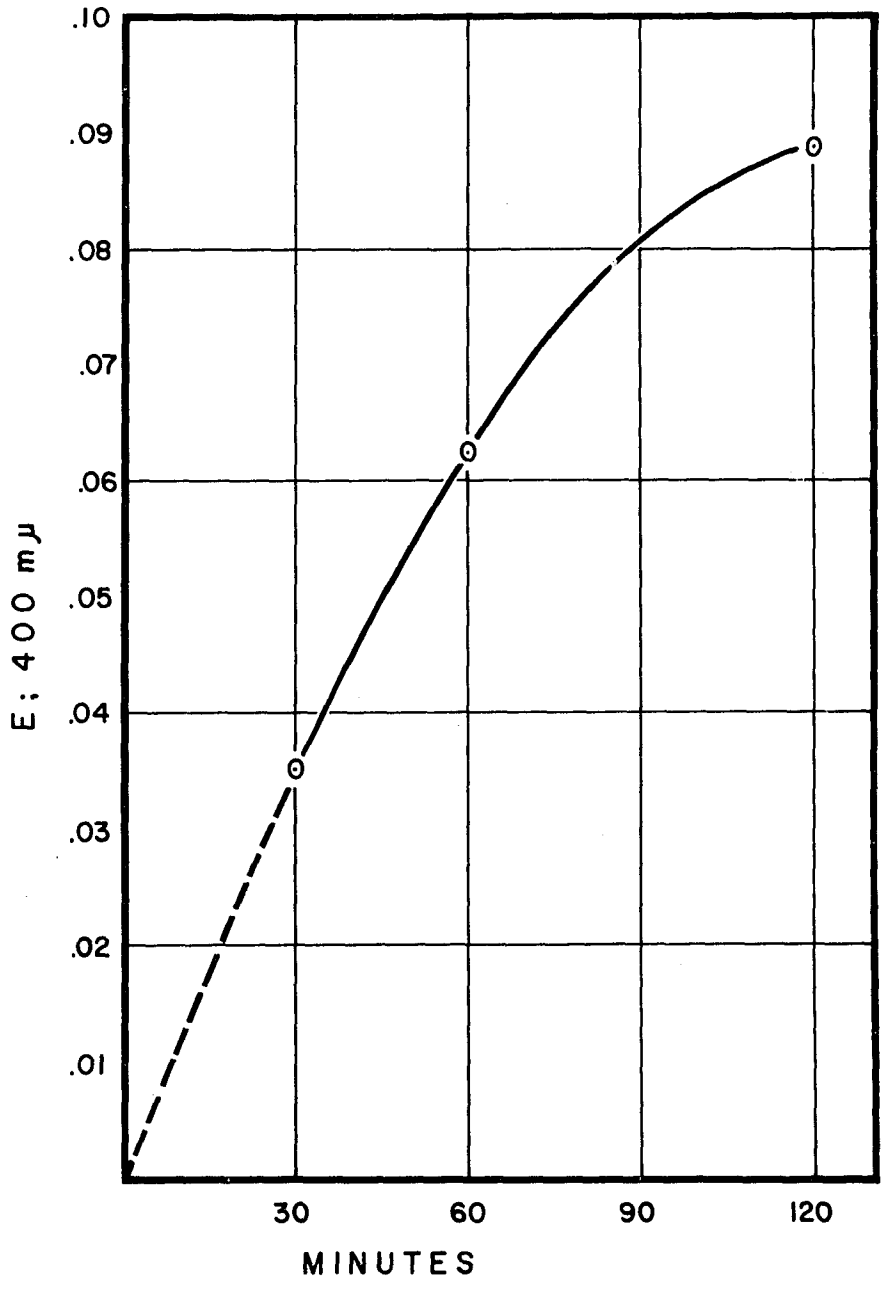
TURBIDITY OF AGGREGATED γG AS A FUNCTION OF WAVELENGTH AND HEATING TIME



Extinction of aggregated γG prepared with different heating times at 63.0 C vs $(1/\lambda^4) \times 10^{10}$. Heating times: 30 min □; 60 min △; 120 min ○.

FIGURE 21.

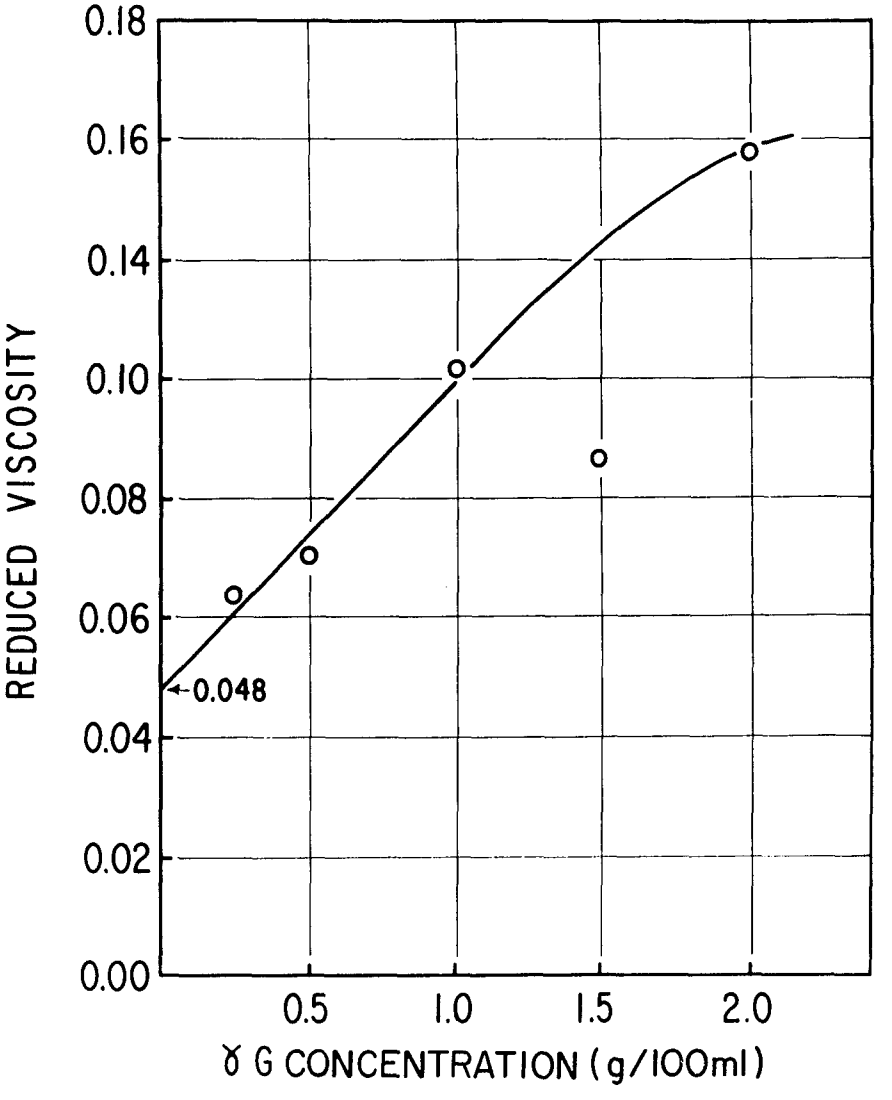
EFFECT OF HEATING TIME ON TURBIDITY OF AGGREGATED γG



Extinction of aggregated γG at 400mμ, vs heating time, min.

FIGURE 22.

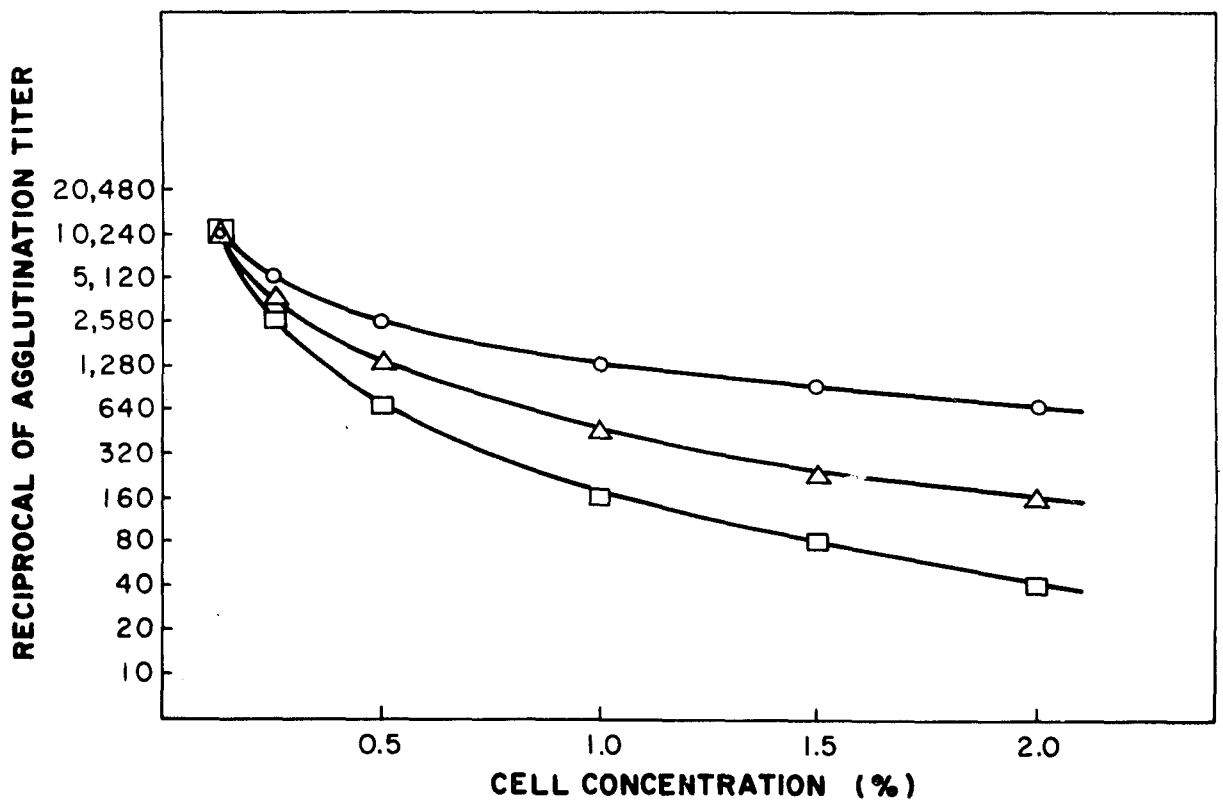
VISCOSITY OF γG IMMUNOGLOBULIN



Reduced viscosity as a function of γG immunoglobulin concentration. Solvent, 0.15M NaCl.

FIGURE 23.

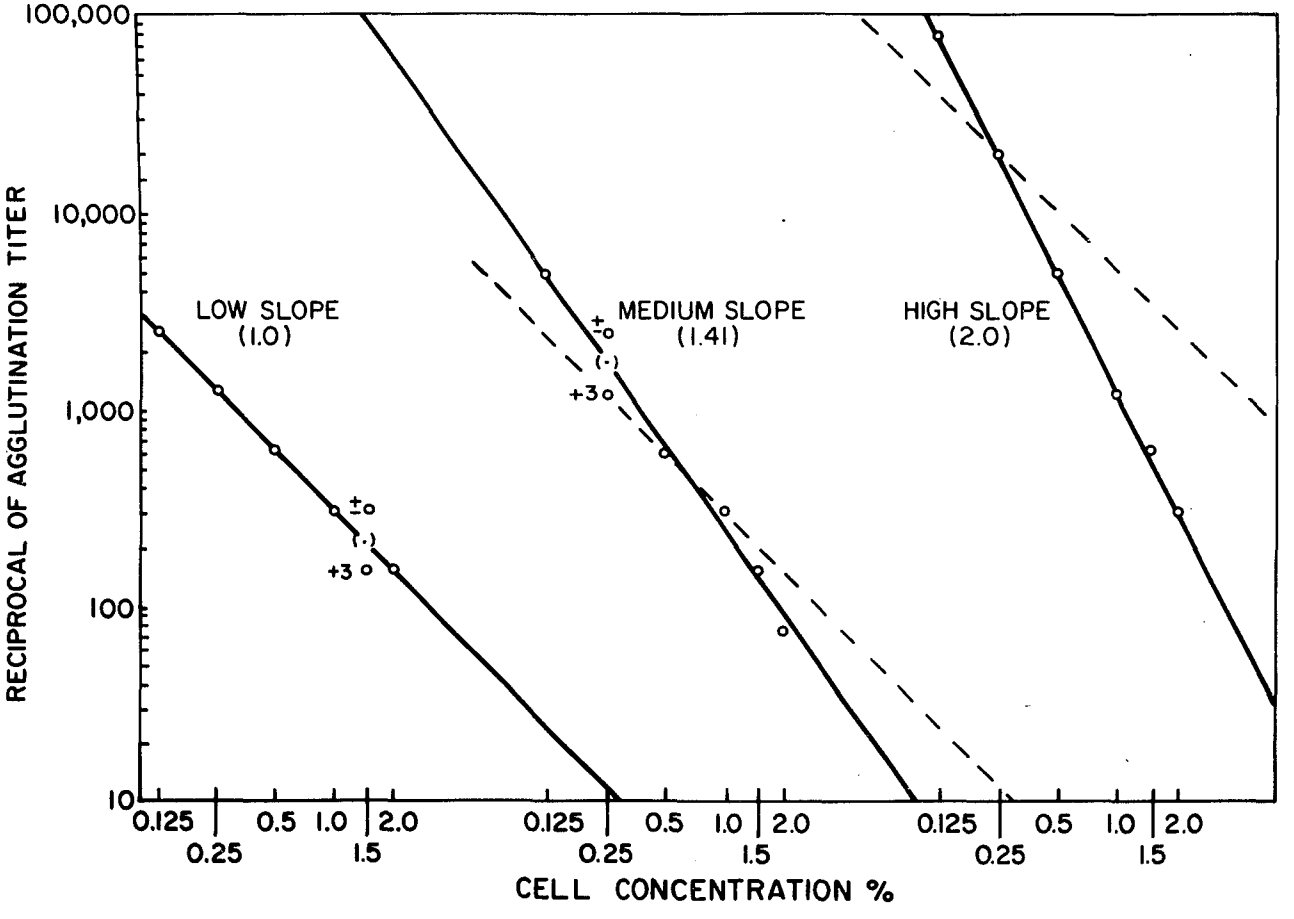
RELATION OF RF AGGLUTINATION TITER TO TANNED CELL CONCENTRATION



Agglutination titers (TSC) vs. cell concentration of three types of rheumatoid factor sera: Plus-minus tubes plotted as "half" values. O, type (a); △ type (b); □ type (c).

FIGURE 24.

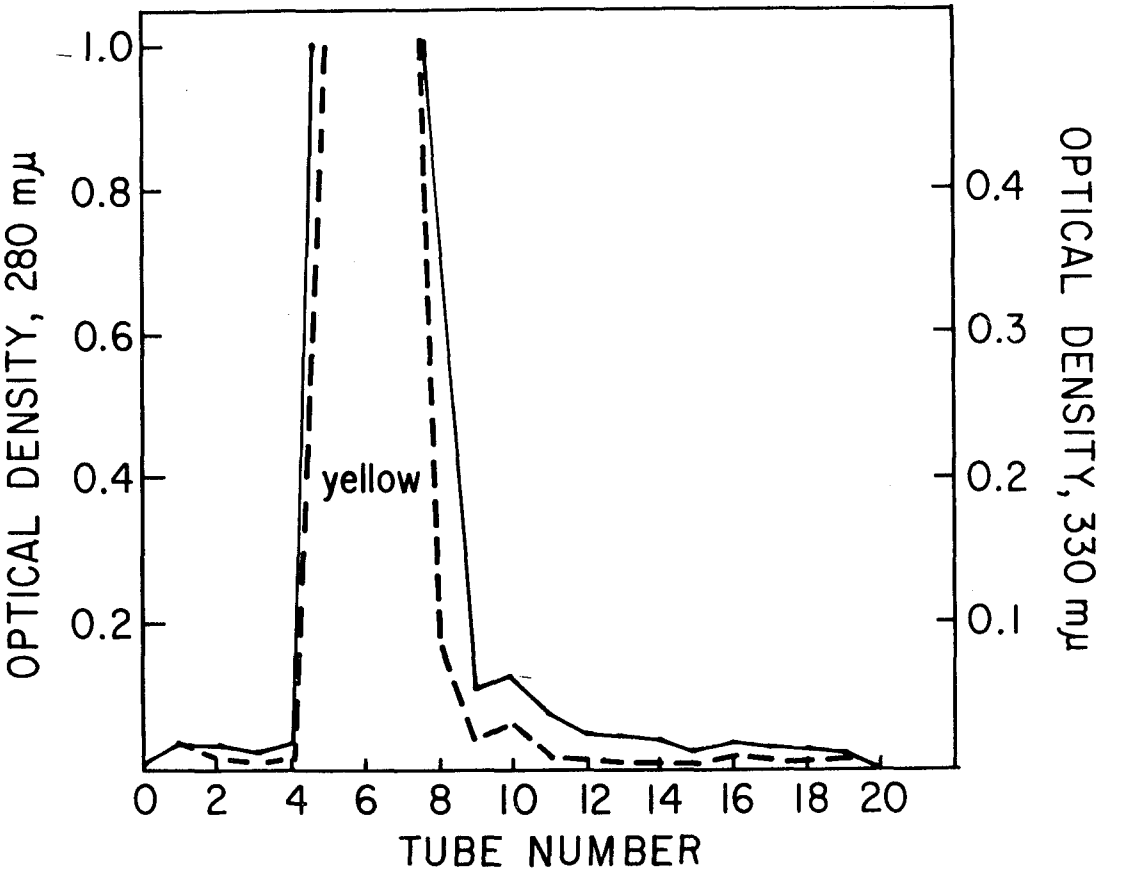
SLOPES OF TITERS VS TANNED CELL CONCENTRATION



Double logarithmic plots of agglutination titers vs. cell concentration for three RF positive sera. (•), calculated titer; mean of the logarithms of the highest serum dilution yielding complete agglutination and of the next higher serum dilution yielding weak (±) agglutination.
------, hypothetical -1.0 slope line for comparison. Calculation of slope: $S = \log T_2 - \log T_1 / \log C_2 - \log C_1$ (from equation 7).

FIGURE 25.

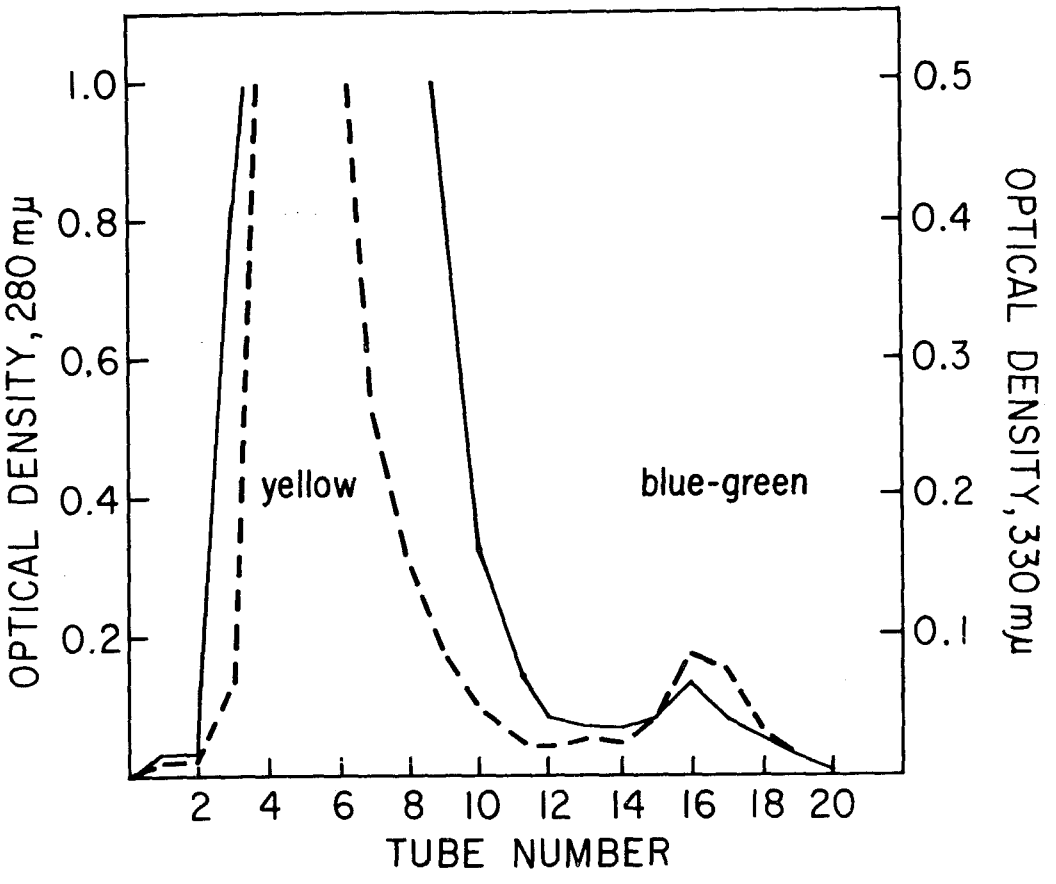
GEL PERMEATION CHROMATOGRAPHY OF DNS-γG



Fractionation of DNS-γG on Sephadex G-25. Eluting solvent, phosphate buffer, pH 7.5, 1.0 M NaCl. Flow rate 40 ml. per hour. Time collection, 15 min per tube._____, OD, 280mμ.----- OD, 330mμ.

FIGURE 26.

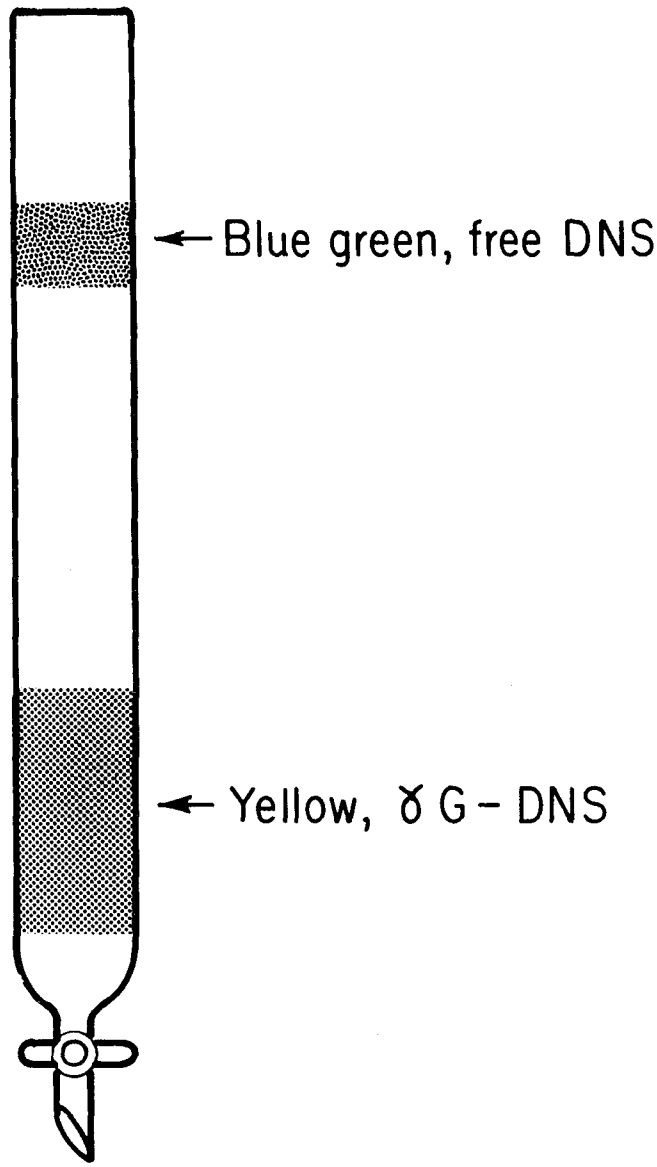
GEL PERMEATION CHROMATOGRAPHY OF DNS-γG



Fractionation of DNS-γG on Sephadex G-75. Eluting solvent, phosphate buffer, pH 7.5, 1.0 M NaCl. Flow rate 40 ml. per hour. Timed collection, 15 min per tube.——— OD, 280mμ.----- OD, 330mμ.

FIGURE 27.

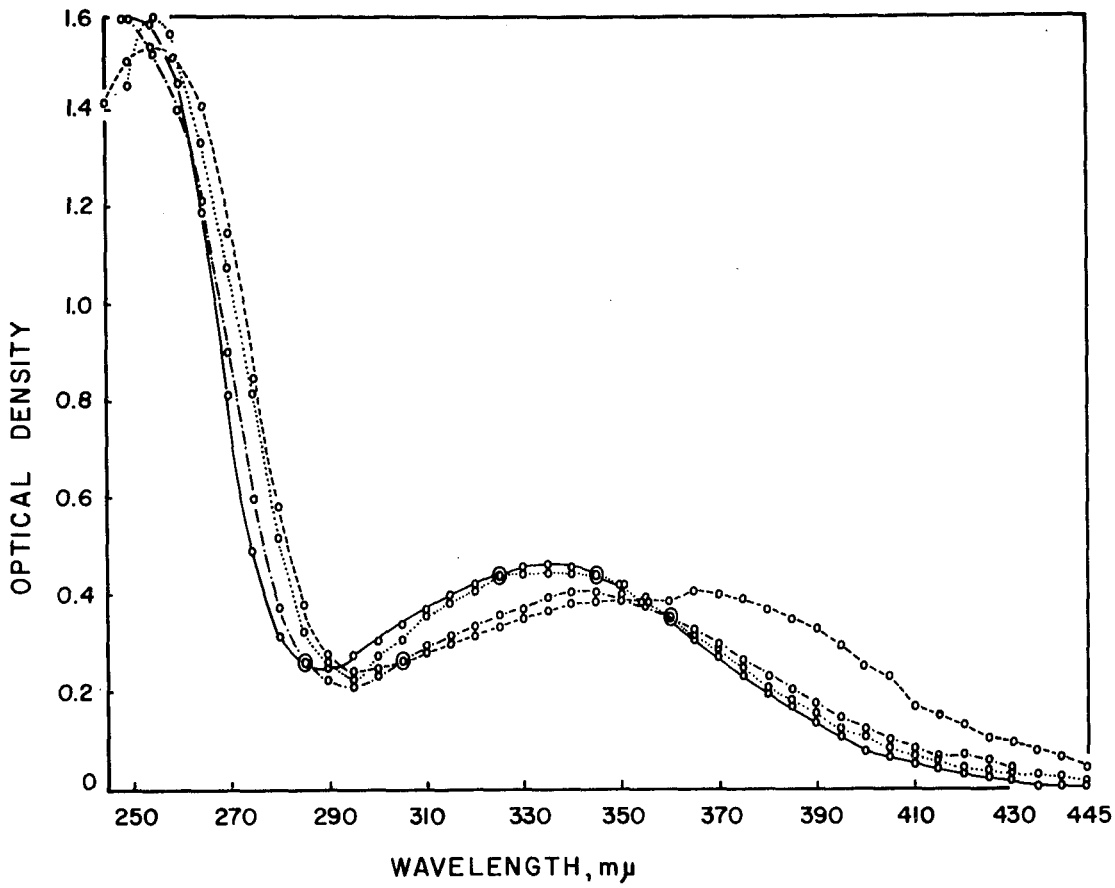
VIEW OF SEPHADEX G-75 COLUMN



GPC of mixture of DNS-γG and free DNS. Bands as seen under long wavelength ultra-violet lamp after collection of tube 2 (Fig. 26).

FIGURE 28.

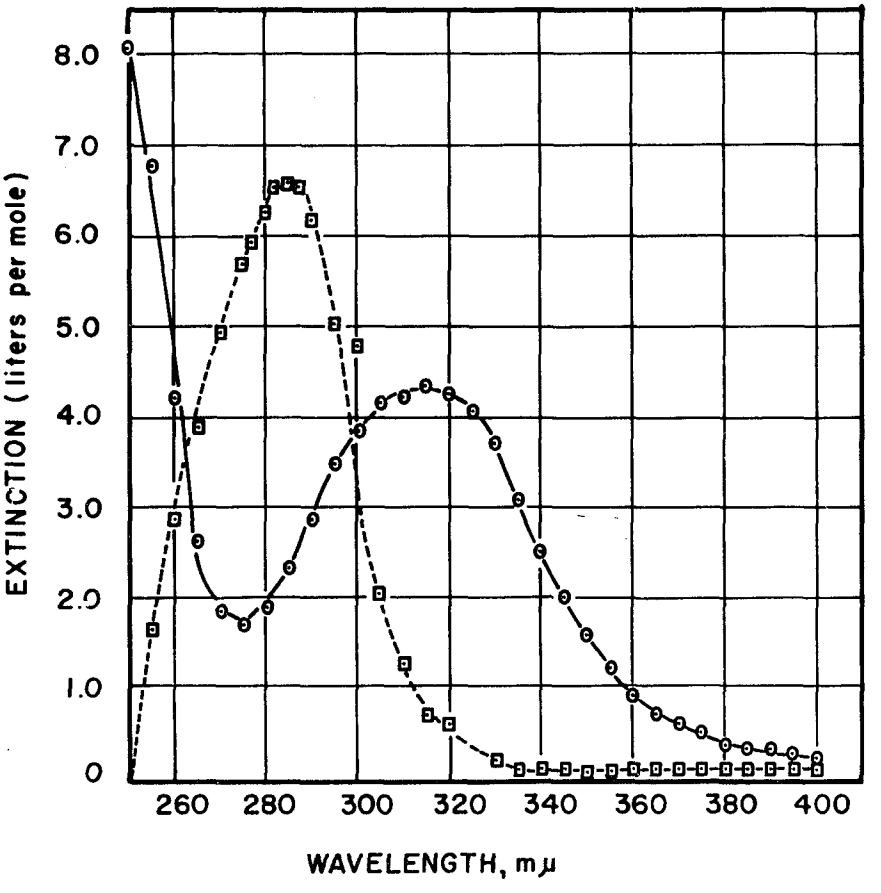
ABSORPTION SPECTRUM OF DIMETHYLAMINO NAPHTHALENE SULPHONYL CHLORIDE



Solvent 95% ethanol. Conc. DNS-Cl, 33μg/ml. Spectrum measured at various times after solution.----1 hour, -.-.-. 24 hours, ... 48 hours, _____, 72 hours.

FIGURE 29.

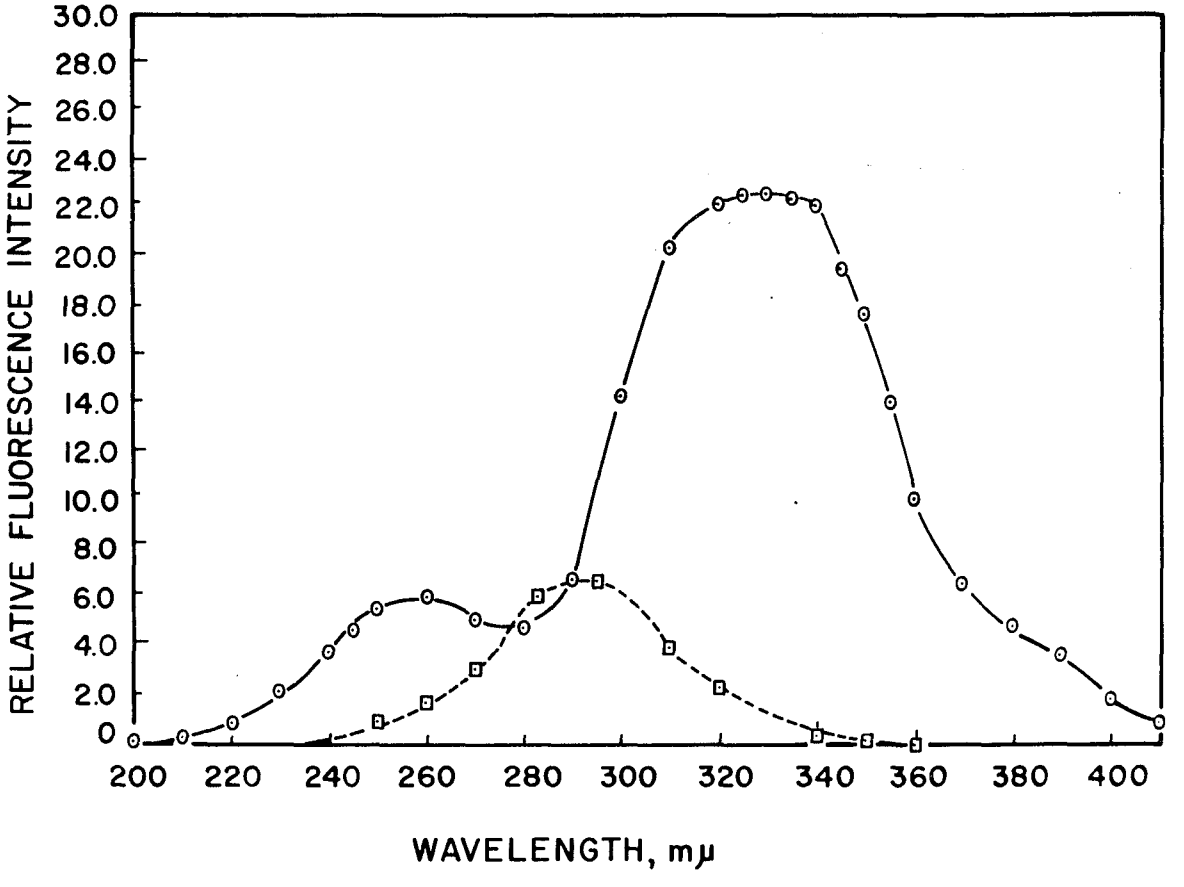
ABSORPTION SPECTRUM OF DIMETHYLAMINO NAPHTHALENE SULPHONIC ACID



Solvents:—⊙—, 0.01 N NaOH;-⊡-0,01N HCl.

FIGURE 30.

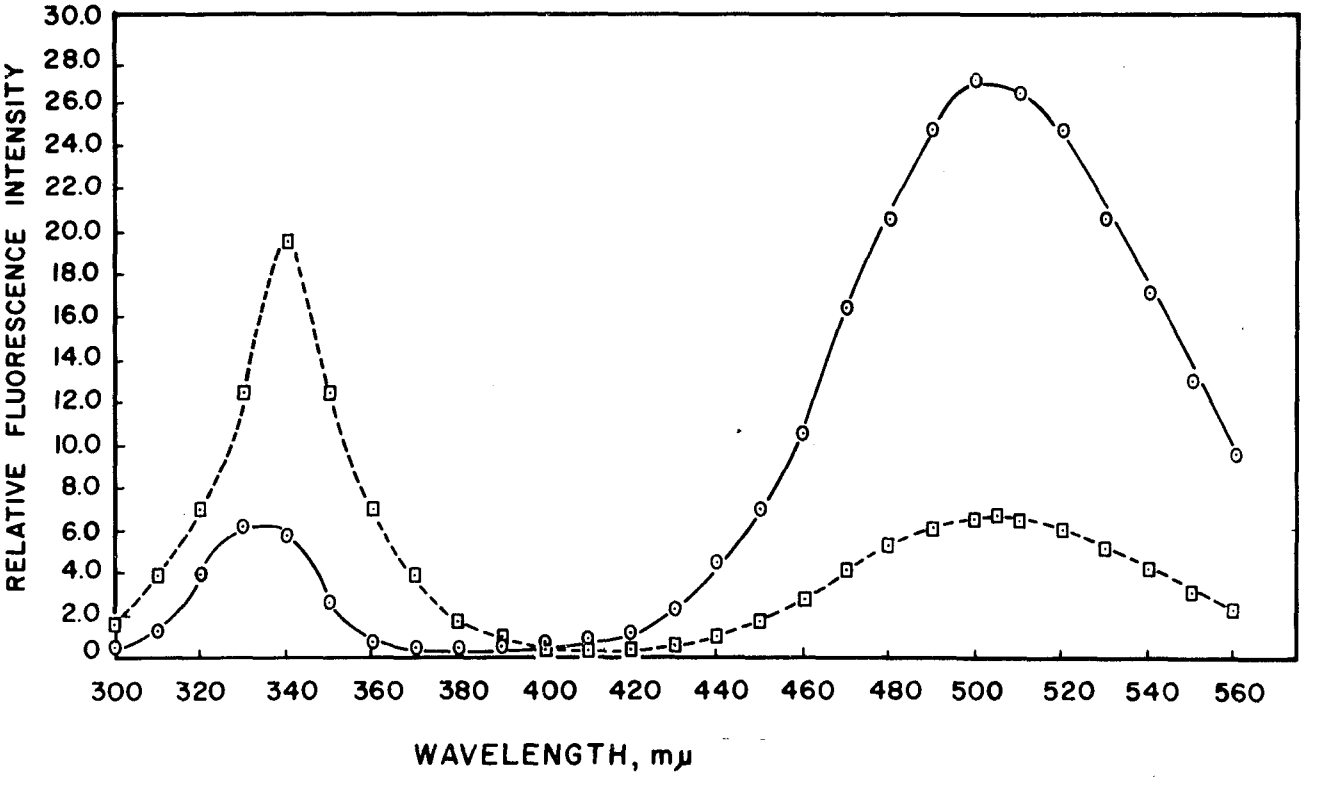
FLUORESCENCE EXCITATION SPECTRUM OF DIMETHYL-AMINO NAPHTHALENE SULPHONIC ACID



Solvents:—⊙—, 0.01N NaOH;--⊡--,0.01N HCl. Fluorescence emission measured at 510mμ.

FIGURE 31.

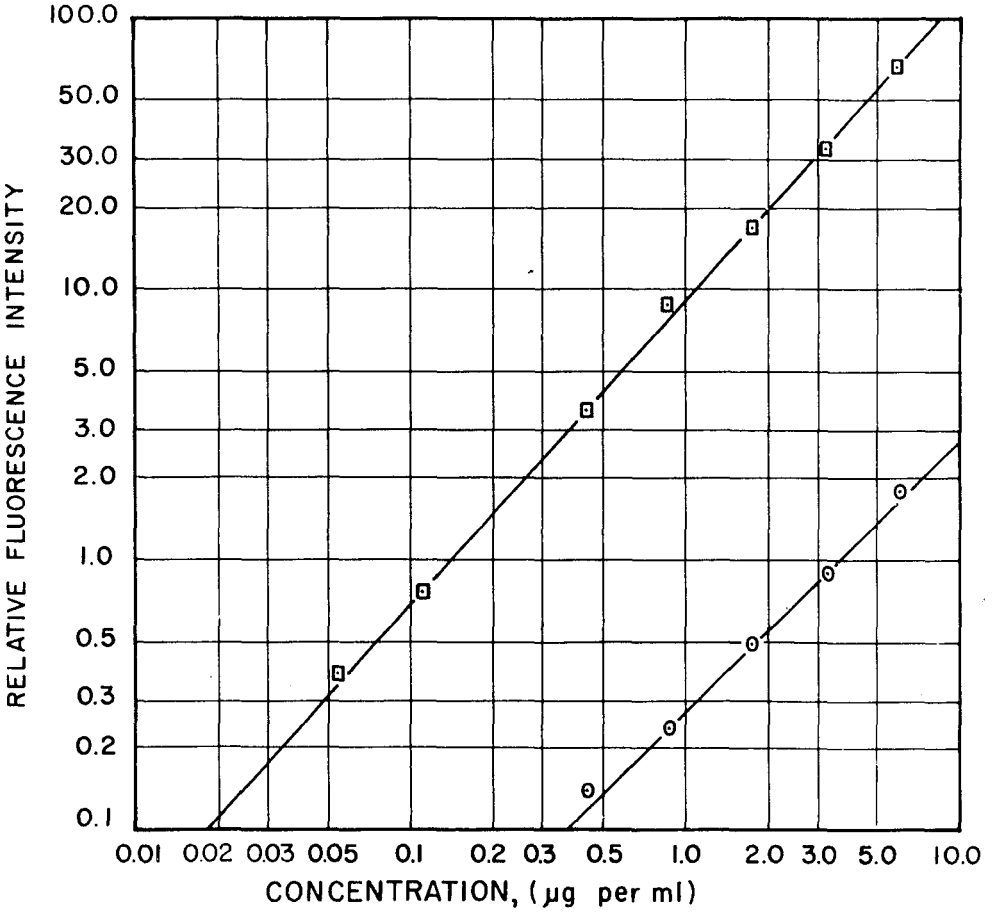
FLUORESCENCE EMISSION SPECTRUM OF DIMETHYL-AMINO NAPHTHALENE SULPHONIC ACID



Solvents: —⊙—, 0.01N NaOH; --⊡--, 0.01N HCl. Fluorescence excitation done at λmax, i.e., 295mμ for acid, and 350mμ for base.

FIGURE 32.

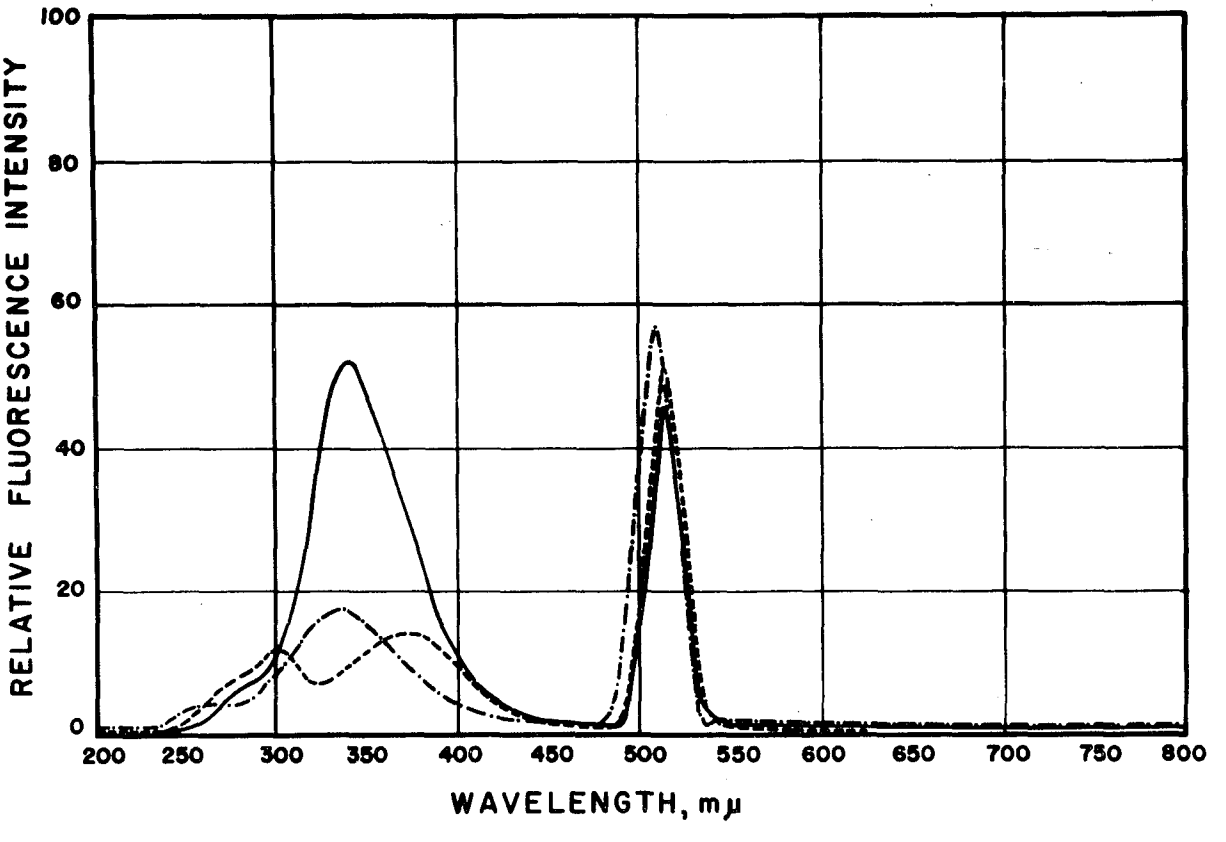
FLUORESCENCE OF DIMETHYLAMINO NAPHTHALENE SULPHONIC ACID



Excitation 350mµ. Solvents: —o— ,0.01N HCl; --□-- ,0.01 NaOH.

FIGURE 33.

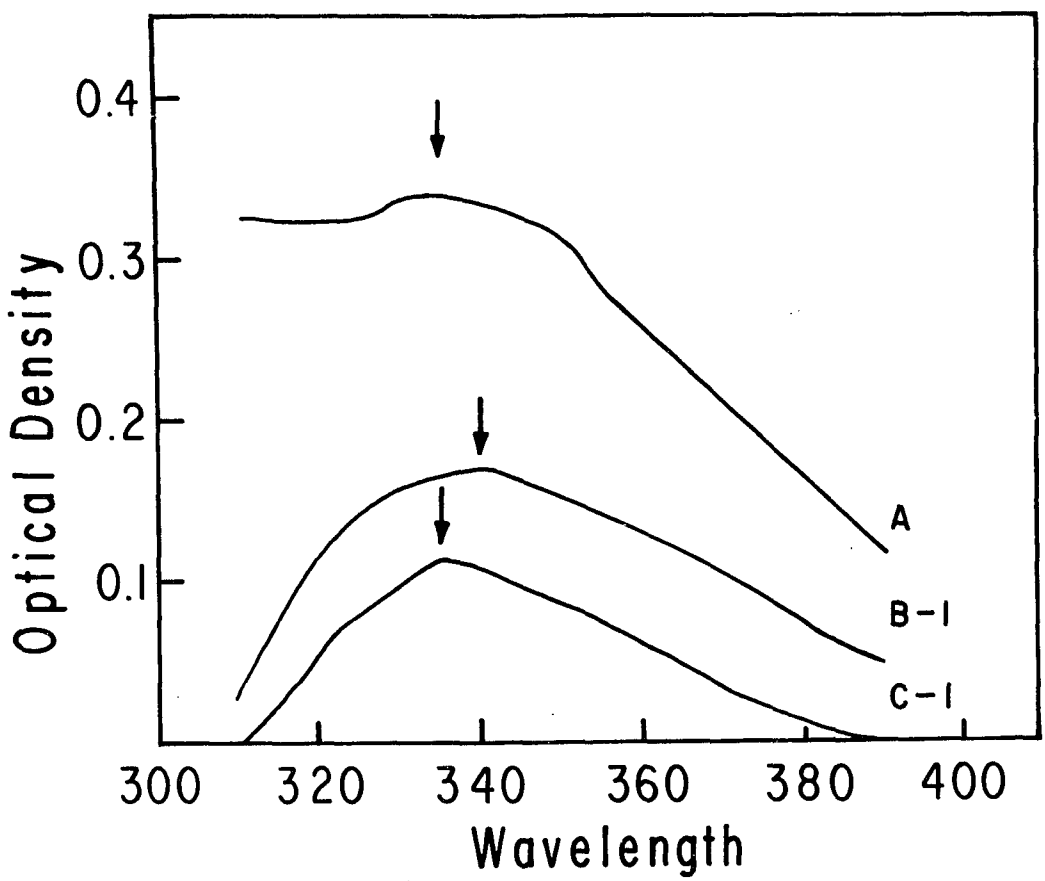
FLUORESCENCE EXCITATION SPECTRUM OF DNS-γG



Emission, 510mμ. Solvents: ———,0.01N NaOH;----,0.01N HCl;-.-.,0.15N NaCl.

FIGURE 34.

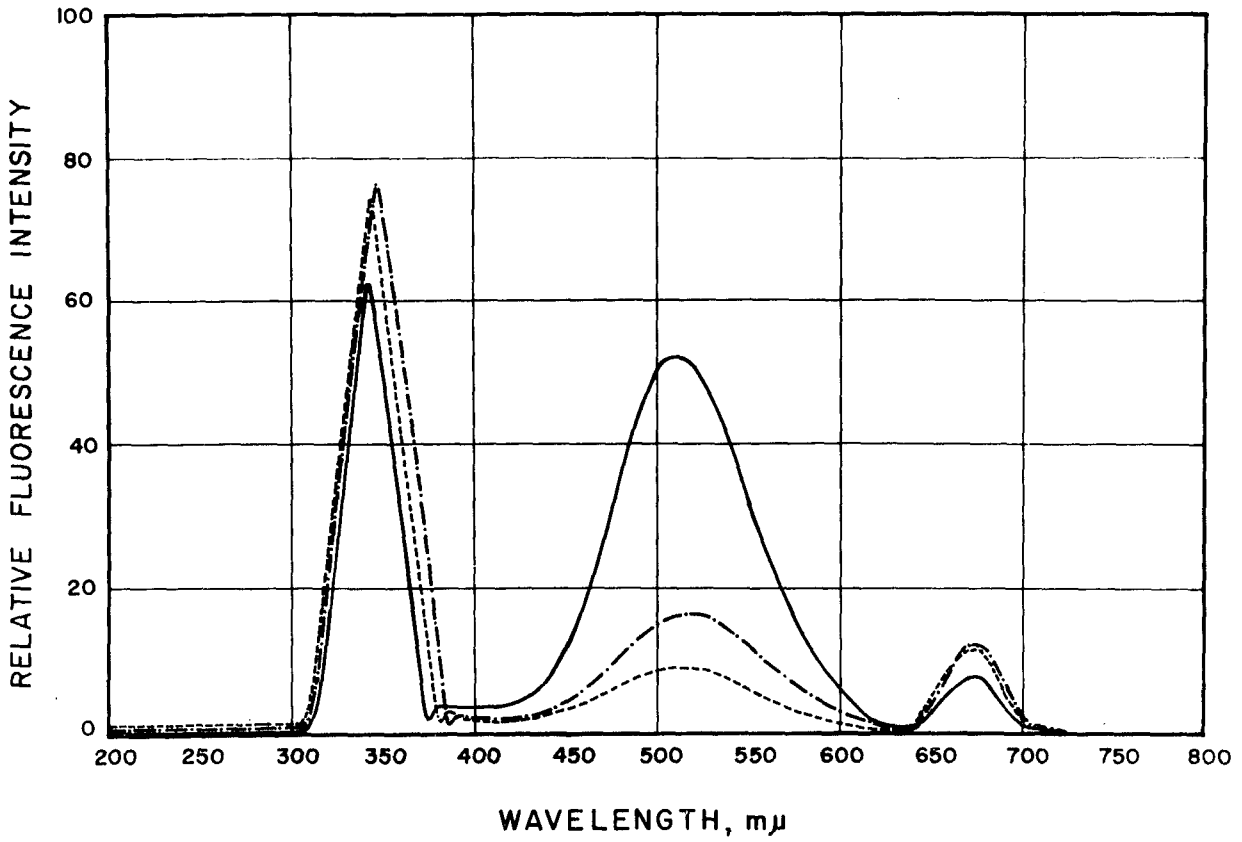
DIFFERENTIAL SPECTROPHOTOMETRY OF DNS-γG



Differential spectrophotometry of DNS-γG vs. untagged γG. Solvent 0.15N NaCl, Beckman DU spectrophotometer, γG concentration (mg/ml) and DNS/γG molar ratio: Batch A: 26.9, 0.37; Batch B-1: 12.4, 0.46; Batch C-1: 1.93, 6.69.

FIGURE 35.

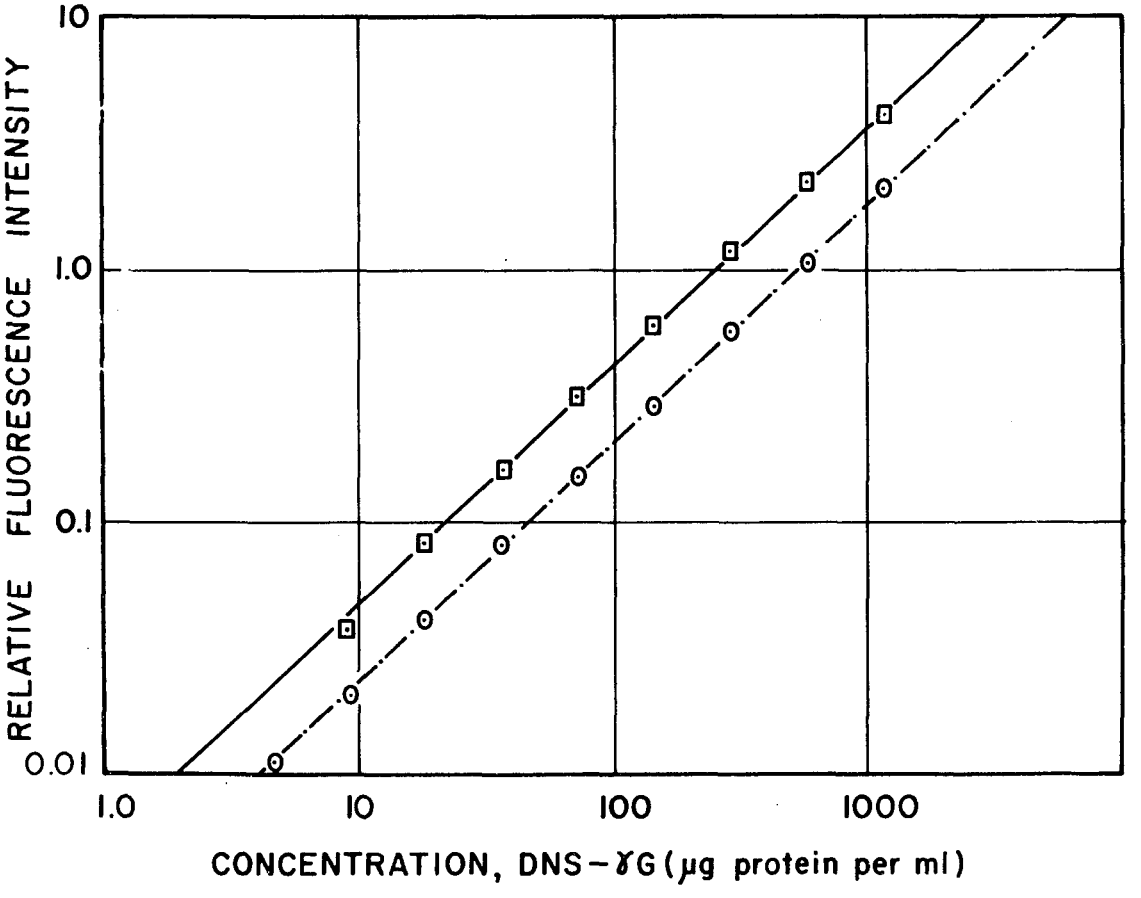
FLUORESCENCE EMISSION SPECTRUM OF DNS-γG



Excitation, 350mμ. Solvents: ——, 0.01N NaOH;-----, 0.01N HCl; -.-.-. 0.15N NaCl.

FIGURE 36.

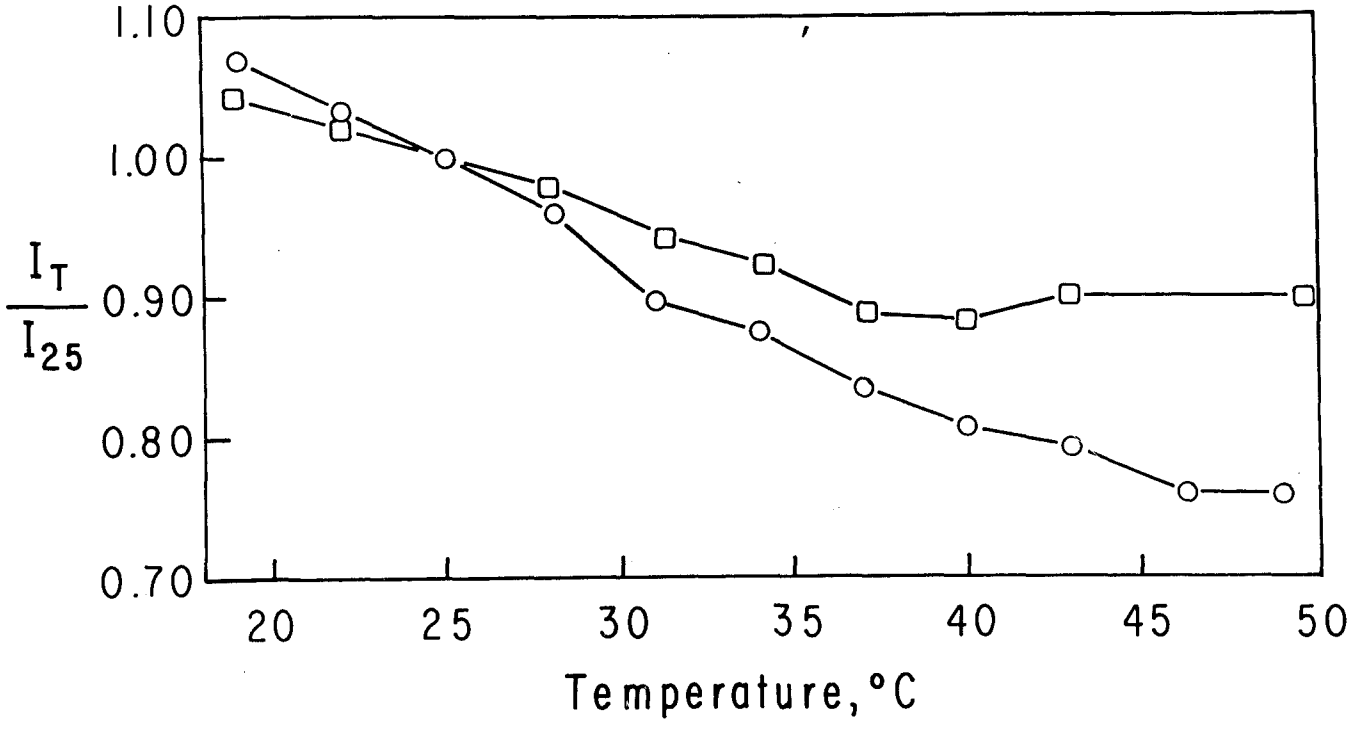
FLUORESCENCE OF DNS-γG



Solvents: _____, 0.01N NaOH; -.-.-.-. phosphate buffer pH 7.5. Excitation, 350mμ. Emission, 510mμ.

FIGURE 37.

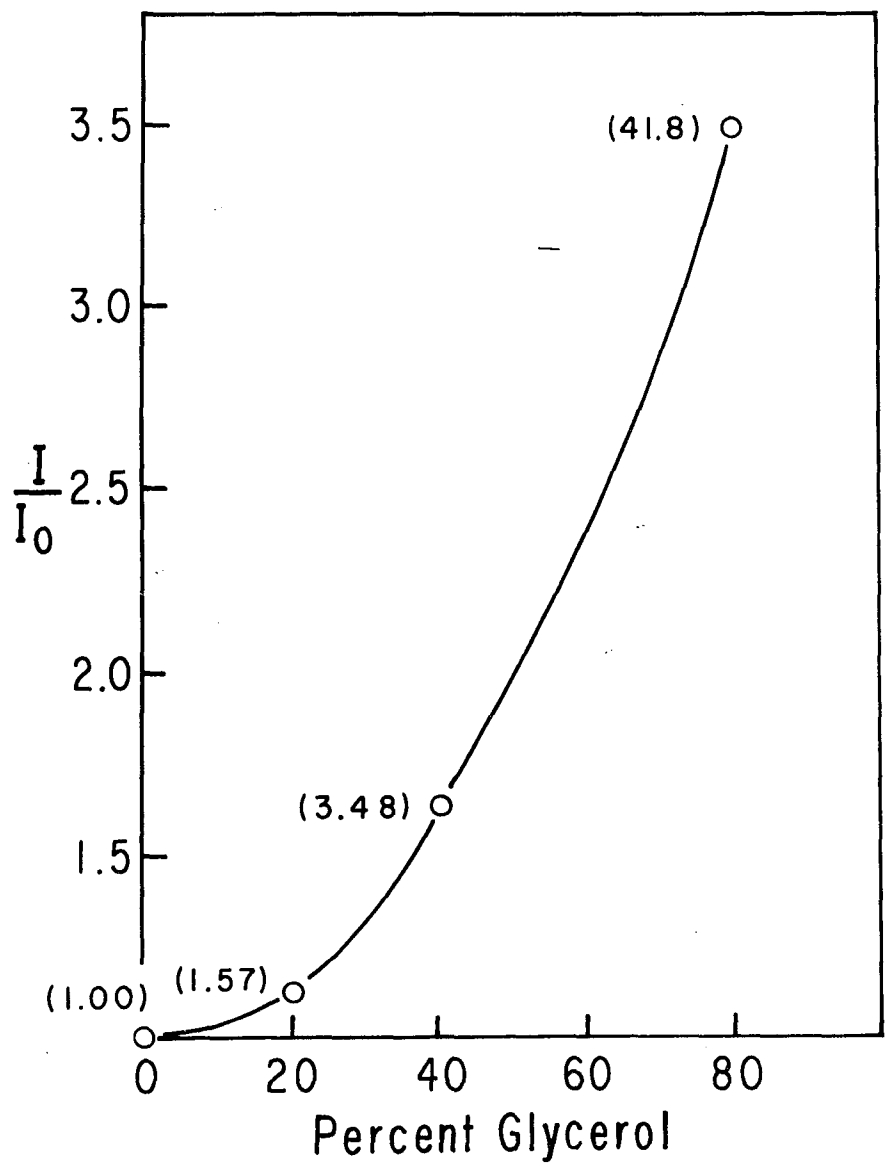
EFFECT OF TEMPERATURE ON FLUORESCENCE INTENSITY



Ratio of fluorescence intensity at various temperature, to fluorescence intensity at 25.0°C (I/I_{25}) as a function of temperature. DNS-γG #5, c=3.31 mg/ml, R=2.47. Solvents: —□— 0.15M NaCl; —⊙— 1.0M NaCl.

FIGURE 38.

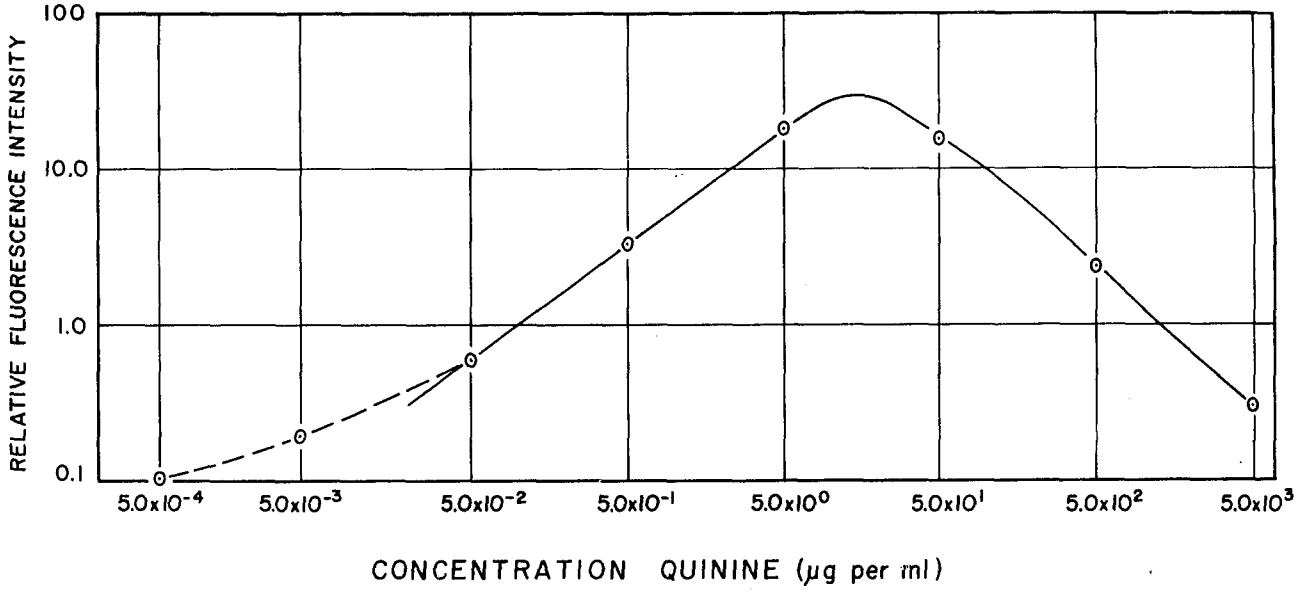
EFFECT OF VISCOSITY ON FLUORESCENCE INTENSITY



Ratio of fluorescence of DNS-γG #C2 at 0% added glycerol to fluorescence at various concentrations of glycerol (I/I_0). Value in brackets is viscosity relative to value at 0% glycerol. T, 15.0°C, solvent 0.15M NaCl.

FIGURE 39.

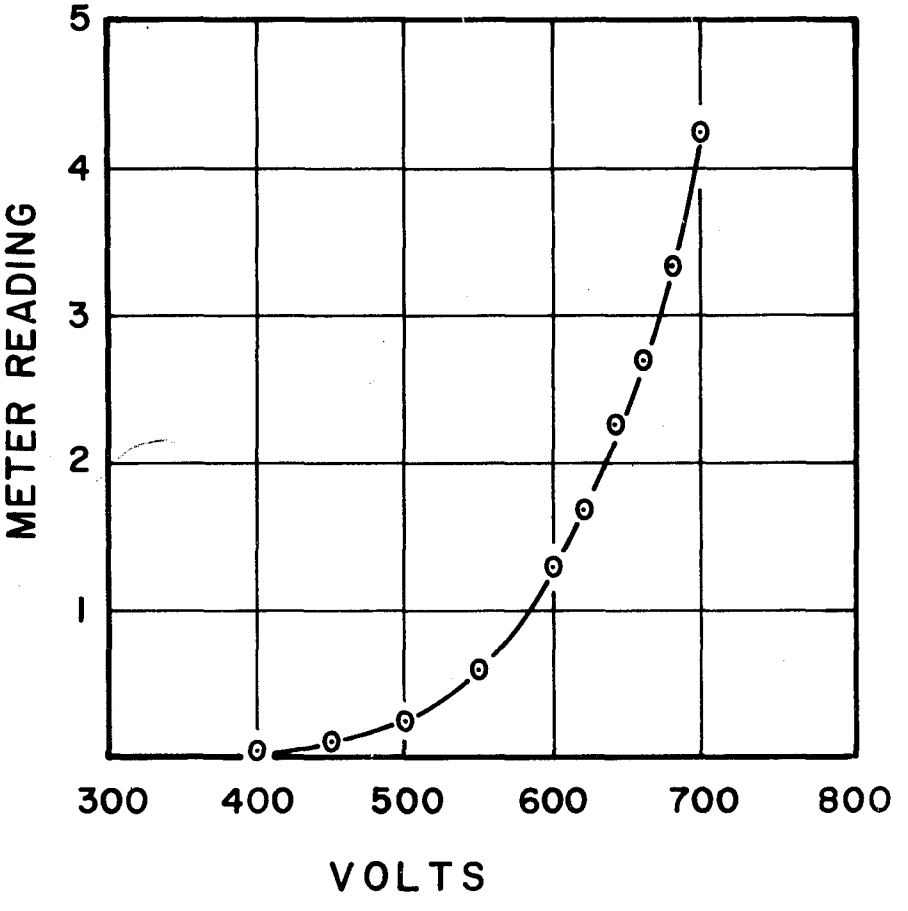
FLUORESCENCE OF QUININE SULPHATE



Solvent: 0.1N H_2SO_4.

FIGURE 40.

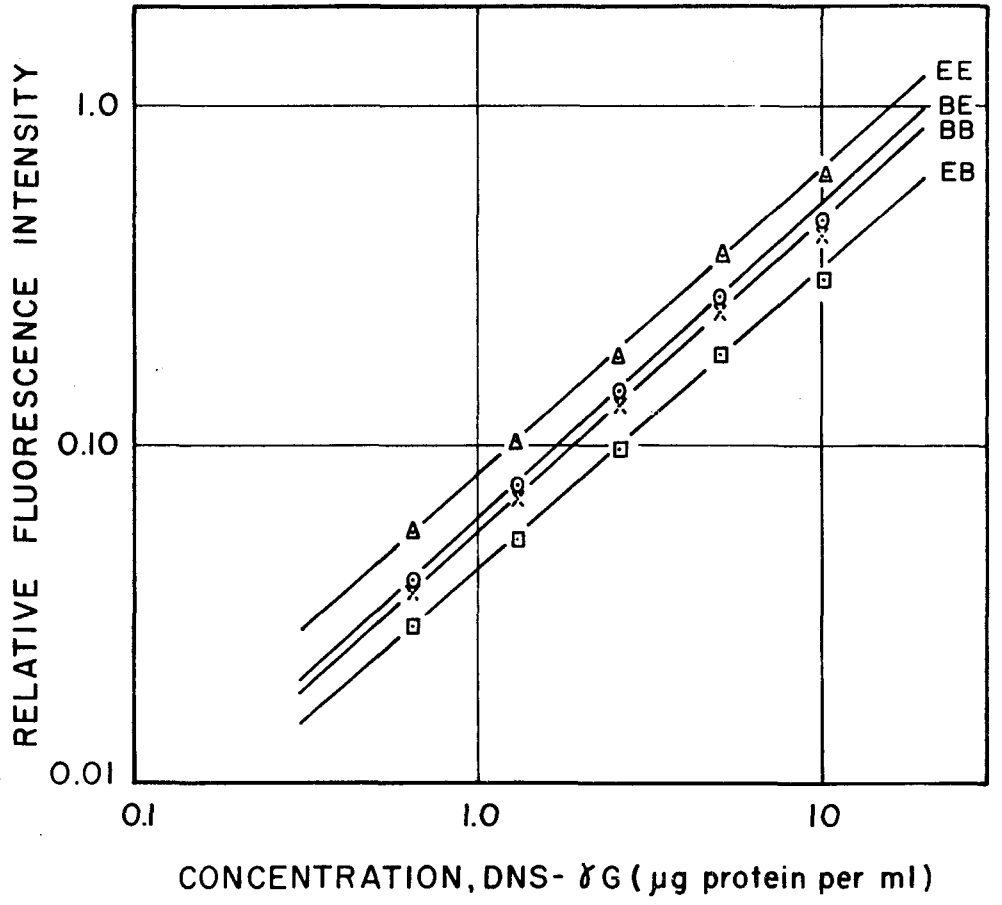
FLUORESCENCE INTENSITY VS PHOTOTUBE VOLTAGE



Experiment performed with no sample and open shutter at 510mμ.

FIGURE 41.

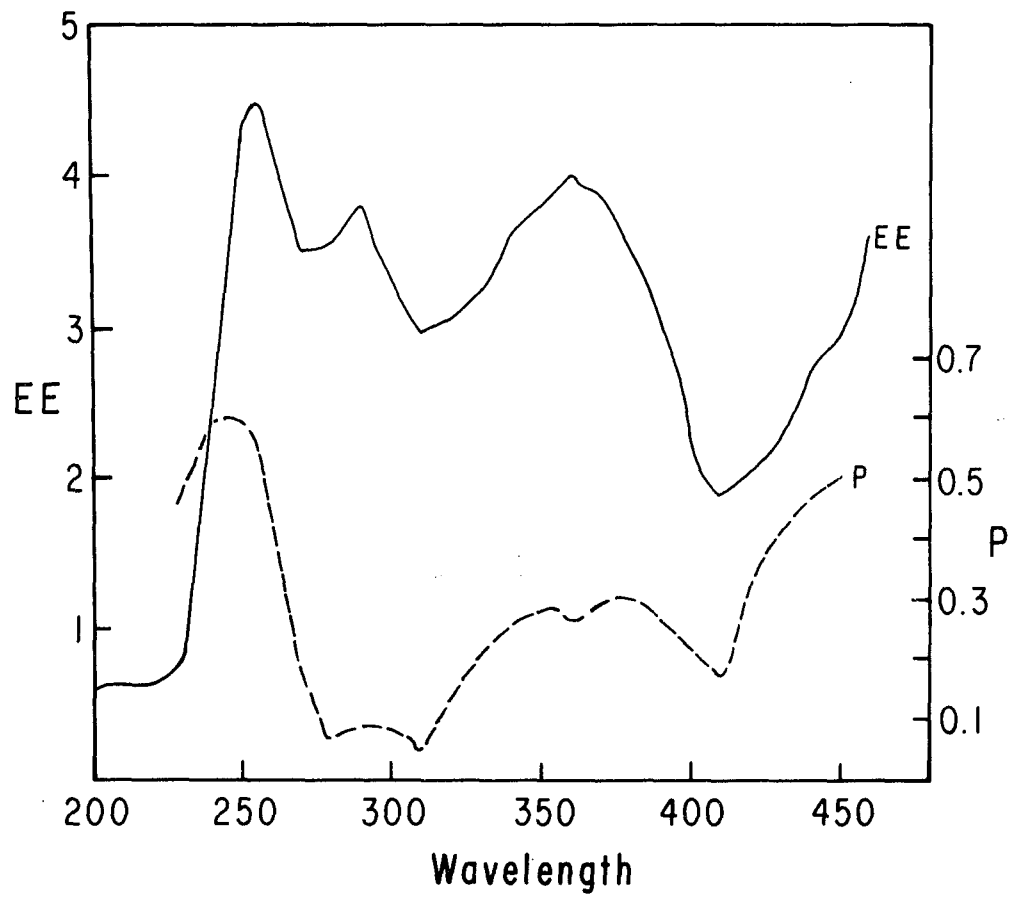
FLUORESCENCE OF DNS-γG WITH POLARIZING ELEMENTS



Done at the four positions of polarizer and analyzer; EE, BE, BB and EB.

FIGURE 42.

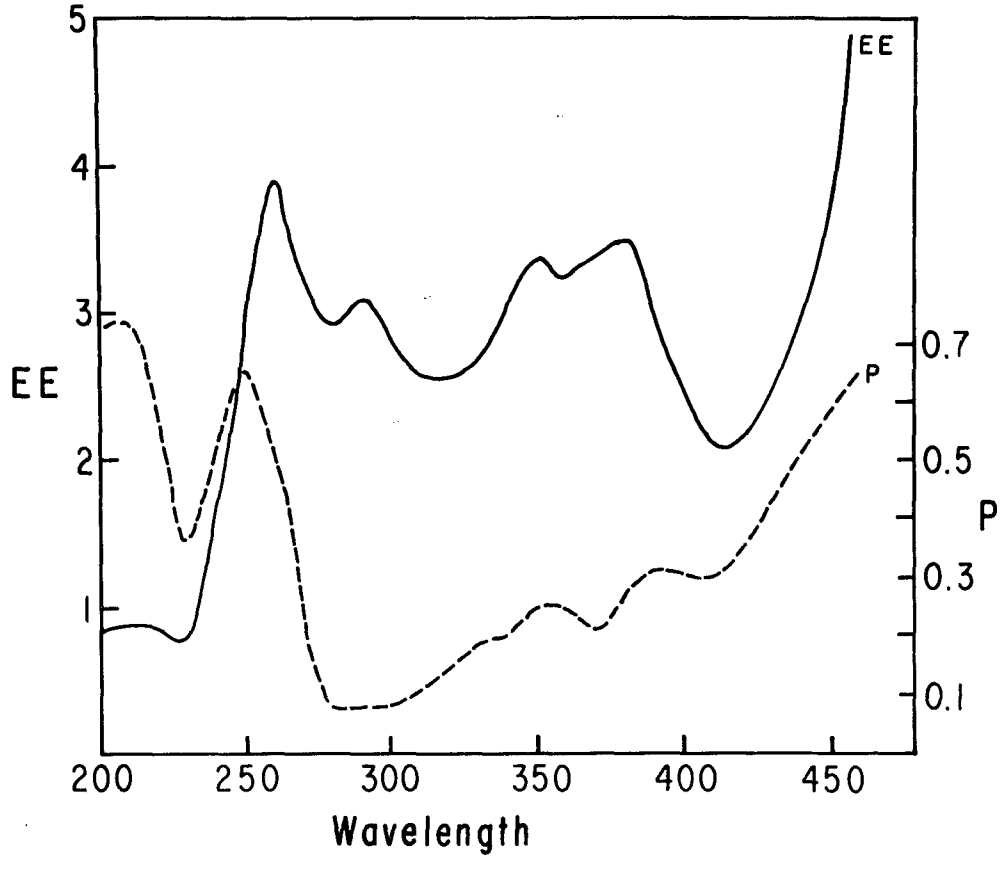
FLUORESCENCE AND FLUORESCENCE POLARIZATION
AT DIFFERENT EXCITATION WAVELENGTHS



Fluorescence (EE,———) and fluorescence polarization (P,----) of DNS-γG batch R-6, as a function of excitation wavelength. Emission, 510mμ. Solvent, 0.1N HCl. γG conc, 0.505 mg/ml, molar ratio DNS/γG, 9.13.

FIGURE 43.

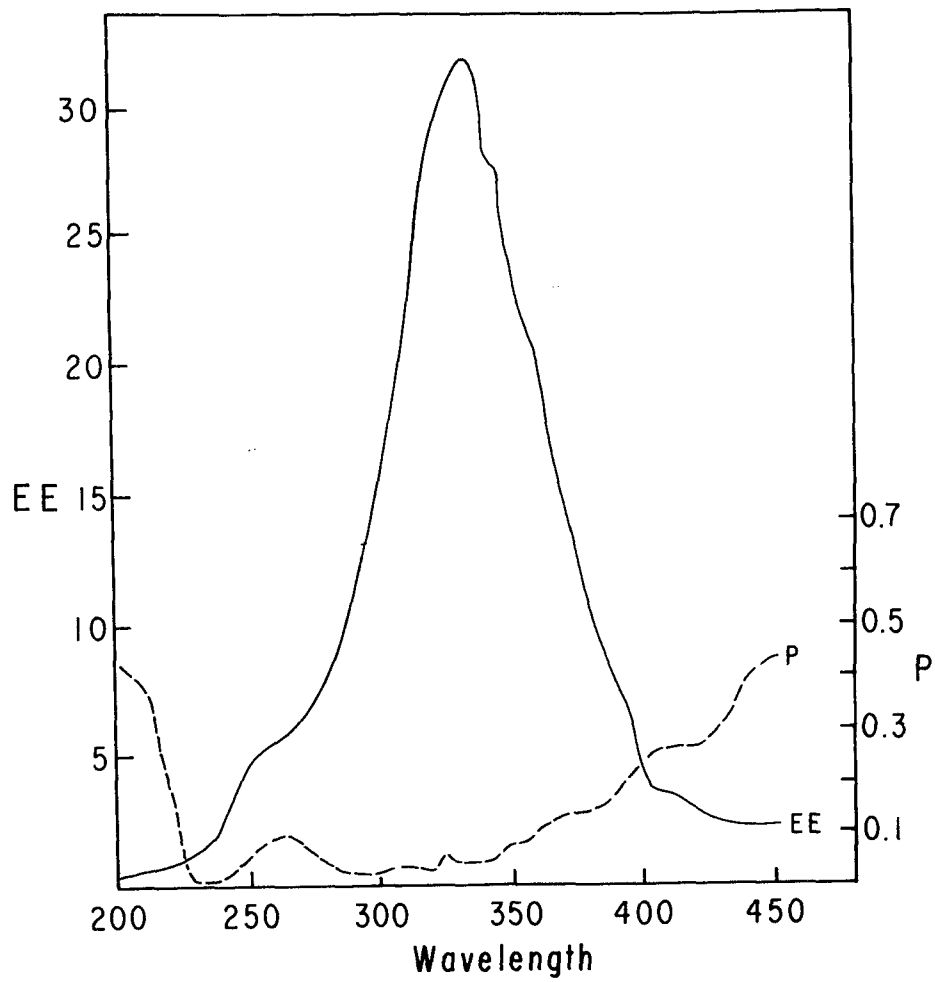
FLUORESCENCE AND FLUORESCENCE POLARIZATION AT DIFFERENT EXCITATION WAVELENGTHS



Fluorescence (EE,———) and fluorescence polarization, (P,----) of DNS-γG, batch R-6 as a function of excitation wavelength. Emission measured at 510mμ. Solvent, pH 8.13 buffer, γG conc. 0.505 mg/ml, molar ratio DNS/γG, 9.13.

FIGURE 44.

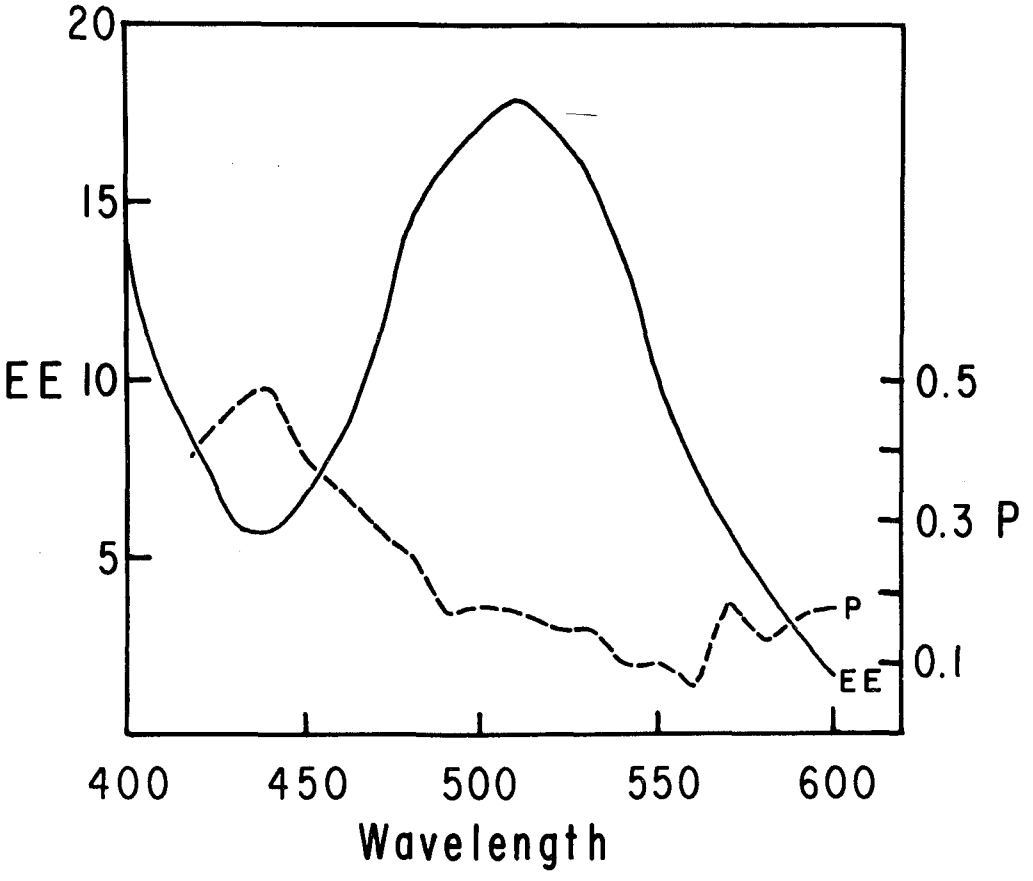
FLUORESCENCE AND FLUORESCENCE POLARIZATION AT DIFFERENT EXCITATION WAVELENGTHS



Fluorescence (EE——) and fluorescence polarization (P,----) of DNS-γG, batch R-6 as a function at excitation wavelength. Emission measured at 510mμ. Solvent, 0.1N NaOH, γG conc. 0.505 mg/ml, molar ratio, DNS/γG, 9.13.

FIGURE 45.

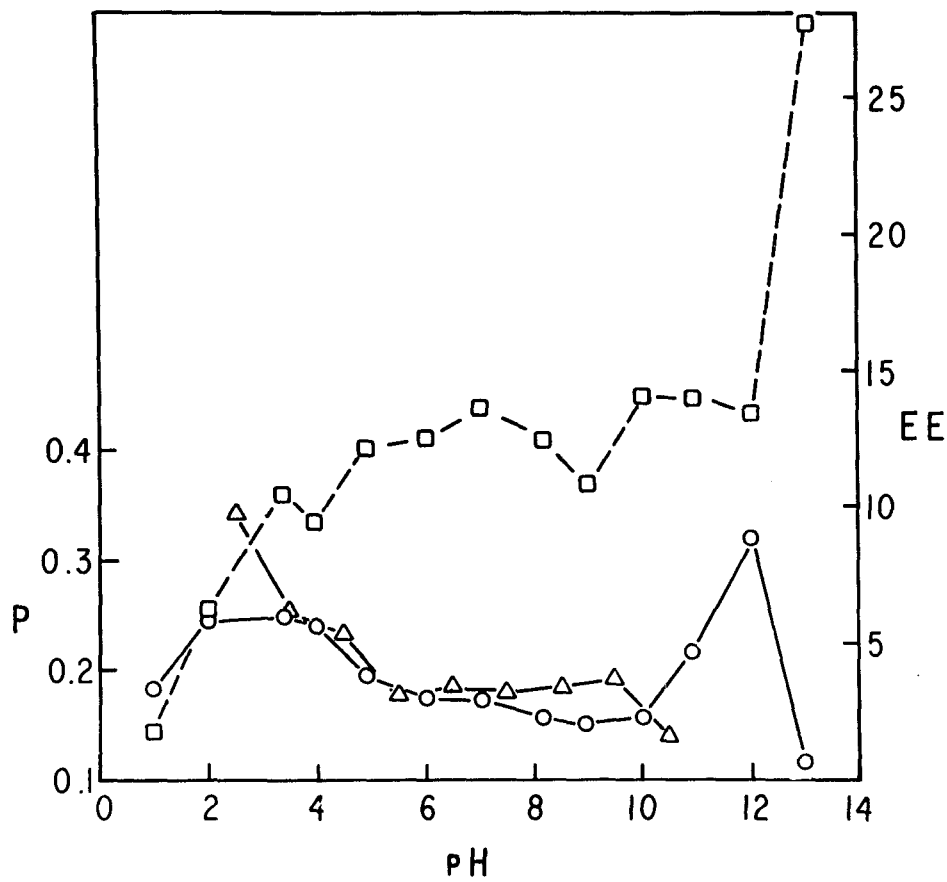
FLUORESCENCE AND FLUORESCENCE POLARIZATION AT DIFFERENT EMISSION WAVELENGTHS



Fluorescence (EE,——) and fluorescence polarization, (P-----) of DNS-γG, batch A, as a function of emission wavelength. Excitation at 350mμ. Solvent, 0.15MNaCl. γG conc. 5.38 mg/ml, molar ratio DNS/γG, 0.37.

FIGURE 46.

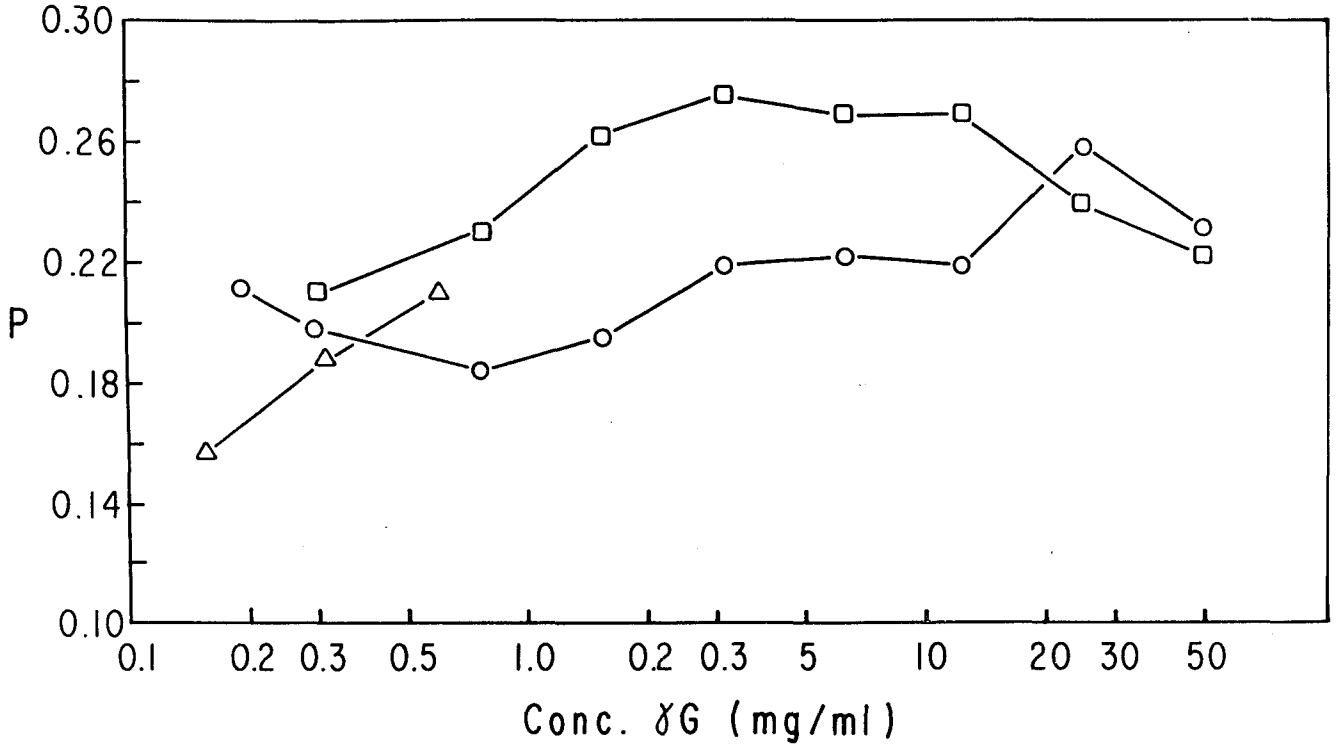
EFFECT OF PH ON FLUORESCENCE AND FLUORESCENCE POLARIZATION OF DNS-γG



Fluorescence intensity (EE, ---) and fluorescence polarization (P, -⊖-) of DNS-δG, batch R-6 0.505 mg/ml, DNS/δG molar ratio, 9.13 in buffers of different pH. Also polarization (P, -⊡-) of same protein originally dissolved in 0.15N NaCl and titrated with 0.01N HCl and 0.01N NaOH to various lower or higher pH valves. pH, untitrated sample, 6.5, fluorescence values corrected for volume changes.

FIGURE 47.

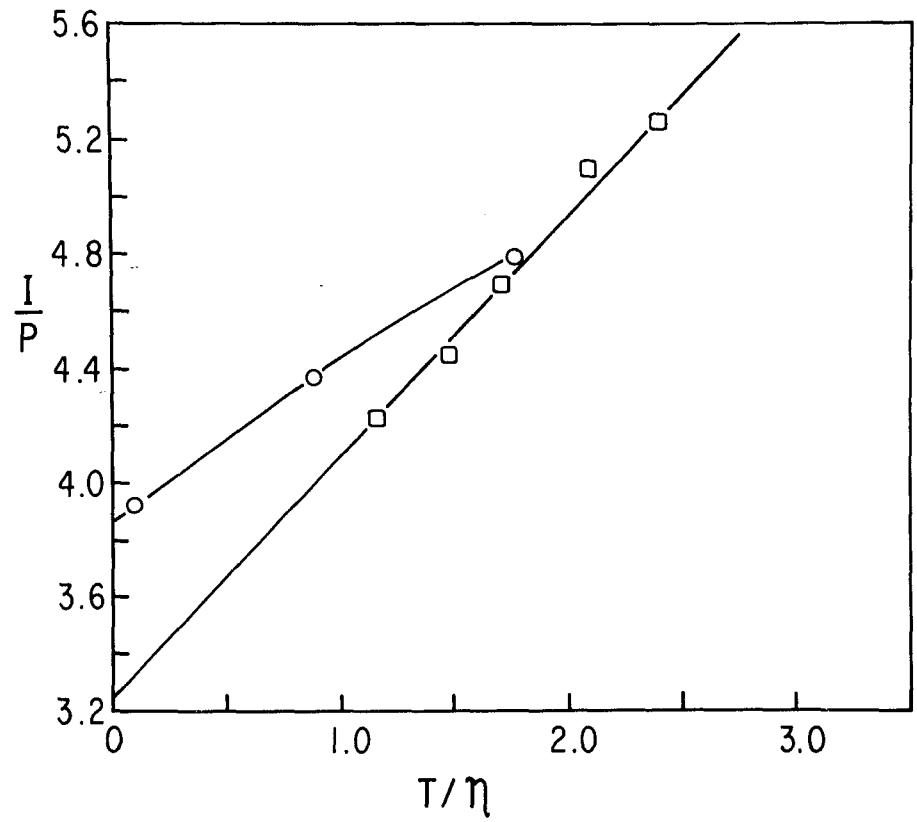
EFFECT OF CONCENTRATION ON POLARIZATION OF DNS-γG



Fluorescence polarization of DNS-γG at various concentrations. DNS-γG, #5 in pH 7.5 buffer, —⊙— ; same in 1.0M NaCl —□— ; R, 2.47. DNS-γG, in 1.0M NaCl —◇— R, 9.13; T, 25.0°C.

FIGURE 48.

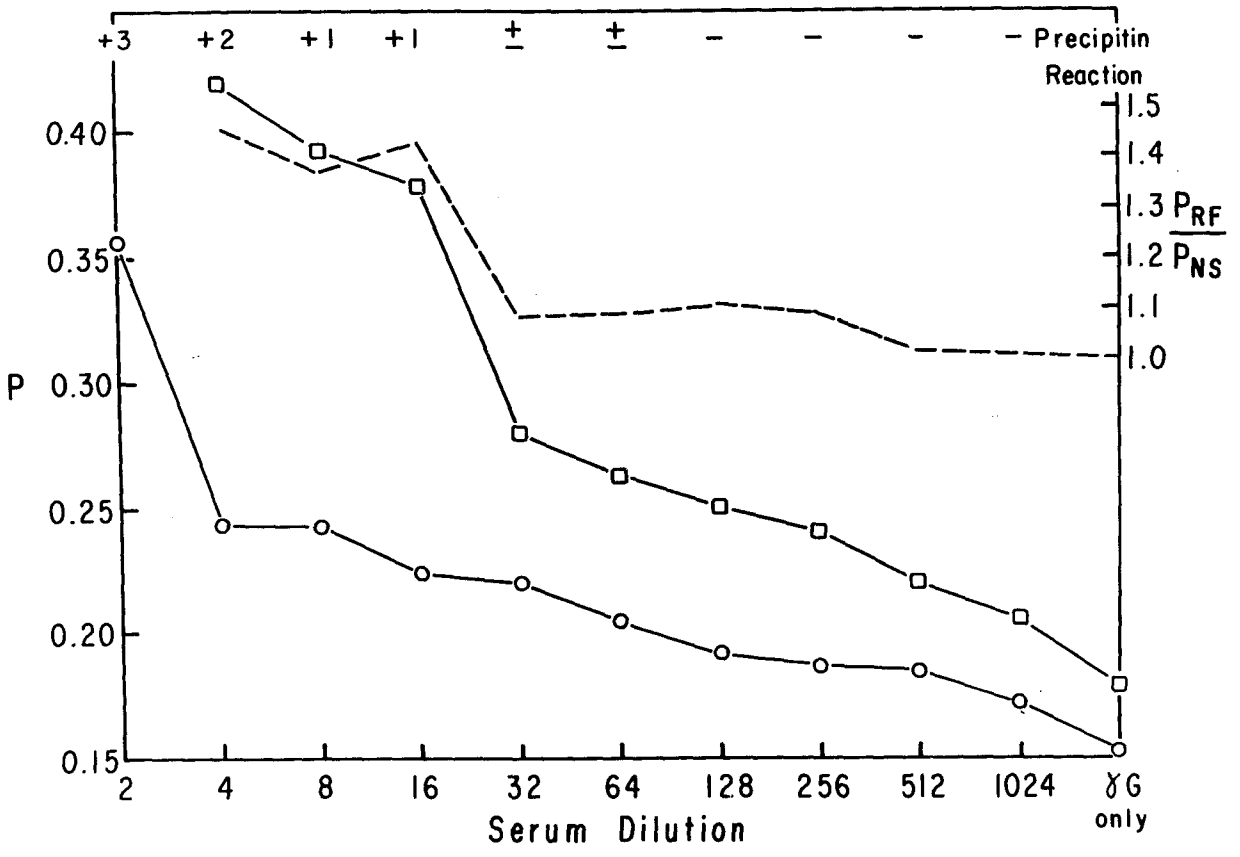
PERRIN EQUATION PLOT OF POLARIZATION



Plot of reciprocal of polarization (1/P) as a function of absolute temperature divided by viscosity (T/η) in deg. poise^{-1} x 10^{-4}) ⊡.DNS-γG #5, conc. 3.31 mg/ml. molar ratio DNS/γG, 2.47. Temperature varied from 19.0 to 49.7 C, P_o 0.3077, relaxation time (ρ_H) 135n sec. ⊙ # C2 conc. 0.250 mg/ml. molar ratio DNS/γG, 6.53.Temperature, 15°C, viscosity varied by adjusting samples to 20,40 and 80% glycerol respectively, P_o 0.2583, relaxation time (ρ_H) 240ηsec.

FIGURE 49.

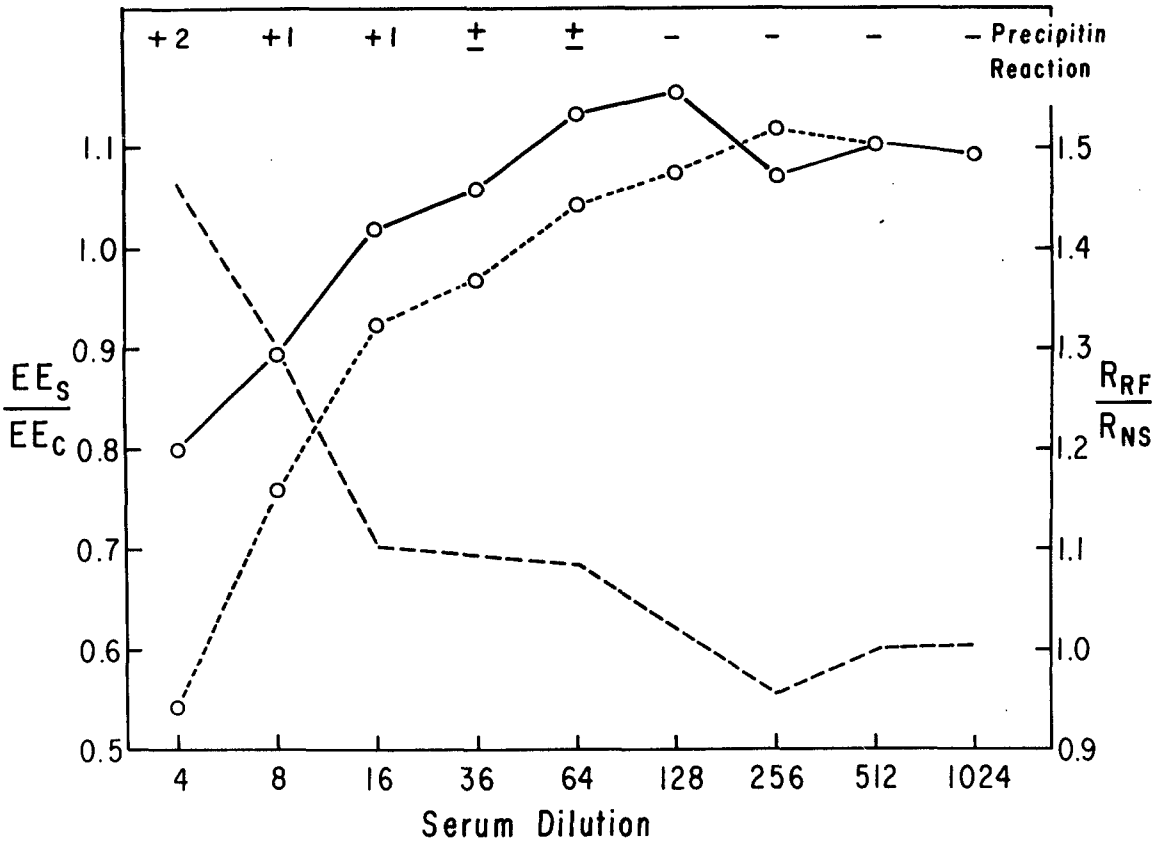
FLUORESCENCE POLARIZATION OF DNS-γG IN THE PRESENCE OF RHEUMATOID FACTOR AND NORMAL SERA



Polarization of DNS-γG, 1a (γG conc. 1.94 mg/ml, DNS/γG, 1.6) in presence of increasing dilutions of normal serum (—○—) and rheumatoid factor serum (—□—), and ratio of polarization valves P_{rf}/P_{ns} adjusted for variation in γG polarization (– – –)

FIGURE 50.

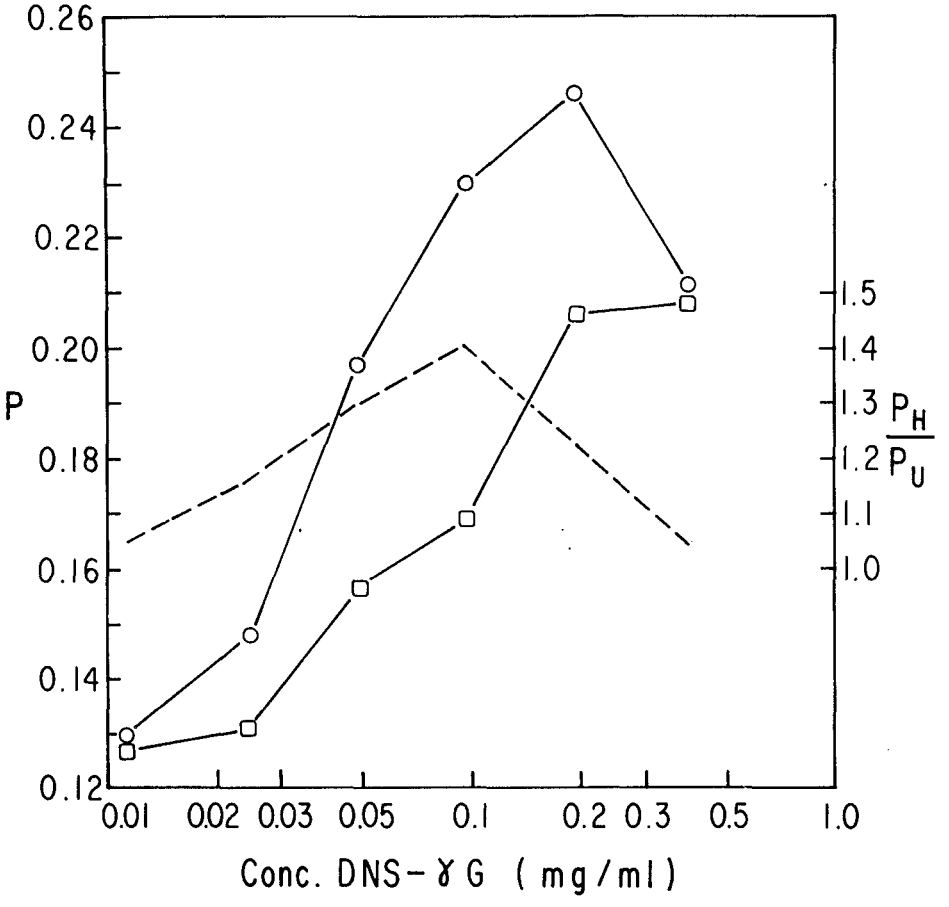
FLUORESCENCE QUENCHING OF DNS-γG IN THE PRESENCE OF RHEUMATOID FACTOR AND NORMAL SERA



Fluorescence quenching of DNS-γG, 1a (γG conc. 1.94 mg/ml, DNS/γG, 1.6) in presence of increasing dilutions of normal serum (—⊙—) and rheumatoid factor serum (- -⊙- -). Data expressed as ratio of DNS-γG fluorescence in presence and in absence of serum (EE_S/EE_C=R). Increment of fluorescence quenching (— · — ·—) expressed as R_{rf}/R_{ns}. Fluorescence data corrected for serum fluorescence.

FIGURE 51.

EFFECT OF RHEUMATOID FACTOR SERUM ON FLUORESCENCE POLARIZATION OF HEATED AND UNHEATED DNS-γG



Fluorescence polarization (P) of DNS-γG, batch 1a, pH 7.5 phosphate buffer mixed with equal volume of 1:100 diluted RF serum. DNS-γG unheated (—□—) DNS-γG heated (—○—) at 63°C for 75'. P_h/P_r (— — —) ratio of heated γG P to unheated γG P, corrected for difference in serum polarization in each experiment.

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BIOGRAPHICAL SKETCH

The author was born in Chicago Ill. on June 30, 1926. He is married and the father of four children. He served in U.S. Army during 1944-1946. He received a B.S. in Chemistry from the College of the City of New York in 1949, and a M.A. in Physical Chemistry from Brooklyn College in 1956. He completed the requirements for a Ph.D. in Chemistry from the City University of New York in 1968. His past professional experience includes the following positions: control chemist, Technical Tape Co., Bronx, N.Y., 1949; research technician, New York University School of Medicine, New York, 1950-1; research physical chemist, Jewish Chronic Disease Hospital, Brooklyn, N.Y., 1951-7; and research fellow in Chemistry, Polytechnic Institute of Brooklyn, 1957-8. In 1959 he joined the staff of the Mount Sinai Hospital as a research assistant, and later a research associate, in the Dept. of Medicine. He is currently an Associate in Medicine at the Mount Sinai School of Medicine, and director of its Clinical Research Center Core Laboratory. He is a member of the following professional and scientific organizations: The American Chemical Society, Phi Lambda Upsilon, The American Rheumatism Association, The New York Rheumatism Association and the Albert Einstein Research Center of Buenos Aires. He has been the recipient of various grants, as principal investigator, from the National Institute of Arthritis and Metabolic Diseases and the Arthritis and Rheumatism Foundation, and has received from the N.Y. State Medical Society in 1954, its second award for scientific research. He is the author or co-author of more than 40 scientific publications. (See list following)

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44. Application of an Enzymatic Technique for Measurement of Blood Ammonia.

45. Measurement of Ionized Calcium In Various Pathological Conditions.

46. Fluorescence Polarization Studies of the Rheumatoid Factor-γG Immunoglobulin Reaction.

47. Rheumatoid Factor Activity In Patients With Renal Transplants.

48. Rheumatoid Factor In Rheumatoid Arthritis and In Other Diseases.

49. Treatment of Psoriasis With a Dietary Regimen.

50. Ionized Calcium Levels In Patients With Rheumatoid Arthritis and Related Diseases.

ABSTRACT

These studies have generally been concerned with the reaction of the macroglobulin antibody, known as the rheumatoid factor (RF), with human γG immunoglobulin (γG). Four points have been considered: I. purification and fractionation of RF; II. role of denaturation and aggregation of γG in enhancing its reactivity with RF; III. detection of autospecific reactivity between individual's RF and his autologous γG, and IV. application of fluorescence polarization to study RF-γG interactions.

Purification of RF was accomplished by Sephadex G-200 gel filtration but this was attended by considerable loss of antibody activity. In other experiments conjugates of diazotized polyaminostyrene and human γG (PAS-γG) were employed in adsorption and immunochromatographic studies of rheumatoid factor serum (RFS) and antihuman γG antiserum. Between pH 5.25 and pH 4.0 seven fractions of RF agglutinating activity were obtained. In most cases, upon re-chromatography, the RF fractions were recovered at the same pH at which they were originally obtained. Earlier fractions obtained from the column required more aggregated γG to inhibit their agglutination in the tanned sheep cell test than did later fractions.

These results are consistent with previous reports of RF heterogeneity. The variations observed in elution of RF subfractions and inhibition of their agglutination reaction suggest that they differ in their affinity for pooled γG.

The immunochromatographic pattern of an anti-γG antiserum differed from that of RFS in that no antibody was eluted until pH 4.0 and pH 3.2, where two fractions were obtained. These results reflect the greater

affinity of anti γG antibody, as compared to RF, for pooled γG.

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Thermal aggregation of human γG was studied turbidimetrically. γG was aggregated by heating at 63°C for ½, 1, and 2 hours. In all three cases plots of turbidity as a function of $1/\lambda^4$ in the wavelength range 400-600mμ were linear but did not pass through the origin. This suggested that the thermal aggregates were not "small" particles as defined by light scattering theory. By comparison to the turbidity of latex particles of known diameter it appeared that the largest γG aggregates were no more than 0.1 microns in diameter. For a given aggregate sample at a given wavelength turbidity was proprotional to concentration. However, the ratio of turbidity to aggregate concentration increased with increasing heating time. Purified aggregates yielded identical turbidity values as did the same concentration of aggregates when not separated from un-aggregated γG.

Various RFS were titrated with human γG coated tanned sheep cells at six cell concentrations ranging from 0.125% to 2.0%. It was found that in 36 of 63 sera tested there was an abnormally large decrease in titer with increasing cell concentration. Double logarithmic plots of titers vs. cell concentration were linear. While linearity with a slope of -1.0 was expected, it was found that these 36 sera tested yielded slopes greater than -1.0.

Addition of aggregated γG to a -1.0 slope serum caused a rise in slope. Removal of autologous 7SγG, by DEAE cellulose column chromatography, from

a high slope serum lowered the slope. Addition of 7S γG, prepared from a high slope serum, to a -1.0 RF serum served to raise the slope. Partial removal of RF by adsorption of a low slope serum also caused an increased slope.

Block titration of rabbit antisera directed against human γG yielded normal -1.0 slopes with the initial bleedings. However, antisera obtained 3 to 4 weeks after initial immunization showed elevated slopes. This was true when agglutination titers were increasing, as well as when such titers were decreasing.

It is suggested that in RF sera at least, elevated slopes are due to a dilution dependent autoinhibition of agglutination which arises from the interaction between at least part of the patient's rheumatoid factor with his autologous γG.

Fluorescence polarization and fluorescence quenching have been applied to they study of RF-γG reaction. γG was coupled to the fluorescent compound dimethylamino naphthalene sulphonyl chloride (DNS). Removal of excess DNS (as the sulfonic acid) was complicated by the fact that denatured or aggregated γG strongly adsorbed unconjugated DNS. Therefore aggregated γG was removed following conjugation with DNS by sodium sulphate precipitation. Filtration through Sephadex-G-25 followed by dialysis yielded preparations containing 2-3 moles DNS bound per mole γG, and substantially free of unbound DNS.

Constant amounts of DNS γG were mixed with serial dilutions of normal serum (NS) and RFS and polarization (P) of fluorescence measurements were made. In both cases P increased with increasing serum concentration, in

part due to viscosity effect. At a serum dilution of 1:16, P was 43% higher with RFS than with NS. At this dilution an appreciable precipitin reaction was later obtained. This increment in P was evidently due to the reaction of DNS-γG with RF. The reaction was also characterized by a considerable degree of fluorescence quenching.

In other experiments RF serum was titrated with increasing amounts of DNS-γG. P values rose to a maximum and then declined. The shape of the P curve was similar to typical immunological precipitin curves.

These results show that the reaction bwtween RF and γG was immediately detectable by fluorescence polarization or quenching whereas agglutination or precipitin techniques required hours or days for completion.

Summing up, the heterogeneity of RF is confirmed and techniques for subfractionation have been devised. Evidence has been presented that the reactivity of RF with aggregated γG is related to the particle size of the aggregates. The view that RF is an autoantibody is supported by evidence of interaction between an individuals RF and his own γG. Finally the RF-γG reaction has been studied by fluorescence polarization and quenching. This reaction appears to be similar to other antigen antibody reactions.