DEVELOPMENT AND COMPARISON OF A TURBIDIMETRIC METHOD AND A NEPHELOMETRIC METHOD FOR QUANTITATING IMMUNOGLOBULINS ON A CENTRIFUGAL FAST ANALYZER

by

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This work is dedicated to my children, Chris Kenny and Aaron Kenny who have been a constant diversion and source of joy and energy while I have been in school.

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INTRODUCTION

A. Methods of Protein Quantitation.

Many different analytical methods have been used to quantitate proteins in biological fluids. Many of these first involve a physical separation step such as salt and solvent fractionation, electrophoresis, chromatography, ultracentrifugation and gel filtration. Using these techniques, protein mixtures can be separated into fractions based on the physical properties of the individual proteins such as charge or molecular weight.

After separation, quantitation of protein in the fractions can be accomplished in a variety of ways. In the Kjeldahl method introduced in 1883, the nitrogen of the protein and all other nitrogen containing compounds is converted to NH_4^+ by oxidation using a digestion mixture of H_2SO_4, a catalyst, and a salt. After alkalinization, the NH_3 is titrated with standard acid and protein nitrogen is calculated. In the biruet reaction (1) protein forms a colored complex with Cu^{++} in alkaline solution and the absorbance is read an 545 nanometers. For detecting lower quantities, turbidimetric determinations using sulfosalicylic acid or trichloroacetic acid (2) and the Folin-Lowry colorimetric method (3,4) have been used. In this latter procedure, two reactions are employed. The first is an interaction of the protein with Cu^{++} similar to the biruet reaction. The second phase is a reduction of phosphotungstic and phosphomolybdic acids to molybdenum blue and tungsten blue, by the Cu-protein complex and by the tyrosine and tryptophan of the protein. Protein can also be quantitated by

specific gravity measurements, refractive index, and dye binding methods. Most of these methods are relatively nonspecific, and often tedious.

Immunochemical methods, on the other hand, have the advantage of being highly specific, simple, and have the potential for great sensitivity. Immunologic techniques for identifying and quantitating proteins are based on the ability of an individual protein alone, or in a mixture of proteins, to react with a specific antiserum. The protein acts as an antigen in this two phase reaction. In the primary phase antigen and antibody molecules combine to form small particles consisting of one antibody and one antigen molecule. In the secondary phase of the reaction, large complexes form as the smaller particles interact with each other, and, under proper reaction conditions, these complexes remain insoluble and precipitate out. This two phase reaction is known as the precipitin reaction and is shown graphically in Figure 1 (5) where antibody precipitated is plotted vs. antigen added. It can be seen that in the initial phase of the reaction, all of the antigen added is bound by the excess antibody in solution. As more antigen is added, the equivalence point is reached, and it is at this point where maximum precipitation occurs. Addition of large amounts of antigen results in antigen excess and dissolution of the precipitated complexes. The existence of soluble aggregates in the antigen excess region was verified by Goldberg and Campbell (6) when they determined the molecular weight of the particles left in the supernate when antibody and excess antigen were mixed. Most immunologic techniques for protein quantitation measure the amount of precipitate formed when antigen and antibody are mixed at the equivalence point

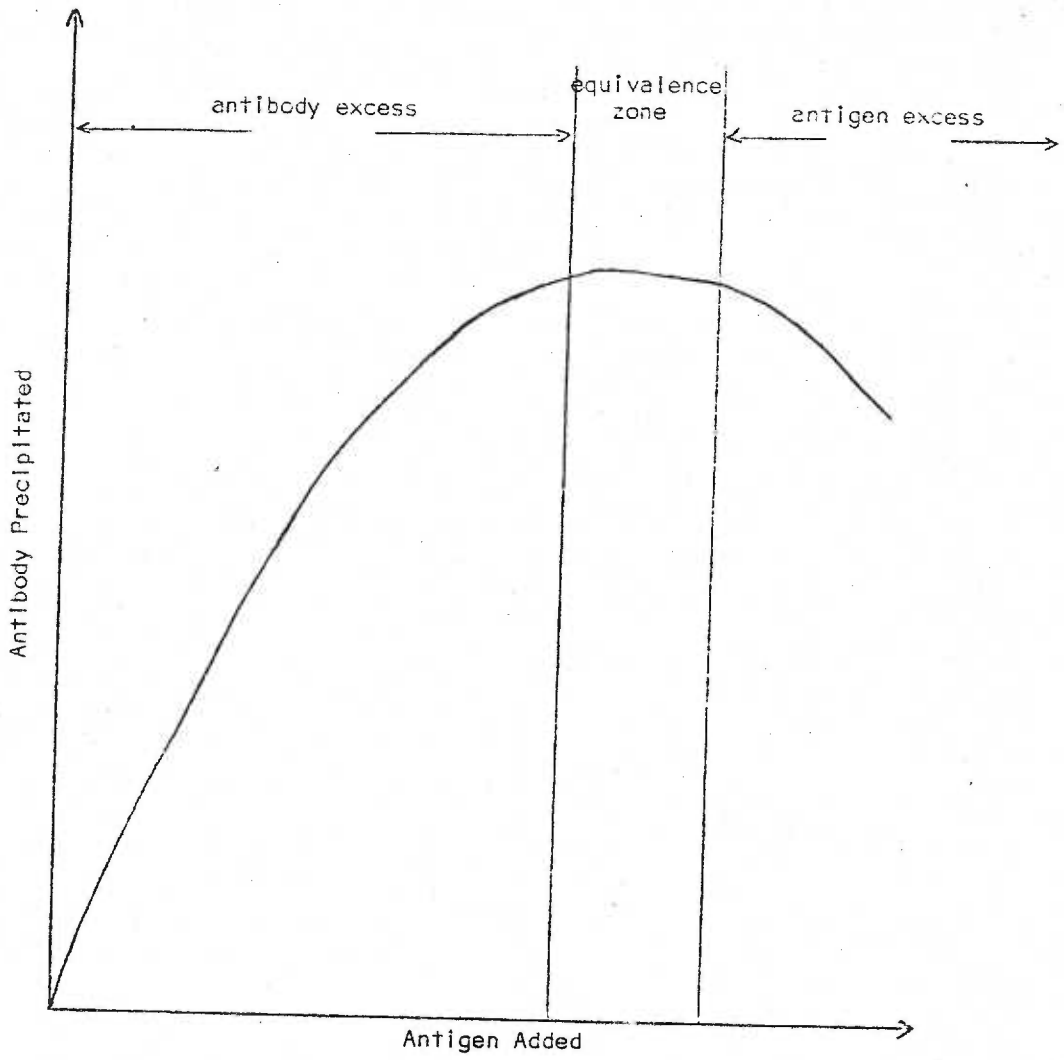


Figure 1: Precipitin Curve for a Monospecific System: One Antigen and the Corresponding Antibody (5)

since this is related to the concentration of protein or antigen in the original solution.

A number of techniques have been described for quantitating proteins based on performing the precipitin reaction in a semisolid medium - usually agarose gel. The techniques are based on the diffusion of antigen and antibody through the gel to form a precipitin line. Single immunodiffusion (SID), first described by Oudin (7), is a technique in which antigen is allowed to diffuse into agar impregnated with antibody. In double immunodiffusion (DID), both antigen and antibody migrate through the semisolid medium. In both SID and DID, speed of diffusion and total distance of diffusion are basically governed by the concentration of reactants.

Radial immunodiffusion (RID) is the most commonly used of the immunologic techniques utilizing gel and is an outgrowth of SID and DID. Antigen is allowed to diffuse from a well cut in agar containing specific antibody, and a precipitin ring forms around the antigen well. Standards are run at the same time as the unknown sample, and the diameter of the precipitin ring, or the area of the circle within the ring is proportional to the concentration of antigen. Two specific procedures are used which allow different reaction times. In 1965, Mancini et al (8) described a method of RID based on the observation that the area of a given precipitin ring will reach a maximum size depending on the concentration of antigen in the well. With further incubation, the size of the precipitated ring will not increase once the maximum size is reached. This is called an equilibrium or end-point technique since the reaction is allowed to go to apparent equilibrium. Fahey, and McKelvey (9) in 1965, proposed a method of

RID in which the precipitated rings were measured before their full development. The concentration of antigen is then a function of the rate of precipitin formation. This is called a non-equilibrium technique. The Fahey technique requires less time than the Mancini technique, but reaction time and temperature must be very carefully controlled. In either case, 18 hours to several days are required for each determination. Kalff (10) has reviewed the theoretical and practical aspects of the various RID methods.

Quantitation of protein can also be accomplished by single one- or two-dimensional electroimmunodiffusion (EID). In the one-dimensional test, first applied to protein quantitation by Laurell (11) in 1965, antigen is allowed to migrate electrophoretically into agarose containing appropriate antiserum. Precipitation occurs along the lateral edges of the path of antigen migration and as antigen is lost by precipitation, the concentration of antigen at the leading edge of the path decreases and finally the precipitin bands come to a point when all of the antigen has been precipitated. The shape of the final precipitin band resembles a rocket, hence, the term "rocket electrophoresis" has been utilized. Laurell showed that the height of the rocket is proportional to the concentration of antigen originally applied. This technique is capable of sensitivity comparable to that of RID but has the advantage of being faster than RID.

B. Light Scattering Theory and Applications.

Light scattering methods also have been used to quantitate proteins immunologically in solution by measuring the amount of light scattered by the antigen-antibody aggregates present at the equivalence point.

Since this study will deal with these techniques, some of the theory of light-scattering and turbidity will be discussed.

When a light beam passes through a solution of particles, a portion of the light will be diffracted or scattered by the particles. If the scattered light is of the same wavelength (λ) as the incident light, Rayleigh scattering is said to occur. For isotropic particles whose dimensions are small compared to the wavelength of the light, Rayleigh, in 1871, worked out the following relationship (12).

$$\text{Equation (1):} \quad \frac{I_s r^2}{I_o} = \frac{8\pi 4\alpha^2}{\lambda^4} \quad (1 + \text{Cos}^2\theta)$$

Where I_s is the intensity of the scattered light at angle θ;

I_o is the intensity of the incident light;

r is the distance from center of scattering to detector;

α is the polarizability of the particle which is roughly proportional to the size of the particle;

λ is the wavelength;

$\frac{I_s r^2}{I_o}$ is the Rayleigh ratio, $R\theta$.

This equation tells us that the scattered intensity is inversly related to the fourth power of the wavelength and predicts that the scattering will increase strongly as the particles become larger. Debye, extended the Rayleigh equation to apply to a solution of macromolecules (13):

$$\text{Equation (2):} \quad R\theta = KCM$$

Where K is a constant for any given solution at wavelength λ and angle θ;

M is molecular weight;

C is concentration in mass per unit volume.

and the theory was further extended to allow for angular dependence of scattering from large particles by introducing the particle scattering factor $P(\theta)$ which takes into account the size and shape of the particles. This yielded the following equation (13):

Equation (3): $R\theta = K\theta\ P(\theta)MC$

The intensity of light scattered at any angle θ varies only with the concentration of molecules in solution, the molecular weight of the molecules, and the size and shape of the molecules.

An actual measure of total light scattered in all directions from the incident beam as it passes through a solution is the turbidity (t) which is defined by:

Equation (4): $I = I_o e\text{-}tb$

Equation (5): $t = \frac{2.303}{b} \log_{10} \frac{I_o}{I}$

Where I_o is the incident intensity, and I the intensity after passing through a length b of solution. Turbidity can be related to the Rayleigh equation in the following way (14):

Equation (6): $t = HCM$

Where H is a constant which is independent of the angle of measurement. Turbidity then depends on the concentration and molecular weight of particles in solution.

When particle size exceeds 1 to 1 1/2 wavelengths, some radiation is reflected in addition to being scattered, and destructive and constructive interference occurs between two or more scattering points on

one particle (14). The pattern of scattering is also different for small and large particles. The intensity of scatter from a particle which is small in relation to the wavelength should be distributed symmetrically around an axis which is perpendicular to the incident beam. It has been known for some time that the light scattered through 90° is less intense than at any other angle due to the polarization of some of the light (15) (Figure 2). For particles larger than the wavelength, the scattering pattern becomes asymmetrical, and the greatest scattering occurs in the foreward direction while much less scattering occurs at angles greater than 90° because the destructive interference is increased at higher angles.

Cambiaso (16) states that for maximum nephelometric or light scattering effect, particle size ought to approach the wavelength value of the incident light. Buffone and Savory (15) determined that the radius of gyration (R_G) of the antibody-antigen complex ranged from 350-600 nm over a variety of antigen/antibody ratios by doing angular dependent light scattering measurements. They also showed that the population of complexes is a polydisperse system with respect to size since a series of light scattering maxima or minima were not observed as a function of angle of observation. Marrack and Richards (17) demonstrated that the dysymmetry ratio (I_{45}/I_{135}) which is a qualitative measure of complex size was nearly constant over the range of antibody excess and equivalence and then decreased in antigen excess. This shows that maximum complex size is achieved in antibody excess and at equivalence.

There are two methods of detecting scattered light. In a nephelometric system, light which is scattered at an angle to the incident

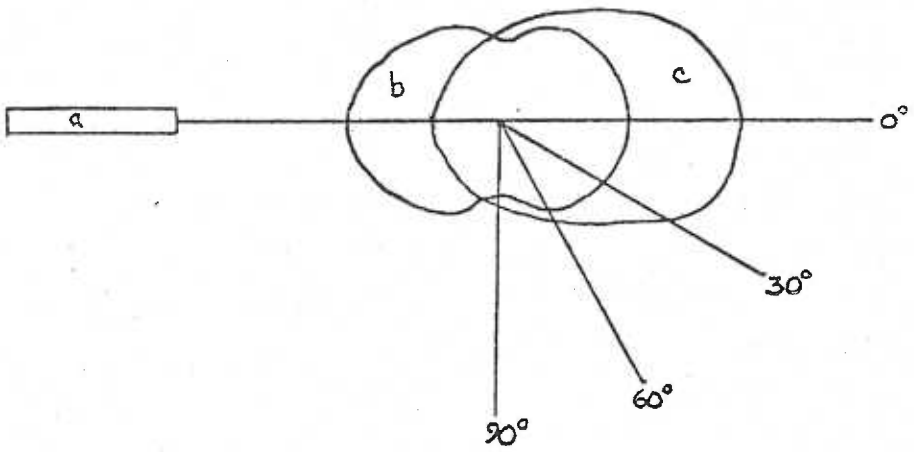


Figure 2: Angular Dependence of Light Scattering as a Function of Particle Geometry

a Source of light.
b Relative scattering envelope for small particles.
c Relative scattering envelope for large particles.

From: Buffone, Savory, Cross, Hammond, Clin Chem 21/12 1737 1975.

ray is detected -- and 90° is the angle most commonly used. Nephelometry allows for great sensitivity at low turbidities. This is because the instrument measures slightly increased light levels compared to very low levels in the blank. From Figure 2 it is apparent that an angle other than 90° would give greater intensity and therefore yield a more sensitive assay. This is why several workers in the last few years have adopted nephelometric systems using angles less than 90°. Blume (18) states that moving the detector from 90° to 20° "results in about a 1000 fold increase in the intensity of light scattered from particles the size of erythrocytes." Measuring light scattered at low angles (or angles close to the incident beam) has been termed near front surface light scattering. At higher turbidities, nephelometry is less desirable, however, because of the interparticle interference. The size of the particles, the angle of detection, and the wavelength are important variables to control.

Turbidimetric measurements determine the amount of light that is scattered by a suspension by detecting a decrease in the incident light which reaches the detector. In this system particle size and shape also affect the results, but the angle of detection is always 0°. Inherent in a turbidimetric system is the problem that, when dealing with very low concentrations of particles, the turbidity reading comes from a small difference between two large signals from the detector, and, therefore is a less sensitive and precise measurement than a nephelometric measurement. From Rayleighs law (Equation 1), it is evident that more scattering is produced at shorter wavelengths of incident light, and in a turbidimetric system, particle settling or further aggregation has to be controlled by very accurate timing to get good results.

In practice, in either a turbidimetric or nephelometric system, concentrations can be determined accurately if standards are used which are similar to the unknown being measured. For both types of systems any light source may be employed.

Libby, in 1938 (19,20) described a sensitive antigen-antibody turbidimetric assay with a special device for the determination of antipneumococcal antibodies. In 1947 Boyden (21) first showed that precipitin curves could be characterized by measuring the turbidity that develops in mixtures of antigen and antibody. Ritchie (22) used a standard spectrophotometer to measure albumin and total immunoglobulin in serum turbidimetrically at 420 nm. Ritchie also suggested, and several other workers have shown, that this procedure could be applied to specific immunoglobulins as well as other proteins (23,24,25,26,27, 28).

Several automated systems have been adapted to the measurement of antigen-antibody complexes formed in an immunochemical reaction. The first of these was a continuous flow system used by Eckman (29) in 1970 to determine transferrin in human serum with a fluorometer as a nephelometer. Killingsworth and Savory (30,31,32), developed an automated continuous flow procedure for measurement of C'3 and the immunoglobulins in serum and CSF. The introduction of the centrifugal fast analyzer (33) (see following section) opened up a new type of automated system which Tiffany (34) adapted for right angle light scattering measurements. Buffone, Savory and Cross (35,36) substituted a laser as the light source on a centrifugal fast analyzer in order to perform kinetic near front surface light scattering determinations. In 1975 Blom (37) and in 1976 Finley (38) used an unmodified centrifugal fast analyzer to do end-

point turbidimetric analyses using light at 340 nm as the incident beam. During the course of this investigation, workers at the Hyland Division of Travenol Laboratories (39) and workers at Beckman Instruments (40) published reports of dedicated instruments for specific protein analysis. The Hyland instrument methodology involves polymer-enhanced endpoint determinations with a laser nephelometer. The Beckman instrument uses a tungsten lamp and filter in a rate-nephelometric system. Both of these instruments are currently available commercially.

C. The Centrifugal Fast Analyzer.

The Centrifichem® centrifugal fast analyzer (CFA) is a general purpose spectrophotometric analyzer. The central component of the instrument is a temperature controlled multiple cuvette rotor assembly which rotates at approximately 1000 rpm. The analyzer uses centrifugal force to mix and transfer measured volumes of samples and reagents from radially arranged compartments into the spinning cuvettes where the resulting reaction is monitored by a special-purpose computer, and continuously displayed on an oscilloscope screen. As the rotor spins, the cuvettes pass through a fixed optical system consisting of a light source, lenses, filter and photomultiplier tube. The absorbances generated by the individual cuvettes are referenced to a blank cuvette and the average of eight consecutive passes through the light path is measured and processed by a sample and hold circuit before being passed on to the A/D converter and oscilloscope display. Rotation continues throughout the entire reaction period and at preprogrammed time intervals during the analysis, the result for each cuvette is calculated and automatically printed out by a digital printer

on the instrument. The analyzer can be programmed to do kinetic or end-point types of analyses and results can be printed in absorbance or concentration units.

The Centrifichem pipettor automatically measures and transfers pre-programmed volumes of reactants from sample cups and reagent wells into a transfer disc. The transfer disc consists of a solid teflon ring containing 30 separate sample and reagent cavities arranged radially and fits into the center of the rotor assembly on the analyzer.

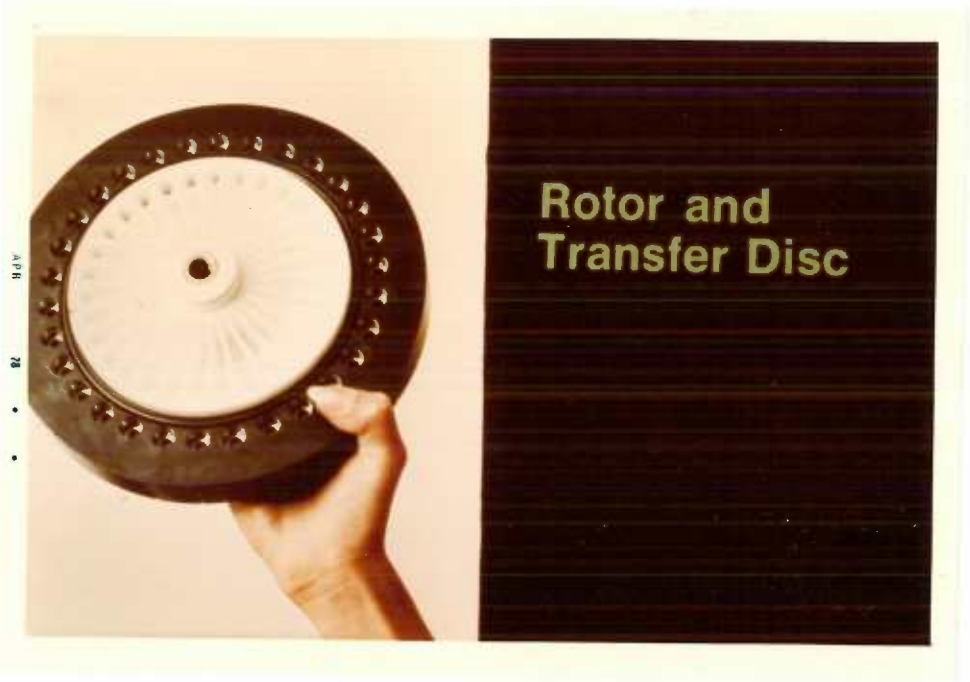
There are several features of the CFA which makes it useful for laboratory and research purposes. Simultaneous mixing of samples and reagents in all cuvettes eliminates technical problems associated with timing and physical handling of reactants. Taking absorbance readings at precise time intervals in all cuvettes and averaging eight readings results in a high degree of accuracy and precision. The system is also very stable, fast, requires a minimum of operator input, and is capable of generating large amounts of data via the digital print out. The analyzer, pipettor and rotor and transfer disc are shown in Photographs 1, 2, and 3.

D. Laser Modification to the Centrifichem 300.

In 1975, a Centrifichem 300 in this laboratory was modified for use in light-scattering determinations (41) similar to that reported by Buffone, Savory, and Cross (35) (see Figure 3 and Photograph 4). A He-Ne laser at 632 nm was mounted on an aluminum frame and the frame itself attached to the centrifuge support plate with 2 guide pins and 3 bolts. The standard light source was disengaged by removing its fuse and illumination tube. The laser beam was aligned, using a mirror attached

Photograph 1: Centrifichem Fast Analyzer

Photograph 2: Centrifichem Autopipettor®



Photograph 3: Centrifichem Transfer Disc (white area) and Rotor

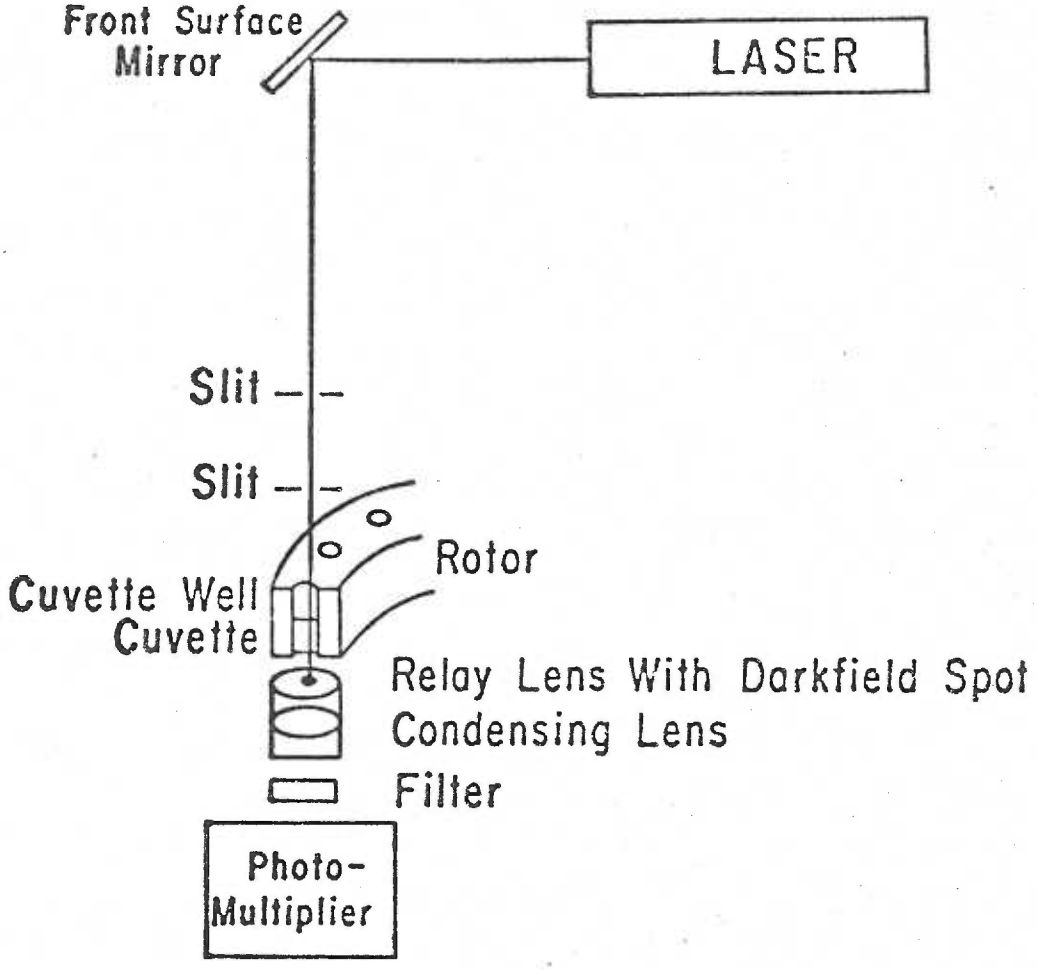


Figure 3: Diagram of Optical System of Modified Centrifichem 300

Photograph 4: Centrifichem with Laser Attachment in Place

to the frame, so that it passed through the cuvettes and impinged on the external photomultiplier lense. A precut black tape spot 1.91 mm in diameter was placed on the center of the lense to block the incident beam of the laser. This darkfield spot could be left on the lense at all times so regular chemistries could be run, and the laser alignment checked. Only the light scattered in the foreward direction at small angles was detected by the photomultiplier tube. The whole laser assembly could be set up or taken down in a few minutes, or the instrument used in the standard mode with the laser in place if the light tube and fuse were replaced, and the instrument cover left open.

An electronic modification was also made on the instrument. Under normal operating conditions, the signal from the PMT is converted into a log function by the log amp. For use with the laser attachment, only the actual intensity of the light reaching the PMT was of interest. Therefore, the log amp circuit was by-passed, and the intensity signal sent directly on to the sample and hold circuit. This provided the proper signal levels to the peak detector in the sample and hold circuit. A switch was installed on the log amp board so that conversion to intensity mode could be accomplished easily when the laser attachment was used.

STATEMENT OF THE PROBLEM

In the fall of 1976 the laser modification of the Centrifichem had been completed and some preliminary work had been done with the system to measure albumin and IgG immunologically on the instrument. For the preliminary work only the analyzer was used -- all pipetting was done by hand. As was previously mentioned, Finley, in August 1976, introduced a turbidimetric procedure for quantitating immunoglobulins G, A, and M using a different centrifugal fast analyzer. This method required no instrument modifications. These fast immunochemical techniques could replace RID for measuring these proteins in serum since RID has been found to be very time consuming, relatively expensive, and relatively imprecise. The initial aim of this research project was to evaluate the laser modified Centrifichem. The second aim was to take advantage of the speed and precision of our Centrifichem and autopipettor and the specificity of the immunologic methods to develop a fast method for determining immunoglobulins in serum.

The problem was broken down in the following way: Procedures were developed for measuring IgG in serum on the Centrifichem, both turbidimetrically (without the laser) and nephelometrically (with the laser attachment in place). Each of these methods was evaluated and they were compared in terms of sensitivity, precision, limits of detection, convenience, and other parameters. Based on these comparisons, it was determined that the turbidimetric method was most useful for the clinical laboratory, and the turbidimetric method was further evaluated and procedures for IgA and IgM were developed. In the course of this

study it was necessary to calibrate the 340 nm filter on the Centrifichem, check the accuracy and precision of the autopipettor, check the purity of commercial antibody preparations, perform correlation studies, as well as to set reaction conditions. The findings of this project are described below.

MATERIALS

A. Equipment.

Centrifichem®300 Fast Analyzer with Autopippetor, Union Carbide Corp., Tarrytown, New York. Includes sample cups, sample cup rings, pipettor tips, and transfer discs.

Beckman Acta C III U.V. - Visible Spectrophotometer, Beckman Instruments, Inc., Fulerton, California.

Eppendorf Push-Button Microliter Pipettes with Disposable tips, Brinkman Instruments, Inc., Westbury, New York.

5-500 µl disposable micropipettes (TC), Dade, Miami, Florida.

Corning ACI Electrophoresis Cell, Corning Medical, Palo Alto, California.

Corning ACI Universal Electrophoresis Film-Agarose, Corning Medical, Palo Alto, California

Millipore Filter Holder and Filters (0.22 µm), Millipore Corp., Bedford, Mass.

Analytical Balance AB-4, Christian Becker, Clifton, N.J.

Beckman Model 25 Spectrophotometer, Beckman Instruments, Inc., Fullerton, California.

B. Reagents.

PEG (Polyethylene glycol): PEG solution was prepared by dissolving 45 g polyethelyne glycol 6000 from Matheson, Coleman, Bell Manufacturing Chemists, Norwood, Ohio, and 21 g NaF, in 1 liter of 0.5 mol/liter NaCl. This solution was used to dilute the antisera to make the antibody reagent. The PEG solution was stored at 4°C.

Antibody Reagents: Antihuman IgG, IgA, and IgM produced in goats was obtained from Meloy Laboratories, Inc., Springfield, Virginia. Each individual antiserum was diluted appropriately with PEG solution. These dilutions were stored at 4°C and made in quantities from 20-100 ml and used for 1-8 days, except where indicated.

Saline: Saline solution was made by dissolving 9 g NaCl in 1 liter water.

Phosphate Buffered Saline (PBS): PBS was made from IFA powdered buffer from Electronucleonics Labs., Inc., Bethesda, Md.

Universal Barbitol Buffer: was made by dissolving 20.6 g barbital from Sigma Chemical Co., St. Louis, Mo. and 0.7 g disodium EDTA in 2 liters of deionized water. This was stored at 4°C.

Amido Black 10B: This stain was supplied by Corning Medical, Palo Alto, Ca. 2 g were dissolved in 1 liter of 5% acetic acid and stored at room temperature.

NADH: 5.0 mg of β-diphosphopyridine nucleotide, reduced form, disodium salt, from Sigma Chemical Co., St. Louis, Mo. was dissolved in 5.0 ml of Tris-HCl buffer (0.2 mol/liter, pH 7.6).

Biuret Reagent: Alpkem Corp., Portland, Oregon.

1 liter contains:	NaK tartrate·(H_2O)	14 g
	Copper sulfate·$5H_2O$	3 g
	KI	5 g
	NaOH	8 g
	Brij 99-20%	1 ml

Biuret Blank Reagent

1 liter contains:	KI	4 g
	NaOH	6 g
	Brij 99-20%	1 ml

IgG: Human IgG-lyophylized, supplied by Miles Labs. Inc., Eckhart, In.

IgM: Solution supplied by ICL Scientific, Fountain Valley, Ca.

Protein Standard Solution: Crystalline bovine albumin, College of American Patholisists, Chicago, Il.

METHODS

A. Instrument Calibration.

1. Calibration of the Centrifichem 340 interference filter.

Calibration of the 340 nm filter was accomplished by determining the absorbance potentiometer setting on the instrument. This potentiometer attenuates the signal coming from the PMT so that true absorbance values are printed for each test. The following procedure was used to determine the proper setting for this potentiometer according to the manufacturers instructions.

Stock NADH solution was prepared by dissolving 5.0 mg of NADH in 5.0 ml of Tris-HCl buffer (0.2 mol/liter) (pH 7.6). Three working NADH solutions were prepared as follows:

#1 4 ml of the stock NADH solution was pipetted into a test tube. 30 ml of Tris-HCl buffer was added and mixed. 0.151 mM/L.)

#2 10 ml of #1 working NADH was added to 10 ml of Tris-HCl buffer and mixed. (0.075 mM/L.)

#3 5 ml of #2 working NADH was added to 5 ml of Tris-HCl buffer and mixed. (0.038 mM/L.)

These three working NADH solutions were first read on the Beckman Acta Spectrophotometer. The spectophotometer was blanked with air in both of the compartments. Each of the three solutions was read in the sample cell (S) compartment against Tris-HCl buffer in the reference cell (R) compartment, and the absorbances recorded.

A water blank was stored in the Centrifichem Fast Analyzer by filling the transfer disc evenly with water and programming the analyzer as follows:

Filter:	#1, 340 nm
Temperature:	30°C
T_o:	10 seconds
ΔT:	0.25 minutes
# Prints:	2 and all
	Write
	Terminal
	Operate
	Absorbance

The filled transfer disc was placed in the rotor, covered, and SPIN initiated. When all of the cuvettes read 0002 or less, the program was changed from WRITE to STORE, the transfer disc emptied, and removed. This stored the water blank in the memory of the computer.

Another transfer disc was filled by placing each of the three working NADH standards in at least 6 positions using a 500 µl Eppendorf. Tris-HCl buffer was placed in the 0 cuvette. The transfer disc was positioned in the instrument, covered, and SPIN initiated. After obtaining the printout, the rotor was left spinning.

The arithmetic mean of the #1 NADH solution readings was computed. If this was the same as the result obtained on the Beckman Spectrophotometer, no instrument adjustment was made. If the Centrifichem result was different from the result on the Beckman Spectrophotometer, an adjustment of the absorbance potentiometer was necessary and the following formula was used to calculate the new setting:

$$\text{New absorbance pot setting} = \frac{\text{Old absorbance pot setting} \times \text{Beckman absorbance reading}}{\text{Centrifichem computed mean}}$$

The absorbance potentiometer was then changed to the new setting, and logic reset pressed to rerun the program. Calibration was complete if the printout showed the #1 NADH solution absorbance value to be the same as the Beckman Acta value.

2. Accuracy and Precision Check of the Centrifichem Automatic Pipettor.

On the C.C. Autopipettor, volumes of reagents and samples are set via several controls on the face of the instrument. Reagent volume can be selected by a simple toggle switch which, when in the up position, delivers 350 µl of reagent, and, when in the down position, delivers 250 µl of reagent to the transfer disc. The sample volume selector switch is a digital thumb wheel capable of being set to deliver 1-50 µl although the manufacturer recommends not using volumes less than 5 µl for the sake of precision. The sample and diluent volume switch is also a digital thumb wheel which permits the operator to select the total volume of sample plus diluent to be loaded into the transfer disc. Volumes up to 100 µl per sample may be selected. These switches activate a stepping motor which controls the sample and diluent syringe. To check the accuracy and precision of these various controls photometric and gravimentric procedures were employed.

a. Photometric method using potassium dichromate.

In this procedure a stock solution of potassium dichromate was manually diluted to give a final concentration which was the same as the final concentration achieved by setting the autopipettor and loading a transfer disc automatically. The absolute difference in

absorbance values between the manual or reference technique and the automatically pipetted technique was considered as an accuracy error in pipetting either sample or reagent. Relatively large volumes and volumetric pipettes were used in the manual dilution to eliminate or diminish the error in this part of the procedure. The photoelectric uncertainty, that is, how much imprecision is due strictly to instrumental variations was indicated by the amount of variation or C.V. between several cuvettes in the reference or manually pipetted transfer disc, since every cuvette contained exactly the same concentration of dichromate. The precision of the pipettor was indicated by the C.V. of the absorbances of the automatically pipetted transfer disc.

Stock $K_2Cr_2O_7$ was prepared by dissolving 2.25 g in 1 liter of 0.01 N H_2SO_4.

For the manually diluted or reference procedure, 2.0 ml of stock dichromate solution was pipetted into a 100 ml graduated cylinder, and deionized water was added to the 80 ml mark. About 0.5 ml of this solution was pipetted into positions 1-29 of a clean, dry transfer disc. Deionized water was placed in the 0 cuvette. A water blank was stored in the Centrifichem analyzer as in the previous procedure. The Centrifichem was programmed as follows:

Filter:	#1, 340 nm
T_o:	3 seconds
ΔT:	0.25 minutes
# Prints:	1

Write
Terminal
Operate
Absorbance

The transfer disc containing the dichromate solution was placed in the rotor, SPIN initiated, and absorbance values obtained.

For the automatically pipetted procedure, the autopipettor switches were set as follows:

10 µl sample
50 µl sample + diluent
350 µl reagent

About 10 positions on the transfer disc were loaded with stock dichromate from the sample cups, and water from the reagent and diluent wells. Water was placed in the 0 cuvette. Absorbances were obtained on the Centrifichem Analyzer using the same program as was used in the reference procedure.

b. Gravimetric method.

This method of checking the accuracy of the autopipettor was used to determine the absolute volumes of liquid being dispensed by the pipettor.

Sample cups were numbered and weighed on an analytical balance and the weights recorded. The switches on the autopipettor were set as follows:

0 µ sample
0 µ sample + diluent
250 µl reagent

The CYCLE of the autopipettor was initiated, and sample cups were held under the dispensing tip to collect the deionized water which was dispensed. The temperature of the water was determined with an NBS

thermometer, and the sample cups + water weighed to determine the actual amount of water in each cup. The mean and S.D. and C.V. were calculated and the mean divided by the density of the water at ambient temperature. Using the following formula, the % error in reagent pipetting was calculated:

$$\frac{\text{Difference between theoretical and actual volumes in ml}}{\text{Theoretical volume}} \times 100 = \% \text{ error}$$

B. Standard Preparation.

A pooled human serum was made from 1 day old, clear, non-icteric specimens collected in the routine chemistry laboratory. These were centrifuged to get rid of any contaminating RBC's then mixed well in a large Erlenmeyer flask and aliquoted into 5 ml plastic tubes with tight-fitting lids. The aliquots were frozen at -20°C and taken out as needed. The longest period of time that the pool remained frozen before use was about 8 months. The pool was standardized in two different ways.

1. Standardization by RID.

Before freezing, and several times after freezing, the immunogobulins in the pool were quantitated by RID. This test was performed in the serology department of the laboratory using Behring Diagnostics Tripartigen plates, standards, and controls. This is an equilibrium technique. According to the manufacturer, these standard immunoglobulins and control sera were compared with the WHO International Referance Prep (67/86). Any immunogobulins which were below the assay range of the tripartigen plates were repeated using Kallestad Quantiplate low level immunoglobulin test system. These test kits used

the Fahey and McKelvey non-equilibrium technique. Plates and references were included in the test kit. According to the manufacturer, each reference sera was prepared from pooled human serum and calibrated against the WHO International Immunoglobulin Reference Prep.

2. Standardization of the pool using the turbidimetric method.

In addition to RID, standardization of the pool was accomplished using the turbidimetric procedure on the Centrifichem. The WHO Immunoglobulin Reference Prep # 67/95 was reconstituted with 1.0 ml of deionized water, allowed to stand for 1 hour, then thoroughly mixed. The final volume after reconstitution was 1.06 ml. About 0.56 ml was used immediately, and about 0.5 ml was frozen. This preparation contained the following published amounts of immunoglobulin:

IgG 766 mg/dl
IgA 135 mg/dl
IgM 81 mg/dl

Appropriate dilutions of the WHO standard were made in PBS to cover the normal range of the standard curve. Two dilutions of the pool were made for each immunoglobulin, one by hand using disposable capillary pipetts and one using a repipetting dispenser. The dilution of pool chosen was the same as that being used routinely for unknowns. The transfer disc was loaded, and run on the Centrifichem and the absorbances recorded. The absorbances of the WHO Standards were plotted as a standard curve and the pool was read off of this curve.

C. Evaluation of the purity of commercial antisera.

Immunoelectrophoresis was used to evaluate the purity of the commercial antisera preparations used in this study. 1.0 µl of pooled

serum or control serum was applied to the surface of Corning ACI Agarose film. The film was electrophoresed at 90 volts for 30 minutes in the Corning ACI Electrophoresis chamber using 95 ml of Universal barbital buffer in each side of the cell. After electrophoresis, 40 µl of antisera was added to each of the antisera troughs. Following a 24 hour incubation in a moist chamber, the film was removed and rinsed for 6 hours in saline with constant stirring. The film was then stained for 2 minutes in Amido Black 10B stain, washed until the background was clear with 5% acetic acid, and dried.

D. Total protein determination on pure immunoglobulin solutions.

Total protein was determined on solutions of IgG and IgM by the biuret method using essentially the method of Weischelbaum (42). A CAP protein standard was diluted to give standards of 1, 2, 3, 4, and 5 mg/ml. Human IgG was weighed on an analytical balance and dissolved in PBS to give a solution of approximately 5 mg/ml by weight. Pure IgM solution was diluted 4 fold and 20 fold. To each of two tubes labeled "t" and "b," 0.5 ml of standard or unknowns was added. 0.5 ml of biuret reagent was pipetted into each of the tubes labeled "t" and 0.5 ml of blank reagent was pipetted into each of the tubes labeled "b." The tubes were mixed and allowed to stand for 15 minutes at room temperature. After standing, each test was read against a test reagent blank and each blank was read against a blank reagent blank in the Beckman Model 25 Spectrophotometer at 555 nm. A standard curve was plotted and unknowns were read off of the standard curve.

E. General Nephelometric Method.

The laser was attached to the Centrifichem, plugged in to a constant voltage regulator, and turned on. Alignment was checked by removing the rotor, and using a flashlight to visualize placement of the beam on the lens. The darkfield was placed so that it completely blocked the laser beam, i.e., no laser light reached the photomultiplier tube. The switch on the log amp board was changed to the intensity mode. A warm up period of approximately 30 minutes was allowed. In order to reduce cleaning problems, a different rotor from the one used for laboratory chemistries was placed in the instrument. A plastic scattering standard which had been found to scatter sufficient light to give a satisfactory blank value was placed in the 0 cuvette. This scattering standard acted as the blank reference for all immunoglobulin runs by the nephelometric method.

Standards were prepared by making appropriate dilutions, in saline or PBS, of the pooled serum, which had an IgG value of 1200 mg/dl by RID. For most of the evaluation and comparison work, standards of 40, 200, 600, 1200, 1500, 2000, and 2400 mg/dl were prepared and run on each transfer disc. Unknowns were diluted 1:50 in saline and run in duplicate. Antibody reagent was made by diluting Meloy antisera 1:30 in PEG.

The autopipettor was programmed to load the sample wells of the transfer disc with 28 µl of sample, 78 µl sample + diluent, and 350 µl of reagent. The diluent used was water, and the reagent was the antibody reagent. The final dilution of antibody in the reaction cuvette was 1:37, and the final serum dilution was 1:764.

The Centrifichem was programmed as follows for the nephelometric method:

Filter:	#7, 632 nm
T_o:	30 seconds
ΔT:	1 minute
# Prints:	4-10
Temperature:	30°C

Write
Terminal
Operate
Absorbance

To obtain the intensity readings, the loaded transfer disc was placed in the Centrifichem rotor, covered and SPIN initiated. After the program was complete, the standards were plotted on standard graph paper as ΔI/2 minutes vs. mg/dl and unknowns were read off of the standard curve.

F. General Turbidimetric Methods.

Standards were prepared by making appropriate dilutions of the pooled serum in saline or PBS. Standards were prepared to cover approximately the following ranges:

IgG	100-2700	mg/dl
IgA	50-700	mg/dl
IgM	30-500	mg/dl

Unknown samples were diluted 1:30 or 1:50 for IgG and 1:15 for IgA and IgM in saline or PBS. Anti IgG antisera was diluted 1:30 in PEG and anti IgA and IgM antisera were diluted 1:20 in PEG. These antisera dilutions constituted the antibody reagent. The diluent

well was filled with water. The autopipettor was programmed for loading the transfer discs as follows:

IgG method A:
1:30 antibody dilution
1:50 sample dilution
28 µl sample
78 µl sample + diluent
350 µl reagent

IgG method B:
1:30 antibody dilution
1:30 sample dilution
17 µl sample
72 µl sample + diluent
350 µl reagent

IgA
25 µl sample
80 µl sample + diluent
250 µl reagent

IgM
46 µl sample
99 µl sample + diluent
250 µl reagent

Under these conditions, the final dilutions of serum and antibody in the reaction cuvettes were:

IgG method A	antibody 1:37	serum 1:764
IgG method B	antibody 1:36	serum 1:745
IgA	antibody 1:26	serum 1:201
IgM	antibody 1:28	serum 1:114

Antibody reagent was placed in the 0 cuvette. The Centrifichem was used with no modifications and was programmed as follows:

Filter:	#1, 340 nm
Temperature:	30°C
T_o:	4 sec
ΔT:	1.0 minutes

Write
Terminal
Operate
Absorbance

The loaded transfer disc was placed in the Centrifichem, covered, and SPIN was initiated. After the program was complete, the absorbances of the standards were plotted. The absorbance at 4 minutes was used for

IgG and the absorbance at 5 minutes was used for IgA and IgM. The absorbances were plotted on standard graph paper against mg/dl and the unknowns were read off of the standard curve. Because an initial reading was taken at T_o = 4 seconds, the 4 minute reading was actually the absorbance at 4 minutes and 4 seconds minus the absorbance at 4 seconds, and the same holds true for the absorbance at 5 minutes.

RESULTS

A. Instrument Calibration.

1. Calibration of the 340 nm Filter.

Table 1 shows the results of an experiment performed to calibrate the 340 nm filter on the Centrifichem Fast Analyzer. Absorbance values of three NADH solutions obtained on the Beckman Acta Double Beam Spectrophotometer were compared with those obtained on the Centrifichem. Calibration was accomplished by adjusting the absorbance potentiometer on the instrument to a value calculated as in the methods section. After the adjustment was made, the program on the Centrifichem was rerun to check the absorbance values after calibration. The final values agree well with those obtained on the Beckman.

Working NADH Solution	NADH Conc.	Absorbance Beckman	C.C*. Absorbance Before Calibration	C.C. Mean Absorbance After Calibration	C.C. N	C.C. S.D.	C.C. C.V.
#3	.038 mM	.237		.228	6	.010	4%
#2	.075 mM	.482		.474	7	.006	1.2%
#1	.151 mM	.965	1.025	.956	15	.010	1%

Table 1
Calibration of the Centrifichem 340 nm Filter
Using 3 Different NADH Solutions

* Centrifichem

2. Accuracy and Precision Check of the Autopipettor by a Photometric Method.

To assess the accuracy and precision of the autopipettor, 0.5 ml of a standard potassium dichromate solution was manually pipetted into the appropriate wells of a transfer disc and the absorbance values ob-

tained. This manual technique was used as a reference method to check the precision and accuracy of the autopipettor. For the automatic procedure, 10 µl of 2.25 g/L potassium dichromate was automatically pipetted into the appropriate wells of the transfer disc along with 40 µl of water, and their absorbance values were determined. The accuracy was expressed as a % difference between the absorbance of the reference procedure, and the absorbance of the autopipettor procedure. A % difference of 2.3% was observed. The C.V. between repeats on the same transfer disc was 2.1% for the automatically diluted procedure when the cuvette #1 absorbance value was not included in the results. It was found that cuvette #1 was consistently in error and was not used in the experiments in this study.

The C.V. of the cuvettes on the reference transfer disc is an indication of the photo electronic uncertainty of the instrument. Since a manual dilution was made and every cuvette was filled with exactly the same solution, any variation was due strictly to photo electronic noise and Centrifichem inaccuracies. The C.V. for the reference procedure was 1.7%. Table 2 summarizes these results.

	Mean Absorbance	S.D.	C.V.
Reference Method	.658	.011	1.7%
Automatic Method	.673*	.014	2.1%

* without cuvette #1

Table 2
Precision of Autopipettor (Automatic Method)
and Photoelectronic Uncertainty of Analyzer (Reference Method)

If the photo electronic uncertainty is taken into account, then the actual precision of the autopipettor is 2.1% - 1.7% = 0.4%.

3. Pipettor Accuracy for Reagent Dispensing by the Gravimetric Method.

Table 3 summarizes the results of a gravimetric method to determine the accuracy and precision of the autopipettor. The reagent volume was checked using the procedure in methods. The C.V. was .62%, and the % difference from theoretical value was 3.1%.

No.	Weight of Cup and H_2O (g)	Weight of Cup (g)	Weight of H_2O (g)
1	1.5165	1.2749	.2416
2	1.5088	1.2659	.2429
3	1.5214	1.2766	.2448
4	1.5170	1.2750	.2420
5	1.5076	1.2663	.2413
6	1.5167	1.2763	.2404
7	1.5139	1.2733	.2406
8	1.5044	1.2664	.2380
9	1.5140	1.2732	.2408
10	1.5202	1.2787	.2415
11	1.5119	1.2676	.2423
12	1.4997	1.2579	.2418
13	1.5344	1.2928	.2416
14	1.5297	1.2888	.2409
15	1.5105	1.2704	.2401

Temperature = 25°C
Density = .9970 g/ml

N = 15
Mean = .2414
1SD = .0015
C.V. = .62%

Table 3
Results of Gravimetric Method for Determining
Accuracy of Centrifichem Autopipettor

$$\text{Calculated mean reagent volume} = \frac{\text{mean}}{\text{density}} = \frac{.2414 \text{ g}}{.9970 \text{ g/ml}} = .2421 \text{ ml}$$

Theoretical volume = .2500 ml
Calculated volume = .2421 ml
Difference = .0077

$$\frac{\text{Difference}}{\text{Theoretical valume}} \times 100 = \frac{.0079}{.2500} \times 100 = 3.1\%$$

Table 3 (Continued)
Results of Gravimetric Method for Determining
Accuracy of Centrifichem Autopipettor

B. Results of Evaluation of Purity of Antibody.

Immunoelectrophoresis showed the Meloy Antisera to be monospecific when reacted against a normal pool and a normal control.

C. Nephelometric Method Results.

1. Evaluation of Darkfield Shape.

Two darkfield shapes were tried. One was a spot 1.91 mm in diameter, and the other was a rectangle 1.91 mm x 12 mm. Three standards were run. The higher intensity reading was considered more satisfactory since this would indicate more light reaching the PMT and would give the most sensitive assay. Figure 4 shows the results of this experiment where ΔI/2 minutes is plotted vs. mg/dl. Standards of 100, 600, and 1200 mg/dl were run at least 7 times each, and all other variables were as in the methods section. It is clear that the circular darkfield gives a higher ΔI. The within run C.V.'s are shown in Table 4.

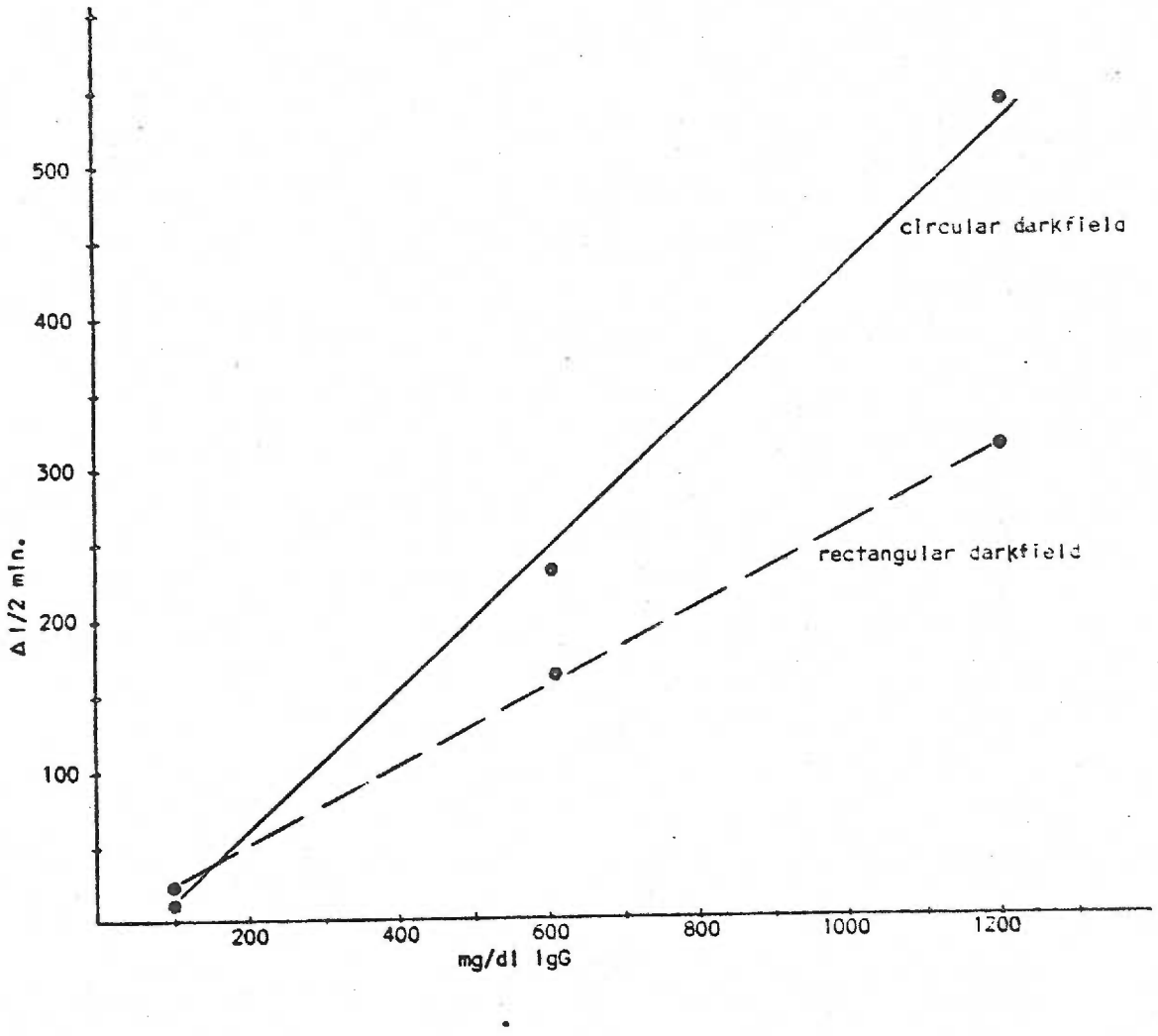


Figure 4: Curves Resulting from Two Different Darkfield Shapes Under Identical Reaction Conditions

Standard	N	Circular Darkfield	Rectangular Darkfield
100 mg/dl	16	15%	8%
600 mg/dl	14	6%	4%
1200 mg/dl	18	4%	3%
H_2O & Reagent Blanks	10	19%	25%

Table 4
Summary of within run C.V.'s for Standards and H_2O Blanks Using Two Different Darkfield Shapes

2. Standard Curve.

Figure 5 shows a typical standard curve by the nephelometric method. All instrumental variables were essentially the same as those stated in the methods section. Value for the pool was 1200 mg/dl by RID. Dilutions of the pool were made to give standards of 200, 600, 1200, 1500, 2000 and 2400 mg/dl. The curve does not go through 0 and is linear until about 2000 mg/dl.

3. Reaction Time Course.

Figure 6 shows the reaction of a normal IgG standard when Intensity is plotted vs. time. The reaction conditions are the same as those stated in the methods section.

4. Limit of Detection.

The lowest amount of IgG consistently detectable by the nephelometric method is approximately 200 mg/dl. Any standards lower than this are indistinguishable from the blank. A noise or blank level up to 36 Intensity Units was observed. Using more dilute antibody solutions did not correct this situation.

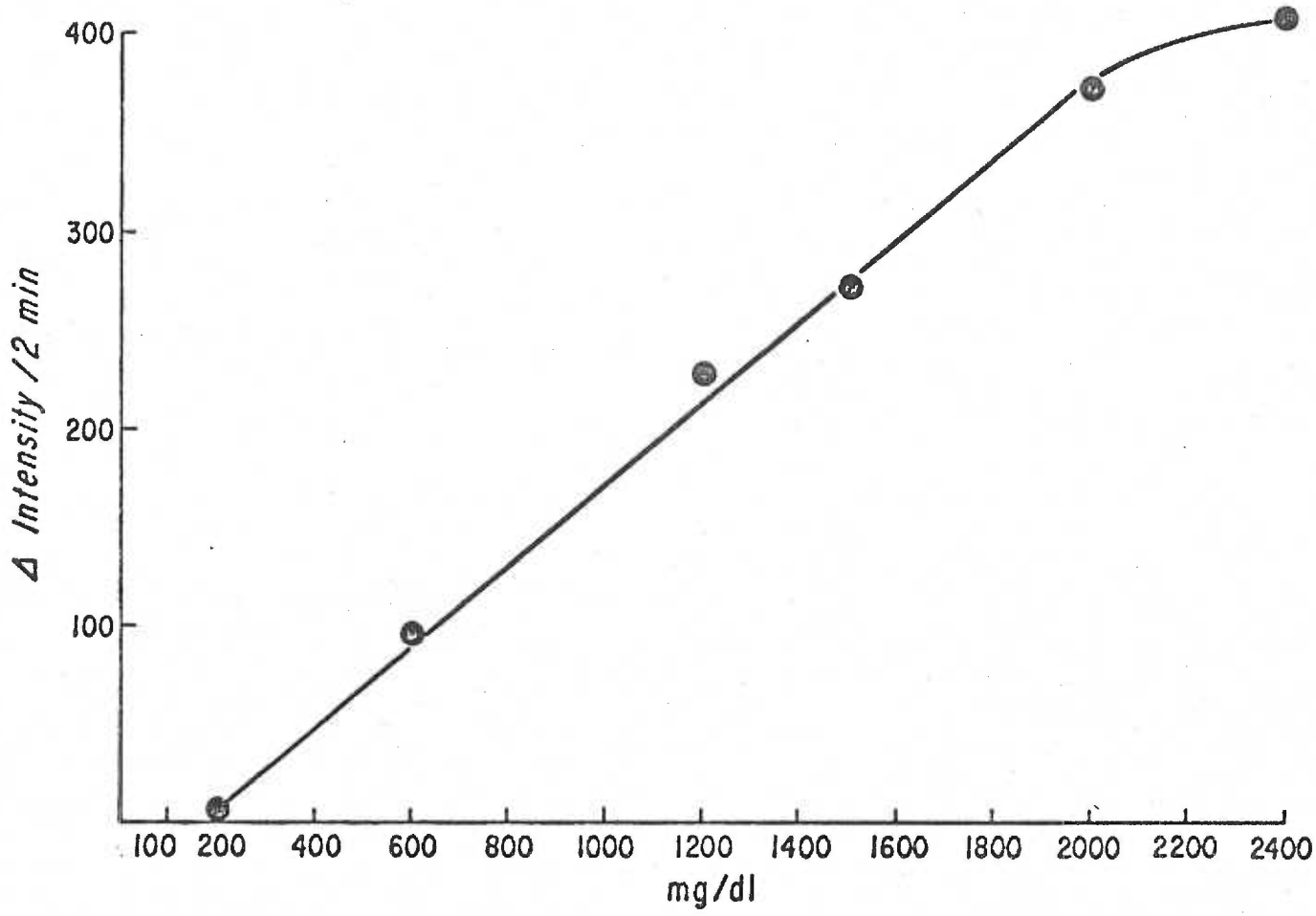


Figure 5: IgG STANDARD CURVE BY NEPHELOMETRIC METHOD

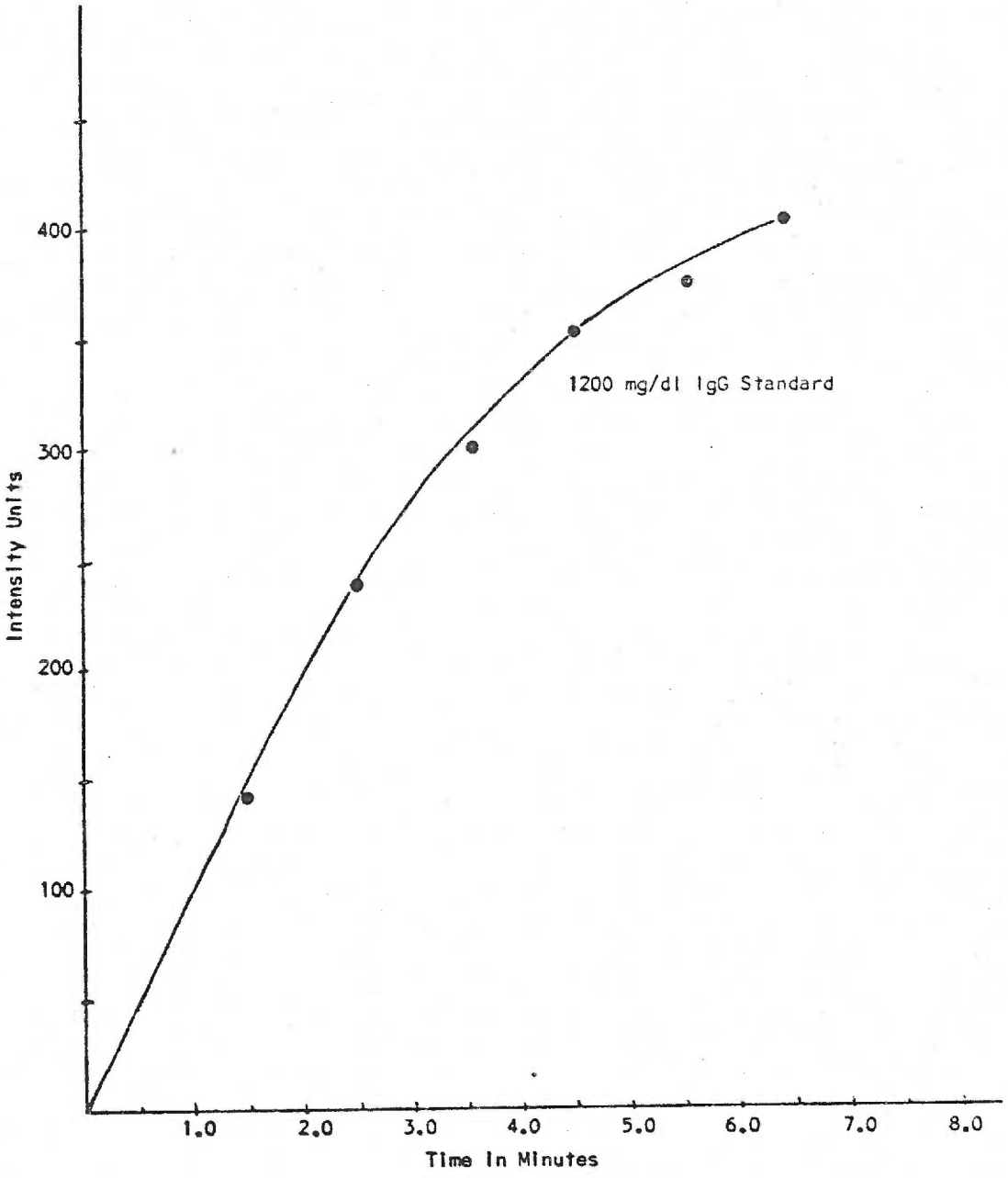


Figure 6: Plot of Intensity Units vs. Time for 1200 mg/dl Standard by Nephelometric Method

5. Precision of the Nephelometric Method.

The precision of the nephelometric method was determined in two different ways. The first method was by running duplicates and computing s using the following formula (43):

$$s = \sqrt{\frac{(\Sigma d^2)}{N}}$$

Where d is the difference between duplicates

N is the total number of determinations.

For 21 duplicate determinations in the range 310-2290 mg/dl a mean of 1178 mg/dl was obtained. The S.D. was 130 mg/dl, and the C.V. was 11%.

The second method of determining within-run precision was by running three transfer discs each with standards and one serum in replicate for the entire transfer disc. Three different ranges of samples were chosen. The C.V.'s for repeats of the 3 samples ranged from 5.6% to 6.6% and the results are shown in Table 5. Reaction parameters were those stated in the methods section.

	Mean mg/dl	S.D. mg/dl	C.V.
Low Range	316 (N=18)	21	6.6%
Mid Range	1420 (N=17)	79	5.6%
High Range	1927 (N=16)	120	6.2%

Table 5
Within-run Precision of the Nephelometric Method
Using Three Different Samples

D. Turbidimetric Method.

1. General Method Development.

a. ΔA of Antibody Reagent.

It was found during the course of developing the turbidimetric method that the absorbance of the antibody reagent increased as the

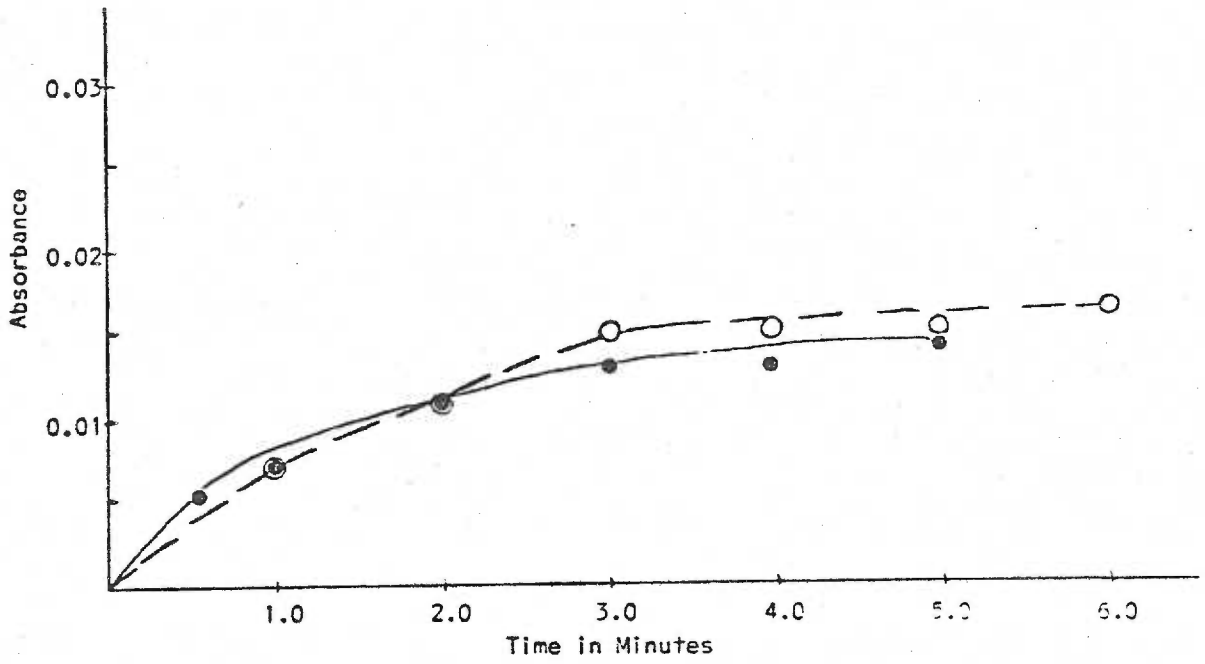


Figure 7: Change in Absorbance with Time of the Antibody Reagent Read Against PEG o- - -o and Deionized Water ●——●

reaction progressed. The increase is shown graphically in Figure 7. Two different curves are shown. In one the absorbance of the antibody reagent was read with water in the 0 cuvette. In the other, the absorbance of the antibody reagent was read with PEG in the 0 cuvette. In both cases, the absorbance was plotted vs. time. It is clear that a significant increase in absorbance occurs in the antibody reagent with time whether it is read against water or PEG. Placing antibody reagent in the 0 cuvette effectively corrects for this change in every cuvette.

b. Stability of the antibody reagent.

Stability of the antibody reagent was also investigated. Several authors had stated that the antibody diluted in PEG was only stable for 1 day. However, Finley found the reagent to be stable for at least 3 months at 4°C. To test the reliability of antibody reagent which had been stored for a long period of time, human anti IgG was diluted 1:30 in PEG as in the methods section. After 8 weeks at 4°C the antibody reagent was taken out and filtered through a 0.22 µm Millipore filter. Standards of 40, 100, 300, 600, 1200, and 2000 mg/dl were run on each of two transfer discs along with 5 unknown samples which had been previously run using fresh antibody reagent. Results are shown in Table 6. There is good agreement between the two values for each unknown whether run with fresh antibody reagent or 8 week old antibody reagent. The two standard curves are also comparable.

Stds & Unks	Abs. Using Fresh Ab. Reag.	Results mg/dl	Abs. Using Old Ab. Reag.	Results mg/dl
40 mg/dl	.015		.006	
100 mg/dl	.033		.036	
300 mg/dl	.090		.096	
600 mg/dl	.160		.156	
1200 mg/dl	.255		.247	
2000 mg/dl	.337		.340	
#14359	.091	340	.092	265
#14452	.168	660	.169	660
#14463	.280	1365	.283	1365
#14490	.055	160	.053	140
#14696	.207	840	.195	850

Table 6
Absorbances and mg/dl of Standards and Unknowns
Using Fresh and 8 Week-Old Antibody Reagent

c. Ionic Strength of PEG Solution.

In the literature there is controversy over the ionic strength of the PEG solution used to dilute the antiserum (15,17,38,44). Three different solutions were compared in this study:

PEG 1: PEG 50 g/l in 0.5 M NaCl and 0.5 M NaF
PEG 2: PEG 50 g/l in 0.15 M NaCl and 0.5 M NaF
PEG 3: PEG 50 g/l in 0.15 M NaCl

Measurement of each of the three immunoglobulins IgG, IgA, and IgM was performed using each of the above three solutions of PEG to dilute the respective antiserum. Final dilutions of serum and antiserum in the reaction cuvettes were:

IgG antibody 1:38 serum 1:763
IgA antibody 1:28 serum 1:210
IgM antibody 1:28 serum 1:105

These are only slightly different from those dilutions stated in methods. The Centrifichem was programmed as in the methods section. The maximum absorbance reached for each of the immunoglobulins was used as an indicator of which PEG solution gave the best results. These results are shown in Table 7 and Figures 8, 9 and 10. PEG 1 gave the best results

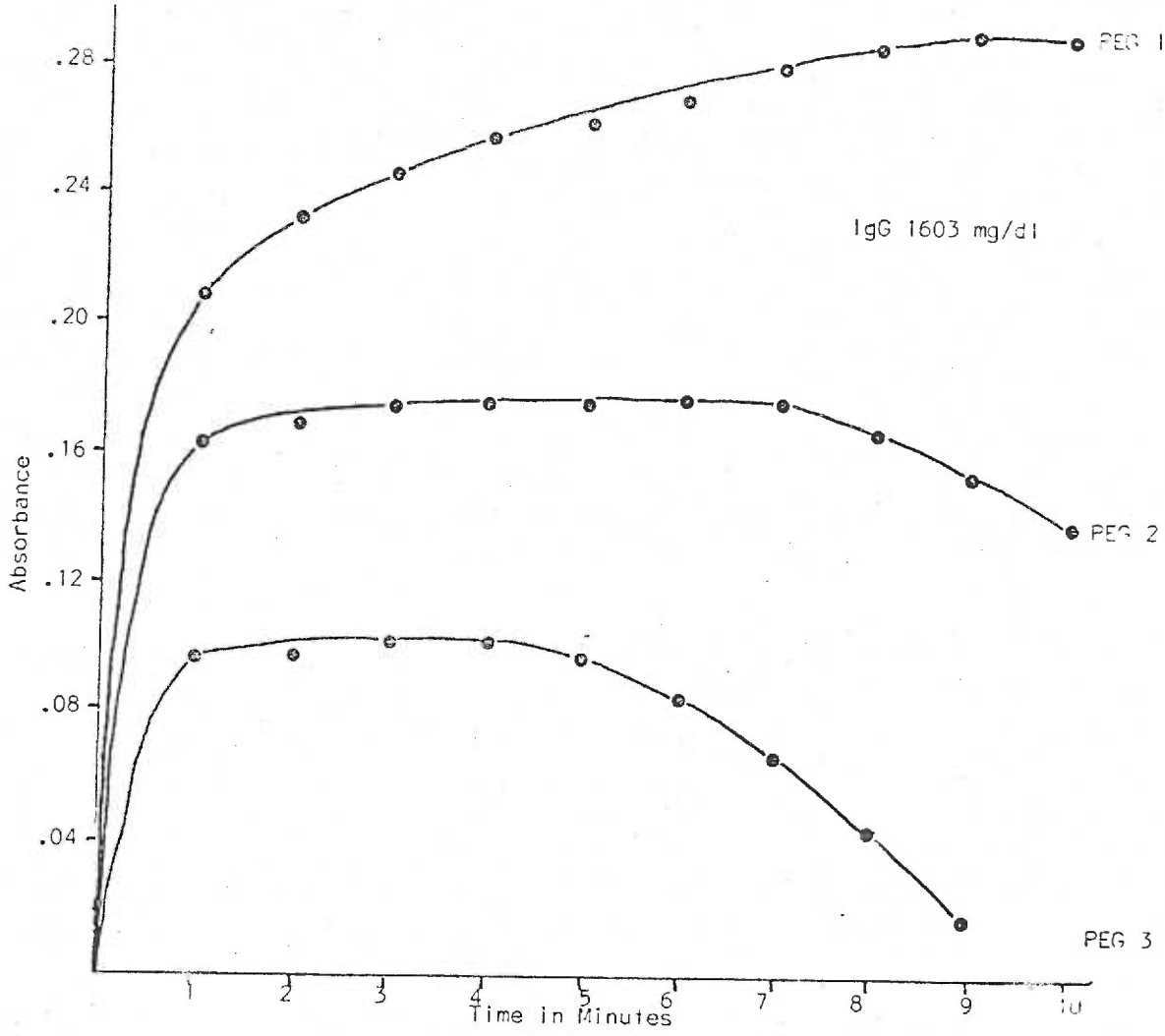


Figure 8: IgG Time Course Curves by the Turbidimetric Method Using Three Different PEG Solutions

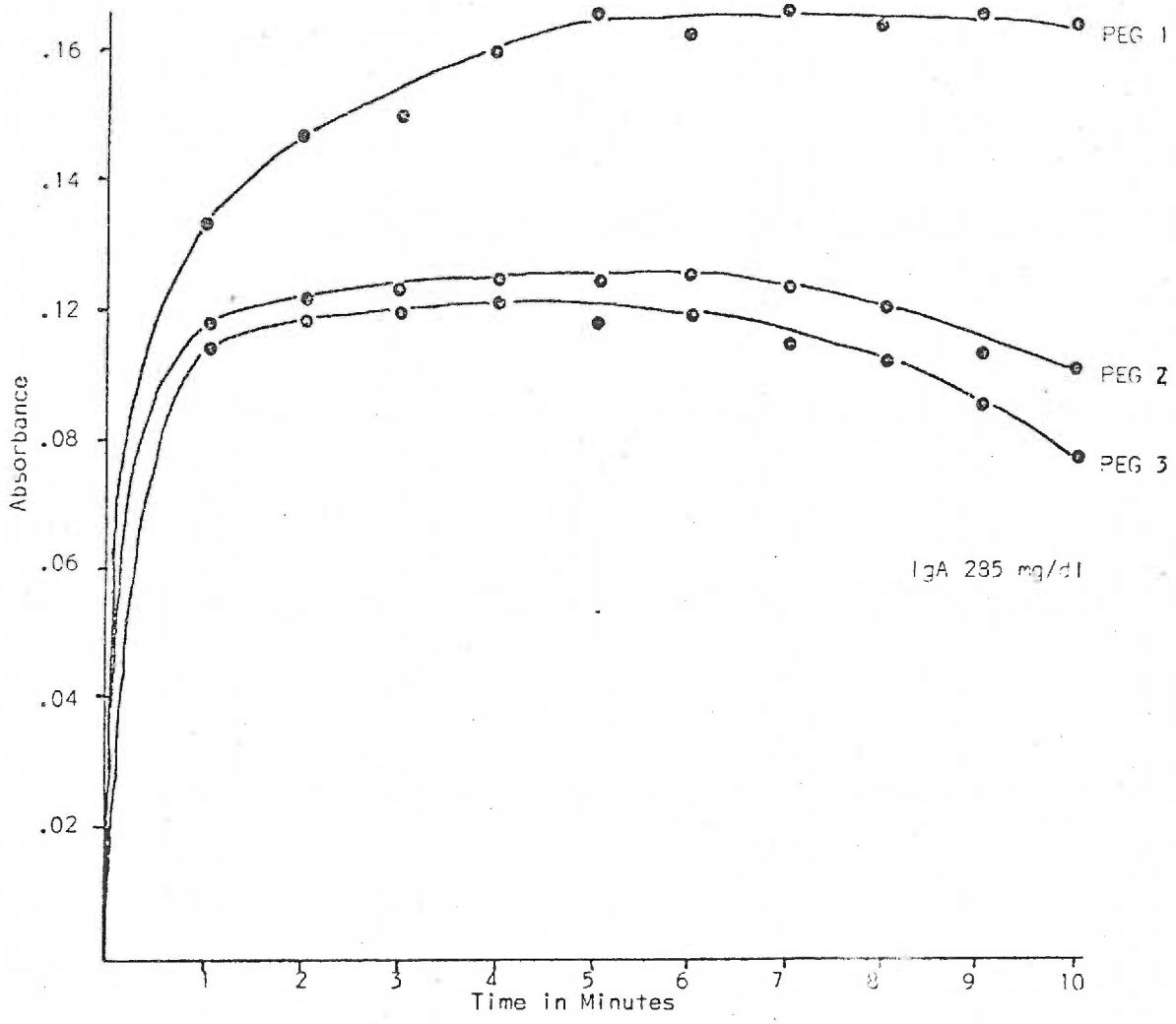


Figure 9: IgA Time Course Curves by the Turbidimetric Method Using Three Different PEG Solutions

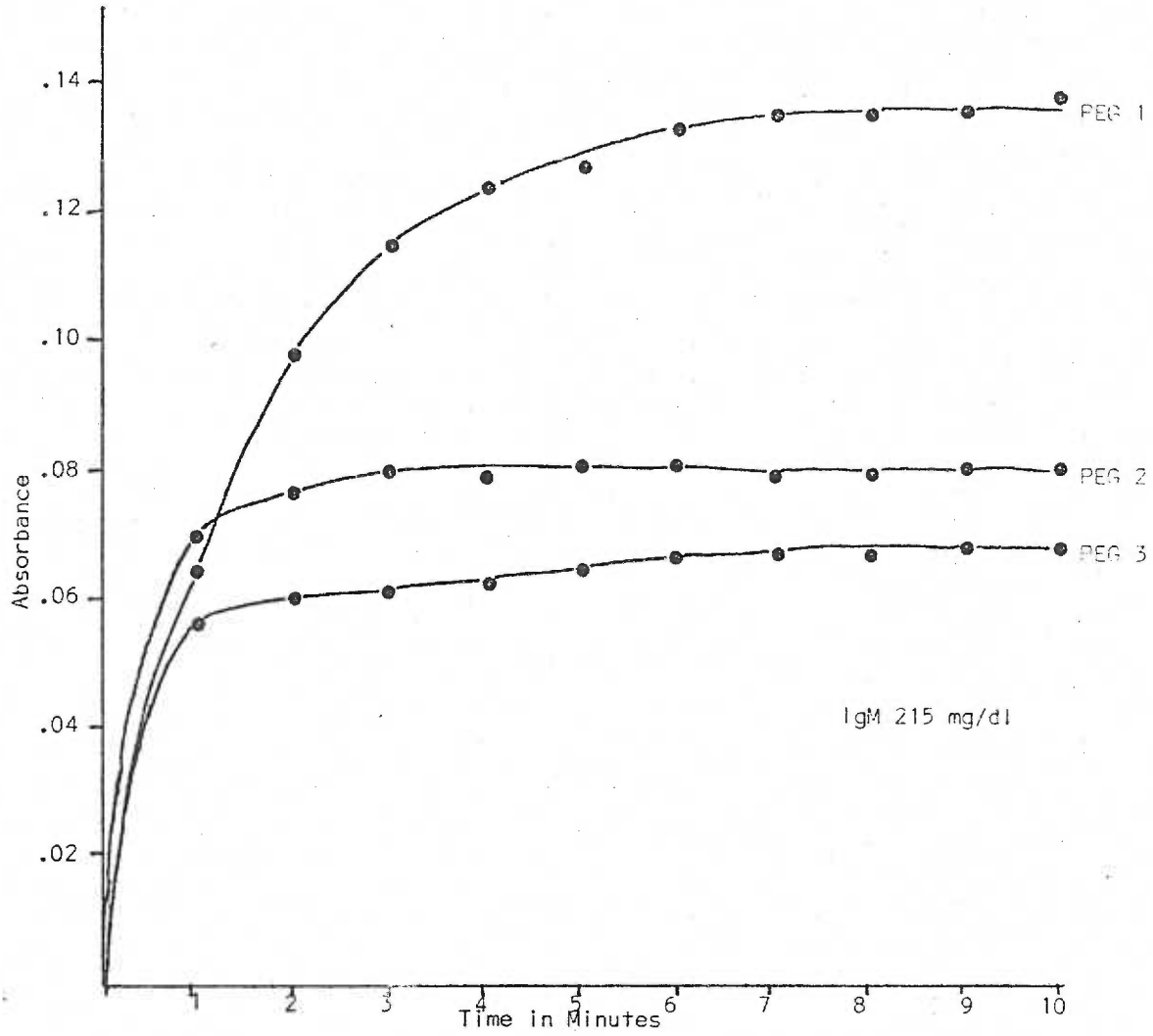


Figure 10: IgM Time Course Curves by the Turbidimetric Method Using Three Different PEG Solutions

and was used in this study. PEG 45 g/l and 50 g/l gave no significant difference in results and PEG 45 g/l was used routinely.

	IgG (1603 mg/dl)	IgA (285 mg/dl)	IgM (215 mg/dl)
PEG 1	.291	.145	.136
PEG 2	.178	.105	.080
PEG 3	.101	.101	.068

Table 7
Maximum Absorbance Reached for each Immunoglobulin
Using 3 Different PEG Solutions

d. General Reaction Conditions for the Turbidimetric Method.

In order to determine the best reaction conditions for the turbidimetric method, time course curves and standard curves were run using a variety of different concentrations of reactants. As was previously mentioned, the maximum amount of light scattering is achieved at the equivalence point of the antigen-antibody reaction. The concentrations of antigen and antibody suggested by Buffone and Savory (45) and Finley (38) were used in early experiments in this study and then these conditions were varied in order to get the maximum turbidimetric signal for the antibody being employed.

Figure 11 shows examples of curves run using different ratios of antigen/antibody. The final dilutions of antibody and serum (antigen) in the reaction cuvette are indicated. Several of these curves were eliminated for various reasons. Curve #1 gave a very high signal; but the serum concentration was so high that antigen excess was a problem for samples with very high antigen concentrations which would shorten the usuable range of the method. Curve #5 did not give enough signal;

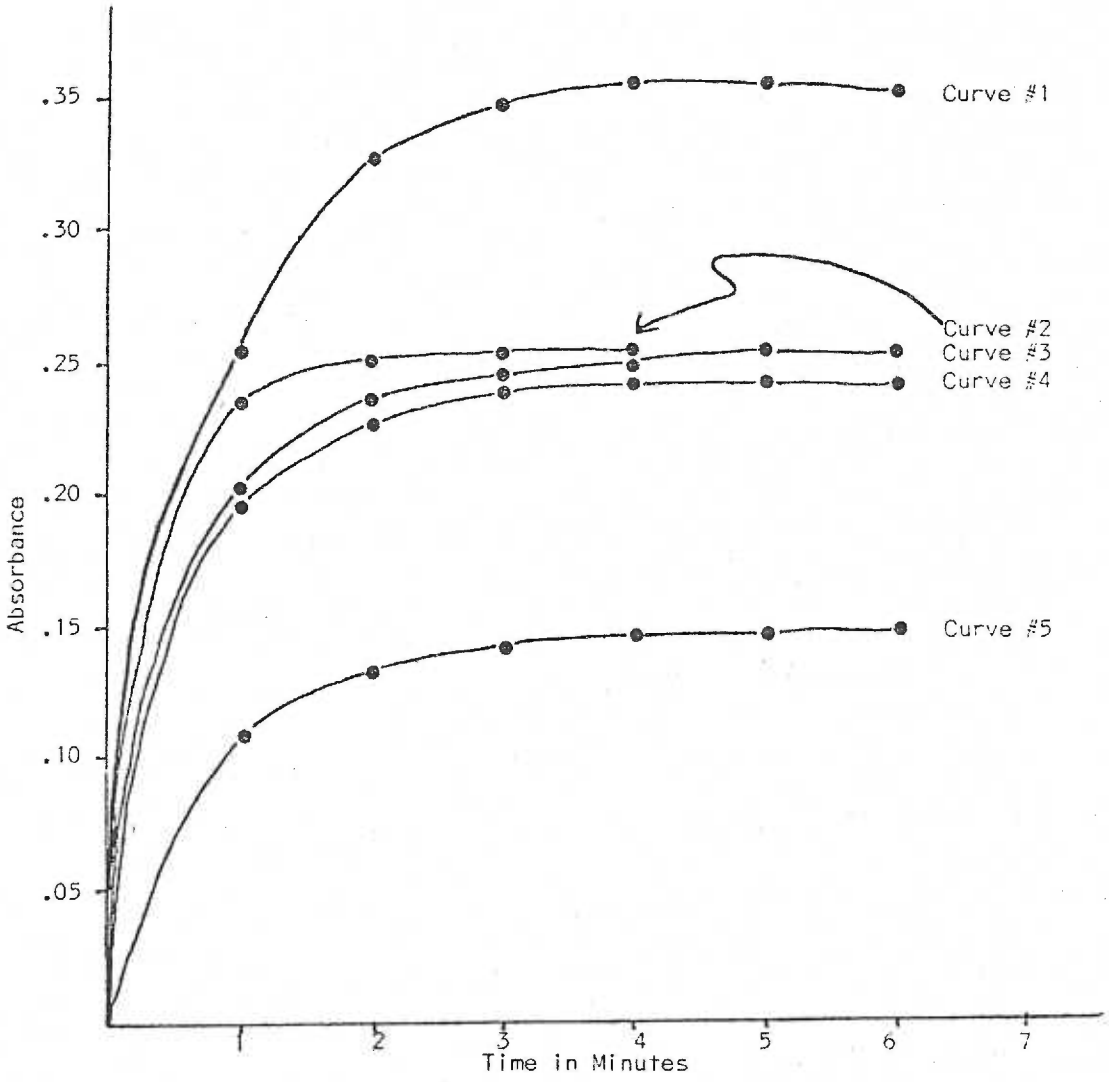


Figure 11: IgG Time Course Curves Using Different Dilutions of Antigen and Antibody

Curve #1	Antibody 1:37	Serum 1:382
#2	Antibody 1:35	Serum 1:725
#3	Antibody 1:38	Serum 1:704
#4	Antibody 1:37	Serum 1:764
#5	Antibody 1:37	Serum 1:1528

therefore sensitivity was lessened. Curve #2 used slightly more antibody and the peak occurred at 2 minutes which was too fast for this assay. Curves #3 and #4 gave similar results and both would be acceptable. Curve #4 was chosen since the pipettor settings allowed for more diluent volume; therefore, more wash between samples and less carry over. Once the final ratio of antigen/antibody was established for the antisera being used, then initial serum dilutions and autopipettor settings were changed if necessary for convenience, always maintaining approximately the same final concentrations of antigen and antibody.

Similar studies were done for IgA and IgM. Different antigen/antibody ratios were tried and reaction time course curves were examined to monitor the reaction. The highest absorbance reached, and the time of the peak of the reaction were used as criteria to pick the most suitable final concentration of reactants. Tables 8 and 9 show results of several experiments. Final dilutions of serum and antibody are listed with the peak A reached with each combination of reactants. Centrifichem parameters and all reagents are those listed in methods.

IgA	Final Antibody Dilution	Final Serum Dilution	Peak Absorbance	Time of Peak
(230 mg/dl by RID)	1:23	1:273	.125	7 min.
	1:26	1:225	.120	3 min.
	1:26	1:201	.154	5 min.
	1:28	1:140	.135	4 min.
	1:28	1:175	.127	3 min.
	*1:28	1:210	.158	7 min.
	1:28	1:280	.099	7 min.

Table 8
Maximum Absorbance and Time of Reaction Peak
with Different Antigen/Antibody Ratios for IgA

* Antibody and serum dilutions chosen for routine use.

IgM	Final Antibody Dilution	Final Serum Dilution	Peak Absorbance	Time of Peak
(170 mg/dl by RID)	1:25	1:180	.105	8 min.
	1:26	1:180	.080	8 min.
	1:26	1:225	<.050	4 min.
	1:28	1:70	.120	7 min.
	1:28	1:105	.117	6 min.
	*1:28	1:114	.093	6 min.
	1:28	1:140	.075	8 min.
	1:28	1:175	.070	10 min.+

Table 9
Maximum Absorbance and Time of Reaction for IgM with Different Antigen/Antibody Ratios

* Antibody and serum dilutions chosen for routine use.

2. Standard Curves for IgG, IgA, and IgM by the Turbidimetric Method.

Figures 12 and 13 show typical standard curves for IgG, IgA, and IgM by the turbidimetric method. Reaction conditions for IgG were those stated in the methods section under IgG method A, and for IgA and IgM were those stated in the methods section. The standard curves were run with every transfer disc and values for unknowns were read off of the standard curve.

3. Reaction Time Course.

Figure 14 shows the time course of the reactions between antigen and antibody. Absorbance is plotted vs. time. Reaction conditions are those stated in methods (IgG method A).

4. Limit of Detection.

To determine the smallest amount of IgG detectable by the turbidimetric method, a series of very high dilutions of the pool were made. These dilutions gave values of 30, 40, 60, 75, 150, 200, 300, 600, 1200, 1500, and 2000 mg/dl of IgG. Dilutions of antibody and serum and autopipettor settings were those stated in IgG method A in the methods

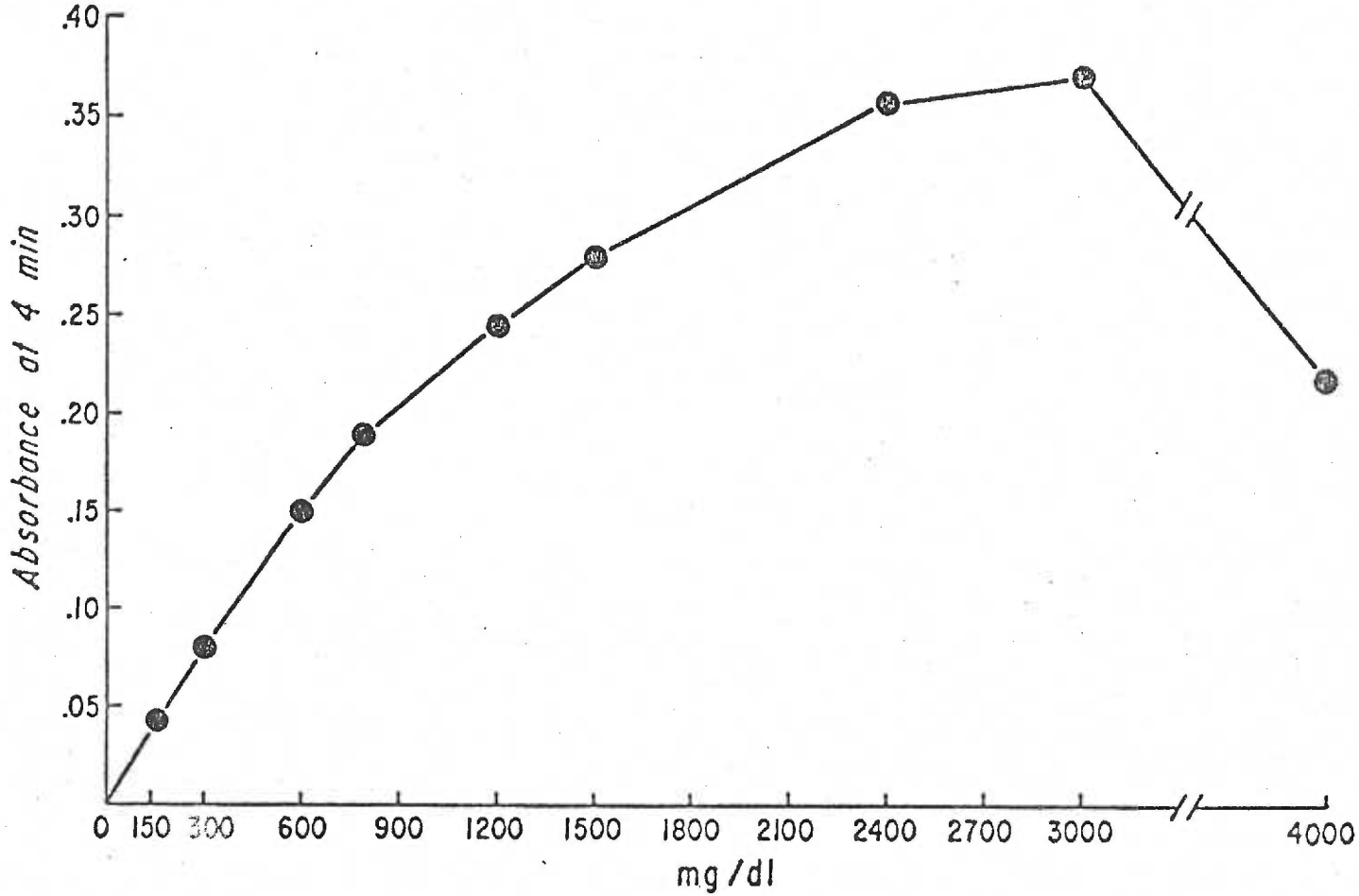


Figure 12: IgG STANDARD CURVE BY TURBIDIMETRIC METHOD

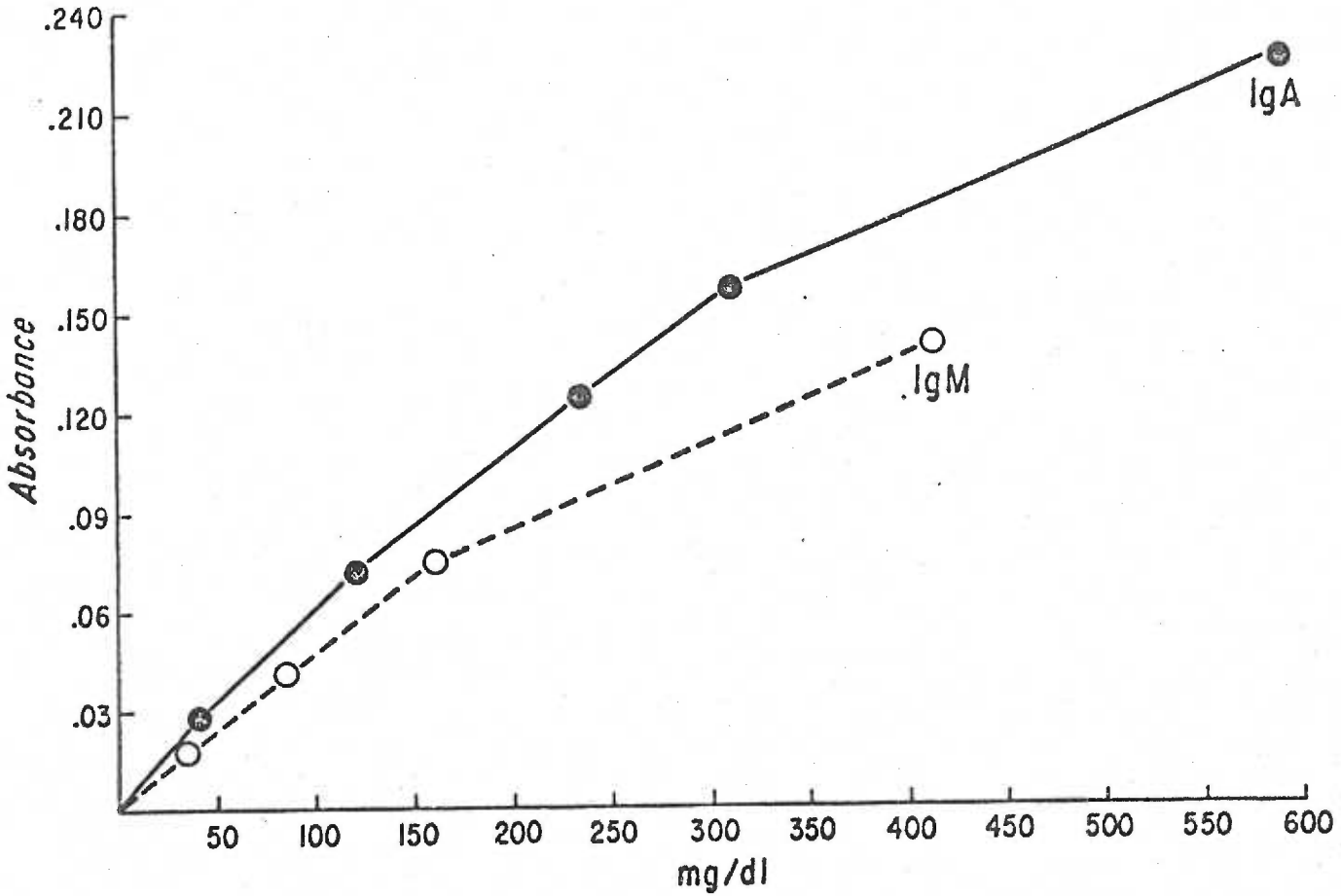


Figure 13: STANDARD CURVES BY TURBIDIMETRIC METHOD

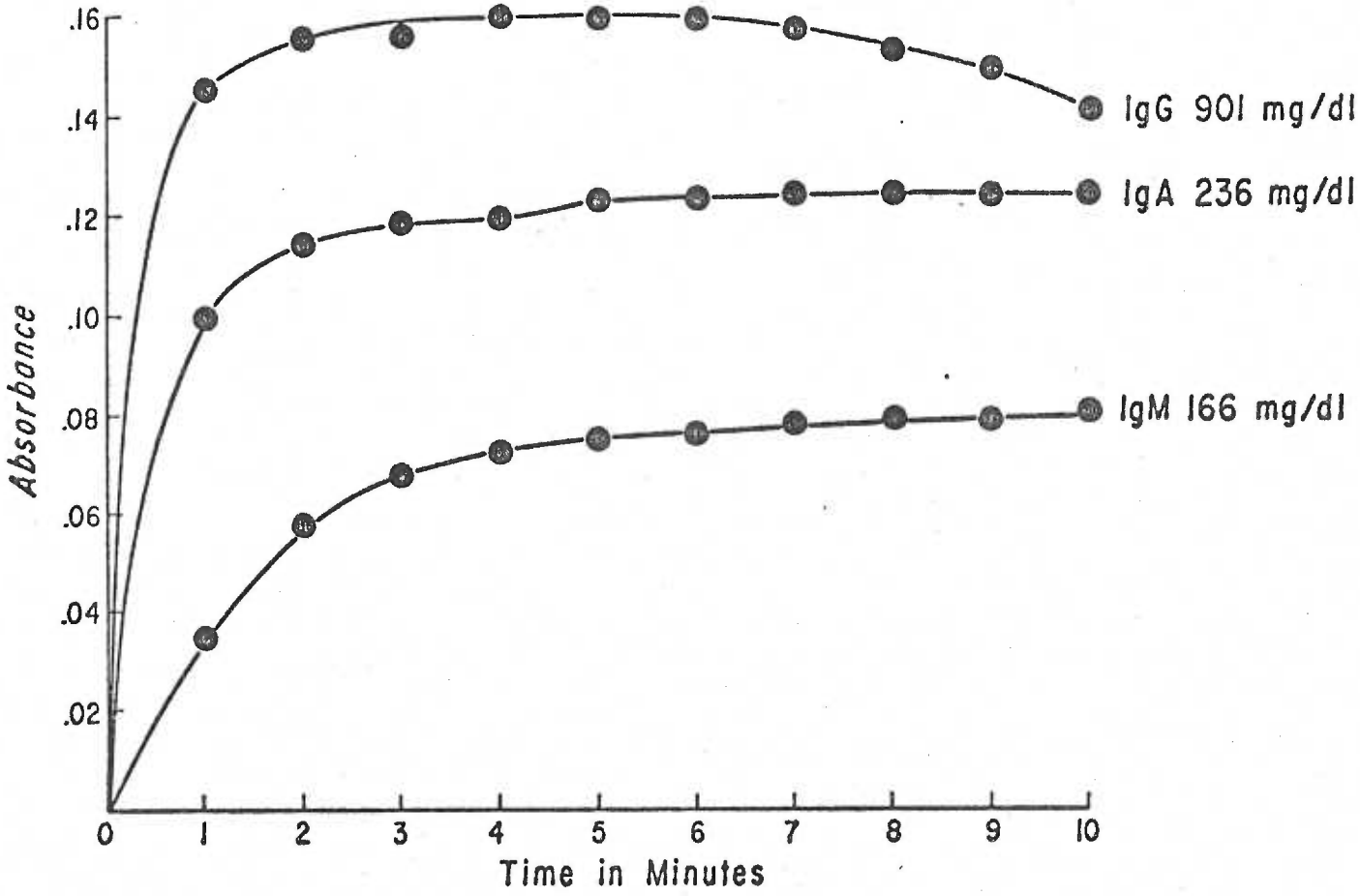


Figure 14: REACTION CURVES

section. The Centrifichem was programmed as in methods. The results of this experiment are shown in Figure 15. From this graph it is clear that the method is sensitive to a level of at least 30 mg/dl and differences of 10 mg/dl between samples can be detected.

5. Calibration of the Pool.

Table 9a shows results of the calibration of the pool by two different methods. For the turbidimetric results, the pool was run by turbidimetric method using the WHO reference material to make the standard curve. The results by RID were obtained in the serology laboratory by the normal RID procedure and, in every case, are higher than those obtained turbidimetrically.

	Turbidimetric Results mg/dl	RID Results mg/dl
IgG	995	1351
IgA	180	236
IgM	127	166

Table 9a
Calibration of the Pool by Turbidimetry and RID

6. Antigen Excess Detection.

It was noticed during the course of this study that the rate of reaction was different for samples with very high concentrations of IgG--concentrations that could be considered antigen excess. These samples had a very fast initial reaction rate which then leveled off. For IgG, the antigen excess region was >3000 mg/dl and below this value the rate of reaction did not vary significantly. Data from 58 patients and standards with IgG values from 2000 to 8000 mg/dl were examined. This data (Table 10) indicated that if the difference between the 1 minute and 4 minute readings was taken, and this Δ absorbance was >.100 absorbance units, then the sample was in antigen excess.

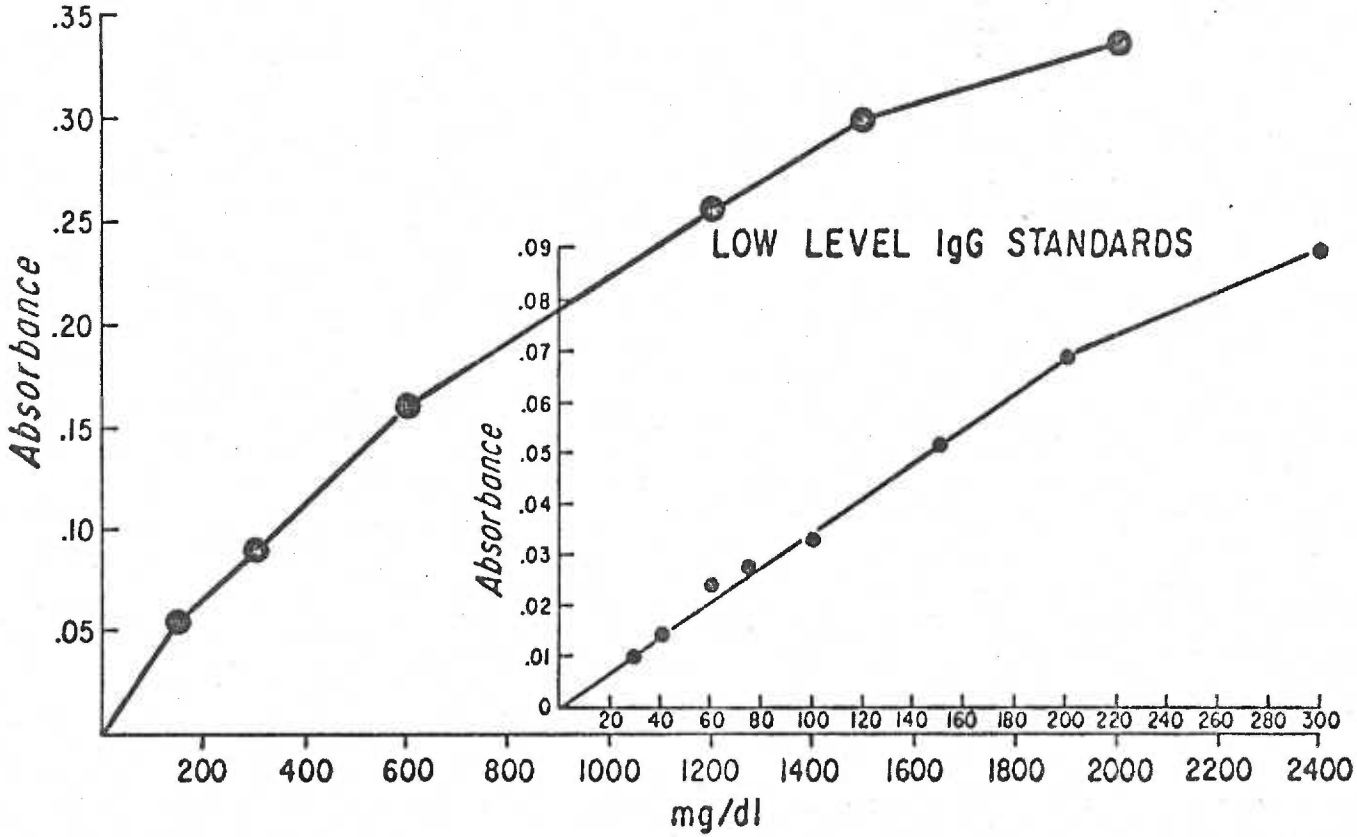


Figure 15: IgG STANDARD CURVES

with Expanded Scale Graph Showing Lower Limit of Detection

IgG Concentration	N	Average Δ Abs. (1-4 min.)
2000 mg/dl	14	.051
2400	16	.065
3000	9	.086
3600	4	.122
4000	5	.161
5000	3	.179
6000	2	.182
7500	2	.118
8000	2	.103
	58	

Table 10
Average Absorbance from 1 Minute to 4 Minutes
for Samples with 2000-8000 mg/dl of IgG

Samples found to be in antigen excess were diluted and run again. Figure 16 is an example of a family of curves from samples of differing antigen concentrations with absorbance plotted vs. time. By just examining the 4 minute absorbance value for the 4000 mg/dl standard, for example, it was not clear that this solution had an extremely elevated IgG value. However, the Δ absorbance from 1 to 4 minutes was .242 - .042 = .200 which was clearly indicative of antigen excess. At 4 minutes, the 3000 mg/dl absorbance value was higher than the 2400 mg/dl absorbance, but the difference between the 1 and 4 minute readings was .370 - .225 = .145 indicating antigen excess. An unknown sample exhibiting a Δ absorbance >.100 would be run again at a higher dilution if it was out of the range of the standard curve.

Antigen excess detection for IgA and IgM was determined by running both the 1:15 and a 1:30 dilution of each unknown.

7. Within-run Precision.

The within-run precision for IgG by the turbidimetric method was evaluated by the method of duplicates (43) and by running repeats of samples on the same transfer disc. For the first method, duplicate samples were run and the two results compared using the formula:

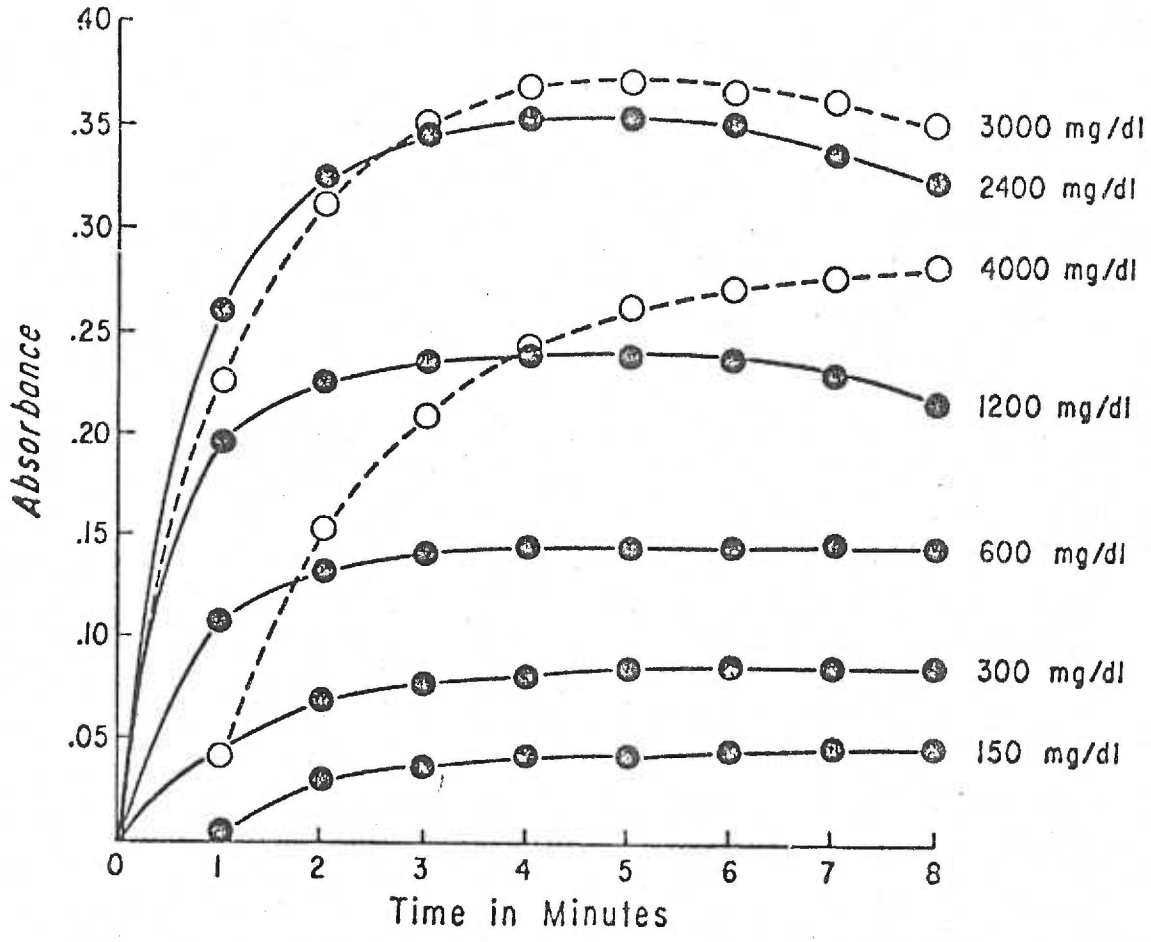


Figure 16: REACTION CURVES FOR IgG

In Antigen Excess The Reaction Rate Is Not The Same As At Equivalence. Absorbance Changes Greater Than .10 Between 1 min. and 4 min. Indicates Antigen Excess.

$$S = \sqrt{\frac{(\Sigma d^2)}{N}}$$ Where d = difference between two duplicates
N = total number of determinations

For 27 duplicate determinations in the range 115-2320 mg/dl, a mean of 992 mg/dl was obtained. One S.D. was 25 mg/dl and the C.V. was 3.5%.

The second method used to determine precision was by running repeats. The results are summarized in Table 11. Three different samples were chosen to represent low, mid, and high values in the range of the method. The C.V.'s for the low, mid, and high range samples were 2.7%, 4.2% and 6.0% respectively.

	Mean mg/dl	1 S.D. mg/dl	C.V.
Low Range	437 (N=19)	12	2.7%
Mid Range	1396 (N=18)	58	4.2%
High Range	2743 (N=19)	167	6.0%

Table 11
Within-run Precision of the Turbidimetric Method

8. Run-to-run Precision for IgG, IgA and IgM.

The run-to-run precision was determined as follows: Several sera were pooled and aliquoted into 0.5 ml aliquots and frozen. One pool was made low range by diluting slightly with saline before freezing and one was in the normal range for IgG, IgA and IgM. For each run of the immunoglobulins a separate aliquot was used. Dilutions of serum and antibody, Centrifichem, and Autopipettor settings were as in the methods section (IgG method A). Results are shown in Table 12.

	Low				Normal			
	N	Mean	S.D.	C.V.	N	Mean	S.D.	C.V.
IgG	12	412	30	7.2%	11	921	64	7.0%
IgA	11	87	3.2	4.4%	11	168	7.1	4.2%
IgM	10	41	3.4	8.3%	10	65	6.4	9.8%

Table 12
Results of the Run-to-run Precision Study for IgG, IgA, IgM

9. Correlation between Nephelometric, Turbidimetric and RID Methods.

Patient or unknown samples which were run by the nephelometric or turbidimetric methods were also run by RID in the Serology Laboratory. 53 patient samples were analyzed for IgG by all three methods: RID, nephelometry and turbidimetry. Standards from the pooled serum were run on every wheel and the reaction conditions were those stated in methods (IgG method B). The results were evaluated using Pearsons r. Table 13 summarizes these results. Figure 17 shows a scatterplot of the correlation data between turbidimetry, and nephelometry.

Turbidimetric to Nephelometric	r = .95	N = 53
Turbidimetric to RID	r = .94	N = 50
Nephelometric to RID	r = .90	N = 50

Table 13
Correlation between Methods for IgG

In another correlation study, IgG, A, and M were run on a group of unknowns by both the turbidimetric method, and by RID and evaluated using Pearsons r. Table 14 and Figures 18, 19, and 20 summarize these results.

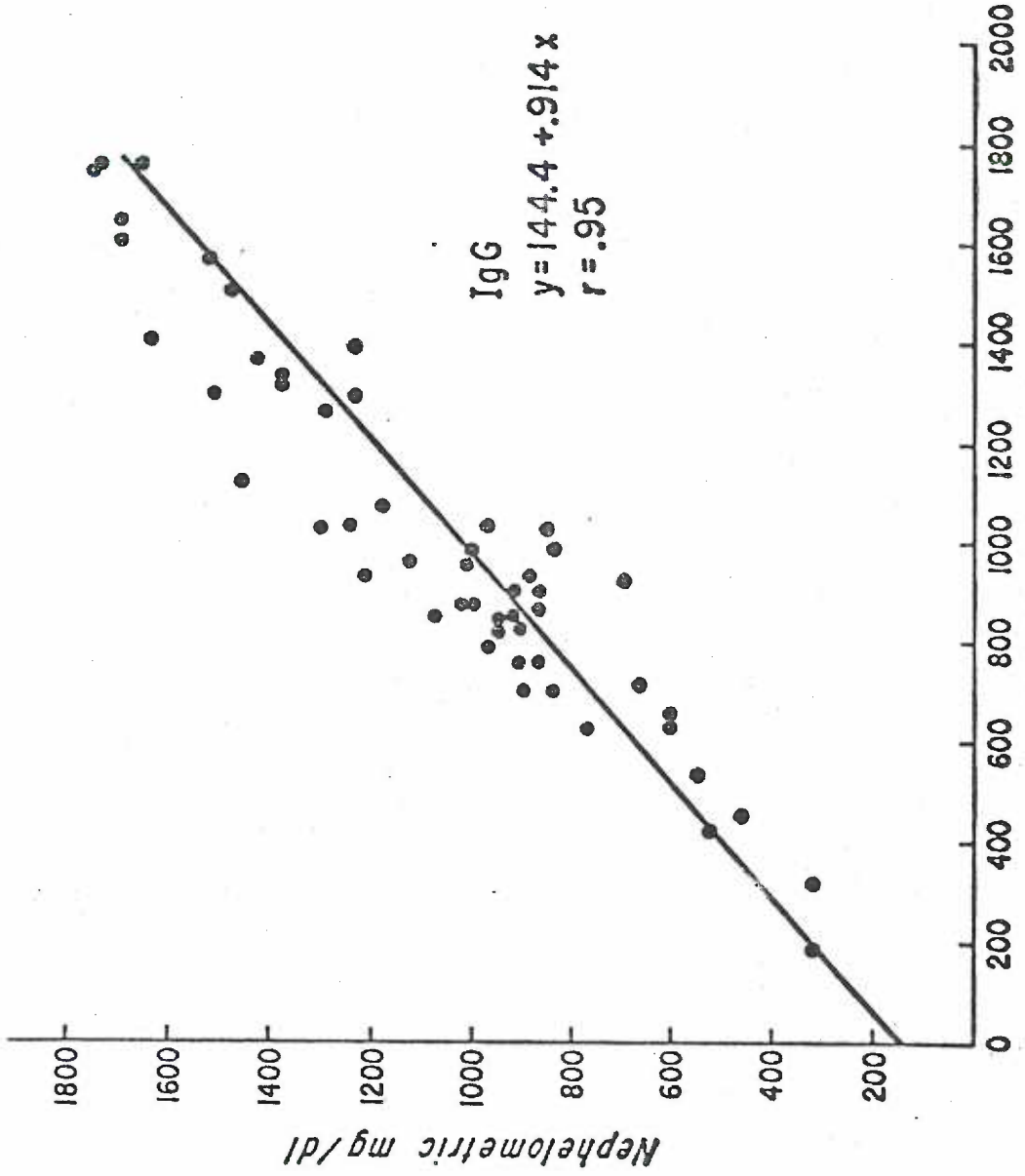


Figure 17: Correlation between Turbidimetric IgG and Nephelometric IgG.

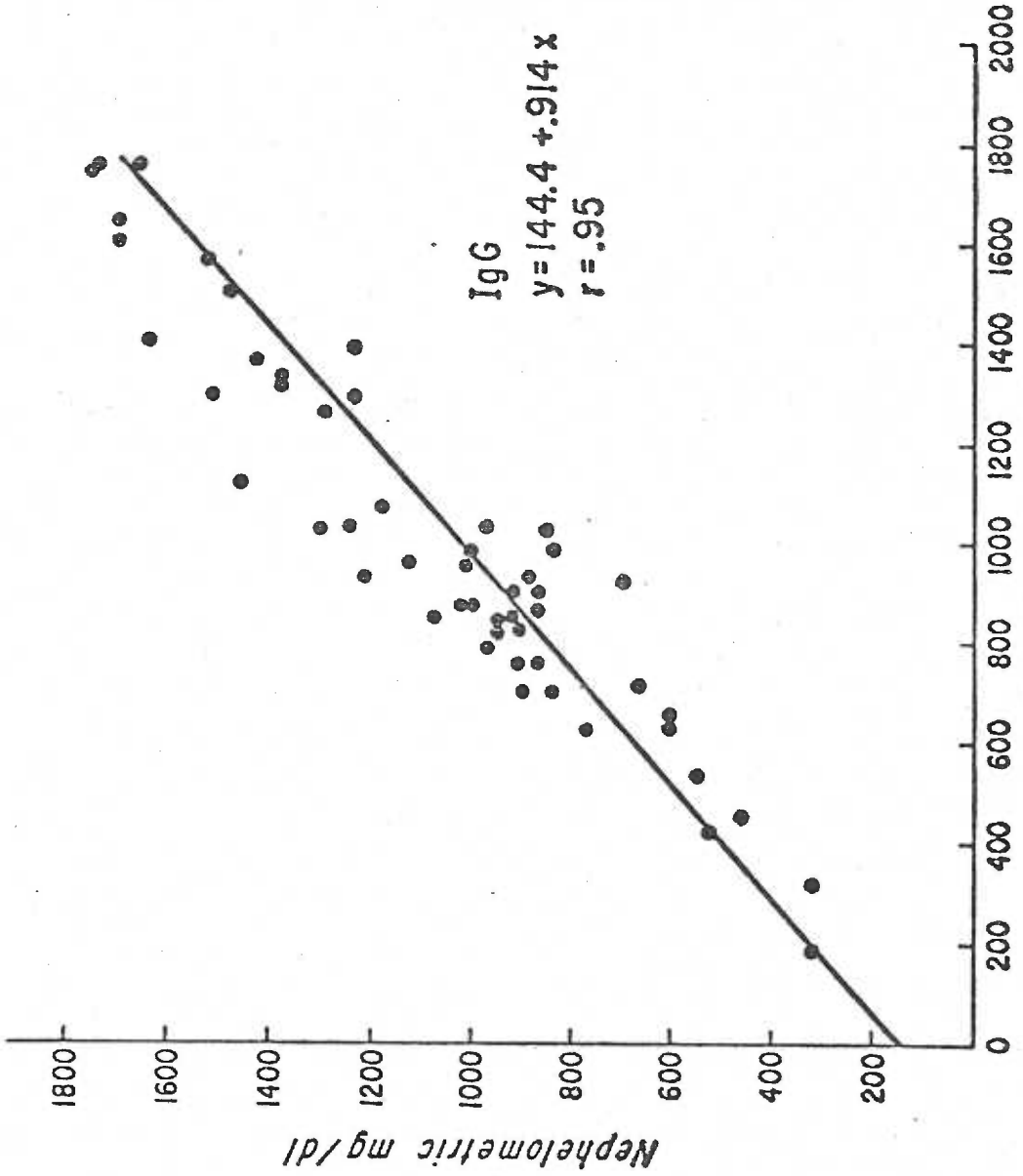


Figure 17: Correlation between Turbidimetric IgG and Nephelometric IgG.

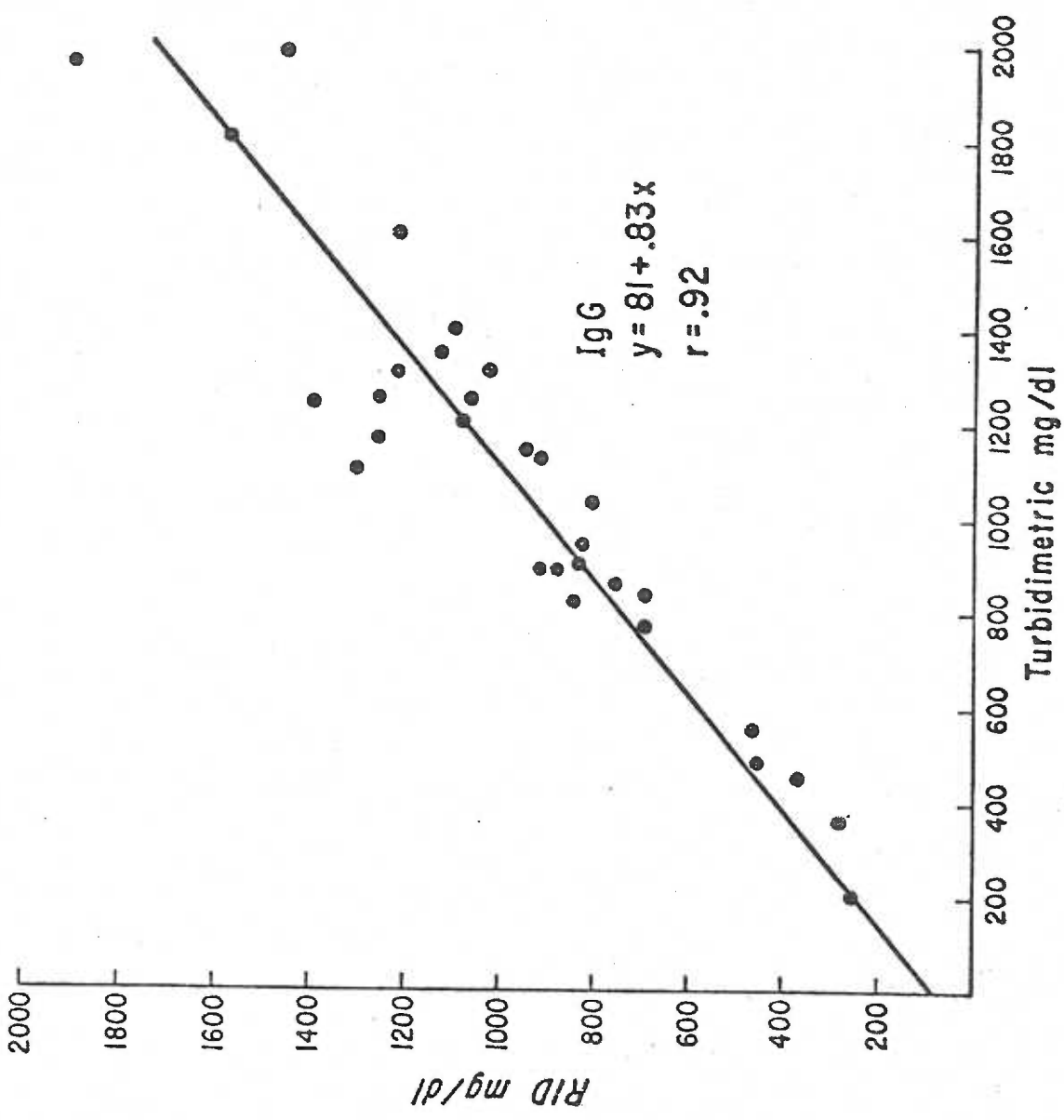


Figure 18: Correlation between Turbidimetric IgG and IgG by RID

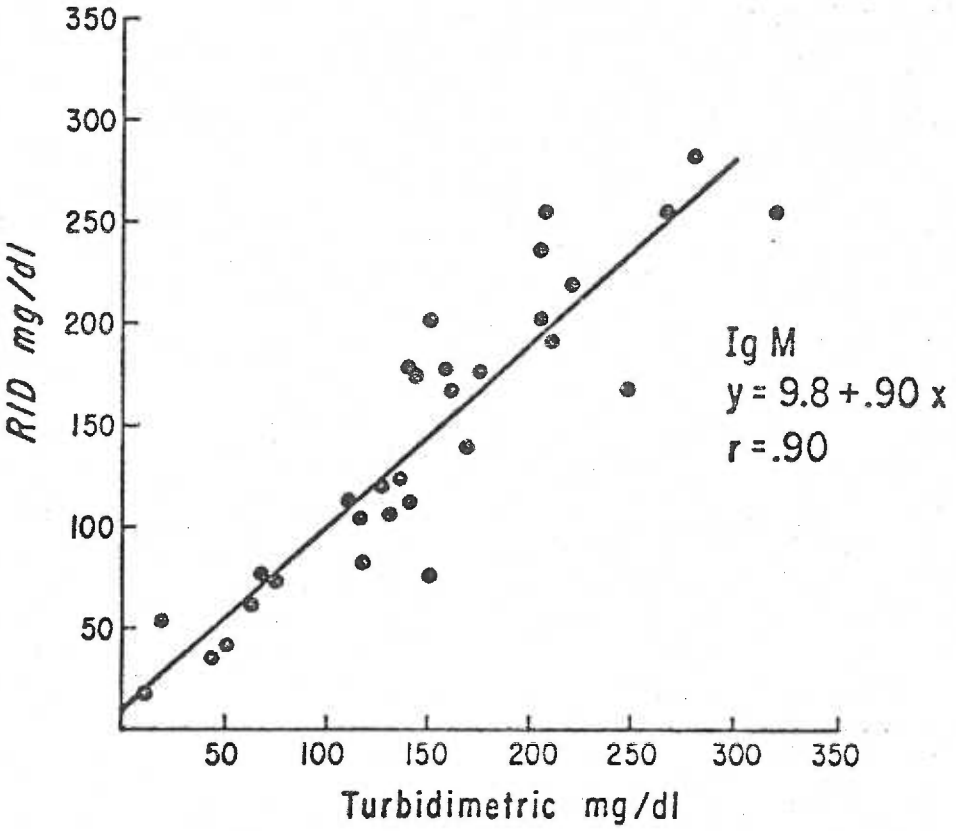


Figure 19: Correlation between Turbidimetric IgM and IgM by RID

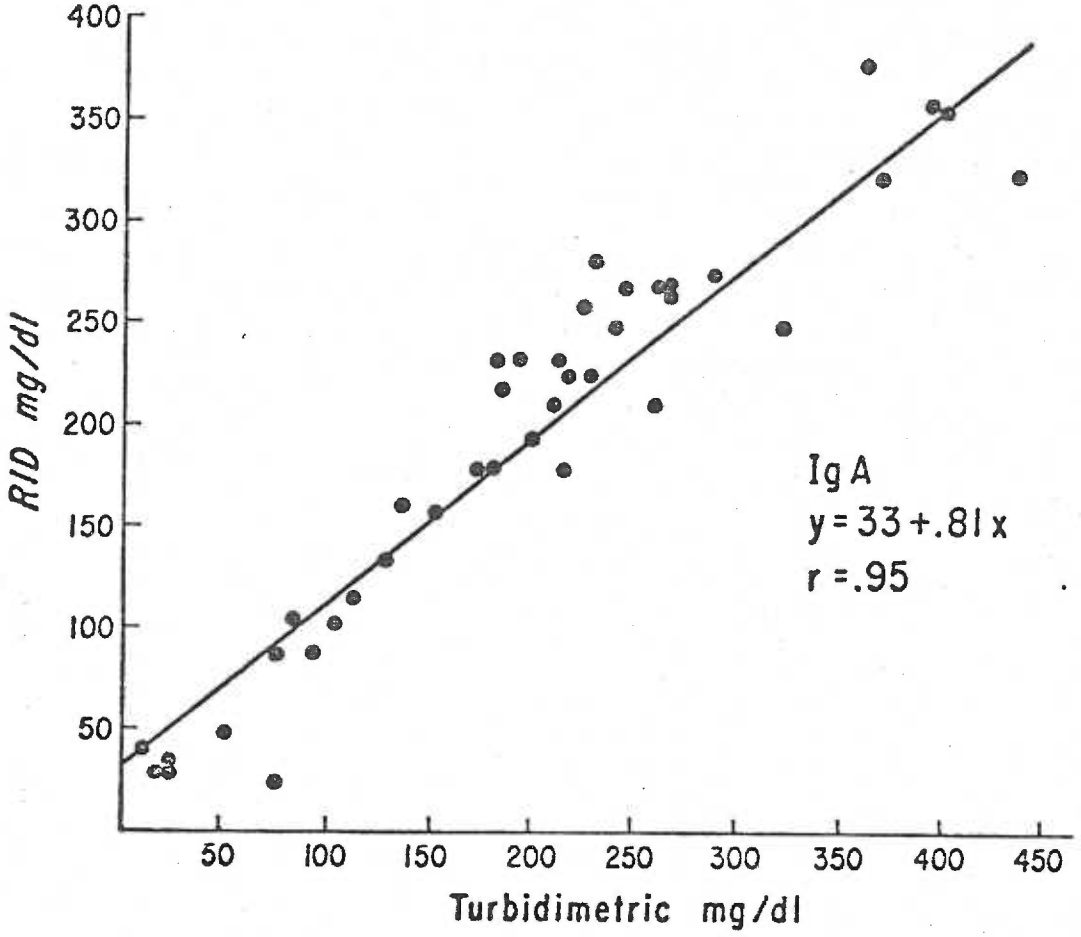


Figure 20: Correlation between Turbidimetric IgA and IgA by RID

IgG	r = .92	N = 29
IgA	r = .95	N = 40
IgM	r = .90	N = 30

Table 14
Method Correlation between RID and Turbidimetry for IgG, IgA and IgM

10. Recovery of IgM and IgG Added to a Pool.

To measure recovery, known amounts of pure IgG and pure IgM were added to the pool and run by the turbidimetric method. The pure IgG solution was made by weighing out Human IgG (Miles Laboratories) and dissolving in PBS to make a concentration of approximately 5 mg/ml. Pure IgM solution was supplied in liquid form from the manufacturer (ICL Scientific). Both solutions were analyzed as in methods for total protein by the biruet method. These two solutions of "pure" protein were also checked for purity by IEP. The solution of IgG was found to be monospecific. The IgM protein solution was found to react slightly with anti IgG antiserum which indicated contamination with IgG protein.

Three mixtures of pooled serum and pure protein were made for both IgG and IgM. These consisted of 20%, 50% and 80% protein in pooled serum. These mixtures as well as the straight protein solutions were then run by the turbidimetric procedure (IgM and IgG method B).

Theoretical or predicted values were calculated. For IgG these were calculated in two different ways: First by using a value for the pure IgG solution obtained by the biuret method, and secondly by using the value obtained when the straight IgG solution was run turbidimetrically. For IgM, since the solution was not found to be pure, only the value derived turbidimetrically was used in calculating the predicted values. These predicted values were then compared to the actual values obtained

in the turbidimetric analysis and the percent recoveries calculated using one of the following formulas:

$$\frac{\text{Experimental result}}{\text{Theoretical result}} \times 100 = \%\ \text{recovery or}$$

$$\frac{\text{Theoretical - Experimental}}{\text{Theoretical}} \times 100 = \%\ \text{difference}$$

The results of these experiments are shown in Tables 15, 16 and 17.

IgG Pool-Protein Mixtures	Theoretical Value of Mixture Calculated Using Value of Pure Protein Solution Obtained by Biuret	Measured Values by Turbidimetry	% Difference from Theoretical
20% Pure Solution in Pooled Serum	874 mg/dl	910 mg/dl	4%
50% "	690 mg/dl	648 mg/dl	6%
80% "	512 mg/dl	426 mg/dl	17%
100% "	391 mg/dl	205 mg/dl	48%

Table 15
Comparison of Theoretical and Experimental Results when Pure IgG as Quantitated by the Biuret Method is Added to Pooled Serum

IgG Pool-Protein Mixtures	Theoretical Value of Mixture Calculated Using Value of Pure Protein Solution Obtained Turbidimetrically	Measured Values by Turbidimetry	% Recovery
20% Pure Solution in Pooled Serum	838 mg/dl	910 mg/dl	109%
50% "	603 mg/dl	648 mg/dl	108%
80% "	368 mg/dl	426 mg/dl	116%
100% "	Measured	205 mg/dl	-

Table 16
Recovery of Pure IgG Protein Quantitated Turbidimetrically and Added to Pooled Serum

IgM Pool-Protein Mixtures	Theoretical Value of Mixture Calculated Using Value of "Pure" Protein Solution Obtained Turbidimetrically	Measured Values by Turbidimetry	% Recovery
20% Pure Solution in Pooled Serum	168 mg/dl	150 mg/dl	89%
50% "	230 mg/dl	265 mg/dl	87%
80% "	288 mg/dl	330 mg/dl	87%
100% "	Measured	332 mg/dl	-

Table 17
Recovery of IgM Protein Added to Pooled Serum

Table 15 shows that when the pure IgG solution was measured by the biuret method, a value of 391 mg/dl was obtained. When it was analyzed by the turbidimetric method, the result was 205 mg/dl. The percent recovery of pure IgG protein was inversly proportional to the amount of pure protein added to the pooled serum. When the theoretical value was recalculated using the value for the IgG protein solution obtained using the turbidimetric method and compared to the measured results, the percent recoveries ranged from 108% to 116% (Table 16). For IgM recoveries of 87-89% were obtained when the theoretical value was calculated using the value for the IgM solution obtained turbidimetrically and compared to the measure value.

DISCUSSION

A. Comparison of Nephelometry and Turbidimetry.

This study was undertaken to evaluate the laser modification of the Centrifichem. This type of instrument modification was investigated not only to see if it was useful for the clinical laboratory, but to see if it would provide an analytical tool which was superior to other methods for quantitating various serum proteins. RID is time consuming, costly, and relatively imprecise. Other protein quantitating procedures are tedious and non-specific. In theory, using a laser as a light source and detecting light scattered from antigen-antibody complexes should be a highly precise and sensitive analytical technique. The laser beam provides a source which is highly collimated and very intense, and detecting near front surface scattering results in the highest intensity readings compared to measuring at other angles. However, results obtained using nephelometry as it was developed in this laboratory were inferior to results obtained using a turbidimetric method.

The nephelometric method was determined to be inferior to the turbidimetric method for several reasons. First of all, there was a very high noise level which resulted in high blanks with the nephelometric technique. This decreased the sensitivity and caused the limit of detection to be high (Figure 5), whereas, with the turbidimetric method, very small amounts of immunoglobulin were detectable (Figure 15). Using a rectangular darkfield shape did not improve the signal-to-noise ratio (Figure 4, Table 4). The within-run precision of the nephelometric method was not as good as the turbidimetric method either by the

method of duplicates or by running repeats (Tables 5 and 11). Also, the correlation coefficients between RID and nephelometry were lower than those for the turbidimetric method (Table 13), although the correlation between nephelometry and turbidimetry was relatively good (Table 13 and Figure 17). Finally, the nephelometric method involved a modification of the Centrifichem, and slightly more time was required for setting up and taking down the laser attachment than was necessary for the turbidimetric method. Both the turbidimetric and nephelometric methods cost the same -- about 10¢ per cuvette.

There are several possibilities of ways to improve the nephelometric method which were not tried since the laser modification was developed before this study was started. Using a shorter wavelength laser should, according to light scattering theory, give more light scattering and therefore could result in better sensitivity. Decreasing the nonspecific light scattering should also improve the method. High light scattering in the blanks could be from a variety of sources. Since the Centrifichem consists of a centrifugal system which is always in motion during analysis, vibrations of the laser could cause some of the high noise levels and general instability of signal. Any reflection of laser light off the sides of the cuvette would also increase light scattering in the blanks. It was suggested that the interior walls of the cuvettes be painted black in order to cut down on this reflection if it were taking place, but this did not seem feasible.

B. Use of the Polymer.

PEG was used as a reaction enhancer to provide for short analysis times. The introduction of rapid manual and automated immunochemical

methods was made possible by the discovery of Hellsing (48), in 1966, that PEG (MW 6000) and other polymers accelerated and enhanced the precipitation of antigen-antibody complexes. Without the addition of polymer, these reactions take hours or days to go to completion. The effect of PEG on the precipitin reaction enables a reduction of total reaction time to 2 minutes in some cases. It has also been shown that the mean complex size is much larger with PEG in the reaction medium. One current theory that has been offered to explain this phenomenon of polymer enhancement of the precipitin reaction, is that exclusion of antigen-antibody complexes from the domain of the polymer decreases the solubility of the complexes and drives the equilibrium toward the formation of large complexes (48).

C. Turbidimetric Method.

There were several criteria used in choosing the various reaction parameters for the immunoglobulin methods. First, the autopipettor was to be used for as much of the sample and reagent diluting and loading as possible. Previous work done in this laboratory with IgG and albumin had been done completely with hand pipetting. Final serum and antibody concentrations obviously had to approach the equivalence point in order to get the maximum signal and therefore the maximum sensitivity for the test. Reaction time course curves were run to determine the maximum signal and the time at which the reaction peaked (Figure 11 and Tables 8 and 9). Also, combinations of antibody and serum dilutions were chosen which gave good standard curves, and covered as wide a range as possible to avoid having to repeat analyses on samples out of the range of the curves. Another consideration was keeping the total amount of

antibody used per test as low as possible to keep the cost per test down, since the antisera is the most expensive reagent used in any immunochemical test. As was stated previously, antisera cost per cuvette is about 10¢ whereas RID costs about $1.00 per well.

Use of the autopipettor imposed several constraints. First of all, only 250 or 350 µl of reagent could be dispensed by the Centrifichem autopipettor. Since the total capacity of the sample/diluent syringe was 1.65 ml, a maximum of 55 µl of diluent per cuvette could be used. It was decided that a volume of diluent should be dispensed which was at least equal to the volume of sample picked up in order to get adequate washing and eliminate carryover between samples. Past experience in this laboratory indicated that the minimum sample volume that could be used was 10 µl. Using a smaller volume resulted in poor precision although the manufacturer claimed that the pipettor should operate with a C.V. of 1% at 5 µl of sample. Finally, the accuracy and precision of the pipettor affected the final accuracy and precision of the method (Tables 2 and 3).

Another important consideration, since this procedure was being developed for possible routine laboratory use, was ease and convenience of making dilutions. Making one dilution of serum and being able to run all three immunoglobulins would have been ideal. However, after developing the turbidimetric and nephelometric IgG methods and comparing them, it was found that the serum dilution chosen (1:50) was not suitable for IgA and IgM. This necessitated a change in IgG method and both dilution schemes for IgG are listed in the methods section. Approximately constant final dilutions of antibody and serum were retained in order to remain at equivalence. The final dilution process for IgG, IgA and IgM

requires that 1:15 and 1:30 dilutions be made on each serum. These dilutions are proportional and can be made serially or by using an automatic pipetting device. The antibody dilutions of 1:30 for IgG and 1:20 for IgA and IgM can be made accurately and conveniently.

The only other limitations were those built-in to the Centrifichem analyzer itself. According to the manufacturer, the minimum T_o or initial reading time was 3 seconds, in order to give the contents of the cuvettes adequate time to combine and mix. The analyzer required a minimum time between sample readings and prints of 0.25 minutes and a maximum number of prints of 10. The actual precision and accuracy of the Centrifichem analyzer obviously affected the precision and accuracy of the whole method (Table 1), and this has to be taken into account when evaluating the within-run and run-to-run precision shown in Tables 11 and 12.

An unexpected finding was the fact that the absorbance of the antibody reagent increased with time when it was read against water or PEG as shown in Figure 7. This could be due to nonspecific scattering or to particles present in the water used as diluent. A more probable explanation is that dilution of the antibody reagent by the diluent water in the reaction cuvette changes the solubility of the antibody present and increases the light scattering slightly. The antibody reagent itself is filtered through 0.22 μm pore filters to eliminate contaminating particles. When the PEG is added to the antiserum there is often a slight amount of visible precipitation formed which is then filtered out.

The data shown in Table 6 supports Finleys (38) findings that the antibody reagent is stable for long periods of time.

Three different PEG solutions were compared, and the results are shown in Table 7 and Figures 8, 9 and 10. PEG 1 contained the same concentrations of reagents used by Finley (38). Figures 8, 9 and 10 show that for each of the immunoglobulins, PEG 1 solution gave higher absorbance readings. Several workers (44,45) had used the lower concentration of NaCl (0.15 moles/liter) with or without fluoride as in PEG 2 and 3. In this study inferior results were obtained using this concentration of NaCl. Marrack and Richards (17) showed that the addition of fluoride to the reaction medium enhanced the light scattering of the antigen-antibody complexes.

D. Recovery.

Recovery was measured by adding known amounts of pure IgG and pure IgM to the pooled serum. A large discrepancy was noted between values for IgG obtained on the pure solution by the biuret total protein method and values obtained turbidimetrically (Table 15). The 100% pure IgG solution contained 391 mg/dl protein as measured by the biuret method and only 205 mg/dl when measured immunochemically. One possible explanation is that the human IgG is fragmented in the purification process and these fragments are not immunologically active yet are measured in the total protein method. The effect of dilution of the pool by the pure solution decreases as the percentage of pure protein decreases so that a solution containing only 20% pure protein and 80% pooled serum gives a difference of only 6% (Table 15). When the value for the pure protein is obtained turbidimetrically and then this value is used to calculate recovery, consistent results are obtained as in Tables 16 and 17. IgM and IgA were not readily available in a purified form and it was beyond the

the scope of this study to attempt to make pure solutions of these immunoglobulins.

E. Detection of Antigen Excess.

Antigen excess detection is a difficulty inherent in immunochemical systems. The problem arises when an intensity value is measured which can be representative of either antibody excess or antigen excess. This phenomenon is demonstrated in Figure 11 where an absorbance value of about .220 can indicate either 4000 mg/dl of IgG or 1000 mg/dl of IgG. A number of approaches have been used to get around this problem. Two different sample dilutions can be used to indicate whether or not the sample is in the antigen excess region. Continuous flow analyzers have a built in detection system for antigen excess since the peaks on the strip chart recorder invert in antigen excess (46). This inversion eliminates the need to use two sample dilutions for every sample. Tiffany, et al (34) took advantage of the reversible equilibrium phenomenon associated with antigen-antibody reactions, by dynamically injecting a small quantity of antibody after initial equilibrium was reached. A rise in light scattering indicated antigen excess because the unbound antigen reacted with the added antibody to form antigen-antibody complexes. Buffone and Savory (45) screened their samples by serum protein electrophoresis to detect antigen excess.

In this study two different approaches for antigen excess detection were used. For IgG, a variation in the reaction time course was noted for antigen excess samples compared to equilibrium or antibody excess samples (Figure 16). Savory, Buffone, and Reich (47) observed an initial rapid increase in light scattering in samples in antigen excess using

very short time intervals in a PBS medium (without PEG). Tengerdy (44), also using a PEG-free medium, demonstrated that light scattering techniques measure the rate of formation of antigen/antibody complexes (i.e., the secondary reaction) in the antibody excess zone, but measure the overall reaction (i.e., both the primary and secondary reaction phases) at the equivalence point and antigen excess zones. Furthermore, in the antigen excess zone the reaction may consist of only the primary reaction and little or no secondary reaction. This would explain the initial very rapid rate of reaction noticed in antigen excess where large amounts of antigen initially combine with antibody, but, when all of the antibody binding sites are taken up, no lattice formation can occur, and there is no further rise in intensity readings. The method for antigen excess detection described, (Figure 16, Page 62) takes advantage of this initial rapid reaction rate in antigen excess samples. The Δ absorbance for a 3 minute period (1 minute to 4 minutes) indicates the initial rate of reaction. If this Δ absorbance value exceeds .100, then antigen excess is indicated and the sample is diluted further and rerun.

For IgA and IgM, two dilutions are run on each sample. The 1:30 dilution should give a mg/dl result which is approximately 1/2 of the value obtained on the 1:15 dilution. If this is not the case, or if the 1:30 dilution gives a larger answer (as would be the case in antigen excess), then further dilutions of the sample can be made and run again. Many patient samples on which immunoglobulin determinations are requested also have serum protein electrophoresis requested and therefore antigen excess can be noted before the immunoglobulin is run and the sample can be handled appropriately when the immunoglobulins are quantitated.

F. Method Calibration.

Standardization of immunoglobulin methods poses an unusual analytical problem. The same sample run by RID and turbidimetry or nephelometry can give very different results. This variation in results depends both on the method being used and the type of standard used in the method. In this study when the pool was run by RID in the serology laboratory, values for IgG, IgA, and IgM were 23-26% higher than those obtained when the same pool was run turbidimetrically using the WHO reference material (with published concentrations of immunoglobulins) as the standards. (See Table 9a.) This phenomenon occurred even though the Behring and Kallestead kits used in serology contained secondary standards which have been calibrated against the same WHO preparation by the manufacturer. This would indicate a methodological bias since the results are different even when the standards are the same.

In the correlation studies shown in Figures 18, 19, and 20, and Tables 13 and 14, the values used for the pool were those obtained by RID. Fairly good correlation results under these conditions. Comparison betwen methods can yield an evaluation of accuracy which is only relative. Since no appropriate reference methods are available, it is impossible to predict which method will give the most accurate results (45). In each laboratory, when a procedure is set up for routine use, a decision has to be made regarding the method of standardization. By standardizing against an established laboratory method such as RID, some consistency in results within the laboratory can be achieved.

SUMMARY AND CONCLUSIONS

A method is described for quantitating immunoglobulins in serum. The method utilizes a centrifugal fast analyzer to monitor an antigen-antibody reaction turbidimetrically at 340 nm. This method is compared to a nephelometric method developed for the same instrument with the addition of a helium-neon laser attachment.

The turbidimetric method was found to be more suitable for routine laboratory use than the nephelometric method. The turbidimetric method is fast, relatively precise and accurate and with proper standardization, compares well with RID. It is an excellent application for a centrifugal analyzer since very fast and precise timing and measuring are characteristic of this instrument. The turbidimetric method for immunoglobulin quantitation also is less costly and much faster than RID.

The disadvantages of the turbidimetric method are its lack of ease of antigen excess detection and the common problem of standardization of the method.

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