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A STANDARDIZED IMMUNOCHEMICAL METHOD FOR QUANTITATIVE DETERMINATION OF THE IMMUNOGLOBULINS IN SERUM*

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Introduction

The three classical immunoglobulins,[13] gamma-G (7s-γ), gamma-A (7S-Beta$_2$A) and gamma-M (19S-Beta$_2$M), have been well defined and established as of major biologic importance in humoral immunity and resistance. The clinical values of gamma-G are well recognized in agamma-, hypogamma- and hypergamma-globulinemic states. However, gamma-A and gamma-M globulin values are also necessary to understand dysgamma-globulinemic conditions associated with the *globulin deficiency* syndrome.[2,3,6,7,8,11]

Of the methods by which immunoglobulins are evaluated, three are used in this laboratory: electrophoretic mobility studies and immuno-electrophoresis which have been well established as laboratory procedures; and an immuno-diffusion method which has been adapted in this laboratory for the quantitation of the immunoglobulins. *Electrophoretic mobility* studies of the serum proteins demonstrate conditions of agamma-, hypogamma- and hypergamma-globulinemia. The immunoglobulins of the gamma-A and gamma-M classes cannot be ascertained by electrophoresis alone as they are overshadowed in the alpha and beta mobility regions by other plasma components. With use of *Immuno-electrophoretic* technics, the gamma-A and gamma-M globulins can be demonstrated, but the analysis is a subjective interpretation which is expressed in terms of; (a) "apparently normal amounts", (b) "decreased" or (c) "apparently not present". The *immuno-diffusion method* presented in this paper, clearly differentiates all three immunoglobulins in quantitative terms which are expressed in milligrams per 100 ml of serum. In addition, a range of normal values for adults has been established for use in this hospital. The procedure is relatively simple, requires only materials readily available from commercial sources and gives reproducible, easily interpretable results.

In this report, the technical details for the laboratory worker are described to show how to quantitate the immunoglobulins and how to avoid the difficulties encountered. This is in response to requests for the standard values published from this laboratory in the paper entitled "Serum Globulin Dyscrasia".[12]

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Materials and Method

I. Materials

A. *Equipment*

Equipment not found in the ordinary laboratory which is used in this method is illustrated in Figure 1.

1. *Svedmyr syringe* (Schick's X-Ray, Inc., Chicago, Ill.) fitted with a disposable (2) *Pasteur pipet* (Bellco Glass, Vineland, N. J.) and adjusted to withdraw and deliver a constant volume (0.1 ml.) of serum or diluent. It is used to make serial dilutions of serum.

3. Disposable *"Microtiter" U plate* (Cooke Engineering Company, Alexandria, Va.). This is a plastic plate with ninety-six cups, each holding a volume of 0.25 ml., used to make serial dilutions of serum.

4. *Capillary tubes* — 1.3 to 1.5 mm. O.D. x 75 mm. long (Scientific Products, Evanston, Ill.) The tubes are fitted with (5) *rubber vaccine bulbs* (The West Co., Phoenixville, Penna.). They are used to fill the wells in the template with diluted serum.

6. *Plastic templates* — These were made in a local machine shop according to the specifications described by Crowle.[5] They are one inch squares of Perspex (Lucite or plexi-glass), one-eighth inch thick, drilled first with four 1/16 inch holes equidistant to each other and 4 mm. from a center hole (center to center). Subsequently, the wells are counterbored by drilling three-quarters through the plate along the axis of the first holes with a 9/64 inch drill. The position of the holes and the size of the wells are carefully controlled. The capacity of each well is approximately 0.025 ml.

Additional equipment required which is not illustrated includes:

1. *Glass microscope slides* — 3x1 inches. No preliminary washing is required.
2. *Wax marking pencil.*

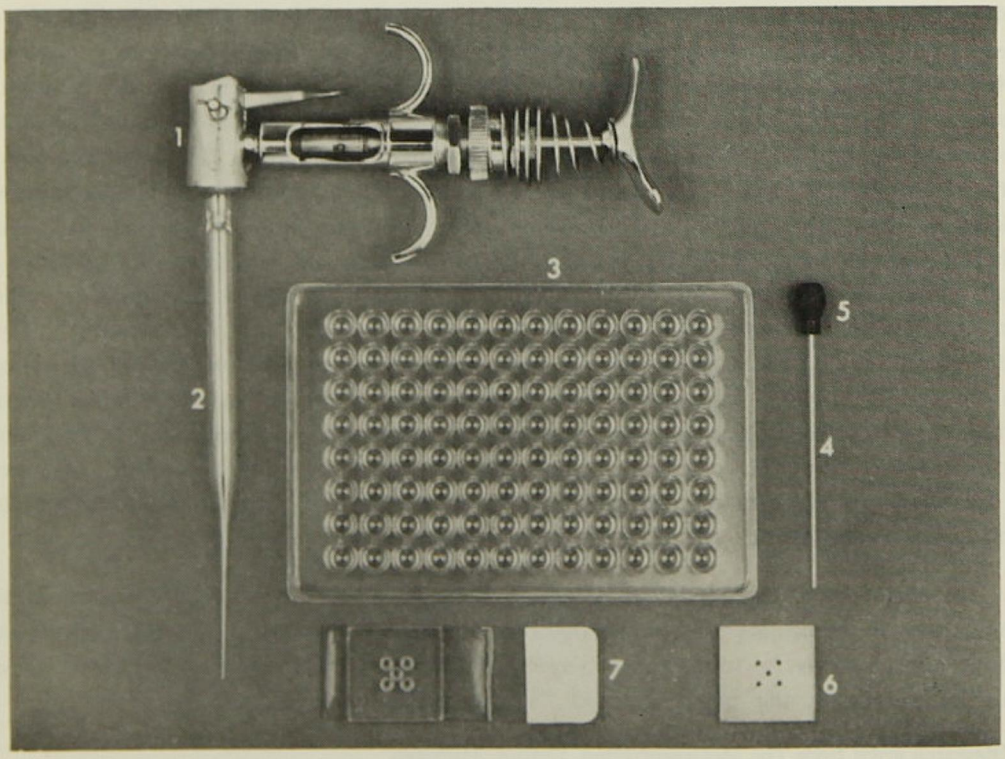

Figure 1

3. *Moist chamber* — an air tight box with moist blotting paper taped to the inner side of the lid.

4. *Magnifying viewer,* for observation of precipitation bands. The choice of viewer is not critical. Very satisfactory results are obtained with the viewer made by the Egaton AG company for use with the Agafor agar electrophoresis apparatus. (Available from National Instrument Laboratories, Inc., Washington, D. C.) A magnification of approximately 24 x is produced.

B. *Reagents*

1. *Stock FTA Phosphate-Saline Buffer, pH 7.3*

FTA Hemagglutination buffer (Baltimore Biological Laboratories, Baltimore, Maryland) prepared as directed by the supplier. One gram sodium azide is added per liter. It has the following formula per liter:

Sodium chloride	— 7.6500 gm.
Disodium phosphate	— 1.2688 gm.
Monosodium phosphate	— 0.1000 gm.
Monopotassium phosphate	— 0.2113 gm.

2. *1% Buffalo Black B* (Naphthol Blue Black) — 1 gm. dye in 100 ml. distilled water.

3. *Stock Barbital Buffer, pH 7.4* — after Crowle[4]

The following formula is made up to one liter:

Sodium barbital	— 6.98 gm.
Sodium chloride	— 6.0 gm.
1 N Hydrochloric acid	— 2.7 ml.
Sodium azide	— 1.0 gm.

4. *1 per cent Agar* (for β_2A and 7Sγ globulin determinations)

In a large beaker is placed 200 ml. stock FTA buffer and 2 gm. Oxoid "Ionagar" #2 (Consolidated Laboratories, Chicago Heights, Ill.). This mixture is brought to a boil and boiled for a few minutes until the agar is dissolved. Then 0.2 ml. 1 per cent Buffalo Black B is added and the molten agar is dispensed to small tubes for storage. Before use, the agar is melted in a boiling water bath. Unused melted agar is discarded.

5. *0.5 per cent Agar* (for β_2M globulin determinations)
Barbital buffer — 200 ml.
"Ionagar" — 1 gm.

C. *Serum and Antiserum*

1. *Antisera to individual human serum fractions* prepared in rabbits or goats (Hyland Laboratories, Los Angeles, California).

2. *Special Clinical Chemistry Control Serum,* Dried (Hyland Laboratories).

3. *Individual Normal Sera* — obtained from 30 adult patients admitted to this hospital for elective surgery and 18 healthy adult laboratory and hospital personnel. The sampling included 15 females and 33 males. All sera had normal cellulose acetate electrophoretic patterns.

4. *Pooled human plasma* — pools of plasma obtained from 80 to 120 healthy blood donors.

II. Method

A. *Principle of the method*

The method is a double diffusion in agar micro technic utilizing a five well template. Antiserum is placed in the central well and diluted serum in the four peripheral wells. After 40 to 48 hours the template is removed and the distance of the inner edge of the resulting precipitation band from the center of the central well is measured under constant magnification. Patient serum is quantitated by comparing the distance of its precipitation band from the center of the central well with the distance of the precipitation band of a reference serum, to which concentrations of the three immunoglobulins have been assigned.

B. *Preparation of slides*

1. A 4.5 cm. space is marked on a glass microscope slide with a wax pencil.
2. Between the wax markings is placed 0.7 ml. of molten (approximately 60° C.) agar. This gives an layer approximately 0.6 mm. thick.

3. Within a few minutes, while the agar is still slightly soft, a plastic template is gently placed on the agar. Care is taken that the template is flat and that there are no air bubbles between it and the agar. Pressure on the template is avoided, as this will cause agar to fill the template wells. If agar enters the wells very erratic results are obtained. Such a slide should not be used. A properly applied template leaves a barely perceptible impression on the agar surface. A completely assembled slide is shown in Figure 1, #7.
4. The prepared slide is placed immediately in a moist chamber until set. It is used within three to four hours. Slides which are allowed to become dry form numerous extraneous bands which interfere with the interpretation of the test.

C. *Dilution of serum*

1. Dilutions of reference and unknown sera are made in the buffer used for agar preparation. For β_2A and 7Sγ globulin determinations, the FTA phosphate-saline buffer is used. For β_2M globulin determinations barbital buffer is used.
2. Using the Svedmyr syringe, 0.1 ml. of the appropriate buffer is placed in each cup of a plastic "Microtiter" plate. Serial dilutions (1:2, 1:4, 1:8, etc.) of the serum are made by adding 0.1 ml. of serum to the first cup, mixing and transferring 0.1 ml. of the mixture to the next cup, continuing until the desired dilution is reached.
3. The dilution series used depends on the particular globulin being determined, on the titer of the antiserum and on the concentration of globulin in the test serum. The proper dilutions to be used for routine determinations are ascertained for each antiserum by setting up preliminary slides with diluted reference serum. The four consecutive dilutions at which the most sharply defined bands are

Table I

Correlation of 7Sγ globulin Concentration in Reference Serum with Distance of the Precipitation Band from the Center of the Antiserum Well.

Serum dilution	Concentration of 7Sγ globulin (mg./100 ml.) (assigned)	Slide No.	Distance (Cm.)	Average distance**
1:4	300	55	wide	
		56	wide	
1:8	150	55	wide	
		56	wide	
1:16	75	55	wide	
		56	wide	
1:32	37.5	55	wide	
		56	wide	
		57	3.9	
		58	3.9*	3.9
1:64	18.75	57	4.4	
		58	4.3*	4.35
1:128	9.4	57	5.1	
		58	5.0*	5.05
1:256	4.7	57	5.6	
		58	5.8*	
		59	5.8	
		60	5.8	5.8
1:512	2.3	59	6.6 (weak)	
		60	6.5 (weak)	6.55
1:1024	1.2	59	weak	
		60	weak	
1:2048	0.6	59	very weak	
		60	very weak	

*Indicates distance measurements of slide photographed (Figure 2.2)
**Average distances were used to plot graph (Figure 5)
Reference serum #369U49; Anti-7Sγ globulin #GP11-63

produced are selected for routine testing of patient serum (Figures 2.2, 3.2, and 4.2). In this laboratory, dilutions of 1:2 to 1:8 for β_2M globulin, 1:8 to 1:64 for β_2A globulin and 1:32 to 1:256 for 7Sγ globulin are most commonly used. Appropriately greater or lesser dilutions are used for abnormal sera.

D. *Charging of the slides*

1. Each slide is set up in duplicate.
2. To prevent contamination with test serum, undiluted antiserum is added to the central well first. Using a capillary tube with rubber bulb, the wells are filled from the bottom upwards. Care is taken to avoid trapping air bubbles in the bottom because these will prevent the serum from diffusing into the agar.
3. After the antiserum well has been filled, the peripheral wells are filled with the pre-determined dilutions of patient or reference serum. Each well should be filled level with the top.
4. Slides filled with diluted reference serum are included with each day's determination.
5. When the slides have been charged, they are sealed into the moist chamber and left at room temperature.

E. *Measurement of the precipitation bands*

1. After 40 to 48 hours, the slides are removed from the moist chamber and the templates are lifted off.
2. With magnification, the precipitation bands and the imprint of the five template wells on the agar surface are clearly seen. (Figures 2, 3 and 4) The bands are measured from the center of the central well to the inner edge of the precipitation

Table II

Correlation of β_2A globulin Concentration in Reference Serum with Distance of the Precipitation Band from the Center of the Antiserum Well.

Serum dilution	Concentration of β_2A globulin (mg./100 ml.) (assigned)	Slide No.	Distance (cm.)	Average distance**
1:2	55	49	wide	
		50	wide	
1:4	27.5	49	wide	
		50	wide	
1:8	13.75	49	wide	
		50	wide	
1:16	6.9	49	wide	
		50	wide	
		51	4.2*	
		52	4.1	4.15
1:32	3.44	51	4.7*	
		52	4.8	4.75
1:64	1.72	51	5.8*	
		52	5.9	
		53	6.0	
		54	5.8	5.9
1:128	0.86	51	6.5*	
		52	6.4	
		53	6.7	
		54	6.3	6.5
1:256	0.43	53	weak	
		54	weak	
1:512	0.21	53	weak	
		54	weak	

*Indicates distance measurements of the slide photographed (Figure 3.2)
**Average distances were used to plot graph (Figure 6)
Reference serum #369U49 Anti-β_2A globulin #GP7964F

band. In some cases, the edge of the band is not clearly seen. In these cases, the measurement is taken to the area of maximum concentration in the band. Figures 2.2, 3.2 and 4.2 illustrate the appearance of the precipitation pattern obtained with the three immunoglobulin antisera. Arrows indicate the actual distance measurements recorded for the photographed slide. Tables I, II, and III show the distance measurements obtained from both the photographed slide and the simultaneously prepared duplicate slide.

3. Distance measurements from the same dilution of serum on duplicate slides are averaged. The average measurement is used to obtain the globulin concentration in the patient serum, using a standard curve prepared from measurements of the reference serum.

4. After the bands have been measured, the slides are dried for storage. A piece of filter paper is placed on top of the agar to draw the buffer fluid and salts from the agar. After the slide is dry, the filter paper is removed by moistening the slide with tap water. The bands are thus permanently fixed to the slide in a thin layer of agar.

F. *Preparation of the standard curve*

1. The Special Clinical Chemistry Control Serum used as the reference serum is assumed to contain the following levels of the immunoglobulins before dilution[10]:

$7S\gamma$ globulin — 1200 mg./100 ml.
β_2A globulin — 110 mg./100 ml.
β_2M globulin — 75 mg./100 ml.

These figures are mean values based on the concentration of immunoglobulins in normal serum reported by Heremans[9].

Table III

Correlation of β_2M globulin Concentration in Reference Serum with Distance of the Precipitation Band from the Center of the Antiserum Well.

Serum dilution	Concentration of β_2M globulin (mg./100 ml.) (assigned)	Slide No.	Distance (cm.)	Average distance**
Undiluted	75	43	wide	
		44	wide	
1:2	37.5	43	4.6	
		44	4.9	
		45	4.9	
		46	5.0*	4.85
1:4	18.75	43	5.7	
		44	5.7	
		45	5.5	
		45	5.8	
		46	5.6*	
		46	5.8*	5.68
1:8	9.4	43	6.0	
		44	6.3	
		45	6.5	
		46	6.3*	
		47	6.1	
		48	6.1	6.21
1:16	4.7	47	weak	
		48	7.2 (weak)	
1:32	2.3	47	weak	
		48	weak	
1:64	1.2	47	weak	
		48	weak	

*Indicates distance measurements of slide photographed (Figure 4.2)
**Average distances were used to plot graph (Figure 7)
Reference serum #369U49 Anti-β_2M globulin #GP 564

2. The concentration of each globulin in mg./100 ml. in the diluted reference serum is calculated. For example, a 1:32 dilution of reference serum is assumed to contain a concentration of 37.5 mg./100 ml. 7Sγ globulin and 3.44 mg./100 ml. β_2A globulin.

3. A standard curve is prepared for each antiserum on semilogarithmic paper. The distance measurement in centimeters is plotted on the horizontal arithmetic scale and the globulin concentration in the diluted reference serum on the vertical logarithmic scale (see Figures 5, 6 and 7).

G. *Calculation of immunoglobulin concentration in patient serum*

1. The concentration of globulin in each of four consecutive dilutions of patient serum is obtained from the standard curve. This concentration is multipled by the dilution to obtain the concentration of globulin in the undiluted serum.

2. The four values obtained from the four different dilutions are averaged to obtain the reported figure.

Results

I. *Characteristic Appearance of the Precipitation Bands*

A. General location and appearance of the specific band

If the proper dilution series has been selected, the precipitation pattern characteristically appears as a single band forming the perimeter of an eccentric four-sided figure about the impression made on the agar surface by the central well. Since each peripheral well contains a different concentration of globulin, the four sides of the precipitation pattern are located at different distances from the central well. The distance of the band from the central well is proportional to the globulin concentration in the diluted serum in the peripheral wells. It is this property which is utilized for quantitation. When the globulin concentration is high (Figures 2.1, 3.1 and 4.1), the band is located close to the central well and as the globulin concentration is reduced by increasing the dilution (Figures 2.3, 3.3 and 4.3), the band is located proportionally further from the center.

The width and density of the band are also affected by the globulin concentration in the peripheral wells. If the globulin concentration in the peripheral well is very high (Figures 2.1, 3.1 and 4.1), the band formed will not only be close to the central antiserum well, but it will also be wide and lack definition. If the globulin concentration is low (Figures 2.3, 3.3 and 4.3), the band formed will be located close to the peripheral well and will be very faint with diffuse edges or the band may fail to form altogether.

Between these extremes is a point, called the point of equivalence, at which the antiserum and its corresponding globulin are present in equivalent concentrations and react to form a narrow, very intense precipitation band. When the globulins and their antisera have approximately the same molecular size, as in the case of the β_2A and 7Sγ globulins, the band at equivalence is located approximately midway between the serum and antiserum wells. Because the β_2M globulin molecule is larger than its antiserum molecule, its diffusion rate is slower than that of its antiserum, and consequently the band formed is located nearer the serum well at equivalence.

The characteristic change in appearance of the precipitation band with dilution of the reference serum is illustrated in Figures 2, 3 and 4. A series of such slides is prepared with each antiserum in a preliminary examination to determine the optimal dilutions for quantitation of each globulin. The dilutions of the reference serum used to construct the standard curves are selected to include the point of equivalence and points directly to either side of the equivalence point. (Figures 2.2, 3.2 and 4.2) The dilutions used for determination of the globulin concentration in the patient serum are the same as those selected for the reference serum, if the globulin concentration of the unknown serum falls in the normal range. If one or more of the globulins are increased in the patient serum, the determination is repeated using greater serum dilution until the point of equivalence is reached. If one or more of the globulins is depleted in the unknown serum, the dilution is reduced or the serum is not diluted.

Tables I, II and III show actual distance measurements obtained with diluted reference serum. One of the duplicate precipitation patterns obtained with each antiserum is shown in Figures 2.2, 3.2 and 4.2. From this data, graphs were prepared for each antiserum (Figures 5, 6 and 7) correlating the concentration of each immunoglobulin in the reference serum with the distance of the inner edge of the precipitation band from the center of the central antiserum well.

B. Appearance of non-specific precipitation bands

All of the antisera we have used have been shown to contain non-specific precipitating antibodies. These are demonstrated by the appearance of one or more bands in addition to the major specific band. The concentration of the non-specific antibodies in the antiserum is low, so that when the reference serum or serum from a normal person is assayed at the dilutions corresponding to the point of equivalence for the immunoglobulins, the non-specific bands are very faint and do not interfere with the specific band or its determination.

Each antiserum differs in its content of non-specific precipitating antibodies. Some idea of their characteristics for a particular antiserum can be obtained by examination of the precipitation pattern obtained with reference serum at varied dilutions, as illustrated in Figure 3. These non-specific antibodies represent a pontential source of confusion when serum from abnormal persons is being assayed for the immunoglobulins.

The non-specific bands become more prominent if the dilution of patient serum is reduced (Figure 3). If the immunoglobulin in the patient serum is present in normal concentration, the specific band will overshadow the non-specfic bands at any dilution. However, if the immunoglobulin is present in low concentration or is absent, and the serum dilution is accordingly reduced, the non-specific band may become sufficiently prominent to be mistaken for specific precipitation.

In addition, in some disease conditions, an increase in prominence of the non-specific band may occur, leading to the presence of two bands of equal density but

different location. In this case, it is impossible to distinguish the specific band from the non-specific band by inspection.

To distinguish the non-specific bands from the specific band in either case, one or two of the peripheral wells are filled with reference serum diluted to a concentration at which it is known that non-specific precipitation is minimal. The remaining peripheral wells are filled with unknown serum. The resulting pattern of fusion of the specific band of the reference serum with the specific band of the patient serum will clearly indicate the identity of the precipitation bands formed. If the immunoglobulin is absent from the patient serum, the specific band of the reference serum will indicate this by extending laterally in a straight line, failing to fuse with any bands on the slide. The two described conditions are illustrated in Figures 8 and 9.

Difficulties arising from these non-specific antibodies have not been encountered with the β_2M and 7Sγ globulin antisera we have used. However, difficulties have occurred frequently with the β_2A antisera. Consequently, it is a routine practice in this laboratory to include reference serum on the slide when assaying for β_2A globulin in low concentration.

C. Characteristics of the β_2M globulins

An agar concentration of one per cent is used for routine determinations of the β_2A and 7Sγ globulins. In our early work, the determination of β_2M globulin was also carried out in an agar concentration of one per cent. However, it became apparent that the location of the precipitation band was not related to the globulin concentration in any consistent way. Frequently, the precipitation band was located at the same distance from the central well irregardless of the serum dilution. Therefore, the agar concentration has been reduced to 0.5 per cent. The equivalence point of the resulting band is closer to the peripheral serum well than the central antiserum well (Figure 4). but the position of the band is proportional to the globulin concentration and consequently can be used as a quantitative measure.

II. Determination of the Normal Range

The normal values obtained with this method depend entirely upon the values assigned to the reference serum, a condition which precludes any appraisal of the absolute concentration of the immunoglobulins in serum. This is unavoidable because preparations of the individual immunoglobulins of sufficient purity to serve as a standard are not available to the average laboratory. As a substitute for a pure immunoglobulin preparation, the Special Clinical Chemistry Control Serum prepared by Hyland Laboratories is utilized. This is a stable, freeze-dried preparation which can be obtained in quantity and stored for extended periods of time in the refrigerator. Thus, change from one lot of reference serum to another is necessary only at infrequent intervals, allowing for maximum continuity of the method.

Assuming the reference serum is representative of an average normal serum, the mean values for the concentrations of the immunoglobulins in normal serum as reported by Heremans[9] were assigned to it. Then, using the reference serum as a standard, the immunoglobulin concentration in the serum obtained from 48 apparently healthy adults was determined. If the concentrations of the immunoglobulins in the reference serum are the same as the mean normal concentrations reported by Heremans, then the normal range from normal persons would be expected to duplicate the range reported by Heremans.

Results of the immunoglobulin assay of serum from normal adults are presented in Table IV. Very close correlation with the normal range of Heremans was obtained for β_2M and 7Sγ globulins. However, our values for β_2A globulin were lower. This would indicate that the concentration of β_2A globulin in the reference serum was considerably higher than the mean concentration of β_2A globulin in our normal sera. It would be necessary to assume a concentration of β_2A globulin of 169 mg./100 ml. in the reference serum, instead of 110 mg./100 ml., in order to obtain Hereman's figures with our sera.

In order to eliminate the possibility that our sera, which represent a relatively small sampling, were not representative of a large population, the method was used to determine the concentration of β_2A globulin in six different lots of pooled human plasma, each obtained from 80 to 120 healthy adult donors. After correction for dilution due to the anticoagulant solution, the mean concentration of β_2A globulin in the plasma was found to be 76 mg./100 ml., a figure in close agreement with the mean concentration of β_2A globulin in our sera. This would indicate that our sampling was representative of a large population and that the discrepancy between our normal range and that of Heremans is due to a higher than expected concentration of β_2A globulin in the lot of reference serum used as a standard.

For this reason, it is essential to establish a normal range for the actual reference serum being used. However, once the normal range has been determined for a particular laboratory, clinically significant variations in immunoglobulin concentration are readily apparent.

Table IV

Normal Immunoglobulin Values in Adult Serum

	β_2A	β_2M	7Sγ
Number of specimens	44	42	47
Mean (mg./100 ml.)	82	69	1004
S.D. (mg./100 ml.)	26	20	237
Range (Mean ± 2 S.D.) (mg./100 ml.)	30 - 134	29 - 109	530-1478
Heremans' normal range (mg./100 ml.) (9)	56 - 195	39 - 117	600-1400

III. Variation between Lots of Reference Serum

Special Clinical Chemistry Control Serum lot #369U49 (Hyland Laboratories) has been used as the reference serum for these studies. Results of the assay of two other lots of control serum using lot #369U49 as the standard are presented in Table V. The immunoglobulin concentration varies between different lots of control serum. In order to retain continuity of the method, it is necessary to assign a new concentration of the immunoglobulins to each new lot of control serum before it is used as a standard.

IV. Reproducibility of the Method

The reproducibility of the method was determined by estimating the concentration of each of the three immunoglobulins in aliquots of the reference serum on different days over a period of one month. Mean and standard deviation (S.D.) were calculated and expressed as a ratio or coefficient of variation (S.D./mean). Results are shown in Table VI. A deliberate attempt was made to simulate the actual working conditions which would be found with repetitive samples of patient's serum. Several different batches of agar and buffer were used. It should be emphasized that individual distance measurements of the bands sometimes varied widely from each other. The reported figure of each assay is an average of eight separate distance measurements.

Discussion

The method developed here for the quantitation of the immunoglobulins is a modification of the method of Beale and Mason[1] which they used for the quantitation of poliomyelitis virus antigen preparations. The method of Beale and Mason in turn was based on the micro double-diffusion agar precipitin technic of Crowle.[5] Quantitative measurement of the immunoglobulins is a much needed routine laboratory procedure for clinical problems in medicine, such as the diagnosis of the *globulin deficinecy syndrome* described by Bruton,[2,3] Janeway,[11] Gitlin,[6,7] Good[8] and others. Moreover, quantitation of the immunoglobulins is necessary in evaluating the immue

Table V

Concentration of the Immunoglobulins in Two Lots of Control Serum Obtained with a Third Lot Used as the Reference Serum

	Reference Sera (Lot Numbers)		
	369U49	369R52	3699X2
	(Assigned Values)		
7Sγ globulin mg./100 ml.	1200	1584	950
β_2A globulin mg./100 ml.	110	161	87
β_2M globulin mg./100 ml.	75	109	60

Table VI

Reproducibility — Estimation of the Immunoglobulins in a Single Sample on Separate Occasions Over a Period of One Month

	β_2A	β_2M	7Sγ
Number of determinations	11	10	20
Mean (mg./100 ml.)	109	75	1155
S.D.	12	4	144
Coefficient of variation (S.D./mean)	11%	5%	8%

humoral status of patients particularly those with *globulin dyscrasia.*[12] Quantitation of the immunoglobulins by immuno-diffusion is not a substitute for electrophoretic mobility studies of the serum proteins. It augments this analysis with data that is not discernable by electrophoresis alone. We have found that electrophoresis may indicate agamma-G and hypogamma-G conditions which actually have a normal quantity of gamma-G globulin. Also, hypergamma-A and hypogamma-G conditions can be clearly shown by immuno-diffusion to be misdiagnosed as a hypergamma-G condition by electrophoresis.[12] These discrepancies occur in disease states when the magnitude of the electric charges on the respective proteins causes them to migrate to other regions in the electrophoretic mobility pattern. By immuno-diffusion, the immunoglobulins are precipitated in the agar by their specific antisera, producing more specific values for the respective globulins. Although the paraproteins in multiple myeloma (gamma-A and gamma-G classes) and in Waldenstroms macroglobulinemia (gamma-M class) have abnormal structural variations, they still react with their specific class antiserum so that they can be quantitated by the procedure described in this paper.[12]

The immunoglobulin values for normal adult serum were found to range (mean $\pm$ 2 S.D.) from 30 to 134 mgs./100 ml. for gamma-A (β_2 A); 29 to 109 mgs./100 ml. for gamma-M (β_2 M); and 530 to 1478 mgs./100 ml. for gamma-G (7S-γ). These values may change due to the variations in the globulin values assigned to the commercial reference serum. For this reason it is essential to establish standard values for a particular laboratory. In this way clinically significant variations become readily apparent.

In the present period of rapid expansion of immunology, the study of immunity is becoming transformed in its application to medical practice as a whole. Quantitative and qualitative evaluation of the classic immunoglobulins are necessary in such studies in order to ascertain the immune humoral status of the individual. The immunological features of various diseases cannot be brought to light until laboratory procedures can better evaluate both humoral and cytological aspects of immunity.

SUMMARY

1) A method for quantitating the three classical immunoglobulins has been adapted from the principles of double immuno-diffusion in agar.

2) The method is relatively simple and readily adaptable as a routine laboratory procedure since all materials and supplies are available from commercial sources.

3) Normal adult serum values for this laboratory were found to range (mean ± 2 S.D.) as follows for the respective immuno-globulins; 30-134 mgs./ml. for gamma-A (7S-β_2A); 20-109 mgs./100 ml. for gamma-M (19S-β_2M) and 530-1478 mgs./100 ml. for gamma-G (7S-γ2).

4) The procedure is of clinical value to augment the data available from electrophoretic mobility studies. Moreover, the two procedures are necessary for diagnosing such conditions as *globulin deficiency* syndrorme and *serum globulin dyscrasia.*

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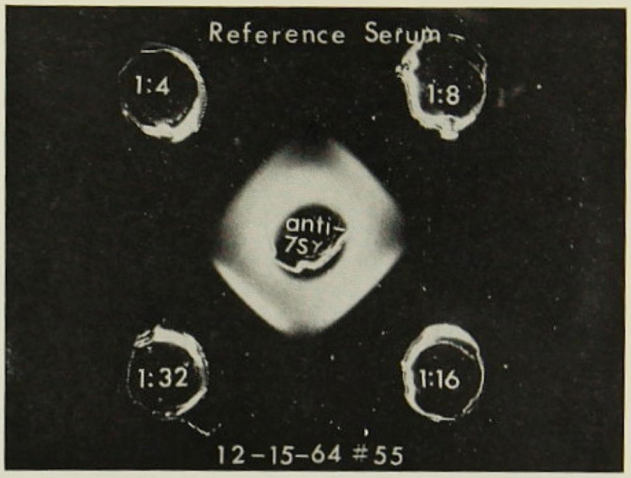
Figure 2

Precipitation pattern obtained with anti- 7Sγ globulin in the central well and diluted reference serum in the peripheral wells.

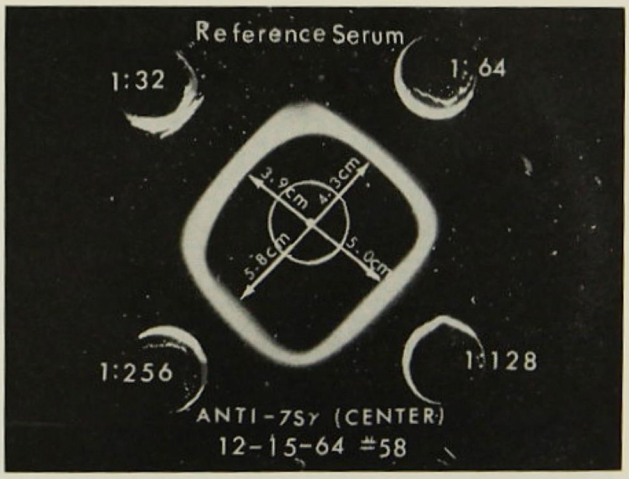
2.1 Pattern obtained when the serum dilution is too low or the 7Sγ globulin concentration is high.

2.2 Pattern obtained when the serum dilution series has been selected so that 7Sγ globulin and its antiserum are present in approximately equivalent concentrations. Actual distance measurements are indicated by arrows.

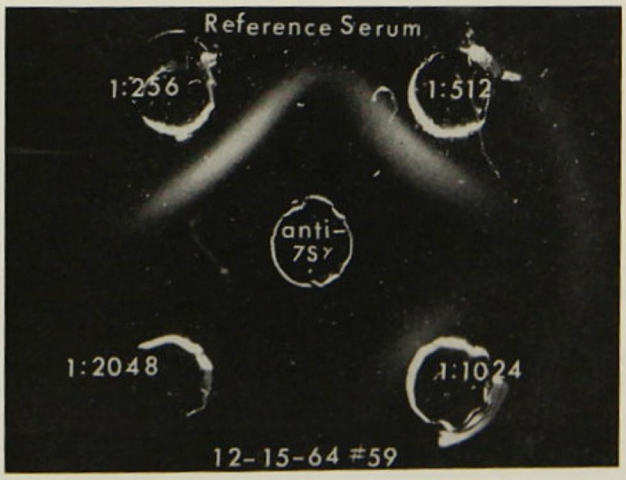
2.3 Pattern obtained when the serum dilution is excessive or the 7Sγ globulin concentration is low.


Reference Serum
1:4
1:8
anti-
7Sγ
1:32
1:16
12-15-64 #55

2.1


Reference Serum
1:32
1:64
3.9cm
4.3cm
5.8cm
5.0cm
1:256
1:128
ANTI-7Sγ (CENTER)
12-15-64 #58

2.2


Reference Serum
1:256
1:512
anti-
7Sγ
1:2048
1:1024
12-15-64 #59

2.3

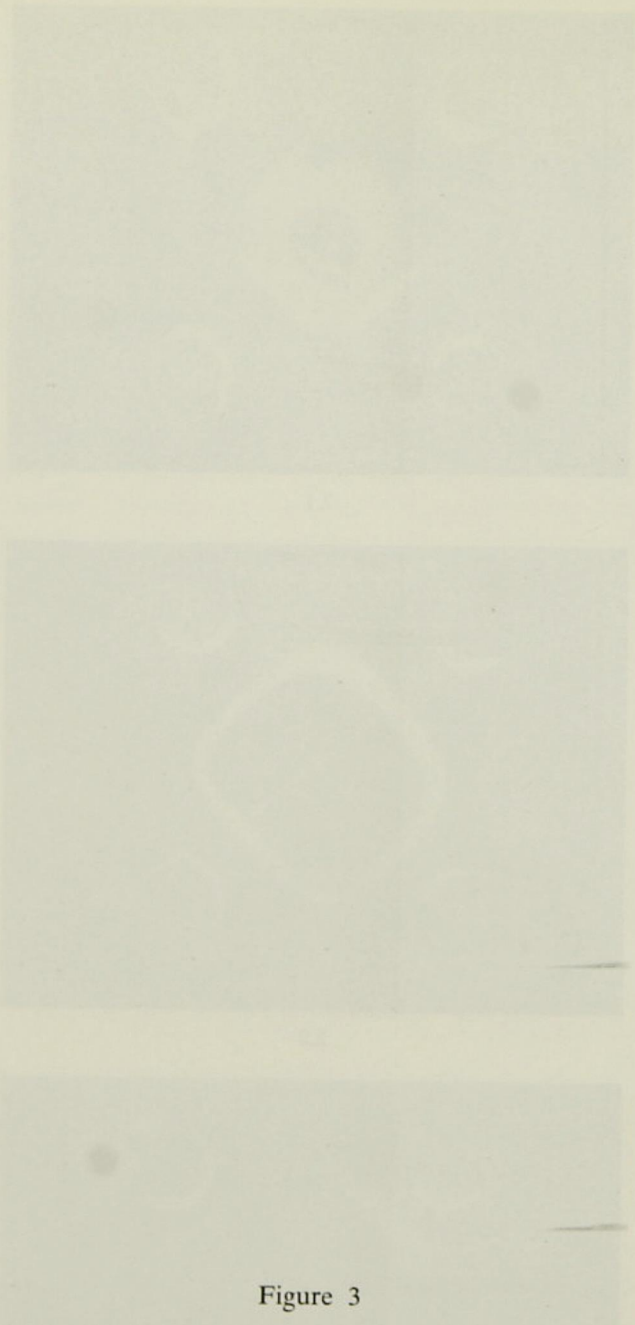


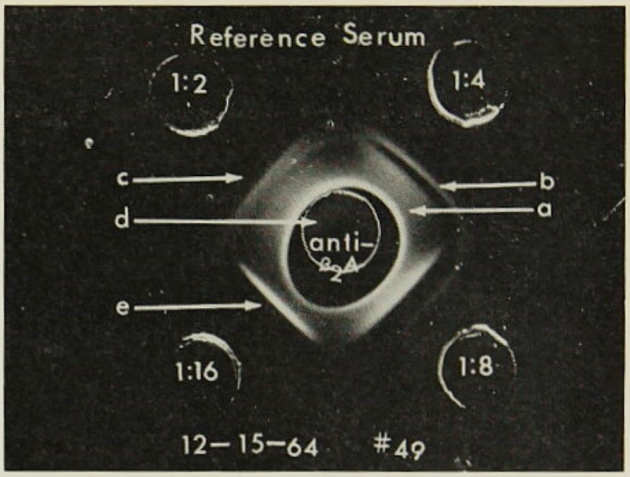
Figure 3

Precipitation pattern obtained with anti-β_2A globulin in the central well and diluted reference serum in the peripheral wells. The specific band (a) and four non-specific bands (b, c, d and e) obtained with this antiserum are indicated by arrows.

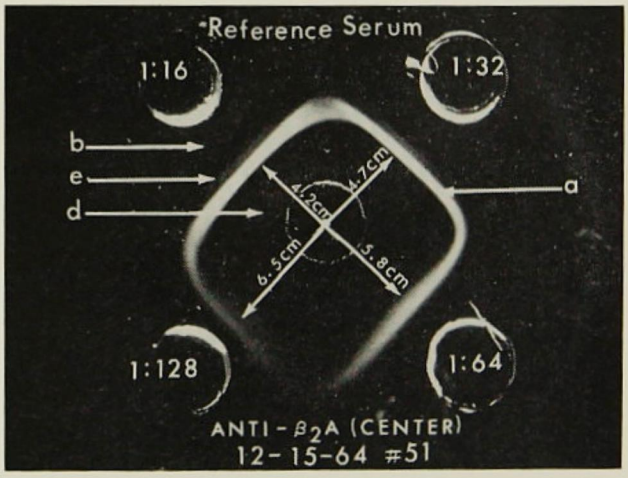
3.1 Pattern obtained when the serum dilution is too low. The specific band is wide and ill-defined and the non-specific bands are prominent.

3.2 Pattern obtained when the serum dilution series has been selected so that β_2A globulin and its antiserum are present in approximately equivalent concentrations. Actual distance measurements are indicated by arrows. Non-specific bands are very faint.

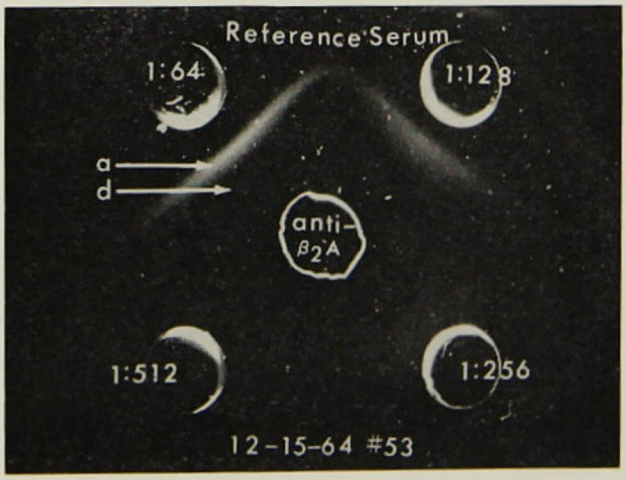
3.3 Pattern obtained when the serum dilution is excessive.


Reference Serum
1:2
1:4
c
b
d
a
anti-
β_2A
e
1:16
1:8
12–15–64 #49

3.1


Reference Serum
1:16
1:32
b
e
d
a
4.2cm
4.7cm
6.5cm
5.8cm
1:128
1:64
ANTI-β_2A (CENTER)
12-15-64 #51

3.2


Reference Serum
1:64
1:128
a
d
anti-
β_2A
1:512
1:256
12-15-64 #53

3.3

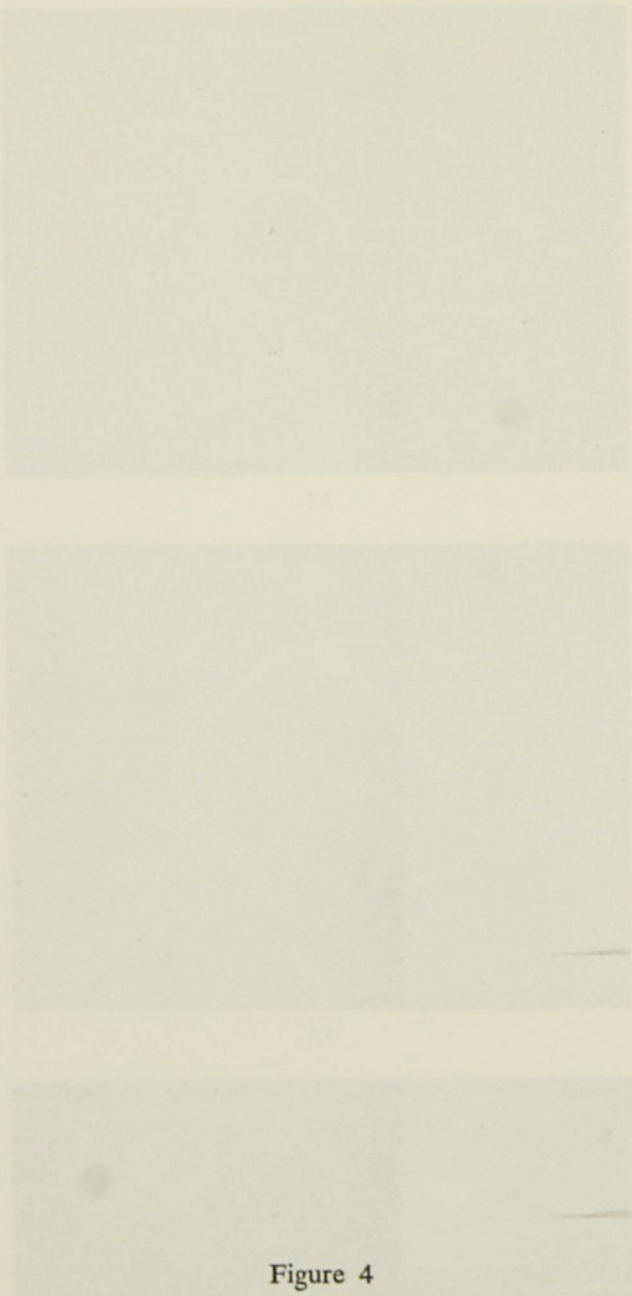


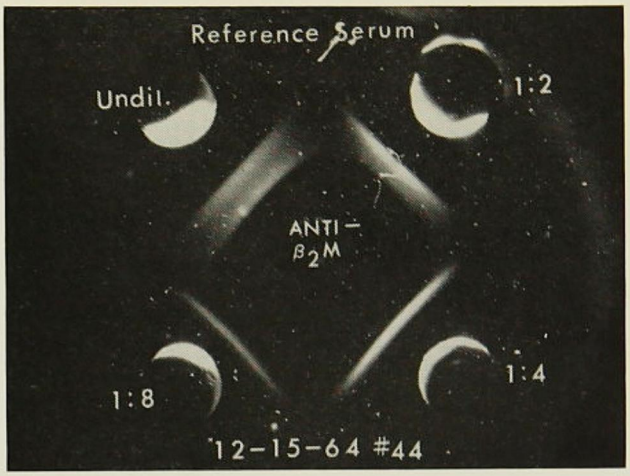
Figure 4

Precipitation patterns obtained with anti-β_2M globulin in the central well and diluted reference serum in the peripheral wells.

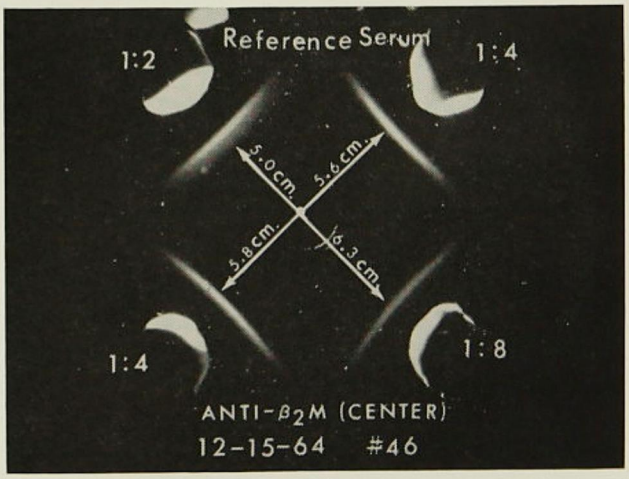
4.1 The concentration of β_2M globulin in normal serum is too low to produce a pattern of β_2M globulin excess with four consecutive serum dilutions. The wide band is obtained only with undiluted serum. The narrow intense band obtained when the serum is diluted 1:4 indicates the equivalence point.

4.2 Pattern obtained when the serum dilution series has been selected so that β_2M globulin and its antiserum are present in approximately equivalent concentrations. Actual distance measurements are indicated by arrows.

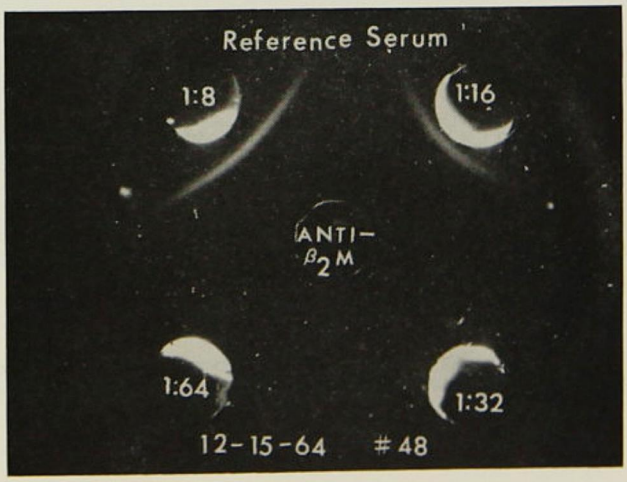
4.3 Pattern obtained when the serum dilution is excessive or the β_2M globulin concentration is low.


Reference Serum
Undil.
1:2
ANTI-
β_2M
1:8
1:4
12-15-64 #44

4.1


Reference Serum
1:2
1:4
5.0cm.
5.6cm.
5.8cm.
6.3cm.
1:4
1:8
ANTI-β_2M (CENTER)
12-15-64 #46

4.2


Reference Serum
1:8
1:16
ANTI-
β_2M
1:64
1:32
12-15-64 #48

4.3

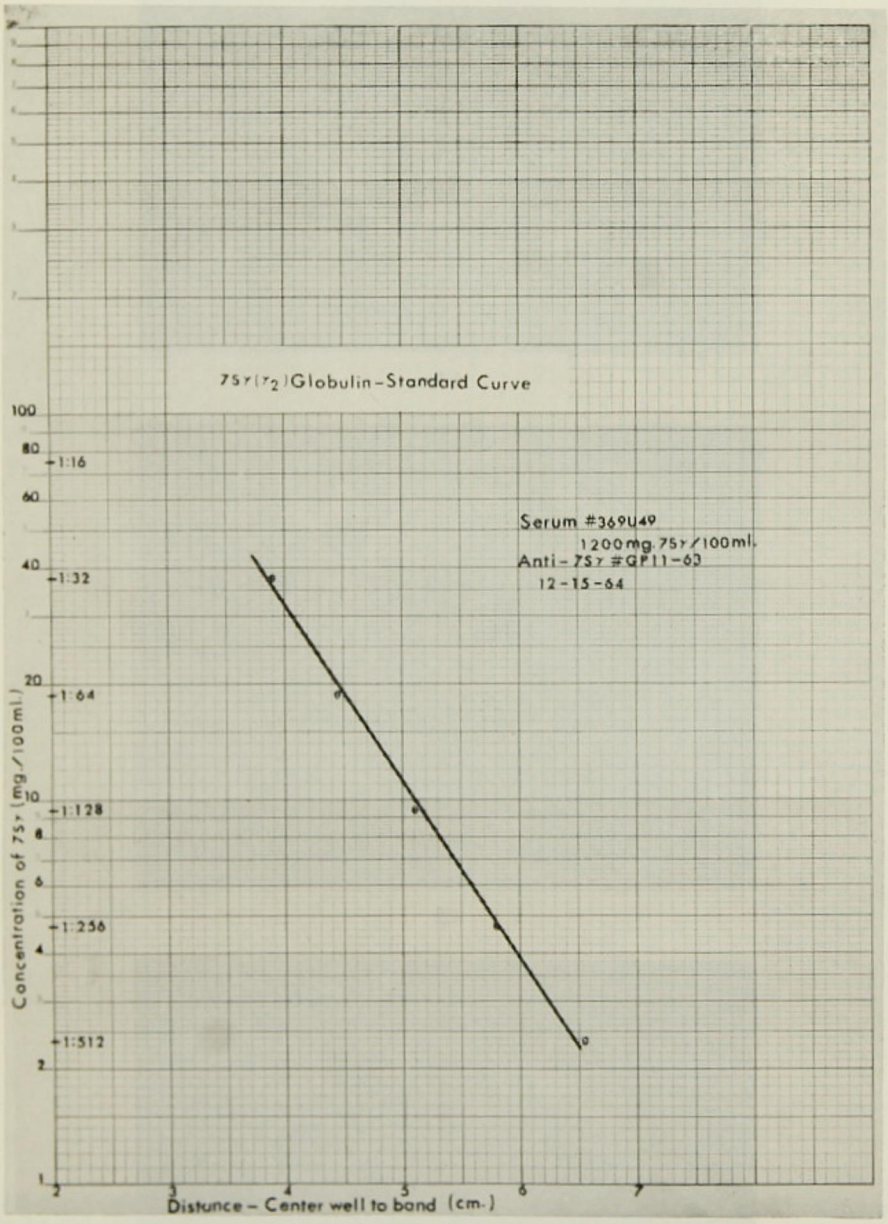


Figure 5

Correlation of 7Sγ globulin Concentration in Reference Serum with Distance of the Precipitation Band from the Center of the Antiserum Well — Standard Curve Prepared from Table I.

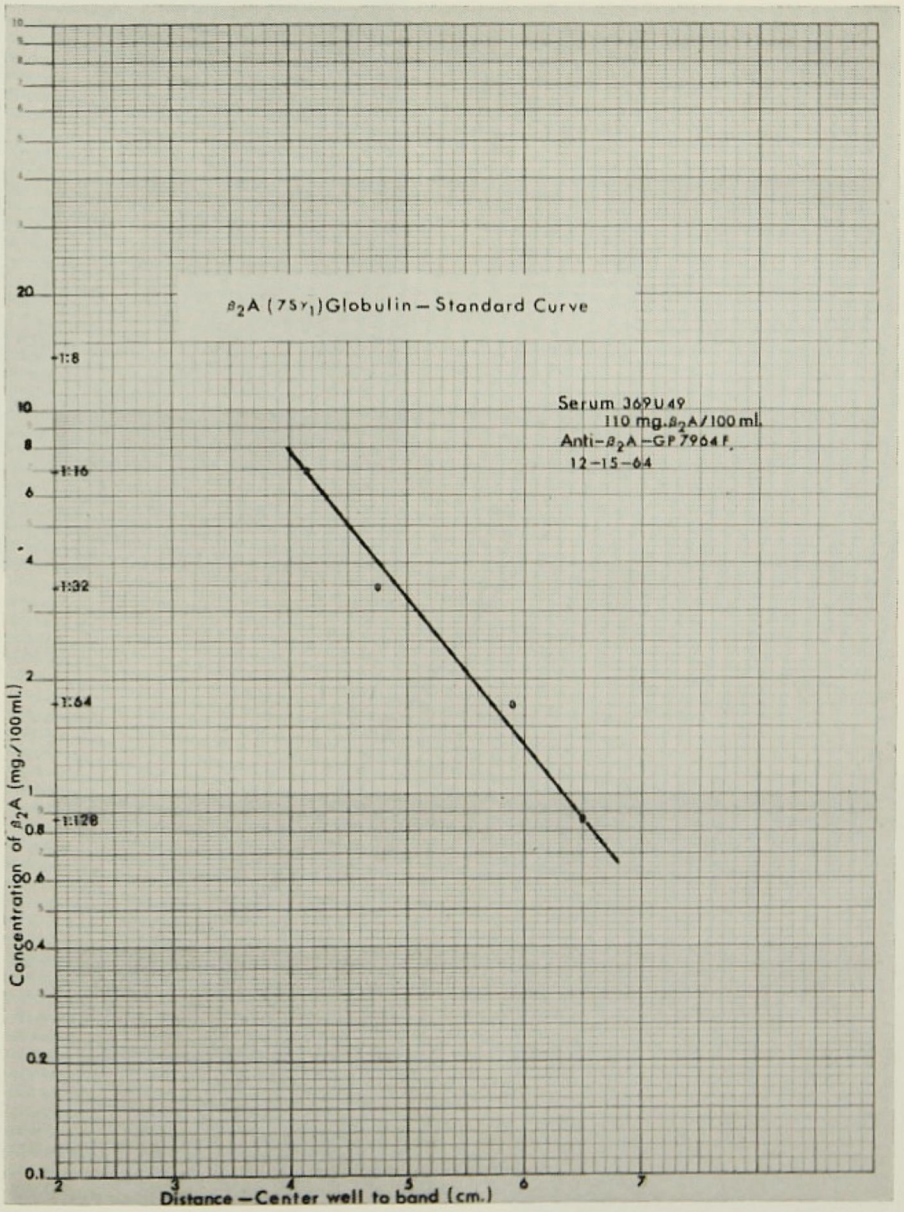


Figure 6

Correlation of β_2A globulin Concentration in Reference Serum with Distance of the Precipitation Band from the Center of the Antiserum Well — Standard Curve Prepared from Table II.

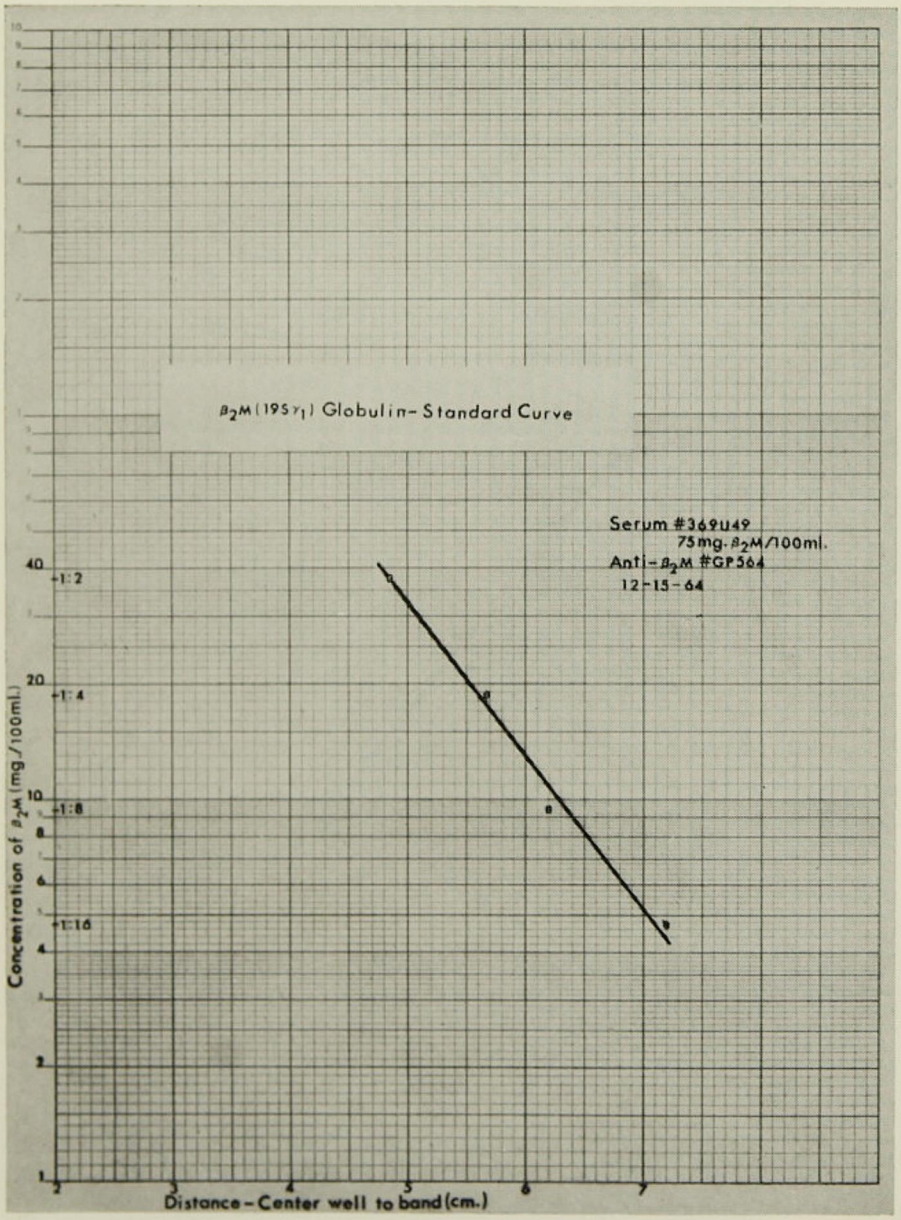


Figure 7

Correlation of β_2M globulin Concentration in Reference Serum with Distance of the Precipitation Band from the Center of the Antiserum Well — Standard Curve Prepared from Table III.

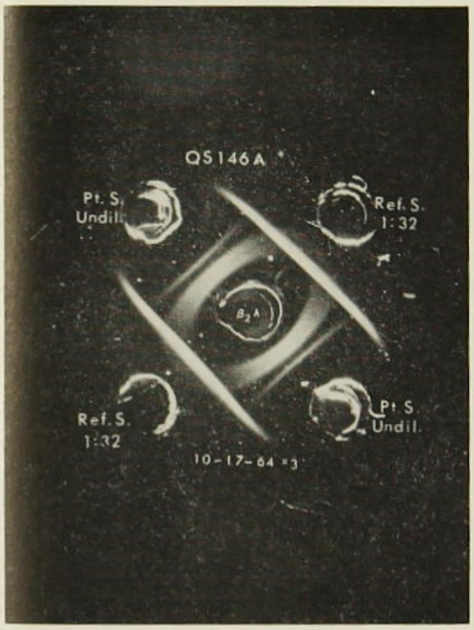


Figure 8

Photograph illustrating the presence of non-specific precipitating antibodies in the antiserum to β_2A globulin. Two peripheral wells contained reference serum diluted 1:32. The other two wells contained undiluted patient serum. Failure of the prominent specific band obtained with reference serum to fuse with any of the bands obtained with patient serum clearly shows that these bands are non-specific. Very slight turning of the tips of the specific band toward the wells containing patient serum may indicate that β_2A globulin is present in very minute quantity, too low to produce visible precipitation. Failure to include reference serum on the slide could lead to erroneous interpretation of a normal β_2A globulin concentration instead of the decrease clearly illustrated.

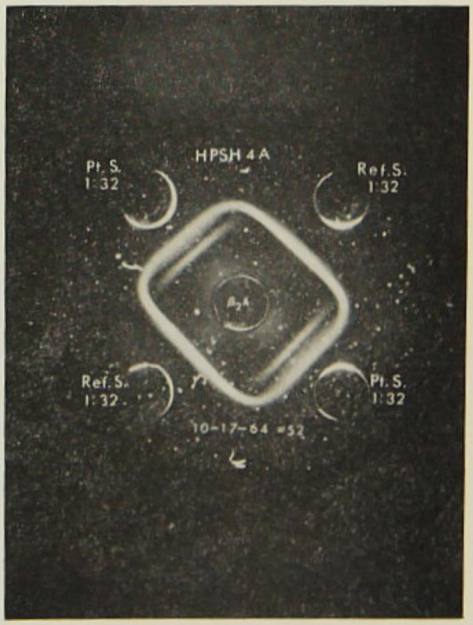


Figure 9

Photograph illustrating the presence of non-specific precipitating antibodies in the antiserum to β_2A globulin. Two opposing peripheral wells contained reference serum diluted 1:32 and the other two wells contained patient serum at the same dilution. Fusion of the prominent specific band obtained with reference serum with only one of the bands obtained with patient serum clearly indicates the specific band to be measured. In the case illustrated, β_2A globulin is present in normal quantity but the normally very faint non-specific band has increased in prominence.