Infant respiration was monitored and recorded during sleep (Fig. 8). Frequency and amplitude of respiration are clearly defined. During the recording period the infant changed from a supine position to sternal recumbency and the record of respiration was momentarily interrupted. Thereafter, a clear record of respiration was obtained with decreased amplitude.

It was necessary to correlate visual assessment of the animal's activity with the recordings obtained by the MTT method for accurate record interpretation. After acquiring proficiency in record analysis one can monitor subject activity without the necessity of constant observation. The authors believe that the MTT can be successfully employed in many other types of activity studies. Perhaps the MTT could be successfully used for patient monitoring, *e.g.*, surgical recovery or the newborn infant. A warning device coupled into the MTT patient monitoring system to alert hospital staff of critical events in the patient's progress would serve a crucial need.

Summary. A motion transducer adaptable to radio telemetry has been designed and described. The transducer was evaluated by studying activity of the infant, sheep, cow and pigeon. Evidence of the motion transducer's versatility is presented by typical recordings from 4 subjects. Activity recorded included walking, trotting, eating, flying and respiration. Observation of the subject and record during initial recording periods permitted interpretation of records from unobserved subjects. Thus, the device was adaptable to monitoring subject activity over prolonged periods without constant observation.

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Separation of 7Sγ_1-Globulin, 7Sγ_2-Globulin and Macroglobulin Guinea Pig Antibodies. (30817)

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Guinea pig immunoglobulins consist of at least 3 distinct types, as indicated by the work of Benacerraf, Block, Ovary and Franklin(1) and Uhr, Finkelstein, and Baumann (2). Two distinct functional types of 7S gamma globulins can be distinguished by their differing electrophoretic mobility in starch block, as shown by Benacerraf and coworkers (1): γ_1 or passive cutaneous anaphylactic type, and γ_2, or cytotoxic type. A third species of immunoglobulin was detected by Uhr and colleagues(2,3) in a fraction rich in macroglobulin and essentially free of 7S gamma globulins obtained by sucrose density gradient ultracentrifugation. This fraction contained the antibody activity against bacteriophage ϕ X 174 during the initial phase of the primary immune response.

These methods are in general quite satisfactory for preparation of small amounts of biologically active materials; however, neither method is capable of readily processing large amounts of serum.

In view of the possibility of separating γ_1 and γ_2 antibodies on the basis of electrical charge, it was of interest to reexamine a variety of well-known immunological phenomena, such as Arthus reactions and the hemorrhagic reaction(4) by passive transfer of large amounts of the fractionated gamma globulins into normal animals. To study the biological activity of macroglobulin antibody

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large amounts of material were also necessary.

With these objectives in mind, the experience of White(5) with guinea pig sera suggested that ion-exchange chromatography on DEAE-cellulose might serve to separate cleanly large amounts of the 2 gamma globulins. Similarly the successful purification of macroglobulins from the sera of other species by gel filtration(6) prompted the use of the same method for macroglobulin preparation.

This paper describes methods for the separation with varying degrees of purification of $7S\gamma_1$, $7S\gamma_2$, and macroglobulin antibody from relatively large volumes of sera.

Materials and methods. 1) *Antigens.* Ovalbumin (Ea, 2 × crystallized (Worthington)) and bovine gamma globulin (BGG) (Equivalent to Cohn Fraction II, Armour Pharmaceutical) were used as soluble protein antigens.

2) *Animals.* Hartley strain albino, random-bred, closed colony female guinea pigs weighing 250-350 g were used for preparation of hyperimmune anti-BGG and anti-Ea antisera and for passive cutaneous anaphylaxis titrations (PCA). Strain 13, an inbred, histocompatible(7) strain of guinea pig, was used for preparation of macroglobulin antibody as described below.

3) *Preparation of antisera.* Hyperimmune antisera rich in γ_1 and γ_2 antibody to soluble protein antigens were prepared by sensitization with antigen in complete and incomplete Freund's adjuvants as previously described (8). To obtain sera likely to have a maximal titer of macroglobulin antibody, donor animals were bled 9 days after having been sensitized with a single dose of soluble antigen in complete adjuvant(8). Serum was separated and pooled after centrifugation at 4°C and subsequently stored at −60°C.

4) *Assay for antibody activity.* Whole serum, undiluted column fractions, and concentrated, pooled fractions were analyzed for the types of antibody activity present. Thus samples were analyzed for total antibody activity by titration of hemagglutinating activity (HA), for specific contribution of $7S\gamma_1$ by PCA titration and for $7S\gamma_2$ by a modification of the hemolysis assay. We used a combination of HA and PCA titrations in searching for macroglobulin antibody activity in appropriate sera based on the fact that in our studies the PCA titer of $7S\gamma_1$ globulin was found to be essentially identical with its HA titer (± 1 two-fold dilutions) and the presence of γ_2 globulins could be demonstrated by hemolysin assay. Early sera (9th day) did not contain hemolytic antibody. Thus in such whole sera or the isolated fractions thereof, whenever HA titers were accompanied by significantly lower or negative PCA titers, the sample was presumed to contain a species of antibody distinct from $7S\gamma_1$ and $7S\gamma_2$ antibodies. This was confirmed by demonstrating the absence of hemolytic activity in pooled concentrated fractions which did have HA titer but lacked PCA activity. Thus it was possible to use a combination of HA and PCA titrations in searching for macroglobulin antibody activity in appropriate sera.

a) *Passive cutaneous anaphylaxis (PCA)* titrations were done following the method described by Ovary(9).

b) *Hemagglutination (HA)* was performed using techniques described in detail elsewhere(8).

c) *Passive immune hemolysis* was carried out by preparing samples (dilution, heating, adsorption) identical to those used for HA (8), except for use of Tris-complement buffer (TCB), pH 7.32, in place of phosphate buffer following the method described elsewhere(8).

5) *Immunoelectrophoresis* was performed by the method of Scheidegger(10) using Veronal buffer at pH 8.6.

6) *Disc electrophoresis on polyacrylamide gel* was done essentially as described by Ornstein and Davis(11).

Gel filtration was performed at 5°C on a column (2.5 × 120 cm) of Sephadex G-200 equilibrated with Tris complement buffer (TCB), pH 7.32. (0.04 M HCl, 0.05 M Tris, 0.098 M NaCl, 1.5×10^{-4} M Ca Cl_2, 5×10^{-4} M Mg $Cl_2 \cdot 6\ H_2O$, pH 7.5; pH adjusted to 7.32 with 1.0 N HCl.) Flow rate of this column was held to 15 ml/hr, consistent with an optimal flow rate for resolution of 3

TABLE I. Biological Activity Profile of Whole Anti-EA Antiserum and γ_1- and γ_2-Globulin Fractions.

	Total hemagglutinin units†	Total PCA units†	Total hemolysin units†
Anti-EA antiserum*	600,000	300,000	16,860
γ_1 fraction (2nd peak, DEAE-cellulose)	280,000	200,000	0
γ_2 fraction (1st peak, DEAE-cellulose)	180,000	0	10,710

* Pooled hyperimmune anti egg albumin serum prepared as described in text.
† Determined by multiplying titer/ml by total volume of starting serum or by total volume of recovered and concentrated fraction.

ml/hr/sq cm. The same column was used in all experiments.

Experimental and results. A. *Separation of γ_2 and γ_1 globulins from guinea pig serum.* Chromatography on DEAE-cellulose resulted in 2 well-separated, distinct peaks as depicted in Fig. 1. The fractions comprising the first peak, even after pooling and concentration, showed no *PCA activity* but contained considerable antibody activity as determined by *hemagglutination titration* and by *passive immune hemolysis* as shown in Table I. Pooled, concentrated fractions comprising the second peak had no hemolytic activity and contained all the PCA activity.

1) *Chromatographic separation of γ_1- and γ_2-globulins by stepwise elution.* In a typical experiment, 15 ml of hyperimmune guinea pig antiserum was dialyzed for 24 hours at 5°C against 2 changes of 1 l each of starting buffer (.009 M K_2HPO_4, .001 M KH_2PO_4), pH 7.9. The dialyzed sample was centrifuged to remove a small amount of precipitate and chromatographed on DEAE-cellulose (Whatman DE-1) containing 1.0 meq N/g. The column (2 × 30 cm) was packed at room temperature with 15 lb air pressure(12) and was thoroughly equilibrated with starting buffer at 5°C. The sample was pumped on the column and eluted with starting buffer at a flow rate of 60 ml/hr until the protein concentration of the eluate was minimal as estimated by measuring absorption at 280 mμ. Limit eluant (0.282 M-K_2HPO_4 0.018 M KH_2PO_4) pH 7.9, was then applied to elute the protein remaining on the column. Eluates were concentrated by ultrafiltration to the volume of the original serum sample applied.

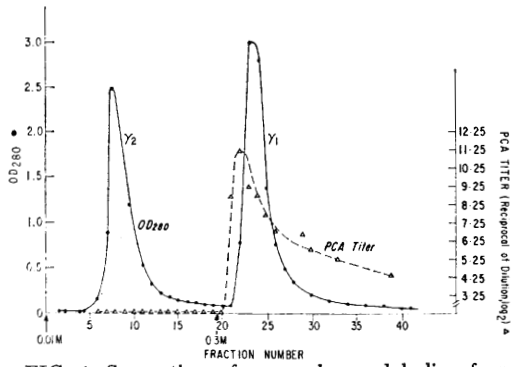


FIG. 1, Separation of γ_2- and γ_1- globulins from guinea pig serum by chromatography on DEAF-cellulose.

2) *Immunoelectrophoresis* of these concentrated peaks as shown in Fig. 2 demonstrated that chromatography had distinctly separated the specific gamma globulin antibody (anti-EA) into the 2 electrophoretic components γ_1 and γ_2 previously described by others(1, 5).

3) *Disc electrophoresis* on polyacrylamide gels (Fig. 3) confirmed the separation of the 2 γ-globulin species and demonstrated the multiple proteins present in the γ_1 and the lack of contamination of the γ_2 fraction with other serum proteins.

B. *Separation of macroglobulin antibody from small amounts of guinea pig immune sera on Sephadex G-200.* Fig. 4 depicts the effluent diagram and the PCA profile of 5 ml of early phase whole serum from donors undergoing a primary immune response following sensitization with ovalbumin. This sera had no hemolytic activity but considerable hemagglutinating activity. The separation of the 2 initial peaks was not complete and there was an overlap of γ_1 activity. The concentrate of the pooled fractions of the first peak, combined up to the point of appearance of positive PCA, showed a substantial amount of hemagglutinating activity without detectable hemolytic titer. Immunoelectro-

phoresis of this same fraction demonstrated precipitin lines with goat-anti guinea pig serum in the regions of lipoproteins and α-globulins; however, no specific precipitate with antigen was detected by either immunoelectrophoresis or Ouchterlony techniques. Analytical ultracentrifugation of this fraction after repeated freezing and thawing, shows a peak approximately 16S, and a relatively broad region comprised of components approximately 2S.

C. *Separation of macroglobulin antibody from large amounts of guinea pig immune sera.* The progressive purification of macroglobulin antibody is depicted in Fig. 3 by disc electrophoretic patterns of the various fractions after salting out, DEAE-cellulose chromatography, and gel filtration on Sephadex G-200.

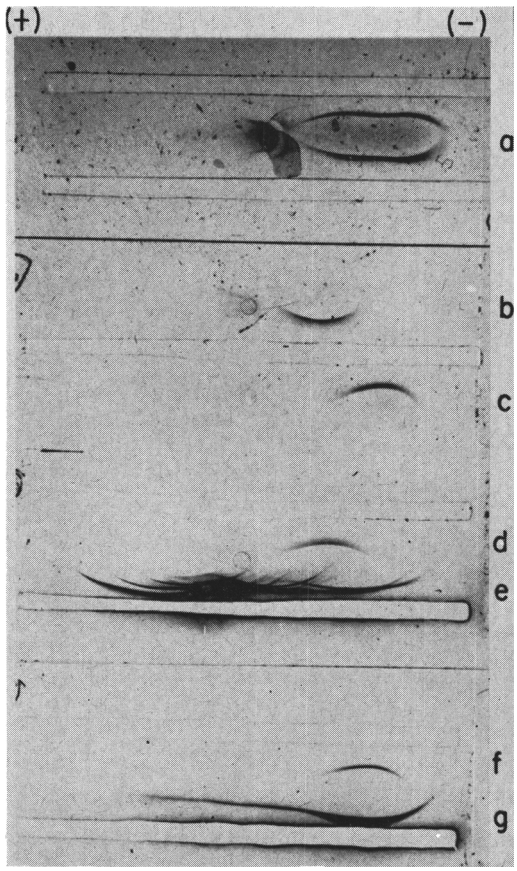


FIG. 2. Immunoelectrophoresis: Well (a): Anti-Ea whole serum; both troughs: Ea. Well (b): γ_1-globulin fraction; trough: Ea; Well (c): γ_2-globulin fraction. Trough (d): Ea; well: γ_1-globulin fraction. Trough (e): goat anti-guinea pig serum. Trough (f): Ea; well: γ_2-globulin fraction. Trough (g): goat anti-guinea pig serum.

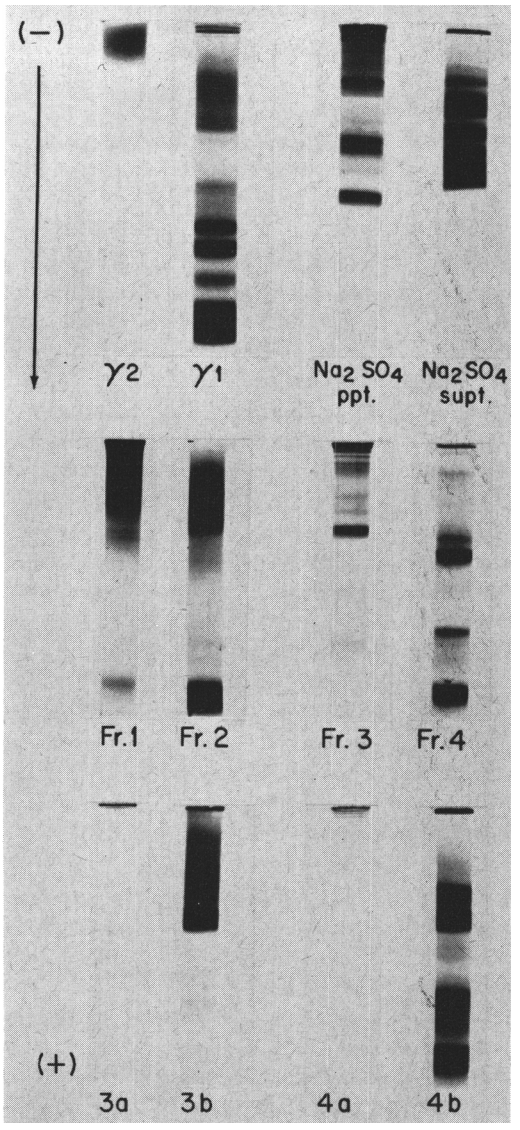


FIG. 3. Disc Electrophoresis patterns of γ_2 and γ_1 globulins obtained by DEAE-cellulose chromatography and fractions obtained during the purification of macroglobulin antibody: salt fractionation, chromatography, (Fr. 1, 2, 3 and 4). Gel filtration, 3a, 3b from Fr. 3 and 4a, 4b from Fr. 4.

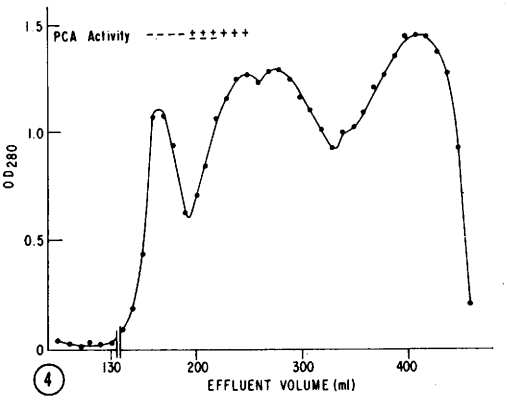
1) *Sodium sulfate precipitation* (18% w/v) of 170 ml of serum left the supernatant free of detectable PCA activity. A small residual amount of hemagglutinating activity remained (Table II). After centrifugation,

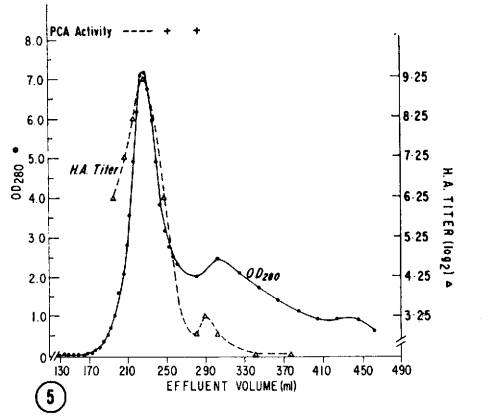
the precipitate was suspended in 10 ml of 0.035 M phosphate buffer, pH 7.5 (0.03 M $Na_2 HPO_4$; 0.005 M $NaH_2 PO_4 \cdot H_2O$) and then dialyzed for 24 hr at 5°C against 2 changes of 4 l each of this buffer.

2) *DEAE-cellulose chromatography.* The dialyzed sample was chromatographed on DEAE-cellulose (Whatman developmental product,† DE 33, 1 meq N/g). The column (1.9 × 40 cm) was packed under a hydrostatic head of 30 cm at room temperature and was then thoroughly equilibrated with the 0.035 M phosphate buffer, pH 7.5 at 5°C. After sample application, the column was washed with this same buffer at a flow rate of 60 ml/hr. The initial protein eluted as a very diffuse, red-colored zone after a hold up volume of 55 ml had passed through the column. After collection of this red-colored zone in an additional 55 ml of effluent (Fraction 1), an intense red-brown band (2 inches in width) remained tightly adsorbed at the top of the column. Limit eluant (0.5 M Tris, 0.5 M $H_3 PO_4$) pH 3.7, was then applied. The tightly adsorbed red-brown band desorbed and separated into a yellow and a red zone. Fraction 2 (40 ml) was collected until the yellow band reached the bottom of the column, whereupon Fraction 3 (55 ml) was collected until the red band reached the bottom of the column. Fraction 4 (red band) was collected in 30 ml of effluent. At this point no visible color remained on the column and optical density measured at 280 mμ was negligible. All fractions were concentrated by ultrafiltration to volumes ranging from 5-8 ml and prior to bioassay were dialyzed against Tris complement buffer (TCB), pH 7.32.

3) *Gel filtration on Sephadex G-200.* The elution patterns, hemagglutination profiles and PCA distributions resulting from individual gel filtrations of DEAE-fractions 3 and 4 are depicted in Fig. 5 and 6, respectively. As shown in both instances, hemagglutination titers followed the relative protein concentration of the initial major peak. Fractions of each first peak devoid of PCA titer were pooled and concentrated. This pu-

† Supplied by H. Reeve Angel & Co., Inc., Clifton, N. J.





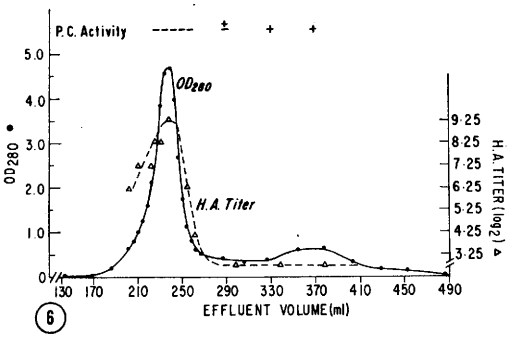


FIG. 4. Gel filtration on Sephadex G-200: Separation of macroglobulin antibody from 5 ml of guinea pig immune sera. Negative PCA titer of undiluted fractions (—); positive PCA titer of undiluted fractions (+).

FIG. 5. Gel filtration on Sephadex G-200: Separation of macroglobulin antibody from DEAE-fraction 3. Negative PCA titer of undiluted fractions (—); positive PCA titer of undiluted fractions (+).

FIG. 6. Gel filtration on Sephadex G-200: Separation of macroglobulin antibody from DEAE-fraction 4. Negative PCA titer of undiluted fractions (—); Positive PCA titer of undiluted fractions (+).

TABLE II. Biological Activity Profile of Fractions Obtained from Early Phase Antisera.

	Total hemagglutinin units†	Total hemolysin units†	Total PCA units†
Whole serum* (anti-EA)	108,800	0	30,000
Na_2SO_4 supernatant‡	18,800	n.d.	0
DEAE-fraction 1	15,000	n.d.	30,000
DEAE-fraction 3 (1st peak, Sephadex G-200)	12,600	0	0
DEAE-fraction 4 (1st peak, Sephadex G-200)	9,000	0	0

* Pooled sera (170 ml) from 28 donors bled 9 days after sensitization (free of hemolytic activity).

† Determined by multiplying titer/ml by total volume of starting serum or by total volume or recovered and concentrated fraction.

‡ Na_2SO_4 precipitate served as starting material for chromatography on DEAE-cellulose; Na_2SO_4 supernatant activity represents loss of antibody activity.

n.d.—not determined.

rified and concentrated material demonstrated a high hemagglutination activity (Table III), showed no PCA activity and in all other particulars behaved identically to the material obtained in the first peak after gel filtration of whole serum. The electrophoretic profiles of these concentrated materials (Fig. 3, 3a and 4a) show that they contain essentially only macroglobulins.

Discussion. Our results demonstrate that it is feasible to obtain relatively large amounts of highly purified γ_2-globulins from guinea pig sera by employing simple 2-step elution chromatography on DEAE-cellulose. This method can easily be modified to handle large volumes of sera. The chromatographic technique leaves the γ_1-globulins distributed among the remaining serum proteins. It is likely that salting out and additional chromatography would produce further purification of the γ_1-globulins. To obtain macroglobulin antibody from whole immune sera the method of gel filtration is somewhat limited. Only relatively small amounts of sera (5 ml) can be handled on large Sephadex columns. However, our results show that salt fractionation of whole serum, followed by ion-exchange chromatography provided a method of obtaining from large volumes of sera (170 ml) highly concentrated fractions greatly enriched in macroglobulin content. Further purification of this material by gel filtration resulted in a fraction which contained essentially only macroglobulin, was rich in specific hemagglutinating activity, and devoid of γ_1- and γ_2-globulins as shown by the lack of PCA or hemolysis activity. The antibody activity found in the macroglobulin fraction could be demonstrated only by hemagglutination and was not detected by precipitin reactions. This is in accord with findings reported by Grey(13).

Table I shows the approximate yields of the $7S\gamma_1$ and $7S\gamma_2$ fractions obtained in one experiment. Relative recovery of total antibody based on HA titer was approximately 70%. The approximate recovery values for $7S\gamma_1$ antibody (PCA) and for $7S\gamma_2$ antibody (hemolysin units) were also in the range of 70%. It can be seen from Table II that only approximately 20% of macroglobulin associated antibody (HA units) was found in fractions resulting from gel filtration of DEAE fractions 1 and 3, respectively. A significant amount of antibody activity ($\sim$ 20%) was left in the sodium sulfate supernatant which could possibly be purified further by ion-exchange chromatography and gel filtration to recover more macroglobulin antibody. Although the relative insensitivity of hemagglutinating titer as means to detect macroglobulin antibody has to be kept in mind, it is possible that the low recovery of HA titer units, aside from losses due to experimental techniques, could be due to the instability of macroglobulin antibody. This seems to be especially the case when repeated freezing and thawing was employed or when samples were not stored at $-60°C$. Instability of macroglobulin antibody was demonstrated when gel filtration peak fractions (3a and 4a) were allowed to remain at 5°C for 24 hours. Disc electrophoretic patterns of these fractions then exhibited an additional 4 to 6 fast

moving electrophoretic components (not shown in Fig. 3). In contrast, these same fractions showed only the 3 or 4 typical slow moving macroglobulin bands (Fig. 3, 3a, 4a) when fractions 3a and 4a were analyzed immediately after gel filtration.

Although passive hemagglutination is a relatively insensitive method for detecting macroglobulin antibody, it lent itself to detection of a significant amount of macroglobulin associated antibody. Those(14) working with natural erythrocyte antigens or bacterial endotoxins adsorbed on erythrocytes have found that immune hemolysis provides enhanced sensitivity in detecting macroglobulin antibody. Under the conditions employed during this study, the macroglobulin associated antibody to soluble protein antigens had no passive hemolytic activity. This particular finding also lent further weight to the specificity of the hemolysin assay for γ_2-globulin antibody.

Our findings demonstrate the appearance of macroglobulin antibody to soluble protein antigens during primary immune response. The biological activity and physical-chemical characteristics of this macroglobulin antibody remain to be further investigated.

Summary. Methods have been described for separating the γ_1- and γ_2-globulin antibodies and the macroglobulin antibody of guinea pig sera. Use of simple stepwise elution chromatography on DEAE-cellulose results in extensive purification of γ_2-globulin antibody. Purified macroglobulin antibody was prepared from relatively large amounts (170 ml) of appropriate sera by combined use of salting out, ion-exchange chromatography and gel filtration.

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Passive Immune Hemolysis: Titration of Hemolytic Anti-Protein Antibody Using Bis-Diazotized-Benzidine to Couple Antigen to Erythrocytes. (30818)

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Recent advances in the separation of antibody moieties on the basis of their biological activity have decreased the importance of precipitin and hemagglutination determinations of antibodies to the understanding of biological phenomena mediated by immunological mechanisms. The macroglobulin antibodies (IgM) in the case of rabbit and guinea pig precipitate poorly in relationship to their hemagglutination or bacteriophage plaque inhibition titers(1-3), and their biological function remains incompletely defined. The guinea

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