

PROPERTIES OF GUINEA PIG 7S ANTIBODIES

I. ELECTROPHORETIC SEPARATION OF TWO TYPES OF GUINEA PIG 7S ANTIBODIES*

By BARUJ BENACERRAF, M.D., ZOLTAN OVARY, M.D., KURT J. BLOCH,† M.D.,
AND EDWARD C. FRANKLIN,§ M.D.

*(From the Departments of Pathology and Medicine, New York University
School of Medicine, New York)*

(Received for publication, January 25, 1963)

Antibodies can be considered to be molecules with two distinct sets of properties: the capacity to react specifically with antigen, and the capacity to mediate a variety of biological reactions. Some of these are: anaphylaxis, which in the guinea pig may depend upon the ability of these molecules to react with unidentified tissue receptors (1) or cell lysis (2, 3) and opsonization (4, 5) which results from their ability to fix complement components. The combination of these two sets of properties on the same molecule ensures that the biological activity mediated by antibodies is selectively directed.

Recent studies have revealed how the structure of 7S mammalian γ -globulins (6, 7) and of specific guinea pig antibodies (8) may explain this dual function of antibodies. Human 7S γ -globulins have been shown to be composed of two types of polypeptide chains (7) designated L (light) and H (heavy) (9) which, after reduction and alkylation, can be separated by starch gel electrophoresis in urea. Guinea pig antibodies of widely different specificities obtained from single animals yielded upon reduction and alkylation L chains which differed in number and electrophoretic mobility in starch gel in urea. The electrophoretic patterns were characteristic for a given specificity (8, 10). Furthermore, the L chains from specific guinea pig antihapten antibodies have been found to be contained in those fractions of the antibody molecule obtained by papain cleavage, which contain the antibody combining site (S fragments, analogous to Porter pieces I and II) (10). It is reasonable to consider that the L chains of antibodies may be concerned with the acquisition of immunological specificity (9). On the other hand, the major portion of the H chains of 7S γ -globulin or specific antibody appears to be contained in that fraction of the papain digest (piece III of Porter, F fragment) which lacks antibody activity but which in the case

* Supported by the United States Public Health Service (Grants AI-04983 and AI-03075) and by the Health Research Council of the City of New York under Contract Nos. I-138 and I-140.

† Special Investigator, Arthritis and Rheumatism Foundation.

§ Senior Investigator, Arthritis and Rheumatism Foundation.

of rabbit 7S γ -globulin has been shown to be able to fix complement when aggregated (11) and to fix to guinea pig skin to mediate reverse passive anaphylaxis (12). These biological properties should therefore depend on the structure of the H chains of 7S antibodies (9).

The question arises whether a single antibody molecule is able to mediate more than one type of biological activity or whether different antibodies with identical immunological specificity are responsible for complement fixation, skin fixation, or lysis. To explore these possibilities, a study was made of the antibodies produced by guinea pigs undergoing hyperimmunization with and without Freund's adjuvants. Guinea pigs were used for this study because (*a*) high levels of antibody could be obtained from single animals (*b*) this animal species is particularly interesting immunologically for the variety and intensity of hypersensitivity reactions it can undergo, and (*c*) previous studies on structure were made with specific antibodies similarly prepared (8, 10).

Irrespective of immunological specificity, two distinct 7S antibody populations with slightly different electrophoretic mobilities have been identified in immune sera from individual guinea pigs (13). Besides sharing common antigens, these antibodies were also found to possess distinct antigenic components. These properties will be discussed in this paper. In the following papers, the ability of the two types of antibodies to transfer passive anaphylaxis and to fix complement will be investigated.

Materials and Methods

Animals.—Hartley strain guinea pigs of either sex, weighing 300 to 500 gm were used for immunization.

Antigens.—Hen ovalbumin, 2 times crystallized (Worthington Biochemical Corporation, Freehold, New Jersey). Bovine gamma globulin, bovine fibrinogen (Armour Pharmaceutical Company, Kankakee, Illinois). Guinea pig serum albumin was prepared by starch block electrophoresis and checked for purity by immunoelectrophoresis.

Conjugated Antigens.—Various protein conjugates were prepared according to the technique previously described (14). All antigens were highly conjugated. 2,4-dinitrophenyl-bovine gamma globulin (DNP-BGG), 2,4-dinitrophenyl-guinea pig albumin (DNP-GPA), and 2,4-dinitrophenyl-beef fibrinogen (DNP-fibrinogen) were prepared with 2,4-dinitrofluorobenzene (Eastman Organic Chemicals, Rochester, New York).

Picryl-guinea pig albumin (Pic-GPA) was prepared with recrystallized picryl chloride (Eastman Organic Chemicals). Pentachlorobenzoyl-guinea pig albumin was prepared with pentachlorobenzoyl chloride (DX-261) obtained from E. I. DuPont De Nemours & Co., Inc. Wilmington.

Immunization.—Most of the animals studied were immunized with complete adjuvants. The antigens, dissolved in 0.15 M NaCl, were emulsified with an equal amount of complete adjuvants (Difco Laboratories, Inc., Detroit) and injected into the 4 foot-pads; 0.1 ml per foot in a dosage ranging from 0.1 to 1 mg per animal. Two weeks later, the animals were reinjected intradermally into each of 4 sites with 0.1 mg of antigen in 0.1 ml of 0.15 M NaCl. This procedure was repeated weekly until severe Arthus reactivity was observed. The animals were exsanguinated under nembutal[®] anesthesia 7 days after the last skin test, usually 4 to 6 weeks from the beginning of immunization; the sera harvested, and kept frozen until used.

Guinea pigs immunized without adjuvants were injected with 1 mg of DNP-BGG intraperitoneally on 3 consecutive days. Four weeks later, and every 10 days for 3 additional doses, the animals were injected intradermally with 0.1 mg antigen in each of 4 sites. A week later, the animals were bled.

Immuno-electrophoresis.—A modification of Scheidegger's technique (15) for agar gel immuno-electrophoresis was generally used. Two per cent agar in a barbital buffer pH 8.6, ionic strength 0.075, was poured on $3\frac{1}{4}$ by 4 inch lantern slides, which had been precoated around the periphery with 10 per cent agar and allowed to dry. In a first series of experiments, 0.01 or 0.02 ml of antiserum was placed in the center well. After electrophoresis for 105 minutes at 24 ma and 200 to 220 volts, 0.1 ml of the corresponding antigen solution containing 0.2 mg/ml was placed in each trough and the slides incubated at room temperature for 2 days. After washing in saline, the slides were dried and stained with amido black 10B by the method of Uriel and Scheidegger (16). In a second series of experiments, samples of purified antibody preparations obtained from single animals were placed in the center wells and the slide developed with antisera prepared in rabbits against pooled guinea pig serum. Two antisera were used, serum R1 and serum R2, which had been prepared by hyperimmunizing individual rabbits with pooled guinea pig serum emulsified in complete adjuvants.

Preparation of Purified Antibodies.—Purified antibodies were prepared as previously described (8). Antibodies specific for the 2,4-dinitrophenyl group were isolated from sera of individual animals immunized with DNP-BGG; antipicryl antibodies were isolated from sera of individual guinea pigs immunized with picryl-GPA. Sera were first decomplexed with rabbit antiovalbumin and ovalbumin. Antihapten antibodies were then isolated by specific precipitation with DNP-fibrinogen or picryl-fibrinogen at equivalence. After washing 3 times in cold saline, the precipitates were dissolved with *N*-DNP- ϵ -L-lysine or with picryl- ϵ -aminocaproic acid at a concentration of 2×10^{-3} M in 0.15 M NaCl. The conjugated fibrinogen was then precipitated by adding 35 mg of streptomycin per ml (17). After dialyzing away excess free hapten, the purified antibody preparations were used for studies. In selected experiments, the amount of picryl-fibrinogen remaining in the purified antibody preparation was measured using I^{131} -labeled picryl-fibrinogen, and was always found to be less than 0.5 per cent of the final protein concentration. Because of the very high binding affinity of the guinea pig anti-DNP and antipicryl antibodies, some bound hapten was always present in our preparations.

Starch Block Electrophoresis.—Electrophoresis on starch block was carried out as described by Kunkel (18) using barbital buffer pH 8.6, ionic strength 0.1. Phosphate buffer pH 7.5, ionic strength 0.2, was used in the electrode jars. The electrophoretic separations were performed for 24 hours at 85 ma and at a voltage of 400 to 440 volts. Then, $\frac{1}{2}$ inch cuts were made, starting at the cathode side generally 5 inches from the origin, and fractions extracted with 7 ml of 0.15 M NaCl. The protein concentration of eluates was measured by the Folin-Ciocalteu technique (19).

Analytical Ultracentrifugation.—Analytical ultracentrifugation was performed in a Spinco model E ultracentrifuge equipped with phase plate schlieren optics. All studies were performed at 20°C in 12 mm double sector cells. To permit direct visual comparison of two solutions examined simultaneously, one cell was fitted with a wedge quartz window.

RESULTS

I. Immuno-electrophoresis of Guinea Pig Hyperimmune Sera Developed with Antigen.—Immuno-electrophoresis of sera obtained from guinea pigs hyperimmunized with ovalbumin or with DNP-BGG in complete adjuvants was carried out and the corresponding antigens, ovalbumin or DNP-GPA, were placed in the trough to identify the mobility of the specific antibodies. A typical

experiment, in which 4 antiovalbumin and 4 anti-DNP-BGG sera were examined, is presented in Fig. 1. With each serum, the antibody activity against ovalbumin or DNP is found to distribute itself into two distinct arcs in the zone of migration of the 7S gamma globulins. Identical experiments were carried

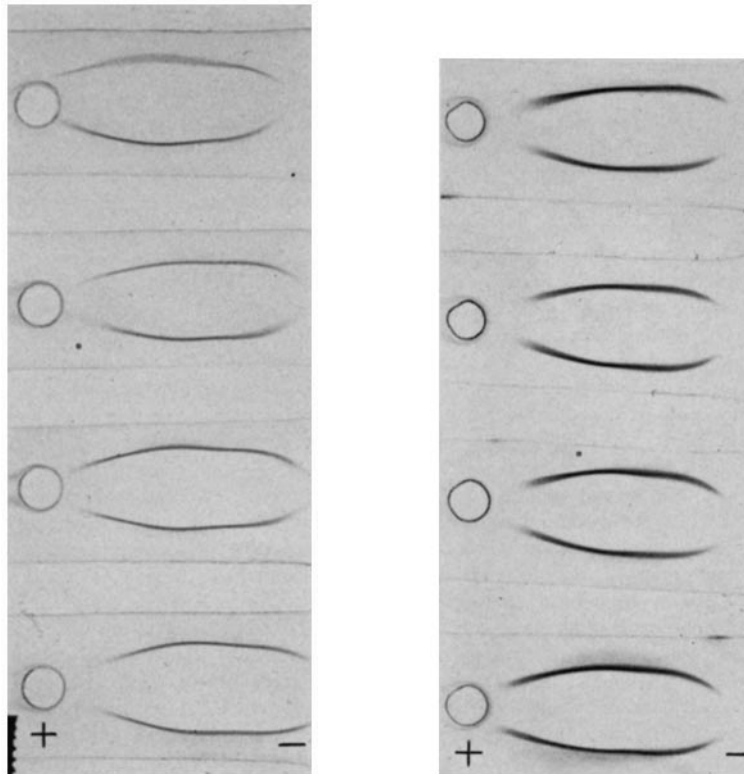
FIG. 1 *a*FIG. 1 *b*

FIG. 1. Immunoelectrophoresis of 4 guinea pig antiovalbumin (*a*) sera, Ea 22, 23, 25, 26, and 4 anti-DNP-BGG sera (*b*), DNP 5532, 5533, 5534, 5539. Wells were filled with 0.01 ml of guinea pig antisera; troughs were filled with 0.1 ml of ovalbumin or DNP-GPA, 0.2 mg/ml.

out with antisera from animals similarly immunized against picryl-GPA and pentachlorobenzoyl-guinea pig albumin. More than 50 sera were studied and similar patterns were found in all cases. This observation suggested that guinea pigs hyperimmunized against protein antigens or hapten conjugates emulsified in complete adjuvants were able to synthesize two types of antibodies with slightly different electrophoretic mobilities.

The time of appearance of these antibodies in the course of immunization was also investigated. Guinea pigs were immunized with DNP-BGG in com-

plete adjuvants and bled at weekly intervals starting 2 weeks after initial immunization. The immunoelectrophoretic patterns of sera from a representative animal are shown in Fig. 2. "Slow" migrating antibody generally appeared earlier and "fast" migrating antibody later in the course of immunization with adjuvants.

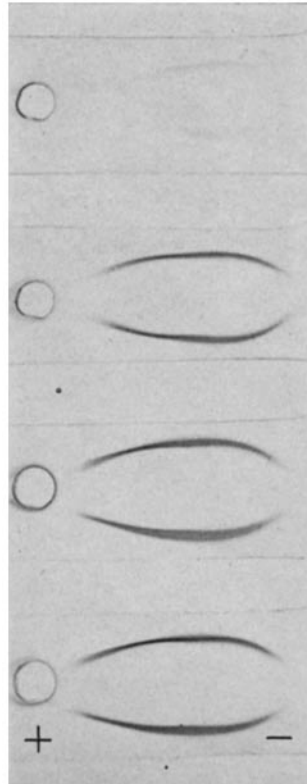


FIG. 2. Immunoelectrophoresis of serum from guinea pig W6 immunized with DNP-BGG in complete adjuvants and bled at weekly intervals starting 2 weeks after initial immunization. Wells were filled with 0.01 ml serum obtained 2 (top), 3, 4, and 5 weeks (bottom) after start of immunization; troughs were filled with 0.1 ml of DNP-GPA, 0.2 mg/ml.

A few guinea pigs were initially immunized with DNP-BGG administered intraperitoneally without adjuvants, and were repeatedly boosted intradermally. Four of these animals made a sufficient amount of anti-DNP antibodies to allow the sera to be studied and the antibodies to be isolated. The results of immunoelectrophoresis performed with these sera and developed with antigen are presented in Fig. 3. In contrast with animals immunized with the same antigen emulsified in complete adjuvants, animals initially immunized

by the intraperitoneal route appeared to produce primarily fast migrating antibodies.

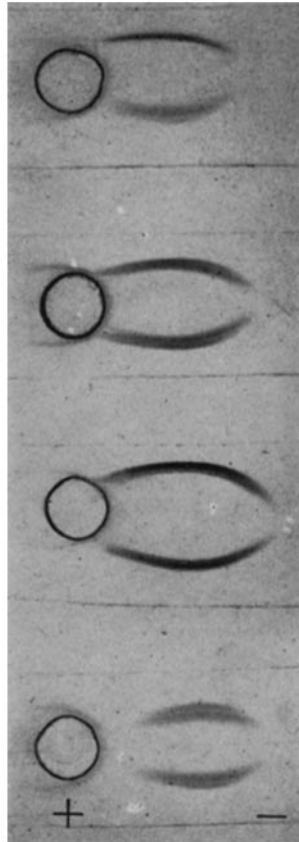


FIG. 3. Immunoelectrophoresis of 4 guinea pig anti-DNP-BGG sera I.P. 1, 2, 3, and 4 produced in animals initially immunized intraperitoneally without adjuvants. Wells were filled with 0.02 ml guinea pig antisera, troughs were filled with 0.1 ml of DNP-GPA, 0.2 mg/ml.

II. Studies with Purified Antihapten Antibodies.—Purified antibodies from individual guinea pigs immunized with either picryl-GPA or DNP-BGG in complete adjuvants, or with DNP-BGG without adjuvants were isolated. These purified antibody preparations were subjected to electrophoresis on starch block and agar gel. In the latter experiments, rabbit anti-guinea pig sera were used to determine whether fast and slow migrating antibody fractions could be shown to possess distinct antigenic components.

Fig. 4 presents a comparison of the migrations on separate starch blocks of purified antibodies isolated from serum of an animal immunized with complete

adjuvants (antipicryl-GPA preparation B) and of antibodies isolated from serum of an animal immunized without adjuvants (anti-DNP-BGG preparation I.P.3).

Differences in the relative concentrations of slow and fast migrating fractions in these two preparations are apparent. Antibodies produced by guinea pigs immunized without adjuvants are almost entirely of the fast migrating type, although slow and fast components are present in both preparations.

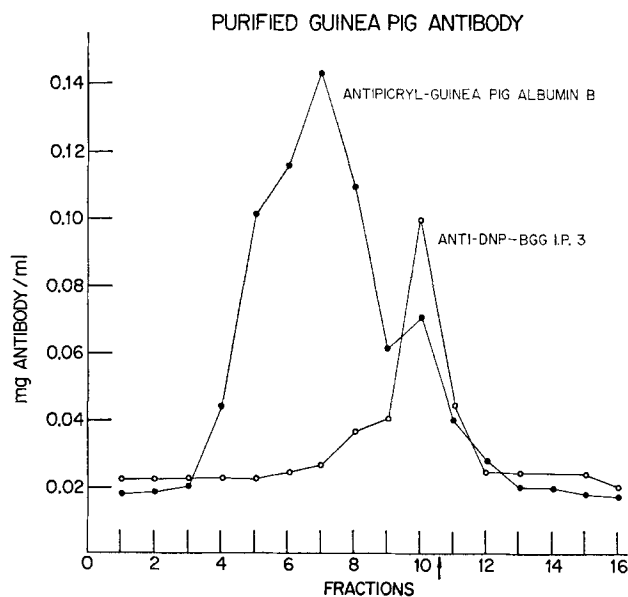


FIG. 4. Comparison of electrophoretic mobility on separate starch blocks of purified guinea pig antipicryl-GPA antibodies (preparation B) obtained following immunization with adjuvants and anti-DNP-BGG antibodies (preparation I.P. 3) obtained after immunization without adjuvants. Arrow indicates site of application of antibody on starch blocks.

Immunoelectrophoresis of purified antipicryl-GPA preparation B was performed and rabbit anti-serum R1 (obtained from a rabbit hyperimmunized with pooled guinea pig sera) was used to develop precipitin lines; the patterns obtained are presented in Fig. 5 *a*. Two distinct arcs, corresponding to two antipicryl antibodies of different electrophoretic mobilities can be seen in the region defined by the precipitin lines of the main 7S gamma globulin component of serum. Furthermore, with rabbit serum R1, the two arcs are seen to cross each other in a reaction of only partial identity. The fast and slow migrating antibodies, which may be designated guinea pig γ_1 and guinea pig γ_2 therefore possess certain different antigenic components. In addition, they also have certain antigens in common. This is more clearly demonstrated by the patterns

obtained with another rabbit anti-serum R2 (Fig. 5 *b*), which is weak with respect to antibodies against the distinct antigenic component of the fast antibody.

Similar immunoelectrophoretic studies were made with isolated antibody

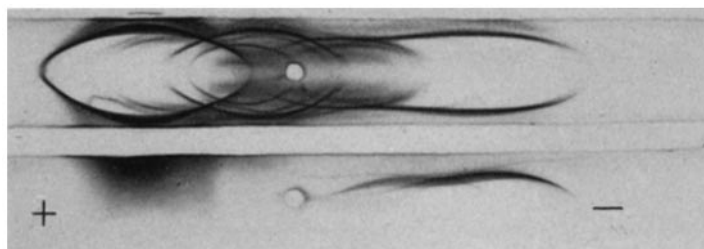


FIG. 5 *a*

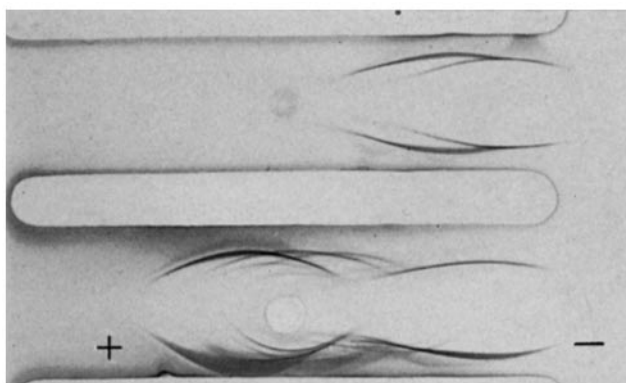


FIG. 5 *b*

FIG. 5 *a*. Immunoelectrophoresis of normal guinea pig serum (top) and purified guinea pig anticryl-GPA antibodies (preparation B) (bottom), developed with rabbit anti-guinea pig whole serum R1 in troughs.

FIG. 5 *b*. Immunoelectrophoresis of purified guinea pig anti-DNP-BGG antibodies (DNP 68) (top) and normal guinea pig serum (diluted 1:5) (bottom), developed with rabbit anti-guinea pig whole serum, R2 in troughs.

preparations from animals immunized with DNP-BGG without adjuvants. The patterns obtained are found in Fig. 6. Although the concentration of the slow migrating antibody fraction is low, the two types of antibodies (γ_1 and γ_2) can still be identified in each serum with the help of a suitable rabbit anti-serum. In some of the preparations, additional precipitin lines can be seen in the slow and fast antibody region. These extra lines cannot be interpreted at this time.

The molecular weights of the two types of guinea pig antibodies γ_1 and γ_2 , were compared by performing ultracentrifugal analysis of slow and fast migrat-

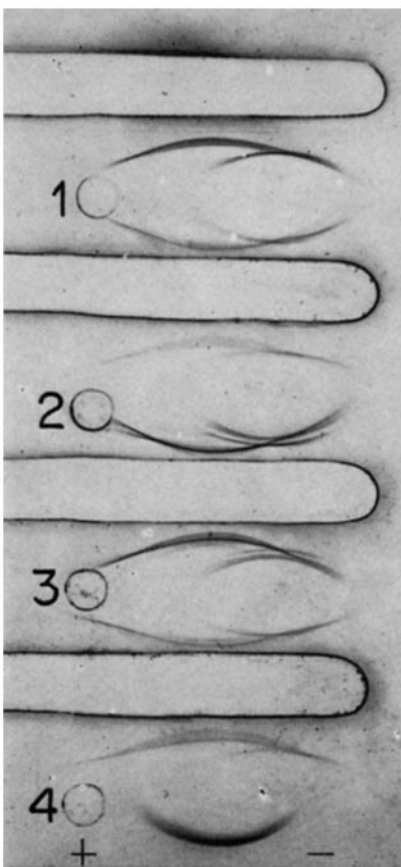


FIG. 6. Immunoelectrophoresis of purified guinea pig anti-DNP-BGG antibodies from individual animals initially immunized intraperitoneally without adjuvants developed with rabbit anti-guinea pig serum or antigen. Wells were filled with 0.01 ml of antibody solutions from guinea pig I.P. 1 (well 1), guinea pig I.P. 2 (well 2), and guinea pig I.P. 3 (wells 3 and 4). Troughs were filled with 0.1 ml of serum from rabbit R2 (top trough), serum from rabbit R1 (second trough), serum from rabbit R1 (fourth trough), and 0.1 ml of DNP-BSA, 0.2 mg/ml (in bottom trough).

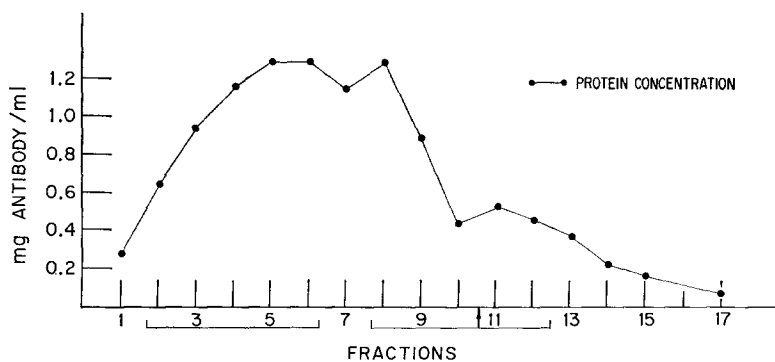


FIG. 7. Electrophoretic separation on starch block of guinea pig anti-DNP antibodies (pool 3, 7, 9). Fractions 2 to 6 and fractions 8 to 12 were combined in order to study the sedimentation behavior of slow and fast antibodies (Fig. 8). Arrow indicates site of application of antibody on starch block.

ing fractions obtained from starch block electrophoresis of an isolated anti-DNP antibody pool prepared from sera of guinea pigs 3, 7, 9, immunized with DNP-BGG in complete adjuvants. In selecting the fractions to be included in

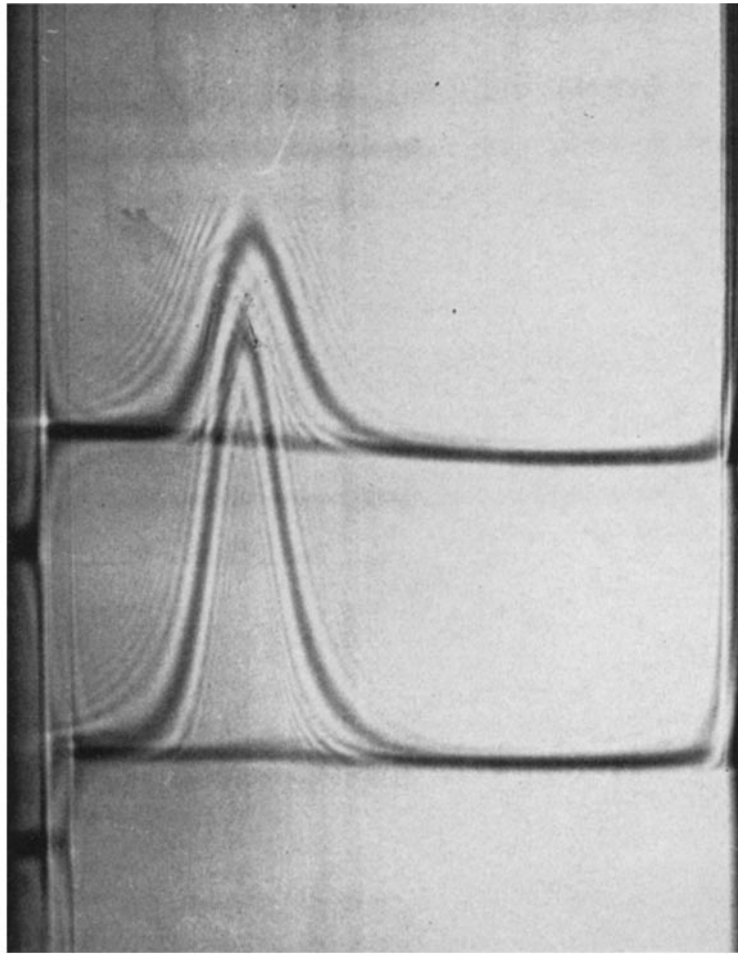


FIG. 8. Ultracentrifugal patterns of slow (fractions 2 to 6) (top) and fast (fractions 8 to 12) (bottom) guinea pig anti-DNP antibodies (pool 3, 7, 9). (See Fig. 7).

Photograph taken after 80 minutes at 52,640 RPM.

the two pools, we were guided by the results of passive cutaneous anaphylaxis (PCA) tests obtained with these fractions. It will be later shown that the ability to sensitize guinea pig skin for PCA is a property of γ_1 antibodies (20). Fractions 8 to 12 contained antibodies able to sensitize guinea pig skin for PCA; antibodies present in fractions 2 to 6 lacked this property (Fig. 7).

The analytical ultracentrifugation patterns of these slow and fast fractions are shown in Fig. 8. Both fractions sedimented as a single homogeneous peak. The observed sedimentation coefficients were 6.3S and 6.5S respectively. Slight differences in sedimentation coefficient may be attributed to differences in protein concentration of the two samples. These values indicate that the two types of guinea pig antibody belong to the 7S group of gamma globulins.

DISCUSSION

Specific antibodies produced by individual guinea pigs hyperimmunized against a variety of soluble antigens have been found to be composed of two distinct antibody populations with sedimentation coefficients of approximately 7S and different electrophoretic mobility. These two types of antibodies which we propose to call guinea pig 7S γ_1 and 7S γ_2 possess common as well as distinct antigenic determinants. Similar results have been reported by Yagi, Maier, and Pressman (21) who used I¹³¹I-labeled insulin to identify two types of guinea pig antiinsulin antibodies which differed in electrophoretic mobility.

Within a given immunological specificity, these two types of guinea pig antibodies will be shown in our experiments to mediate different biological reactions in this animal species (20, 22). Since these two antibody types can be produced against the same antigenic determinant, they may be considered, in keeping with the role assigned to the L and H chains of 7S mammalian gamma globulins (9), to differ primarily in their H chains.

Slow migrating γ_2 antibody is formed in largest amounts in animals initially immunized with antigens emulsified in complete adjuvants and seems to appear earlier in the course of hyperimmunization. Sera of animals immunized intraperitoneally without adjuvants have been found to contain predominantly fast migrating γ_1 antibody. Several problems deserving investigation must be considered: Are the two types of antibody synthesized by the same cells? Are they both formed in lymph nodes and spleen? How does the method of immunization determine the type of antibody produced?

It is also pertinent to discuss the relationship of the two types of guinea pig antibodies to different types of 7S gamma globulin produced in other mammalian species.

Relyveld has described the production of two types of 7S antibodies in horses hyperimmunized with diphtheria toxin (23). After short periods of immunization, antibody activity appeared in the γ_2 fraction but on continued immunization, antitoxin migrating as γ_1 (T component) was formed. It is now well established that in man (24) and in the mouse (25), there are two types of 7S γ -globulins which differ in their electrophoretic mobility, slow migrating γ_2 and a fast migrating γ_{1A} (also called β_{2A}), and which differ in the antigenic components (26) of their F fragments. Both types have antibody activity. It is tempting to conclude that the 7S guinea pig γ_1 globulin corresponds to the γ_{1A} of man, horse, and mouse since no other γ_{1A} component has yet been identi-

fied among guinea pig antibodies. However, such a conclusion might be erroneous because additional antibody types may yet be found among the guinea pig 7S γ -globulins. A uniform nomenclature for mammalian 7S gamma globulins should be based on similarities of structure, and biologic function. Until more is known about the biological properties of the various antibody types in the species under discussion, it would seem advisable not to generalize prematurely. We therefore propose that, for the present, the 7S guinea pig antibodies described in this study be referred to as guinea pig 7S γ_1 and 7S γ_2 .

SUMMARY

Guinea pigs hyperimmunized with single protein antigens or hapten conjugates emulsified in complete adjuvants produced two types of precipitating antibodies with different electrophoretic mobilities. "Slow" migrating antibody generally appeared earlier and "fast" migrating antibody later in the course of immunization. Animals initially immunized by the intraperitoneal route with hapten conjugates without adjuvants produced primarily fast migrating antibody. Purified guinea pig antibodies were also separable into slow and fast migrating components by electrophoresis in supporting media. Using suitable antisera prepared in rabbits hyperimmunized with guinea pig serum, it was demonstrated that slow and fast antibodies have both common and distinct antigenic determinants. Analytical ultracentrifugation disclosed that both antibodies have sedimentation coefficients of approximately 7S. These antibodies have been designated guinea pig 7S γ_1 and 7S γ_2 .

We are grateful to Dr. G. Jeanette Thorbecke for the use of serum R1 and for her advice and assistance in performing the immunoelectrophoretic experiments.

BIBLIOGRAPHY

1. Ovary, Z., Immediate reactions in the skin of experimental animals provoked by antibody-antigen interaction, *Progr. Allergy*, 1958, **5**, 459.
2. Mayer, M. M., Studies on the mechanism of hemolysis by antibody and complement, *Progr. Allergy*, 1958, **5**, 215.
3. Green, H., Barrow, P., and Goldberg, B., Effect of antibody and complement on permeability control in ascites tumor cells and erythrocytes, *J. Exp. Med.*, 1959, **110**, 699.
4. Benacerraf, B., and Miescher, P., Bacterial phagocytosis by the reticuloendothelial system *in vivo* under different immune conditions, *Ann. New York Acad. Sc.*, 1960, **88**, 184.
5. Biozzi, G., and Stiffel, C., Role of normal and immune opsonins in the phagocytosis of bacteria and erythrocytes by the reticulo-endothelial cells, *in* Second International Symposium on Immunopathology, (P. Grabar and P. Miescher, editors), Basel, Benno Schwabe & Co., 1962, 249.
6. Porter, R. R., The hydrolysis of rabbit γ -globulin and antibodies with crystalline papain, *Biochem. J.*, 1959, **73**, 119.

7. Edelman, G. M., and Poulik, M. D., Studies on structural units of the γ -globulins, *J. Exp. Med.*, 1961, **113**, 861.
8. Edelman, G. M., Benacerraf, B., Ovary, Z., and Poulik, M. D., Structural differences among antibodies of different specificities, *Proc. Nat. Acad. Sc.*, 1961, **47**, 1751.
9. Edelman, G. M., and Benacerraf, B., On structural and functional relations between antibodies and proteins of the gamma system, *Proc. Nat. Acad. Sc.*, 1962, **48**, 1035.
10. Edelman, G. M., Benacerraf, B., and Ovary, Z., Structure and specificity of guinea pig 7S antibodies, in preparation.
11. Ishizaka, K., Ishizaka, T., and Sugahara, T., Biological activity of soluble antigen antibody complexes. VII. Role of an antibody fragment in the induction of biological activities, *J. Immunol.*, 1962, **88**, 690.
12. Ovary, Z., and Karush, F., Studies on the immunologic mechanism of anaphylaxis. II. Sensitizing and combining capacity *in vivo* of fractions separated from papain digests of anti-hapten antibody, *Immunology*, 1961, **86**, 146.
13. Ovary, Z., and Benacerraf, B., Separation of skin sensitizing from blocking 7S antibodies in guinea pig sera, *Fed. Proc.*, 1962, **21**, 21.
14. Benacerraf, B., and Levine, B., Immunological specificities of delayed and immediate hypersensitivity reactions, *J. Exp. Med.*, 1962, **115**, 1023.
15. Scheidegger, J. J., Une micro-methode de l'immuno-electrophorese, *Internat. Arch. Allergy and Appl. Immunol.*, 1955, **7**, 103.
16. Uriel, J., and Scheidegger, J. J., Electrophorese en gelose et coloration des constituants, *Bull. Soc. chim. biol.*, 1955, **37**, 165.
17. Farah, F. S., Kern, M., and Eisen, H. N., The preparation and some properties of purified antibody specific for the 2,4-dinitrophenyl group, *J. Exp. Med.*, 1960, **112**, 1195.
18. Kunkel, H. G., Zone electrophoresis, *Methods Biochem. analysis*, 1954, **1**, 141.
19. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry*, Springfield, Illinois, Charles C. Thomas, 2nd edition, 1961.
20. Ovary, Z., Benacerraf, B., and Bloch, K. J., Studies on 7S guinea pig antibodies. II. Identification of antibodies involved in passive cutaneous and systemic anaphylaxis, *J. Exp. Med.*, 1963, **117**, 951.
21. Yagi, Y., Maier, P., and Pressman, D., Immuno-electrophoretic identification of guinea pig anti-insulin antibodies, *J. Immunol.*, 1962, **89**, 736.
22. Bloch, K. J., Kourilsky, F., Ovary, Z., and Benacerraf, B., Studies on guinea pig antibodies. III. Identification of antibodies involved in complement fixation and hemolysis, *J. Exp. Med.*, 1963, **117**, 965.
23. Relyveld, E. H., *Toxine et antitoxine diphtheriques*, Paris, Hermann, 1959.
24. Heremans, J. F., *Les globulines seriques du systeme gamma; leur nature et leur pathologie*, Paris, Masson & Cie, 1960.
25. Fahey, J. L., Immunochemical studies of twenty mouse myeloma proteins: Evidence for two groups of proteins similar to gamma and beta 2A globulins in man, *J. Exp. Med.*, 1961, **114**, 385.
26. Franklin, E. C., and Stanworth, D. R., Antigenic relationship between immune globulins and certain related paraproteins in man, *J. Exp. Med.*, 1961, **114**, 521.