USAF Arctic Aeromedical Laboratory, Fort Wainwright, Alaska, for supplying the ground squirrel hearts.

1. Dempster, G., Grodums, E. I., Spencer, W. A., Can. J. Microbiol., 1961, v7, 587.
2. Gustafson, R. C., Petreman, M. C., Can. Med. Assn. J., 1963, v89, 900.
3. Soto, P. J., Jr., Beall, F. A., Nakamura, R. M., Kupferberg, L. L., Arch. Path., 1964, v78, 681.
4. De Wan, M., Henson, J., Dallahite, J., Bridges, C., Am. J. Path., 1965, v46, 215.

Received December 10, 1966. P.S.E.B.M., 1967, v125.

Passive Cutaneous Anaphylaxis Induced in Mice with Rabbit 5S Antibody Fragments.* (32053)

FRANCIS B. CASEY, JR.† AND SEI TOKUDA†
(Introduced by L. C. McLaren)
Department of Medical Microbiology, College of Medicine, University of Vermont, Burlington

When γG-immunoglobulins (7S) molecules are treated with cysteine activated papain or with pepsin some of their original serological activities are retained.

Cysteine-activated papain splits rabbit and human γG-immunoglobulin molecules into 3 fragments which together account for 90% or more of the original molecule(1). Two of these fragments are almost identical and are called Fab fragments(2). Each Fab fragment has a sedimentation constant of 3.5S, contains a single antibody-combining site and behaves as a "univalent" antibody. With regard to specificity and combining power, the antibody-combining sites of the Fab fragments appear to be the same as those of the original intact molecule, indicating that the antibody-combining sites are not appreciably altered by the enzyme(3,4). The third component is called the Fc fragment. This portion does not have any antibody-combining sites.

Pepsin reduces γG-immunoglobulins to about 2/3 of their original size(1,4). The pepsin-digested material has a sedimentation constant of 5S and has two antibody-combining sites per molecule. The 5S antibody fragments are believed to consist of 2 Fab fragments joined together by a single disulfide bond. The portion destroyed by enzymatic digestion corresponds to the Fc fragment. The 5S fragments derived from pepsin digestion behave like bivalent antibodies capable of exhibiting agglutinating or precipitating activities.

Fragments lacking the Fc fragment, whether obtained by either papain(5) or pepsin(6) treatment, do not sensitize guinea pig skin for the passive cutaneous anaphylaxis (PCA) reaction. Although aggregated γ-globulin as well as aggregated Fc fragments cause cutaneous reactions in guinea pigs and fix guinea pig complement, aggregated Fab fragments do not(7). Brambell *et al*(8) demonstrated that while the Fc fragments cross the placental barrier, the Fab fragments do not. It is therefore believed that the Fc fragment contains the site(s) responsible for: a) skin attachment in the cutaneous reaction; b) complement fixation and c) placental transmission of antibody(1).

It is generally accepted that the antibodies responsible for systemic anaphylaxis must "fix" to host tissues at certain critical sites in order to trigger the toxic reaction(9). However, Kind and Goodman(10) reported that pepsin-digested rabbit γ-globulin fractions (presumably containing the bivalent, 5S antibody fragments, which lack the sites responsible for tissue fixation) sensitize the mouse for passive systemic anaphylaxis.

The purpose of our investigation was an attempt to resolve the apparent discrepancy between the inability of 5S antibody fragments to elicit PCA in the guinea pig and

* This investigation was supported in parts by USPHS Research Grant CA 08074 from Nat. Cancer Inst. and by Grant IN-71C, Allotment 31, from Am. Cancer Soc.

† Present address: Dept. of Microbiology, School of Med., Univ. of New Mexico, Albuquerque.

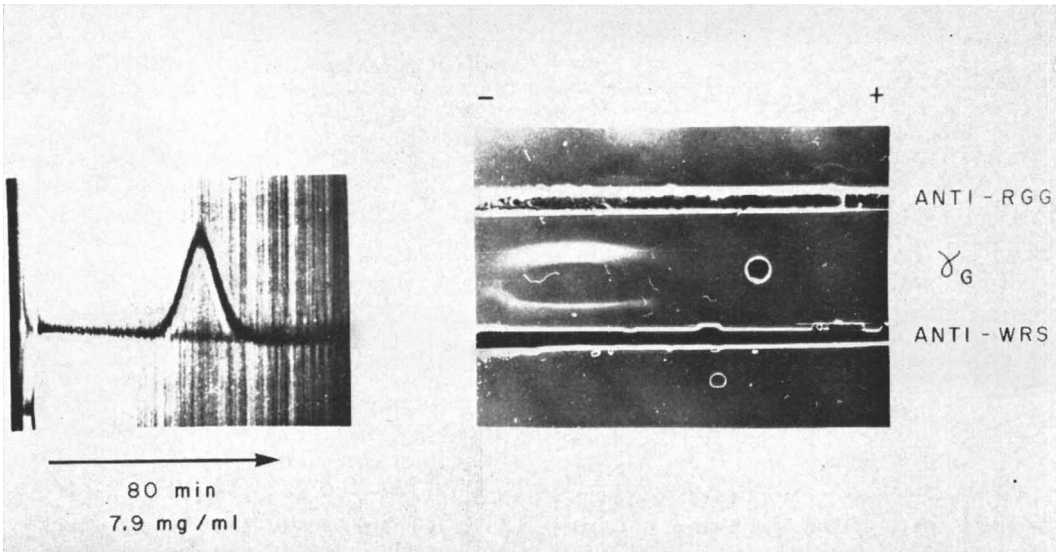

FIG. 1. Analytical ultracentrifugation pattern (left) and immunoelectro phoresis (right) of DE AE-Sephadex separated rabbit γG-immunoglobulin containing anti-BSA antibody. Anti-RGG= Goat anti-rabbit gamma globulin. WRS=Goat anti-whole rabbit serum.

the ability of these same fragments to sensitize the mouse for passive systemic anaphylaxis.

Methods. New Zealand white rabbits were hyperimmunized by multiple injections of bovine serum albumin (BSA) (3x crystallized, Pentex, Inc.) in complete Freund's adjuvant. The sera obtained from several rabbits were pooled. The γG-immunoglobulin fraction containing anti-BSA was separated from whole serum employing the batch method of Baumstark *et al*(11) using DEAE-sephadex A-50 (Pharmacia Fine Chemicals, Inc.). The purity of each preparation was determined by immunoelectrophoresis and by analytical ultracentrifugation. Immunoelectrophoresis was performed according to the method of Scheidegger as outlined by Campbell *et al*(12). The test samples contained 8-10 mg protein per ml and the precipitin bands were developed with goat anti-whole rabbit serum (Colorado Serum Co.) and with goat anti-rabbit-γ-globulin (Antibodies, Inc.). All of the γG-immunoglobulin preparations used in this study appeared homogeneous on analytical ultracentrifugation and gave a single precipitin arc on immunoelectrophoresis at protein concentrations of 8-10 mg/ml (Fig. 1). Quantitative precipitin tests were performed according to the method described by Kabat and Mayer(13). Protein concentration was assayed either by the micro-Kjeldahl method for the determination of protein nitrogen or by the Biuret method.

The 5S antibody fragments were prepared by the digestion of the anti-BSA γG-immunoglobulin with pepsin (2x crystallized, Worthington Biochemical Corp.) according to the method of Nisonoff *et al*(4,14). The 5S fragments were recovered from the digestion mixture by precipitation with 18% (w/v) sodium sulfate. The precipitate was reconstituted and dialyzed against phosphate buffered saline (PBS) (0.02 M sodium phosphate; 0.15 M NaCl; pH 7.4). Analytical ultracentrifugation of this preparation at a concentration of 8-10 mg/ml revealed a single symmetrical peak with a sedimentation constant of 4.8S (Fig. 2a). The bivalent nature of this material was demonstrated by its ability to show a ring precipitin reaction against BSA and its ability to show passive hemagglutination of tannic acid treated sheep red blood cells (SRBC) sensitized with BSA. Passive hemagglutination was performed according to the method of Boyden as outlined by Campbell *et al*(12).

The reduction and alkylation of 5S anti-BSA preparations to form 3.5S Fab frag-

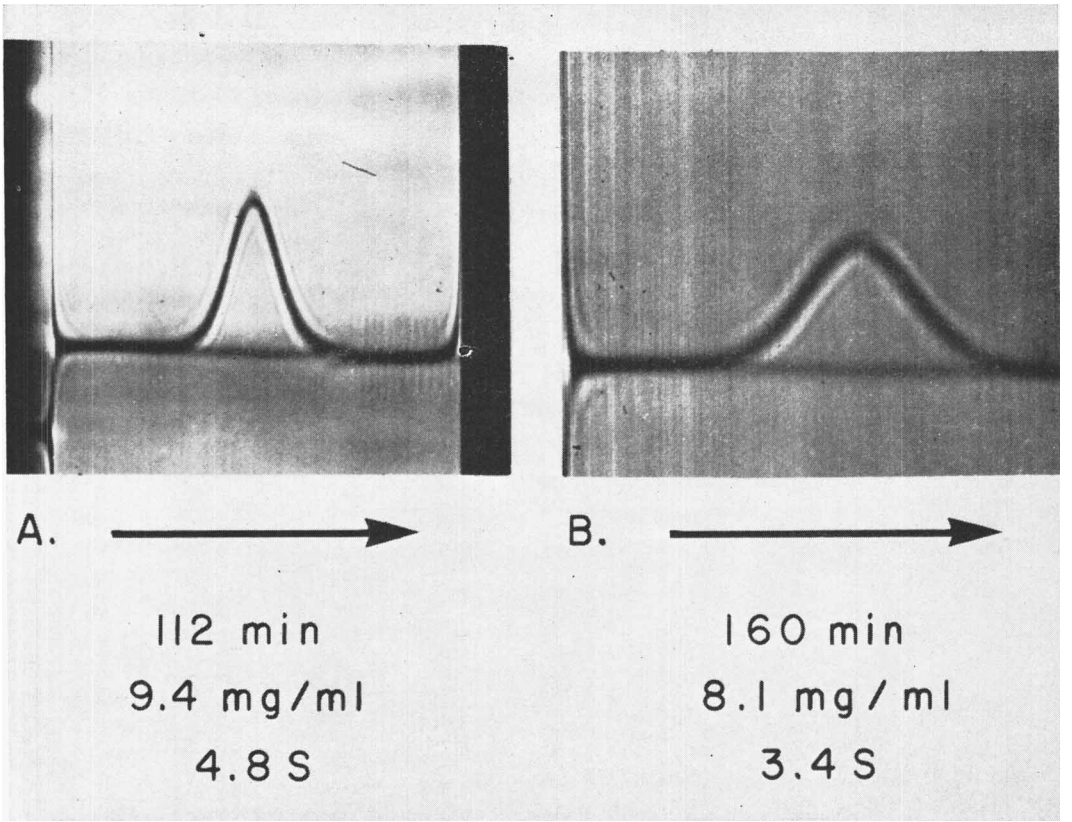

FIG. 2. A, (left), analytical ultracentrifugation pattern of pepsin digested rabbit γG-immunoglobulin. B, (right), analytical ultracentrifugation pattern of reduced and alkylated 5S anti-BSA preparation.

ments was accomplished by a slight modification of the method of Nisonoff *et al*(4,14). Following 2 1/2 hours of room temperature incubation at pH 4.5 with 0.01 M cysteine, the material was alkylated by dialysis against 0.02 M iodoacetamide for 18 hours at 4°C. The resulting material displayed a single, symmetrical 3.4S peak upon analytical ultracentrifugation at 8.1 mg/ml concentration (Fig. 2b). The univalent nature of this material was determined by its inability to precipitate BSA antigen and its ability to cause 100% inhibition of passive hemagglutination of a system involving a diluted preparation of whole anti-BSA rabbit serum and tannic acid treated SRBC's sensitized with BSA. This same univalent preparation failed to inhibit the agglutination of human γ-globulin coated SRBC in the presence of anti-human γ-globulin rabbit serum.

Functionally univalent reaggregated 5S fragments were formed when cysteine reduced 5S fragments were allowed to reaggregate without alkylation. Following reduction with cysteine, the material was returned to neutral pH by dialysis against phosphate buffered saline (14). The univalent nature of this material was determined by its ability to form a precipitin reaction with BSA and its ability to inhibit passive hemagglutination of BSA sensitized SRBC's. The resulting preparation is therefore presumed to be hybrid 5S antibody molecules containing one anti-BSA Fab fragment and one normal Fab fragment.

Passive cutaneous anaphylaxis in mice and guinea pigs was performed according to the method of Ovary(15). Actual injected sensitizing doses and latent periods are given in the *Results* section. Female Swiss-Webster strain of mice weighing 20-25 g and albino guinea pigs of either sex weighing approximately 250-350 g were used in all instances.

TABLE I. Titration of γG Anti-BSA for PCA in the Mouse.

μg protein injected intradermally	Latent period	Challenge dose	Score*
61.0	1 hr	Normal†	8 mm
51.0	"	"	7 mm
38.2	"	"	6 mm
30.6	"	"	5 mm
25.5	"	"	Neg.
306.0	"	Dye only	"
Undiluted NRS‡	"	Normal	"
102.0	3 hr	Normal	14 mm
51.0	"	"	9 mm
30.6	"	"	5 mm
25.5	"	"	Neg.
306.0	"	Dye only	"
Undiluted NRS‡	"	Normal	"

* Diameter of dyed area beneath the skin. Each score is average of at least 10 sites.

† 1.25 mg BSA + 0.5% Evans Blue dye injected intravenously in a volume of 0.25 ml after designated latent period.

‡ NRS = Normal Rabbit Serum.

In all cases, the intravenous challenge dose was: 1.25 mg BSA in PBS containing 0.5% Evans Blue dye in a total volume of 0.25 ml for mice; and 1 mg BSA in PBS containing 1% Evans Blue dye in a total volume of 0.5 ml for guinea pigs. The animals were sacrificed one hour after administration of the challenge dose. Such timing gave the most sensitive and reproducible results. The skin of the animals was then grossly inspected externally and internally with particular emphasis placed on the appearance of the reflected skin site. Reactions were read as the diameter of the dyed area beneath the dermal area.

Results. The rabbit γG-immunoglobulin fractions containing anti-BSA were titrated for PCA activity in female Swiss-Webster mice (Table I). Using latent periods of one to 3 hours between sensitization and challenge, the minimum sensitizing dose was 30 μg of γG-globulin containing 1.72 μg of antibody N (AbN). If latent periods of longer than 3 hours were used, the intensity of the reaction decreased markedly. The same preparation of γG-immunoglobulin containing anti-BSA used in guinea pigs showed a minimum sensitizing dose of 1.5 μg protein (0.087 μg AbN) after a 5-hour latent period. Two control groups of animals were included in this study. One group was sensitized with normal γG-immunoglobulin and challenged with BSA and dye. The other group was sensitized with anti-BSA γG-immunoglobulin and challenged with dye only. Control animals were negative in all instances.

Pepsin digested 5S anti-BSA fractions were also tested for PCA activity in mice and guinea pigs (Table II). In mice, a minimum sensitizing dose of 120 μg 5S protein was required with a latent period of one hour; and a minimum of 500 μg protein was necessary for sensitization when a 3-hour latent period was used. Comparing the minimum sensitizing doses of the 5S with the γG-immunoglobulin anti-BSA fractions on an equimolar basis, sensitization requires 5.3 times more 5S antibody than γG-antibody for PCA in the mouse when the latent period is one hour. Using a latent period of 3 hours, sensitization requires 22 times more 5S antibody than γG-antibody.

In the guinea pig, as much as 875 μg of 5S protein anti-BSA was unable to sensitize guinea pig skin. This was the maximum concentration of 5S anti-BSA available from the preparation used. The finding that 5S antibody is unable to sensitize guinea pig skin is consistent with the earlier observations of Ovary(6).

TABLE II. Titration of 5S Fragment Anti-BSA for PCA in the Mouse.

μg protein injected intradermally	Latent period	Challenge dose	Score*
437.5	1 hr	Normal†	10 mm
218.7	"	"	8 mm
117.5	"	"	4 mm
109.4	"	"	Neg.
54.7	"	"	"
437.5	"	Dye only	"
NRS‡	"	Normal	"
650.0	3 hr	Normal	9 mm
500.0	"	"	4 mm
437.5	"	"	Neg.
650.0	"	Dye only	"
NRS‡	"	Normal	"

* Diameter of dyed area beneath the skin. Each score is average of 6 sites.

† 1.25 mg BSA + 0.5% Evans Blue dye injected intravenously in a volume of 0.25 ml after designated latent period.

‡ NRS = Normal Rabbit Serum.

TABLE III. Detection of Contamination of γG Anti-BSA with 5S Fragment Anti-BSA in the Guinea Pig.

% γG in mixture	μg protein injected intradermally		Latent period	Challenge dose	Score*
	γG	5S fragment			
10.0	1.5	15.0	3 hr	Normal†	10 mm
5.0	1.5	75.0	"	"	8 mm
1.0	1.5	150.0	"	"	6 mm
.3	1.5	495.0	"	"	Neg.
100.0	1.5	0.0	"	"	8 mm
5.0	1.5	75.0	1 hr	Normal	12 mm
1.0	1.5	150.0	"	"	9 mm
.3	1.5	495.0	"	"	3 mm
100.0	1.5	0.0	"	"	7 mm
.0	.0	563.5	"	"	Neg.

* Diameter of dyed area beneath the skin. Each score is average of 3 sites.
† 1 mg BSA + 1% Evans Blue dye in a volume of 0.5 ml injected intravenously.

Because the 5S antibody preparation induced PCA in mice but not in guinea pigs, we considered the possibility existed that the 5S fractions may have been contaminated with intact γG-immunoglobulin anti-BSA. To test this possibility, mixtures of 5S and γG fractions were prepared in varying proportions and tested for their ability to induce PCA in the guinea pig. The guinea pig was chosen because it was not sensitized by the 5S antibody fractions and has proved to be a sensitive indicator for γG-antibody. Latent periods of one to three hours were used (Table III). It was observed that as little as 0.3% contamination by γG-antibody in the 5S preparation could be detected after a one-hour latent period. Using these figures, it was calculated that if the 5S antibody fractions contained intact γG-antibody in amounts detectable by the analytical ultracentrifuge at 8-10 mg/ml protein concentrations, such trace amounts of γG-antibody would not be adequate to provoke PCA in the mouse. Therefore, it can be concluded that the PCA reaction induced in the mouse with 5S antibody fractions is a function of the 5S antibody fraction and is not due to the presence of undigested anti-BSA γG-immunoglobulin.

Because 5S antibody fragments induced PCA in mice, 3.5S "univalent" Fab fragments were also tested for PCA in mice and guinea pigs. It was observed that doses up to 1.05 mg of 3.5S protein failed to sensitize the mouse for PCA and doses up to 2.10 mg protein failed to induce PCA in the guinea pig. The 3.5S Fab fragments were derived from the 5S antibody fragments by the reduction and alkylation of a single disulfide bond(4, 14); thus a Fab fragment is essentially equivalent to one-half of a 5S molecule.

Because the 3.5 Fab fragments have only one antibody-combining site, it was decided to determine whether such "univalent" antibodies would inhibit the PCA reaction induced by bivalent γG-anti-BSA molecules. Therefore, anti-BSA 3.5S Fab fragments and anti-BSA γG fractions were mixed *in vitro* 5 minutes prior to the intradermal injection of the sensitizing dose. In the mouse, the PCA reaction could be provoked if the anti-BSA ratio of 3.5S protein to γG-immunoglobulin protein was less than 1 (Table IV). However, if the 3.5S to γG-immunoglobulin ratio was increased to 1.38 or greater, the PCA reaction was inhibited in the mouse. In the guinea pig, the PCA reaction was not inhibited even when the 3.5S/IgG ratio was 170/1.

Discussion. These results indicate several differences exist between the heterologous PCA reactions in guinea pigs and mice. For instance, we have shown that pepsin-digested rabbit 5S antibody fragments sensitize the mouse for PCA. This same preparation failed to provoke the PCA reaction in the guinea pig. Although it is generally accepted that pepsin digested 5S rabbit antibody fractions lack the site necessary for "tissue fixation" in the PCA reaction of the guinea pig, we are unable to draw any conclusions about this requirement in the mouse. Kind and Goodman

TABLE IV. Inhibition of γG Anti-BSA Sensitization for PCA by 3.5S Anti-BSA Fragments in the Mouse.*

Ratio 3.5S : γG	μg protein injected intradermally 3.5S	γG	Challenge dose	Score†
10 : 1	515.0	51.5	Normal‡	Neg.
6.8 : 1	515.0	76.5	"	"
3.4 : 1	515.0	153.0	"	"
2 : 1	515.0	257.5	"	"
1.38 : 1	515.0	382.5	"	"
1 : 1	515.0	515.0	"	15 mm
—	1050.0	0.0	"	Neg.
—	0.0	51.5	"	13 mm

* Results shown are identical for both a 1 hr and 3 hr latent period.

† Diameter of dyed area beneath the skin. Each score is average of 4 sites.

‡ 1.25 mg BSA + 0.5% Evans Blue dye injected intravenously in a volume of 0.25 ml after end of latent period.

(10) reported that pepsin-digested rabbit γ-globulin fractions provoked passive systemic anaphylaxis in the mouse. They submitted their data as evidence to support the concept that "tissue fixation" is not necessary in the anaphylactic reaction of mice. However, a critical examination of their data raises some questions about the validity of their assumptions. First, no data were presented to indicate that the 5S antibody preparation was not contaminated with undigested γG-antibody. Second, the strongest anaphylactic reactions were induced with pepsin-digested rabbit globulins containing anti-mouse γ-globulin antibody. This antigen-antibody reaction involves host γ-globulin as the antigen. Several years ago, Ovary(16) reported that the induction of PCA in guinea pigs requires that either antigen or antibody must be "fixed" to the tissue. When the host's own γ-globulin is part of the *in vivo* antigen-antibody reaction, it is highly probable that "tissue fixation" is involved and therefore, the arguments presented by Kind and Goodman(10) do not necessarily support the concept that "tissue fixation" is not necessary in the anaphylactic reaction of the mouse.

If we assume that the rabbit 5S fragments are incapable of fixing to mouse tissue, then it can be argued that "tissue fixation" is not a prerequisite for PCA in the mouse. Alternatively, if rabbit 5S antibody fragments possess secondary sites for fixation to mouse tissues, then it is conceivable that rabbit 5S antibody fragments would have a reduced affinity for mouse tissue. Such an occurrence could explain why more rabbit 5S antibody than rabbit γG-antibody is required for PCA in the mouse and it also could explain why more 5S material is necessary for a 3-hour latent period than for a 1-hour latent period. In addition, it can be argued that if the 5S material does fix to mouse tissue, then the reduced and alkylated 3.5S fragments would also fix to tissue. This possibility could explain why rabbit Fab fragments effectively inhibit the PCA reaction in the mouse.

Kabat and Benaceraff(17) reported that non-precipitating "univalent" antibodies passively sensitize the guinea pig for anaphylaxis. Recently, Ovary(18) demonstrated that reduced and reaggregated monovalent "hybrid" antibodies induce PCA in guinea pigs. In addition, there are reports that bivalent haptens provoke PCA, whereas monovalent haptens do not(19,20). Ovary(18) therefore suggests that . . . "the bridging of two combining sites on two different antibody molecules, which are fixed to guinea pig tissues, is necessary and sufficient for PCA reactions." However, in our studies, we have demonstrated that in using rabbit antibody fractions, only the intact γG- and bivalent 5S-antibody fractions provoke PCA in the mouse. Both the Fab fragments and the reduced and reaggregated univalent "hybrid" 5S fragments failed to sensitize the mouse for PCA. To emphasize further the importance of bivalent antibodies, Fab fragments exhibited an impressive inhibitory effect on PCA in the mouse produced with rabbit γG-antibody. The Fab fragments failed to inhibit the PCA reaction in the guinea pig.

An interesting observation is that a small amount of the 5S fragment added to the γG-antibody seems to enhance its ability to produce the PCA reaction in the guinea pig, while increasing amounts of the 5S material are actually inhibitory (Table III). The significance of this observation is not clear because no control experiments were done with non-antibody 5S fragments.

Earlier, we(21) reported that 5-hydroxytryptamine rather than histamine plays a predominant role in the anaphylactic reaction of the mouse. This report points out other differences between the anaphylactic reaction in the mouse and the guinea pig. It suggests that in mice and guinea pigs slightly different molecular mechanisms are involved in the antigen-antibody reaction which leads to the release of the pharmacological mediators that trigger the anaphylactic syndrome when heterologous antibody is the sensitizing agent.

The conclusions reached in this investigation are concerned only with observations using a heterologous antibody and do not refer to the mechanisms involved with the homologous antibody system. The effects of whole and fragmented homologous antibody upon the PCA reaction are being investigated.

Summary. γG-immunoglobulin, obtained from rabbit anti-bovine serum albumin serum, was treated with pepsin and subsequently reduced and alkylated to yield fractions containing 5S and 3.5S Fab fragments, respectively. The 5S fraction had precipitating antibody activity and induced passive cutaneous anaphylaxis (PCA) in mice. Such sensitization has not been demonstrated in the guinea pig. With respect to the dosage and the length of the latent period required, there was a marked difference in the abilities of the 5S antibody fractions and the γG-antibody to provoke PCA in the mouse. The 3.5S Fab fragments possessed "univalent" antibody activity but failed to induce PCA. However, 3.5 S Fab fragments inhibited the PCA reaction in the mouse, but failed to block the reaction in guinea pigs. Hybrid 5S fragments had the same properties as Fab fragments. These results point out additional differences between the PCA reaction of guinea pigs and mice. The findings also show that PCA in the mouse demands functionally bivalent antibodies.

We wish to express our appreciation to Dr. T. B. Tomasi, Jr. and Miss Jane Wark (formerly of the Dept. of Exp. Med., University of Vermont) for perfoming the analiytical ultracentrifugation and to Mrs. Patricia McEntee for technical assistance.

1. Porter, R. R., Brit. Med. Bull., 1963, v19, 196.
2. Nomenclature for Human Immunoglobulin, Bull. Wld. Hlth. Org., 1964, v30, 447.
3. Karush, F., Fed. Proc., 1959, v18, 557.
4. Nisonoff, A., Wissler, F. C., Lipman, N. N., Woernley, D. L., Arch. Biochem. Biophys., 1960, v89, 230.
5. Ovary, Z., Karush, F., J. Immunol., 1961, v86, 146.
6. Ovary, Z., Taranta, A., Science, 1963, v140, 193.
7. Ishizaka, K., Ishizaka, T., Sugahara, T., J. Immunol., 1962, v88, 690.
8. Brambell, F. W. R., Hemmings, W. A., Oakley, C. L., Porter, R. R., Proc. Roy. Soc. B., 1960, v151, 478.
9. Benacerraf, B., Kabat, E. A., J. Immunol., 1949, v62, 517.
10. Kind, L. S., Goodman, J. W., Nature, 1962, v196, 79.
11. Baumstark, J. S., Laffin, R. J., Bardawil, W. A., Arch. Biochem. Biophys., 1964, v108, 514.
12. Campbell, D. H., Garvey, J. S., Cremer, N. E., Sussdorf, D. H., Methods in Immunology, W. A. Benjamin, Inc., New York, 1963.
13. Kabat, E. A., Mayer, M. M., Experimental Immunochemistry, 2nd Ed., C. C Thomas, Springfield, Ill., 1964.
14. Mandy, W. J., Rivers, M. M., Nisonoff, A., J. Biol. Chem., 1961, v236, 3221.
15. Ovary, Z., in Immunological Methods, J. F. Ackroyd, ed., F. A. Davis Co., Philadelphia, 1964, p259.
16. Ovary, Z., Immunology, 1960, v3, 19.
17. Kabat, E. A., Benacerraf, B., J. Immunol., 1949, v62, 97.
18. Ovary, Z., Fed. Proc., 1965, v24, 94.
19. Cohen, W. B., ibid., 1962, v21, 21.
20. Ovary, Z., Compt. Rend., 1961, v253, 282.
21. Tokuda, S., Weiser, R. S., J. Immunol., 1961, v86, 292.

Received February 14, 1967. P.S.E.B.M., 1967, v125.