STUDIES ON THE MECHANISM OF HYPERSENSITIVITY PHENOMENA

II. The Participation of Complement in Passive Cutaneous Anaphylaxis of the Albino Rat*

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In view of the recurrent demonstration that the hemolytic activity of serum may decline during experimentally induced systemic anaphylaxis (reviewed in references 1, 2) and in some naturally occurring disease processes with a presumed allergic component, (3, 4), complement (C′) has been implicated in the mediation of hypersensitivity reactions (5, 6). Recently, this inference gained further support when it was shown that an induced C′ deficiency in albino rats was associated with a diminished reactivity to passive cutaneous anaphylaxis (PCA) (7). The evidence emerging from the latter observations did not permit differentiation between the temporal coincidence of the two events and a possible dependence of cutaneous anaphylaxis upon adequate levels of circulating C′. The present investigation was therefore undertaken in an effort to clarify the relationship between PCA and C′. The data detailed below are in accord with the hypothesis that this immediate type of allergic reaction may be significantly influenced by variation of any one of at least three variables, *viz.*, antigen, antibody, and a host factor which resembles hemolytic C′.

Materials and Methods

Passive Cutaneous Anaphylaxis.—

This type of local reaction was selected as the experimental model because of its relative reproducibility and ease of quantitation in comparison to systemic anaphylaxis. The follow-

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ing considerations led to the choice of rats rather than guinea pigs as the experimental animals. The hemolytic C′ level of rat serum is much lower than that of the guinea pig (approximately 40 as compared to 200 fifty per cent hemolytic units, ($C'H_{50}$), per ml.). An economy was therefore affected in the amount of specific antibody required for the decomplementation procedure which was an integral part of the experimental approach (*cf.* reference 8). Moreover, the resistance of these animals to generalized anaphylactic reactions enabled them to withstand the systemic immune reaction used for C′ depletion without any manifest symptoms of shock.

The PCA reactions were induced as described below (9). Male albino rats of the Wistar strain weighing between 130 and 170 gm. were used in all of these experiments. The hair over the abdominal area was gently clipped with an electric shaver without irritating the skin. Dilutions of serum containing the designated quantities of antibody in a volume of 0.1 ml. were prepared in 0.15 M NaCl and injected intracutaneously with tuberculin-type syringes of 0.25 ml. capacity equipped with gauge 27 hypodermic needles. A maximum of four skin sites per rat were thus prepared. During the latter part of the study we were informed that skin reactions elicited on the dorsal surface were more sharply demarcated than those in the abdominal area (10). This observation was confirmed but use of the abdominal area was continued to facilitate comparisons with the many earlier experiments. Preliminary experiments also confirmed earlier findings that a 2 hour latent period between the intradermal antibody inoculation and the intravenous injection of dye-antigen mixture resulted in maximal skin blueing (11). This latent period was uniformly maintained in all experiments. The dye-antigen mixture was prepared immediately before injection and each animal received 0.25 ml. of a 1 per cent solution of Evans blue and the designated quantity of antigen in isotonic saline. The total volume of injected material was varied between the limits of 1.2 and 2.2 ml. As noted below, the saline diluent for the antigen was replaced in some of the experiments by various serum preparations. The penile vein was found most satisfactory for the intravenous inoculations, although the superficial veins on the paws of the hind feet were also used occasionally. The rats were sacrificed 60 minutes after the injection of the dye and the skin of the abdomen was carefully stripped from the subcutaneous tissues. The reactions were read by measuring the diameter of the blue area on the inner surface of the skin with a transparent millimeter ruler.

The same time relationships were retained in the experiments with C′-deficient rats. For these studies, the animals received an intravenous injection of 1.0 ml. of a saline dilution of serum containing 200 μg. of rabbit anti-ovalbumin (Ea) N and an intraperitoneal inoculation of 80 μg. of Ea N. These reagents were administered immediately following the preparation of the skin sites for the PCA reactions. As noted below, other antigen and antibody preparations were occasionally substituted for the ovalbumin immune system in rendering the animals deficient in circulating C′.

Antisera and Antigens.—

A variety of rabbit and horse antisera and corresponding protein or polysaccharide antigens were employed for the induction of PCA reactions and for decomplementation. Details as to the source and properties of each immune system are described below. All antisera were heated at 56°C. for 30 minutes, clarified by centrifugation at 0°C. and preserved with 0.01 per cent merthiolate at −20°C. Antibody N estimations of the sera were carried out by the quantitative precipitin method of Heidelberger and Kendall as described in reference 12. Stock solutions of all antigens except chicken ovalbumin (Ea) were prepared in 0.15 M NaCl containing 0.01 per cent merthiolate and stored at −20°C. The Ea solutions were also prepared in merthiolated saline but were stored at refrigerator temperatures and were freshly made at frequent intervals.

Complement.—

Fresh guinea pig and rat sera were used as the source of C′. For the estimation of hemolytic potency in sera from individual rats, blood was obtained by puncture of the infra-orbital sinus with a clean, dry pipette (13). Fresh normal rat serum for parenteral use was obtained by cardiac or jugular puncture of 10 to 20 animals. The pooled serum was clarified by centrifugation at 0°C., and stored in clean, tightly stoppered pyrex test tubes (13 x 100 mm.) at −50°C.

Fresh guinea pig serum was obtained commercially in the frozen state, absorbed twice at 0°C., with 1 ml. of washed, packed sheep erythrocytes per 30 ml. of serum, and stored at −50°C. as described above. For some of the experiments, the guinea pig serum was also absorbed with washed, packed rat erythrocytes but this practice was discontinued when comparative studies showed that this procedure did not affect the results.

Estimations of the hemolytic activity of C′ and of the C′-fixing potencies of the several antisera described below were carried out as described in reference 14 with the veronal buffer diluent containing 5×10^{-4} M Mg^{++} and 1.5×10^{-4} M Ca^{++}. Similar determinations with rat serum as the source of C′ necessitated some minor modifications of these procedures. Thus, preliminary experiments indicated that the hemolytic potency of rat C′ was slightly diminished when calcium salts were added to the assay reaction mixtures in final concentrations as low as 10^{-6} M. Further, a 90 minute reaction period at 37°C. was required to achieve maximal lysis in titrations of rat C′ activity. Finally, it was necessary to adjust the level of rabbit hemolysin for optimal sensitization of the sheep erythrocytes in experiments with rat C′. The need to institute these minor adjustments indicates that the cation and antibody requirements for maximal efficiency of the hemolytic system comprising sheep erythrocytes, rabbit amboceptor, and guinea pig C′ are not necessarily optimal for hemolytic studies with C′ derived from other species.

C′ Component Reagents.—

These reagents were prepared and tested essentially as described in reference 12 except for the modifications noted below:

1. Heated C′.—Guinea pig and rat sera in volumes of 5 to 20 ml. were initially heated at 56°C. for 60 minutes to destroy the first and second components of C′, (C′1 and C′2). It was subsequently found that exposure at this temperature for a period of 15 minutes sufficed to abolish hemolytic activity. For example, one pool of fresh rat serum contained sufficient hemolytic activity so that 0.4 ml. of a 1 → 100 dilution lysed 50 per cent of 10^8 sensitized sheep erythrocytes in a volume of 1.5 ml. When an aliquot of this pool was heated at 56°C. for 15 minutes, 1.0 ml. of a 1 → 10 dilution produced no visible lysis with the same number of sensitized red blood cells. C′ component titrations showed that this heated serum had no demonstrable C′1 or C′2 activity and only about half of the initial C′3 and C′4 potency.

2. Zymosan-Treated C′.—Two hundred mg. of zymosan (Fleischman, lot No. 369) were suspended in 100 ml. of 0.15 M NaCl in a Waring blendor. The suspension was then heated in a boiling water bath for 60 minutes with intermittent shaking and centrifuged for 30 minutes at 3000 *G* (15). The clear supernatant was carefully aspirated and the sediment suspended in 10 ml. of isotonic veronal buffer. Preliminary titrations were then performed to determine the minimal quantity of this zymosan preparation necessary for the preferential destruction of C′3 in fresh guinea pig or rat serum at 37°C. It was found that 1.5 to 5.0 mg. of the zymosan suspension per ml. of serum rendered C′ devoid of hemolytic activity through the inactivation of C′3. However, these quantities of zymosan also destroyed varying amounts of the C′1 and C′4 activities as estimated by component titrations (*cf.* references 15, 16 with human serum).

3. Ammonia and Hydrazine-Treated C'.—When NH_4OH at a final concentration of 2×10^{-2} M or hydrazine at 3.6×10^{-2} M were added to fresh guinea pig or rat serum, virtually all of the C'4 was destroyed (17, 18). This treatment proved more selective than that for the other C' component preparations in the sense that hemolytic activity of ammonia or hydrazine-treated sera could be restored to the initial levels by the addition of the appropriate reagents.

4. Specific Decomplementation.—Guinea pig and rat sera were added to washed, preformed specific precipitates for the selective uptake of C'1, C'4, and C'2. Thus, 5 mg. of rabbit anti-bovine gamma globulin N were reacted with 0.6 mg. of homologous antigen N for 24 hours in the refrigerator. The precipitate was collected and washed four times with 25 ml. of cold isotonic NaCl. The sedimented immune aggregate was then evenly suspended in 25 ml. of fresh guinea pig or rat serum and stored in the refrigerator overnight with intermittent shaking. The reaction mixtures were then clarified by centrifugation at 0°C. for 60 minutes at 15,000 *G* and the supernates stored at −50°C. Several preparations of this type were devoid of lytic activity at levels of 1.0 ml. of a 1 → 10 dilution.

It should be emphasized that although the procedures employed for the preparation and estimation of C' components conform to current practice, they are subject to several serious limitations. The reagents obtained following treatment of fresh normal serum with zymosan, ammonia, or heat are neither reproducible in a strictly quantitative sense, possibly because of the difficulties of the assay procedures, nor are they uniquely specific for the selective destruction of any given C' component (19–21). These observations are borne out, in part, by the continuing efforts over the past two decades, directed towards the purification and isolation of the individual components of C' (22–26). In this light, the results obtained with these reagents in the present studies are considered as providing essentially qualitative evidence directed towards the provisional identification of a host factor which participates in PCA of the albino rat.

EXPERIMENTAL RESULTS

A. PCA Response as a Function of Antigen and Antibody Concentrations

The data in Table I and Fig. 1 summarize the results of numerous experiments in which the magnitude of the dermal reaction was studied at several levels of rabbit anti-pneumococcus Type III serum and increasing quantities of the type-specific capsular polysaccharide, SIII. These findings, as well as those obtained with rabbit anti-Ea reacting with intravenously injected Ea, indicate a reciprocal relationship between the levels of antigen and antibody required to elicit blueing of the rat skin. Several observations emerge from these data. For the experimental conditions employed in this study, it appears that 6.0 μg. of SIII represents the threshold antigen level which will produce an unequivocal PCA reaction in the rat skin injected 2 hours previously with at least 3.5 μg. of anti-SIII N. The magnitude of the response at each antibody level increases as more antigen is used reaching an apparent maximum at approximately 48 μg. of SIII. These data also confirm previous findings in the demonstration that with a large excess of antigen, the rat requires about 100 times more antibody than does the guinea pig for a minimal PCA reaction (11). As judged from examination of the rat skin in the living animal, the rate of blueing at the site increases with greater quantities of immune reactants.

Since the total amount of antibody N in the four skin sites for each rat depicted in Table I approximates 16 μg., it appears that maximal PCA responses require a considerable excess of antigen in terms of the precipitin reaction, if it is assumed that all of the injected antigen is available for the reaction. Similar observations have been reported for systemic and passive cutaneous anaphylaxis in the guinea pig (27, 9).

TABLE I

PCA Responses in Normal Rats with Varying Quantities of Rabbit Anti-SIII N (R_3 — 175) Reacting with SIII‡ Dissolved in Saline§*

SIII in saline intravenously	Rabbit anti-SIII N injected intradermally, μg. per skin site					
	10.5	5.3	3.5	2.6	1.2	0.4
	Average diameter of response, *mm.*					
μg						
1.5	0.4 (8)‖		0 (8)		0 (8)	0 (8)
3.0	1.4 (22)		2.2 (22)		1.1 (22)	0.4 (22)
6.0	8.5 (57)		6.3 (57)		3.2 (57)	1.7 (57)
12.0	13.8 (56)		10.8 (52)		5.8 (52)	2.1 (48)
24.0	18.1 (33)		14.8 (25)		9.3 (29)	5.1 (25)
48.0	21.5 (4)	22.8 (4)		18.5 (4)	14.8 (4)	
96.0	18.5 (4)	14.8 (4)		14.0 (4)	8.8 (4)	
192.0	19.5 (4)	15.5 (4)		14.5 (4)	11.0 (4)	

* This serum was obtained through the courtesy of the New York State Department of Health and contained 5.26 mg. anti-SIII N per ml. after absorption with the "C" substance of Type II pneumococci.

‡ We are grateful to Dr. M. Heidelberger for the type-specific pneumococcus polysaccharides used in these studies.

§ The degree of variation in PCA reactions between rats diminished as the amount of antigen and antibody used to elicit them was increased. Thus, the range of variation for PCA provoked with 6 μg. SIII and 3.5 μg. anti-SIII N was 0 to 20 mm. with a standard deviation of 6.6 mm. The range for the reactions induced with 24 μg. SIII and 10.5 μg. anti-SIII N was 10 to 25 mm. with a standard deviation of 3.5.

‖ The figure in parenthesis indicates the number of rats used for each determination.

B. PCA Response as Influenced by the Addition of Fresh Normal Serum to the Antigen-Dye Mixture

In earlier studies on PCA reactions in the albino rat, a parallelism was noted between the intensity of the local cutaneous response and the levels of circulating C′ activity (7). If this correlation of the two phenomena implied a casual relationship, it might be anticipated that an increase in available C′ would be associated with enhanced PCA reactions. Accordingly, a series of experiments was designed, similar in all respects to those summarized in Table I except that the antigen-dye mixture was incorporated in fresh rat or guinea pig serum

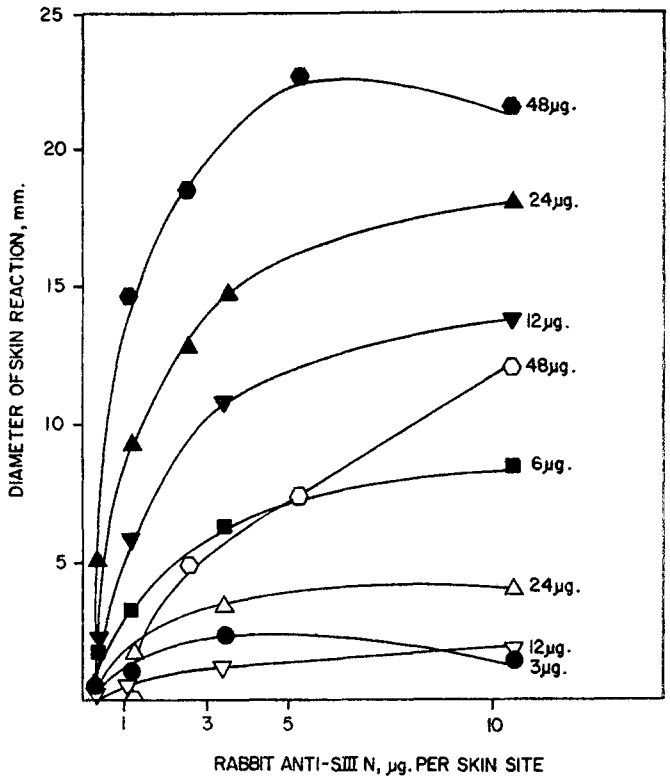

FIG. 1. PCA reactions in normal and C'-depleted rats following challenge with varying amounts of SIII.

TABLE II

*PCA Responses in Normal Rats with Varying Quantities of Rabbit Anti-SIII N (R_s — 175) Reacting with SIII Dissolved in Fresh Rat or Guinea Pig Serum**

SIII injected intravenously with 1 to 2 ml. of fresh rat or guinea pig serum	Rabbit anti-SIII N injected intradermally, μg. per skin site					
	10.5	5.3	3.5	2.6	1.2	0.4
	Average diameter of response, *mm.*					
μg.						
1.5	0.6 (12)‡		0 (12)		0 (12)	0 (12)
3.0	7.7 (20)		6.2 (20)		4.4 (20)	1.6 (20)
6.0	15.4 (28)		9.7 (28)		6.5 (28)	4.0 (28)
12.0	20.4 (18)		17.1 (18)		10.0 (18)	7.1 (18)
24.0	24.5 (12)	21.1 (8)	18.0 (4)	19.5 (8)	14.1 (12)	8.5 (4)

* Since the enhancing effect and the range of variation induced by fresh guinea pig or rat sera were similar, the data have been combined.

‡ The figure in parenthesis indicates the number of rats used for each determination.

instead of isotonic saline. The results of these experiments are tabulated in Table II. Both the rat and guinea pig sera potentiated the local anaphylactic response at each level of antigen or antibody tested (*cf.* Tables I and II, Figs. 2 and 3). Moreover, the threshold SIII level for minimal PCA reactions was reduced from 6.0 to 3.0 μg. when fresh serum replaced the saline diluent for the antigen. It was also of interest to note that guinea pig serum was not more

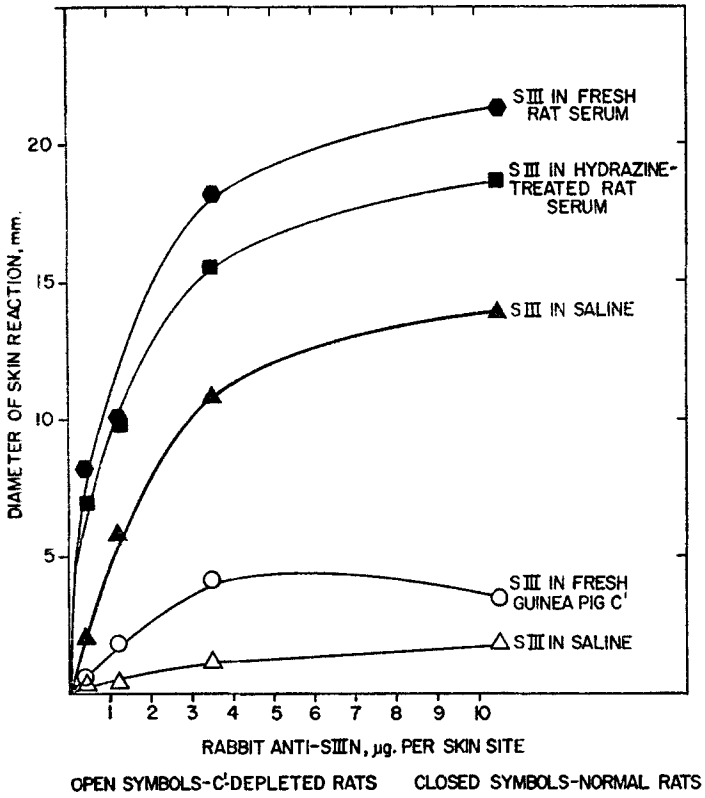

FIG. 2. PCA reactions in normal and C'-depleted rats following challenge with 12 μg SIII.

uniformly effective than that of the rat despite its greater hemolytic activity. This observation may perhaps be attributed to the slight toxicity associated with the intravenous injection of foreign serum.

The enhancing effect of fresh rat serum was also observed in an experiment summarized by the data in Table III.

In this case, each of 20 rats received five intradermal injections of antibody. Two sites were prepared on the left side with 5.0 and 2.5 μg. of homologous anti-SVIII *N* respectively. The right side of each animal was prepared with 7.0, 3.5, and 1.8 μg. of cross-reacting anti-

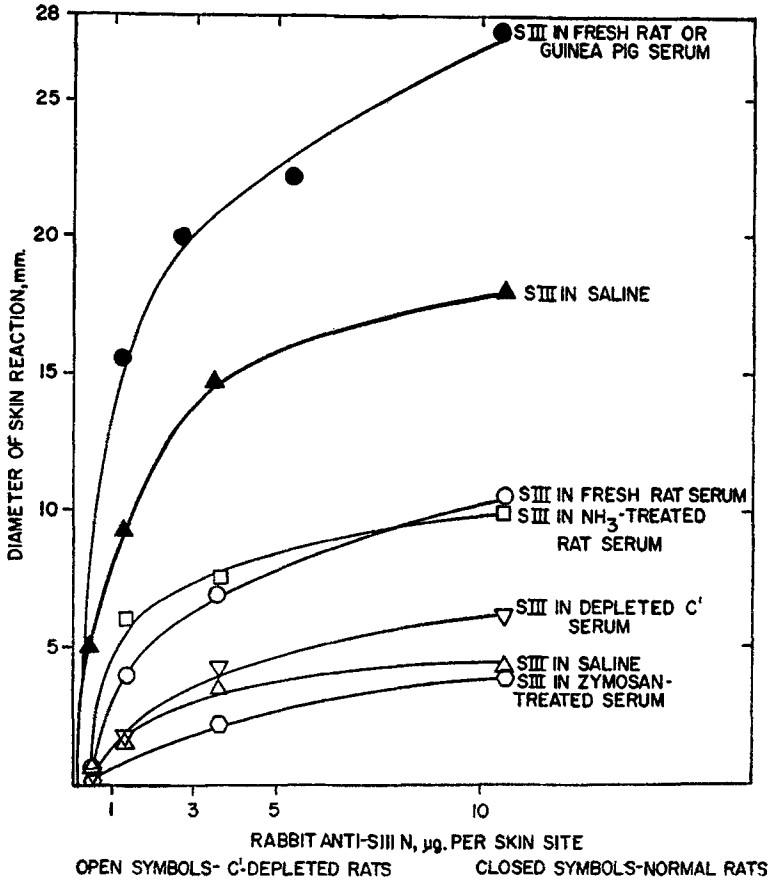

FIG. 3. PCA reactions in normal and C′-depleted rats following challenge with 24 μg SIII.

TABLE III

The Effect of Fresh Serum on PCA Reactions Induced in Rats by Homologous and Heterologous Rabbit Anti-Pneumococcus Sera Reacting with SVIII

Sera*—Rabbit anti-pneumococcus Type III, STD. No. 1 containing 1.36 mg. of anti-SIII N and 0.035 mg. of anti-SVIII N per ml. Rabbit anti-pneumococcus Type VIII, R8-76 containing 3.05 mg. of anti-SVIII N per ml.

SVIII injected intravenously	Heterologous anti-SVIII N (STD. No. 1), μg. per skin site			Homologous anti-SVIII N R8-76, μg. per skin site	
	7.0‡	3.5	1.8	5.0	2.5
	Average diameter of skin response, *mm.*				
μg.					
50 in saline	0 (4)§	0 (4)	0 (4)	17.5 (4)	11.2 (4)
100 in saline	6.2 (4)	2 (4)	2 (4)	19 (4)	14.2 (4)
200 in saline	15.1 (6)	6.2 (6)	2.7 (6)	18.2 (6)	13.7 (6)
200 in fresh rat serum	20.2 (6)	16.1 (6)	12.2 (6)	18.7 (6)	12.7 (6)

* We wish to thank Dr. Dalldorf, Division of Laboratories of the New York State Department of Health for these sera. Both sera were absorbed with the pneumococcus "C" substance prepared from a Type II culture.

‡ Used 0.2 ml. of serum per skin site.

§ Figure in parenthesis indicates the number of animals used for each determination.

SVIII N contained in a rabbit antiserum to Type III pneumococci. Each animal then received an intravenous injection of SVIII dissolved in saline or in fresh rat serum as designated in the table.

It is apparent that for the homologous reactions, an increase in the quantity of SVIII from 50 to 200 μg. per rat produced no appreciable change in the PCA response, confirming the data in Table I. Neither did the presence of fresh serum intensify this response. In contrast, a marked potentiation was observed with the heterologous immune system upon the addition of hemolytically active serum (*cf.* also section *D*, below).

TABLE IV

The Effect of C′ Component Reagents on the PCA Response in Normal Rats

24 μg. SIII injected intravenously with 1 ml. of:	C′ components present*	Rabbit anti-SIII N (R_3-175) injected intradermally, μg. per skin site		
		10.5	3.5	1.2
		Average response, *mm.*‡		
Isotonic NaCl	None	11.2	6.2	0
Fresh guinea pig serum	1, 2, 3, 4	23.5	16.2	10
Ammonia-treated guinea pig serum	1, 2, 3	22.5	14.8	11.8
Specifically de-C′ guinea pig serum	3	20.5	13.8	7.5
Fresh rat serum	1, 2, 3, 4	18.8	14.2	11.5
Heated rat serum (56°C.—15 min.)	3, 4	20.0	8.2	6.0
Zymosan-treated rat serum	1, 2, 4	11.0	2.5	0

* As determined by the methods described in reference 12.

‡ Four rats used for each determination.

C. The Effect of C′ Component Reagents on PCA Reactions in the Albino Rat

The observations recorded in the experiments with fresh sera were extended through the use of guinea pig and rat sera deficient in one or more of the four C′ components. Although precise quantitative interpretations cannot be applied to data obtained with the use of these reagents, it was anticipated that information might emerge which would facilitate the identification of the substance in normal serum which potentiates the PCA response. The data in Table IV summarize the results obtained in one experiment of this type in which PCA reactions were elicited with 24 μg. SIII and varying quantities of rabbit anti-SIII N (R_3-175). As in the experiment described immediately above, the various reagents were injected intravenously together with the antigen-dye mixture. Analogous results were obtained in replicate experiments when 12 μg. SIII were injected with fresh, heated, hydrazine- or zymosan-treated serum. As shown in Table IV, each of the serum reagents which augmented the PCA reactions was shown to possess C′3 activity. Hemolytic C′

titrations on sera of one group of normal rats 2 hours prior to, and 15 minutes after the intravenous injection of 2 ml. of fresh guinea pig serum showed an increase in activity from an average value of 45.9 $C'H_{50}$ per ml. to 79.8 $C'H_{50}$. With respect to the C′ component reagents, the presence of C′3 was verified by two titration procedures, the classical method described in reference 12, and the use of sensitized erythrocytes reacted with C′1, C′4, and C′2 as outlined in reference 28. In some preparations of heated serum, C′3 activity could not be demonstrated with the latter technique because of its anticomplementary activities. Destruction of C′3 by treatment with zymosan abolished the enhancing property of fresh whole serum. These data may be interpreted to indicate that the third component of C′ participates in potentiating PCA reactions in the rat.

D. Parallelisms between in Vitro Fixation of C′ and PCA Induction by Antigen and Antibody

1. In Homologous and Cross-Reacting Immune Systems.—Previous studies have shown that the C′-fixing potency of a cross-reacting polysaccharide or protein immune system is markedly inferior to that of the homologous reactants on an equivalent weight basis, particularly in experiments carried out at 37°C. (29). It was therefore considered pertinent to the problem at hand, to compare the efficacy of homologous and heterologous immune systems in provoking PCA reactions. Quantitative precipitin analyses of rabbit anti-pneumococcus Type III serum R_3-164 with SIII and SVIII at 0°C. indicated levels of 1.93 mg. anti-SIII N and 0.210 mg. of anti-SVIII N per ml. of serum. C′ fixation assays at 37°C. were also carried out with these reagents in the presence of diluted rat C′ containing 5 $C'H_{50}$ (30). Whereas 0.09 μg. of homologous antibody N fixed 4 of the 5 C′ units in the reaction with 0.005 μg. SIII, the same degree of C′ fixation with SVIII required 2.9 μg. of cross-reacting antibody N and 0.04 μg. of the Type VIII polysaccharide. The efficiency of the heterologous antibody was therefore less than 5 per cent of the homologous reactants under these conditions.

When PCA was studied in rats, the homologous SIII–anti-SIII reaction with serum R_3-164 yielded results which were entirely comparable with those summarized in Table I. Further, when 100 μg. of SIII was injected intravenously, PCA reactions could be elicited with as little as 0.48 μg. of homologous antibody N. In contrast, the same amount of SVIII failed to provoke demonstrable skin blueing even with 20.0 μg. of cross-reacting antibody N. When the SVIII level was doubled, to 200 μg., perceptible responses of 6 mm. were observed with 2.5 to 5.0 μg. of heterologous antibody N. These results are analogous to those reported for systemic anaphylaxis in the guinea pig and have been interpreted solely on the basis that fewer combining groups are available for the cross-reaction than for the interaction between SIII and its homologous

antibody (27). It is noteworthy, however, that the diminished efficiency of the heterologous immune system in provoking local or general anaphylactic reactions, is also associated with a markedly reduced C′-fixing potency. In experiments with rabbit anti-pneumococcus Type VIII serum, the SVIII polysaccharide yielded PCA reactions that were entirely comparable to those shown in Table I for the SIII immune system.

2. In the Reaction of Horse Anti-Pneumococcus Antibody with the Homologous Polysaccharide.—In a preliminary report of this study, note was taken of the slight but unexpected diminution of PCA reactions and C′ titers that was observed following the injection of horse anti-pneumococcus serum and its homologous polysaccharide into the rat (7). This finding seemed to conflict with the present working hypothesis that C′ participates in PCA since it has frequently been reported that the horse polysaccharide immune system does not fix guinea pig C′ (31–34). In view of the *in vivo* behavior of this system, the question was reinvestigated. Complement fixation tests of the conventional type with 5 $C'H_{50}$ (30) confirmed the earlier findings in showing that as much as 50 μg. of horse Type III or Type VIII anti-pneumococcus N failed to yield evidence of fixation with a wide range of SIII or SVIII concentrations respectively. However, when the C′ fixation studies were repeated in the presence of 50 or 100 $C'H_{50}$ (35), clear evidence of specific fixation was obtained. Thus, 25 μg. of antibody N from horse anti-pneumococcus Type III serum (No. 141) reacting with SIII in the cold for 20 hours fixed 24 out of the 100 $C'H_{50}$ initially available. The horse antiserum of Type VIII specificity appeared to be more reactive in this regard since 21 μg. of anti-SVIII N fixed 28 of 50 $C'H_{50}$ when reacted with SVIII at 37°C. for 90 minutes.

The data in Table V show that both of these horse sera also conferred PCA reactions in the rat and further, the Type VIII antibody produced more intense responses than did the horse anti-SIII at equivalent levels of antibody N. These findings provide additional evidence for the association of C′ fixation and PCA reactions. Moreover, the horse anti-pneumococcus Type VIII serum showed considerable cross-reactivity with SIII in that 128 μg. of anti-SIII N per ml. of serum was precipitated with the heterologous polysaccharide, SIII. Despite the presence of this precipitable cross-reacting antibody, the C′-fixing potency of the heterologous system comprising SIII and anti-SVIII was virtually negligible at 37°C. Thus, in a reaction mixture containing 50 $C'H_{50}$, 5 μg. of the cross-reacting antibody N fixed only 4 $C'H_{50}$, a value barely exceeding the experimental error of the method (*cf.* also references 35, 29). This inefficiency in fixation of C′ was accompanied by the failure to obtain PCA reactions in rats receiving 5 μg. of heterologous antibody N (average response, 2 mm. in 4 rats). With 13 μg. of cross-reacting antibody N, 5 mm. PCA responses were observed. Data of this type would seem to denote that the antigen-antibody reaction in itself does not necessarily suffice to evoke a PCA reaction.

3. In the Reaction of Rabbit Anti-Ribonuclease with Ribonuclease and the Acetylated and Guanidinated Derivatives of the Enzyme.—The availability of an antiserum to bovine ribonuclease afforded a further opportunity to confirm the association of PCA reactions with the fixation of C′. These studies were carried out with the serum of rabbit 172–1 containing 250 μg. anti-ribonuclease N per ml.[1] This antiserum, at a level of 2.5 μg. antibody N, fixed 81 of 100 guinea pig $C'H_{50}$ in reactions carried out at 0°C. with ribonuclease and 70 $C'H_{50}$ with the guanidinated derivative of the enzyme. The acetylated ribonuclease fixed only 14 $C'H_{50}$ with the same antibody level under optimal conditions. It could also be demonstrated that the acetylated enzyme combined

TABLE V

Induction of PCA Reactions in Rats by Horse Anti-Pneumococcus Sera Reacting with Homologous Polysaccharides

Sera*—Horse anti-pneumococcus Type III 141 (1.07 mg. anti-SIII N per ml.). Horse anti-pneumococcus Type VIII 649 (0.702 mg. anti-SVIII N per ml. and 0.128 mg. anti-SIII N per ml.)

Polysaccharide injected intravenously	Horse anti-SIII N (141), μg. per skin site				Horse anti-SVIII N (649), μg. per skin site				
	20	10	5	2	10	8.3	3.3	2.8	1.0
	Average diameter of response, *mm.*								
μg.									
102 SIII	16 (8)‡	11.2 (8)	8.5 (8)	2 (4)					
102 SIII	2 (4)	0 (4)	0 (4)	0 (4)§					
24 SVIII						9 (4)		8 (4)	2 (4)
100 SVIII					24 (6)		17 (6)		8.3 (6)

* We wish to thank Dr. Dalldorf, Division of Laboratories of the New York State Department of Health for these sera.

‡ Figure in parenthesis indicates the number of animals used for each determination.

§ The serum injected into these rats contained no precipitable anti-SIII following specific absorption of the antibody with SIII.

with the antibody and inhibited fixation of C′ on subsequent addition of the native enzyme. These experiments confirmed similar and prior observations by Van Vunakis *et al.* (36).

As seen in Table VI, the PCA reactions paralleled the *in vitro* fixation of C′ by the three antigen preparations. The acetylated enzyme fixed very few units of C′ and elicited minimal skin blueing only with 120 μg. of antigen N. In contrast, the native enzyme was far more efficient in both respects while the guanidinated derivative showed a slightly diminished C′-fixing potency and a moderately reduced capacity for the induction of PCA. It may also be noted that the acetylated ribonuclease preparation did provoke blueing of guinea pig

[1] We are indebted to Drs. Helen van Vunakis and Lawrence Levine of the New York State Department of Health for a generous supply of these reagents.

skins but to a lesser degree than did the native enzyme. Thus, guinea pigs prepared with 0.05 and 0.025 μg. of anti-ribonuclease N and challenged with 0.25 μg. of either ribonuclease N or its acetylated derivative yielded skin reactions averaging 13 and 10 mm. for the native enzyme. The reactions obtained with the acetylated preparation averaged 3 and 0 mm. at the same antibody levels.

4. In Normal Rats Following the Intravenous Injection of Zymosan.—Several experiments such as the one summarized in Table IV, indicated that zymosan-treated guinea pig and rat sera failed to potentiate PCA reactions in normal rats. In view of the destructive action of zymosan on C'3, an attempt was

TABLE VI

Comparison of PCA Responses and C'-Fixing Potency of Rabbit Anti-Ribonuclease (Serum 172-1) Reacting with the Native and Modified Enzyme Preparations

Antigen N injected intravenously		PCA reactions Rabbit anti-ribonuclease N, μg. per skin site				Maximal C' H_{50} fixed at 0°C. with 2.5 μg. anti-ribonuclease N
		12.6	6.3	3.1	1.6	
		Average diameter of skin response, *mm.*				
	μg.					
Ribonuclease	30		14.8 (13)*	11.0 (13)	7.0 (13)	
	15		11.0 (13)	7.0 (13)	5.1 (13)	81
	5		0 (6)	0 (6)	0 (6)	
Acetylated ribonuclease	120	5 (3)	3 (3)	3 (3)	1 (3)	
	60	1 (3)	1.7 (13)	0 (13)	0 (13)	14
	30		0.3 (13)	1.2 (13)	1 (13)	
Guanidinated ribonuclease	30	14.6 (11)	6.1 (11)	5 (11)	3.2 (11)	70
	15	5.4 (11)	2.5 (11)	1 (11)	0 (11)	

* Figure in parenthesis indicates the number of animals used for each determination.

made to extend these observations. Preparations of zymosan which effectively destroyed C'3 in fresh rat serum, were injected intravenously into normal rats. Some of the animals were bled for hemolytic C' estimations and others were used to study the effect of zymosan on the PCA response. The results of these experiments, as outlined in Table VII, again demonstrate a parallel diminution in the *in vitro* lytic activity and in the response of the rat to cutaneous anaphylactic reactions.

5. In Rats Rendered C'-Deficient by an in Vivo Antigen-Antibody Reaction.—An earlier study demonstrated than an *in vivo* antigen-antibody reaction resulted in a concomitant reduction of both hemolytic C' activity and PCA responsiveness (7). These observations were corroborated by the zymosan

studies described immediately above. The use of immune reactants was subjected to a more detailed study in an effort to explore the relationship between circulating C′ levels and reactivity to cutaneous anaphylaxis.

(*a*) *Time course studies of circulating C′ levels following an in vivo antigen-antibody reaction:* The data in Table VIII summarize the results obtained in three experiments wherein hemolytic activity measurements are given for varying intervals of time following an intravenous injection of rabbit antibody and an intravenous or intraperitoneal administration of the homologous antigen. The several experiments differ with respect to the specificity and quantity of immune reactants utilized for initiating the C′ depletion process. When both antigen and antibody are injected by the intravenous route, an immediate fall

TABLE VII

The Effect of Intravenous Injection of Zymosan on C′ Levels and PCA Responses in Albino Rats

Zymosan injected intravenously	No. $C'H_{50}$ per ml.		PCA reactions, μg. antibody N injected per skin site					
			Rabbit anti-Ea			Rabbit anti-SIII		
	Before zymosan injection	2 hrs. after zymosan injection	15	5	2	10.5	3.5	1.2
			Average diameter of skin response, *mm.*					
mg.								
0			14 (9)	9.4 (9)	5 (6)	17.1 (6)	11.3 (6)	4.5 (6)
1.0*	43 (4)‡	26 (4)						
2.5	41 (4)	11 (4)				0 (8)	0 (8)	0 (8)
5.0	34 (4)	10 (4)				0 (12)	0 (12)	0 (12)
10.0	41 (10)	7 (11)	1 (19)	0.6 (19)	0 (19)			

* The zymosan suspension was injected 30 minutes prior to the intravenous injection of the dye-antigen mixture.

‡ Figure in parenthesis indicates the number of rats used for each determination.

in the hemolytic activity of the sera is apparent. However, when the antigen is injected intraperitoneally, the decrease occurs more gradually, as might be expected. The latter schedule was selected for subsequent studies to ascertain the association of the decline in C′ activity with cutaneous reactivity to passive anaphylaxis. The data in Table IX summarize the results of one such experiment in which PCA and C′ levels were studied at various intervals following the C′ depletion procedure. A marked parallelism is apparent between the circulating C′ levels and the susceptibility of the animals to PCA. Thus, within 15 minutes after the intraperitoneal injection of ovalbumin, C′ levels are perceptibly lower and PCA reactions are essentially abolished at antibody N levels of 0.4 and 1.2 μg. per skin site. The simultaneous decline in both activities continues for at least 2 hours before a return to normal values is observed in the PCA responses as well as in the hemolytic C′ activity.

TABLE VIII

Hemolytic C′ Levels in Rats Following in Vivo Antigen-Antibody Reactions

Time after initiation of *in vivo* immune reaction	Rabbit anti-Ea N; 500 μg. intravenously* Ea 280 μg. N, intravenously	Rabbit anti-SIII N; 420 μg. intravenously SIII, 53 μg. intraperitoneally	Rabbit anti-SIII N; 210 μg. intravenously SIII, 53 μg. intraperitoneally
	Average $C'H_{50}$ per ml.		
hrs.			
0	41 (28)	32 (20)	42 (6)
0.13	3 (4)		
0.17		25 (4)	
0.25			48 (2)
1.0			10 (4)
1.15	7 (4)	19 (4)	
2.0			4 (4)
2.7		4 (4)	
3.4	14 (4)		
4.0			20 (4)
4.7		4 (4)	
5.0	19 (4)		
6.2		13 (8)	30 (4)
7.5	29 (4)		
8.0			22 (2)
22.4	42 (4)		

* Rabbit anti-hen ovalbumin 6456 contained 0.65 mg. of antibody N per ml. Ea, twice recrystallized hen ovalbumin obtained from Worthington Biochemical Company, Freehold, New Jersey.

TABLE IX

Effect of an in Vivo Immune Reaction on Levels of Serum C′ and PCA Responsiveness

Antibody injected intravenously—200 μg. rabbit anti-Ea N (serum 6456). Antigen injected intraperitoneally—80 μg. Ea N.

Time after Ea-anti-Ea injections	Average $C'H_{50}$ per ml.	Rabbit anti-SIII N (R_3 – 175) injected intradermally, μg. per skin site			
		10.5	3.5	1.2	0.4
		Average response, *mm.**			
hrs.					
0	41 (8)‡	18 (4)	14.8 (4)	8.3 (4)	5 (4)
0.25	24 (6)	16 (3)	6 (3)	2.7 (3)	0 (3)
0.75	>4 (4)	8 (4)	5 (4)	0 (4)	0 (4)
2.0	9 (4)	2.8 (16)§	1.6 (16)	0.3 (16)	0.2 (16)
4.0	25 (4)	21 (3)	15 (3)	12 (3)	6 (3)
6.0	26 (8)	16 (3)	17 (3)	4 (3)	3 (3)

* PCA reactions provoked with 24 μg. SIII.

‡ Figure in parenthesis indicates the number of rats for each determination. Different animals were used for C′ and PCA estimations.

§ The results of several experiments are combined. In many instances the PCA reactions are completely abolished under these experimental conditions.

A further demonstration of the possible dependence of PCA reactivity on the hemolytic activity of the animal's serum is indicated by the data in Table X. In these experiments, the PCA tests were all carried out at 2 hours after the *in vivo* decomplementation procedure. However, varying quantities of immune reactants were employed, thus providing a graded diminution of both C′ activity and PCA responsiveness. The intensity of the PCA response varied not only with the hemolytic activity of the C′ but also with the quantity of SIII used to provoke the skin reaction. The data thus show that for a given C′

TABLE X

Comparative Studies of C′ Levels and PCA Reactions in C′-Deficient Rats

μg. Ra anti-Ea N* / μg. Ea N used for decomplementation	Average $C'H_{50}$ per ml.	SIII used for challenge	Rabbit anti-SIII N injected intradermally, μg. per skin site			
			10.5	3.5	1.2	0.4
			Average response, *mm.*			
		μg.				
100/40	22	12	6 (4)	3 (4)	0 (4)	0 (4)
200/80	9	12	2.8 (16)	1.6 (16)	0.3 (16)	0.2 (16)
100/40	22	24	13.5 (4)	9.2 (4)	4.8 (4)	0 (4)
200/80	9	24	4 (26)	3.4 (14)	1.5 (26)	0.6 (14)
400/200	4	24	0 (3)	0 (3)	0 (3)	0 (3)
100/40	22	49	14.5 (4)	9.5 (4)	3.2 (4)	0 (4)
200/80	9	49	7.7 (8)	9.5 (4)	1.6 (4)	0 (4)
200/80	9	98	16.8 (4)	10.5 (4)‡	7.5 (4)	
200/80	9	196	14.8 (4)	11.8 (4)‡	9.5 (4)	
1000/100	3	211	0 (8)	0 (8)‡	0 (8)	

* The rabbit antiserum in the amounts indicated, was injected intravenously and followed immediately by an intraperitoneal injection of the antigen.

‡ Actually used 2.6 μg. anti-SIII N at these sites.

level, *e.g.*, 9 $C'H_{50}$ per ml., the PCA reaction is proportional to the amount of antigen; while at a constant level of the latter, the existing C′ activity would appear to influence the extent of skin blueing. Thus, when C′ levels were reduced to 3 $C'H_{50}$ per ml. with 1 mg. of antibody N, PCA reactions were not demonstrable even when more than 200 μg. SIII were injected as the challenge dose. When the systemic immune reaction was reduced to 0.1 mg. of anti-Ea N, definite PCA reactions resulted when 24 μg. of SIII were injected and in these rats, the C′ levels were substantially higher.

In most of the experiments of this study, PCA reactions were elicited with rabbit antibody to the Type III pneumococcus reacting with the homologous polysaccharide, while C′ depletion was effected through the interaction of hen

ovalbumin and homologous rabbit antibody. The data in Table XI show the effect of decomplementation with several different immune systems on PCA reactions induced with rabbit antibody to Type I pneumococci and the corresponding polysaccharide. Similar findings resulted when rabbit antibovine gamma globulin or albumin were used to elicit the cutaneous anaphylactic reactions.

It should be mentioned that when rats were injected systemically with a rabbit polysaccharide immune system, depressions in cutaneous reactivity to ovalbumin were observed only with 400 to 500 μg. of antibody N. However, decomplementation with 200 μg. of anti-SI N or anti-SIII N, virtually ob-

TABLE XI

Effect of Variations in the Specificity of the in Vivo Immune Reactants on PCA Responses

In vivo immune reactants*	Rabbit anti-SI N, μg. per skin site			
	3.4	1.1	0.4	0.1
	Average diameter of skin response, *mm.*‡			
None	12.5	11.5	9.8	3.8
Rabbit anti-Ea + Ea	4.2	2.5	0	0
Rabbit anti-BGG + BGG	3	2.5	2.5	0
Rabbit anti-SIII + SIII	5.2	3	1.5	0
Horse anti-SIII + SIII§	11.1	5.2	1.2	0

* In each case 200 μg. of antibody N were injected intravenously and a slight excess of antigen intraperitoneally.

‡ Four rats were used for each experiment.

§ The C′ titers in these rats diminished from 47 to 34.5 $C'H_{50}$ per ml. in 2 hours after the systemic immune reaction.

literated PCA reactions to immune systems other than Ea-anti-Ea as typified by the data in Table XI.

(*b*) *In C′-depleted rats injected with C′ component reagents:* The data in Table IV indicated that PCA reactions in normal rats were enhanced when various C′ component preparations were substituted for the saline diluent injected with the antigen. The possibility of confirming these findings in C′-depleted animals was explored in experiments which are summarized by the data in Table XII. In these studies, the rats received an intravenous injection of 200 μg. of rabbit anti-ovalbumin N and an intraperitoneal inoculation of 80 μg. of ovalbumin N. The designated amounts of SIII polysaccharide, mixed with Evans blue, were diluted in saline or in one of the several serum preparations as indicated in Table XII and injected intravenously, 2 hours after preparation of the skin sites with rabbit anti-SIII.

As was observed in the studies with normal animals, the C′-depleted rats

also showed an enhanced response when fresh, heated (56°C.—15 minutes), or ammonia-treated serum was incorporated in the antigen-dye mixture. The potentiation was less pronounced with serum heated for 1 hour or absorbed with washed antigen-antibody aggregates, whereas zymosan-treated serum was ineffectual. The lack of enhancement by zymosan-treated serum in these

TABLE XII

Restoration of PCA Reactions in C'-Depleted Rats

SIII per rat	SIII injected intravenously with 1 ml. of:	Rabbit anti-SIII N, μg. per skin site			
		10.5	3.5	1.2	0.4
		Average diameter of skin response, *mm.*			
μg.					
6	Isotonic saline	0 (8)*	0 (8)	0 (8)	0 (8)
6	Fresh guinea pig serum	8.6 (12)	7.2 (12)	5.1 (12)	3 (12)
6	Heated guinea pig serum (56°C.—60 min.)	2.5 (4)	2.5 (4)	0.5 (4)	3 (4)
12	Isotonic saline	1.8 (12)	1.1 (12)	0.4 (12)	0.3 (12)
12	Fresh guinea pig serum	3.5 (20)	4.1 (20)	1.8 (20)	0.5 (20)
12	Heated guinea pig serum (56°C.—60 min.)	4 (8)	4.5 (8)	2.5 (8)	0.8 (8)
24	Isotonic saline	4.3 (34)	3.5 (22)	1.4 (34)	0.4 (22)
24	Fresh rat or guinea pig serum‡	10.4 (44)	6.6 (20)	3.9 (32)	0.5 (20)
24	Heated serum§ (56°C.—15 min.)	10.5 (11)	5.8 (11)	3.7 (11)	2 (11)
24	Heated serum§ (56°C.—60 min.)	5.5 (14)	4.9 (14)	2.4 (14)	0.7 (12)
24	Ammonia-treated rat serum	9.9 (8)	7.5 (8)	6.0 (8)	0 (8)
24	Zymosan-treated rat serum	3.9 (7)	2.1 (7)	1.4 (7)	0 (7)
24	Decomplemented serum‖	6.1 (12)	4.2 (12)	1.5 (12)	0 (12)

* Figure in parenthesis indicates the number of animals used for each determination.

‡ The injection of fresh guinea pig serum in one group of these rats increased the number of $C'H_{50}$ from a mean value of 9 to 37 per ml.

§ Sera from rats or guinea pigs yielded essentially similar results.

‖ Serum treated with washed specific precipitate (200 μg. rabbit anti-bovine gamma globulin N plus 24 μg. of homologous antigen N per ml. of serum).

animals cannot be adequately explained on the basis of our present knowledge concerning all the factors which might influence PCA. Clearly, this observation necessitates further study.

E. The Association of Variations in PCA Reactivity to Blueing of the Rat Skin by Other Than Immune Reagents

It has been established that the intravenous injection of a dye such as Evans blue may be utilized to demonstrate local permeability changes in cutaneous

sites inoculated with a variety of pharmacologically active substances. Several reagents (histamine, 48/80, dextran, serotonin, normal rat and guinea pig serum) were tested to ascertain whether the variations in PCA reactivity observed in this study could be attributed to an altered permeability of the minute vessels of the skin induced by the experimental procedures (37–39). The evidence obtained in numerous trials clearly indicated that the blueing of the rat skin resulting from these reagents was neither enhanced nor suppressed in those animals showing an altered PCA reaction in response to the injection of fresh serum, C′ component reagents or the *in vivo* decomplementation procedure.

DISCUSSION

The proposed hypothesis linking C′ and PCA in a causal relationship can be considered in terms of three subsidiary problems, (*a*) the detection of a host factor which, together with antigen and antibody, influences the outcome of the cutaneous response, (*b*) the identification of this reactant as hemolytic C′, and (*c*) the elucidation of its role as an essential mediator or as an intensifier of the local anaphylactic reaction.

The evidence accumulated thus far, clearly suggests that the host supplies one of the reagents which participates in PCA. This conclusion emerges from data such as that in Tables I, II, and IV (*cf.* also Figs. 2 and 3) which show that the intravenous injection of fresh serum is associated with a considerable increase of the allergic reaction. Moreover, this effect appears to be specifically related to the antigen-antibody reaction at the skin site since the intravenous injection of serum did not intensify the blueing of the skin produced by intradermal injections of histamine, 48/80, dextran, serotonin, or fresh serum.

Characterization of the serum constituent which participates in the induction of PCA, as C′, presents a somewhat more complex problem. In part, this difficulty stems from the necessity of defining C′ mainly in terms of operational considerations. On this basis, PCA may be regarded as the final step of a series of events initiated as an *in vivo* C′ fixation reaction carried out in the presence of a large excess of C′ supplied by the circulating blood in continuous contact with the reaction site. A logical consequence of this analogy implies that the present findings cannot be adequately interpreted in terms of the antigen-antibody reaction *per se*. The first pertinent line of evidence relates to the magnitude and sensitivity of the allergic response as influenced by the addition of hemolytically active sera. In a comparable fashion, the extent of *in vitro* C′ fixation also varies directly with the level of C′ initially available for the reaction (40). Furthermore, a sharp diminution in circulating C′ levels induced in the rats by zymosan or by specific immune aggregates of known reactivity, is accompanied by a pronounced decline in the PCA response (Table VII, IX, and X). The two events follow a similar time course and, in addition, PCA

reactions are only moderately suppressed when C′ levels are partially reduced (Tables IX, X, and XI). As shown by the data in Table X and Fig. 1, the extent of the PCA reaction can be varied in either direction by simultaneous manipulation of the hemolytic potency of the host's serum or the level of antigen used for the challenge. When the hemolytic activity of C′ is reduced to barely detectable levels (Table X and reference 7), PCA reactions are abolished and fail to appear even with large excesses of antigen.

Experiments with immune systems of varying C′-fixing potencies provide further evidence in accord with the proposed mechanism (Tables V and VI). Thus, the correlation between the efficiency of an immune system to fix C′ and to provoke PCA has been found applicable in comparisons between horse and rabbit antipneumococcus sera reacting with their homologous polysaccharides. A similar parallelism has been demonstrated for rabbit anti-ribonuclease in its reaction with three antigens, the native enzyme as well as the guanidinated and acetylated derivatives. With the first two proteins, comparable efficiencies were noted in C′ fixation and PCA experiments. The acetylated ribonuclease reacting with the same antibody fixed little C′ and was markedly less able to call forth a PCA reaction.

The studies with the homologous and cross-reacting pneumococcus-immune systems further support the analogy that has been drawn between PCA and C′ fixation. It has been well established and confirmed in this study that the cross-reaction between SVIII and rabbit antipneumococcus Type III antibody is far less efficient than are the homologous reagents in the fixation of C′ (Table III and reference 29). A similar deficiency in PCA productivity has now been noted. It would appear difficult to interpret these findings solely on the basis that the antigen has fewer combining groups available for the heterologous than for the homologous antibody. Under experimental conditions in which the skin blueing due to the homologous reactants attained maximal values, (Table III) only the PCA reactions involving the heterologous reagents were potentiated by fresh serum, possibly because the greater availability of C′ resulted in more efficient fixation in the latter situation. With the homologous reactants, fixation may already have reached peak levels so that the PCA reactions were thereby not altered (*cf.* reference 40). These data provide additional evidence that the intravenous serum injection did not produce any generalized changes in cutaneous permeability.

The experiments summarized in Tables IV and XII and Fig. 3 were designed to fulfill some of the criteria currently available for the identification of C′. There emerges from these data the indication that one of the limiting factors for PCA is a serum constituent which is relatively heat-stable and possesses other properties associated with C′3 such as susceptibility to destruction by zymosan but not by ammonia or hydrazine. Furthermore, other reagents containing C′3 activity, such as fresh serum depleted of hemolytic activity by

specific fixation, also retain the capacity to enhance PCA in the normal rat (Table IV). The failure of this latter reagent to exert a similar effect in C′-deficient rats (Table XII) may signify that adequate levels of all four C′ components are required for this reaction as in other immune processes which utilize C′.

In an effort to ascertain whether the properdin system (41) could be implicated in the present findings, titrations for properdin activity were carried out with two pools of serum, one obtained from normal and the other from C′-deficient rats. These assays were performed by Dr. Myron Leon according to the procedure described in reference 42. Both serum specimens yielded essentially similar values. However, the paucity of data with regard to rat properdin levels as estimated by this technique, prevents a rigorous exclusion of this factor at the present time.[2]

Evidence for the participation of C′ in PCA is in line with previous reports based on studies involving other aspects of hypersensitivity such as the Schultz-Dale reaction (43, 44), activation of plasma proteolytic activity (6, 45), and the release of serotonin and histamine from sensitized tissues (46, 47). The *in vivo* role of C′ in cytotoxic phenomena emerges from the recent work of Spear (48) confirming earlier studies referable to the Forssman antigen in chick embryos (49, 50). It should be noted, however, that Mongar and Schild have recently concluded that C′ does not participate in the release of histamine or in the anaphylactic contraction of smooth muscle (51).

A further complexity encountered in this investigation pertains to the variability in response observed in different groups of rats. Although all animals were uniform with respect to strain, sex, weight, and source, the minimal amount of antigen required to elicit PCA in normal rats received at different times, varied from 6 to 12 μg., and on rare occasions to 24 μg. For this reason, the differences between treatments were estimated from experiments performed on the same day with a single shipment of rats. Statistical evaluations[3] estimated by using an error term reflecting the variability of the measurements within individual experiments on a single day were in complete agreement with the qualitative interpretations given above.

The analogy that has been advanced between the *in vitro* fixation of C′ and PCA is limited in several respects. Both of these phenomena are highly complex and involve numerous intermediate stages, only one of which, the final event, has been subjected to systematic study. Moreover, the present studies do not provide unequivocal proof as to the absolute requirement for C′ in the initiation

[2] The authors are grateful to Dr. Leon for his cooperation in the performance and interpretation of these titrations.

[3] We are grateful to Dr. Paul Meier of the Department of Biostatistics of The Johns Hopkins School of Hygiene and Public Health for his advice on the statistical treatment of these data.

of PCA despite the strongly suggestive evidence. Thus, serum C′ may merely serve to potentiate the PCA reaction, not through the utilization of C′ in the classical sense but rather by intensifying the interaction of antigen with antibody as noted in specific precipitation (52, 53). In either case, the ability to inhibit or augment the local anaphylactic reaction by manipulation of serum C′ levels creates further opportunities to elucidate some of the individual reaction steps whose cumulative effect produces the tissue injury associated with hypersensitivity reactions of the immediate type.

SUMMARY

Experiments are described indicating that the magnitude and sensitivity of the passive cutaneous anaphylaxis (PCA) response in normal rats to a given level of immune reagents, may be enhanced by the addition of hemolytically active sera.

A similar enhancement in normal rats has been obtained with C′ component reagents possessing properties associated with the third component of C′.

Parallelisms between *in vitro* fixation of C′ and PCA induction by antigen and antibody are shown. The horse anti-pneumococcus system has low C′-fixing potencies and is also less efficient than the rabbit polysaccharide system in the induction of PCA.

Findings of a similar nature were observed in the reaction of rabbit anti-ribonuclease with ribonuclease, the acetylated and guanidinated derivatives of the enzyme.

The injection of hemolytically active serum into C′-deficient rats was accompanied by a partial restoration of PCA. Restorative effects were also noted with heated and ammonia-treated serum.

The return of hemolytic potency and responsiveness to PCA in C′-depleted rats, follow a similar time course.

The data presented indicate that the PCA reaction can be studied as a function of at least three variables, antigen, antibody, and a serum constituent resembling C′.

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