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A PERMEABILITY FACTOR RELEASED FROM GUINEA-PIG SERUM BY ANTIGEN-ANTIBODY PRECIPITATES

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MANY attempts have been made to identify the mediators of the increased vascular permeability of inflammation. Most of the work has been concerned with the liberation of histamine but, more recently, other substances such as 5-hydroxy-tryptamine, polypeptides and serum globulins have received attention (Spector, 1958). Nearly all the more recent work has dealt with inflammation induced by irritants, burns, releasers of active substances (such as dextran, egg-white, compound 48/80, etc), or with *in vitro* changes of normal serum, plasma or lymph. Little is known about the mediators of allergic inflammation: it is tacitly assumed by many authors that histamine alone is involved. However, hesitation in accepting the histamine hypothesis stems from the failure of anti-histamine drugs to inhibit certain types of allergic response (Brocklehurst, Humphrey and Perry, 1955). This failure is usually explained in terms of Dale's (1948) concept that intrinsically-liberated histamine is less accessible to antagonistic drugs. Rocha e Silva and Rothschild (1955) showed that cutaneous anaphylaxis in rats could still be obtained in animals from which histamine had been depleted by dextran, ovomucoid or compound 48/80. Again, Inderbitzin and Dobrić (1959) found that the pattern of changes in histamine content of skin at sites of passive cutaneous anaphylaxis resembled those at adjacent control areas of abdominal skin. They concluded that the skin-histamine released during cutaneous anaphylaxis could not have mediated the resulting inflammation. As an alternative hypothesis, the possibility that allergic inflammation is caused by other substances is equally tenable and should be explored.

Allergic reactions result from the interaction of antigen, antibody and body constituents and can take many forms. We have selected for study the acute inflammatory response induced by injecting antigen into the skin of sensitized animals. As a first step we have added antigen-antibody precipitates (Ag/Ab) to serum and studied the ability of the treated serum to cause a rapid and sustained increase in vascular permeability when injected intradermally. In order to simplify interpretation of results we have, as far as possible, restricted our

observations to a single species—the guinea-pig—and have found that, under certain specified conditions, the addition of Ag/Ab to guinea-pig serum leads to the formation of a permeability factor which is distinct from histamine or a histamine liberator. We refer to this factor as PF/P.

MATERIALS AND METHODS

Antigen-antibody precipitate.—Sandylop rabbits were immunized with alum-precipitated egg-albumin solution by repeated intravenous injections over a period of 8 weeks. The pooled antiserum was stored at −20°. Antibody N content was determined by the quantitative precipitation technique (Kabat and Mayer, 1948). The Ag/Ab was prepared at equivalence by adding a solution of crystallized egg-albumin (Armour) to a sample of the antiserum and incubating at room temperature for 1 hr. All precipitates were prepared freshly each day, and, before use, washed three times with saline and centrifuged.

Activation of serum.—Blood was obtained from the heart of normal guinea-pigs, of either sex, weighing between 300–800 g. Serum, obtained from this blood was always used on the day of collection. Each ml. of neat or diluted serum was added to a portion of washed Ag/Ab containing 0·05 mg. of protein N as determined by the micro-Kjeldahl method. The Ag/Ab was suspended in the serum by shaking and the mixtures allowed to stand on the bench for ½ hr. before being tested for their ability to increase capillary permeability.

Increased capillary permeability.—All the experiments were done in guinea-pigs. It was essential to use healthy animals given an adequate source of vitamin C. They were fed on crushed oats and cabbage and in addition were given 4 mg. of vitamin C by mouth twice weekly. Size and sex were less important—satisfactory results were obtained with both males and females in the weight-range 200–500 g.

The animals were shaved with electric clippers and a solution of pontamine sky blue (5 per cent in 0·45 per cent saline) 0·15 ml./100 g., given into an ear vein 2–5 min. before the intradermal injection of the solutions under test. The intradermal injections were made with a 20 gauge needle, bevel downwards and introduced into the skin as superficially as possible. No anaesthetic was used—the animals were held by an assistant. Pinching of the skin at the site of injection was avoided. Up to 10 injection sites were used on each animal and in many experiments the order of injection was randomized. The animals were killed by a blow on the head 15 min. after the intradermal injection and portions of skin containing the injection sites were removed and pinned, inside uppermost, on a cork mat. The maximum and minimum diameters of the blue areas were measured on the following day with the aid of dividers and a ruler calibrated in millimetres.

As a rule each group contained 4 control animals and 4 animals which received mepyramine (5 mg./kg. intraperitoneally 15 min. before the intradermal injection) to distinguish between the effects of histamine and those of other substances.

Histamine-release in vitro.—Three methods were used to test the activated serum for histamine-releasing properties. Guinea-pig ilea were mounted in oxygenated Tyrode's solution containing atropine 1 μg./ml.; guinea-pig lungs were perfused by the method of Rocha e Silva and Aronson (1952) and minced guinea-pig lungs were used according to Mongar and Schild (1953). Compound 48/80 gave the expected histamine-release in all these preparations.

Serum kinin.—Rat uteri and duodena were prepared by the methods described by Gaddum and Horton (1959). Serum kinin prepared from ox serum and trypsin contracted the uterus and relaxed the duodenum at 1 μg./ml.

Fractionation of serum.—Serum proteins were fractionated by chromatography on diethylaminoethyl cellulose (DEAE cellulose Eastman Kodak) having a specific adsorption of 44 (determined by the method of Peterson and Sober, 1956) and containing 0·88 m-mole ionizing groups/g. The cellulose was suspended in 0·01 M phosphate buffer, pH 8·0, equilibrated and the pH of the supernatant fluid adjusted to pH 8·0 by cautious addition of phosphoric acid. Columns were packed according to the method of Sober, Gutter, Wyckoff and Peterson (1956). Serum was equilibrated with the starting buffer (0·01 M phosphate buffer pH 8·0) by dialysis for 24 hr. at 2°. The small precipitate of euglobulins which resulted from this dialysis was separated by centrifugation and the supernatant fluid was then applied to the column. The entire separation was performed in the cold room at 2°. A cone-sphere apparatus

was used to give a gradient for elution (Fahey, McCoy and Goulian, 1958). The spherical mixing vessel contained 1 litre of starting buffer and the cone held 500 ml. 0·3 M NaH_2PO_4 pH 4·3. The eluate was collected in fractions of 10 ml.; optical densities were measured at 280 mμ. Fractions were bulked according to the elution pattern; the bulked fractions were dialysed, freeze-dried and examined by paper electrophoresis.

RESULTS

Formation of a permeability factor (PF/P) by the addition of Ag/Ab to serum

The intradermal injection of neat or diluted guinea-pig serum into guinea-pigs produced an increase in capillary permeability only slightly greater than that produced by saline. On the other hand, sera containing Ag/Ab (equivalent to 0·05 mg. protein N per ml.) produced an increase in permeability comparable to that produced by 0·5 μg. of histamine. Furthermore, the effect of the treated serum was only slightly inhibited by 5 mg./kg. of mepyramine given intraperitoneally 15 min. before the intradermal injection : this dose completely inhibited the effect of histamine. Table I shows the results of such an experiment in which groups of 4 guinea-pigs were used.

TABLE I.—*Activation of Guinea-pig Serum by Antigen-antibody Precipitate : Effect of Mepyramine*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
Neat serum + Ag/Ab	16·6	19·3
1 : 10 serum + Ag/Ab	11·3	17·5
Neat serum	5·2	7·3
1 : 10 serum	5·4	6·4
Saline + Ag/Ab	5·6	7·1
Histamine in saline (0·5 μg.)	3·8	18·8
Saline	4·4	4·5

Properties of PF/P which distinguish it from other permeability factors

The mild inhibitory effect of mepyramine on PF/P appeared to rule out histamine as the main cause of increased permeability. No contraction of a guinea-pig ileum, suspended in 20 ml. of Tyrode's solution, was caused by 1 ml. of activated serum, nor did it release histamine from minced or perfused guinea-pig lung. The duration of increased permeability was greater with PF/P than with histamine as is shown in Table II. For this experiment groups of 4 animals

TABLE II.—*Comparative Persistence of the Permeability Increasing Effects of PF/P and Histamine*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)		
	Dye 60 min. after i.d. injection	Dye 30 min. after i.d. injection	Dye 2 min. before i.d. injection
1 : 10 serum + Ag/Ab	6·1	15·3	15·9
1 : 10 serum	5·1	4·2	3·8
Histamine (0·5 μg.)	3·0	3·1	18·1

received dye at various intervals after the intradermal injection. PF/P was still fully active 30 min. after injection whilst the effect of histamine was characteristically short-lived.

PF/P is not a kinin because it did not contract the rat uterus or relax the rat duodenum. Furthermore, the activity was neither dialysable nor extractable with hot ethanol. Holdstock, Mathias and Schacter (1957) have shown that a kinin-like substance is formed when guinea-pig serum is diluted. This kinin is unstable, being rapidly destroyed by the peptidases of serum. A 1 : 10 dilution of guinea-pig serum in saline was prepared and incubated in a water bath at 37°. At various intervals 1 ml. amounts of the dilution were added to an isolated organ bath containing a rat uterus. The period elapsing between addition of the diluted serum and contraction of the uterus was noted. This is a measure of the amount of kinin present. The experiment was then repeated using 1 : 10 serum containing Ag/Ab (0·05 mg. protein N per ml.). Table III shows the mean result of 4 such

TABLE III.—*Lack of Effect of Ag/Ab on the Formation and Destruction of Kinin in Freshly-diluted Guinea-pig Serum*

Time after dilution (min.)	Lag period before contraction of uterus	
	Dilution + Ag/Ab	Control dilution
0	>2 min.	>2 min.
2	1 min.	1 min.
10	40 sec.	40 sec.
20	1 min. 40 sec.	1 min. 40 sec.
30	>2 min.	>2 min.

experiments. Ag/Ab did not alter either the rate of formation or the rate of destruction of kinin.

Contact with a glass surface appears necessary for the activation of some permeability factors (Armstrong, Keele, Jepson and Stewart, 1954). This is not so for PF/P since plasma obtained from blood collected in a siliconed syringe and transferred to a polythene centrifuge tube in the presence of 1 : 10,000 heparin was still activated by Ag/Ab in a similar manner to serum obtained from blood allowed to clot in glass (Table IV).

TABLE IV.—*Formation of PF/P in Guinea-pig Plasma Collected in a Siliconed Syringe and Transferred to a Polythene Tube in the Presence of* 1 : 10,000 *Heparin*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine 5 mg./kg. i.p.	Controls
1 : 10 serum + Ag/Ab	12·5	17·4
1 : 10 plasma + Ag/Ab	11·4	16·4
1 : 10 serum	6·3	7·6
1 : 10 plasma	5·1	6·9
Saline + Ag/Ab	4·9	7·1

Some other properties of PF/P

Only preliminary studies have been made of the conditions necessary for optimal production of PF/P. Continued presence of Ag/Ab is not required for activity since its removal by centrifugation just before injection did not affect the result (Table V). Serum heated at 60° for 30 min. was not activated by Ag/Ab (Table VI).

TABLE V.—*Effect of Removing Ag/Ab from Activated Serum*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
(1) 1 : 10 serum with Ag/Ab still present	14·3	18·6
(2) 1 : 10 serum : Ag/Ab removed by centrifugation	15·2	18·4
(3) 1 : 10 serum	9·6	10·4
(4) Saline with Ag/Ab still present	5·8	7·9
(5) Saline : Ag/Ab removed by centrifugation	4·3	5·8
(6) Control saline	3·6	5·6

All tubes were allowed to stand for 1 hr. at room temperature. Tubes 2 and 5 were then centrifuged for 5 min. of 4000 r.p.m., the supernatants removed and 0·1 ml. amounts injected into guinea-pigs.

TABLE VI.—*Lack of Formation of PF/P in Heated Serum*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
1 : 10 serum + Ag/Ab	15·8	17·1
1 : 10 serum heated 60° 30 min. + Ag/Ab	6·5	10·9
1 : 10 serum	6·0	8·6
1 : 10 serum heated 60° 30 min.	3·3	6·3
Saline + Ag/Ab	3·8	5·2

Activation was inhibited by isotonic buffers prepared from citrate, phosphate, barbiturate, acetate and tris (hydroxymethyl)aminomethane.

When serum was diluted 1 : 5 with saline and allowed to stand 1 hr., then Ag/Ab added and the mixture allowed to stand for a further 30 min., subsequent dilution to 1 : 10 with isotonic phosphate buffers (pH 6·8 and 7·4) did not inhibit PF/P. It seems therefore that phosphate inhibits production of PF/P but not its activity.

Activation was inhibited by heparin at 0·2 mg./ml. or more. Neither Soya bean trypsin inhibitor (1 mg./ml.) nor 10^{-3}M-di-isopropylphosphofluoridate (DFP) inhibited activation.

At the moment little is known about the specificity of Ag/Ab but an effect similar to that of PF/P was produced by the addition of zymosan (1·5 mg./ml.) to serum (mean lesion diam. 11·9 mm. in animals dosed with mepyramine and 14·9 mm. in control animals), but not by kaolin (25 mg./ml.) (mean lesion diam. 5·5 mm. in both groups).

Spector (1957) has shown that inflammatory pleural exudates, collected 24 hr. after intrapleural injection of turpentine in rats, fail to increase capillary permeability when injected intradermally, but can be induced to do so by lung mitochondria. A similar exudate obtained from guinea-pigs was inactive but became active after the addition of Ag/Ab (Table VII).

TABLE VII.—*Activation of 24 hr. Pleural Exudate by Ag/Ab*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
24 hr. exudate . . .	7·5	7·9
24 hr. exudate + Ag/Ab .	12·7	17·9
Ag/Ab in saline . . .	5·3	6·1

Activation of serum by acid

Experiments designed to test the effect of pH changes on the formation of PF/P by the use of conventional buffers were precluded by the inhibitory action mentioned above. Consequently an attempt was made to study this effect by addition of acid or alkali, relying on the buffering power of the serum itself. This attempt was also frustrated since acid alone without the intervention of Ag/Ab led to the activation of guinea-pig serum. A sample of guinea-pig serum was diluted 1 : 10 with saline, allowed to stand 1 hr., adjusted to various pH values with hydrochloric acid (0·01 N) or sodium hydroxide (0·01 N), allowed to stand a further 20 min., neutralized and then injected intradermally into blued guinea-pigs. Those sera which had been at a pH of 5·5 or less caused an increase in permeability similar in size and intensity to that produced by PF/P. Sera which had been at pH values between 6·0 and 11·0 were inactive (Table VIII).

TABLE VIII.—*Formation of Permeability Factors in Serum by Acid*

Initial pH of serum	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
5·0 .	Not done	16·0
5·5 .	9·8	11·8
6·0 .	5·0	6·2
7·0 .	4·0	4·0
8·0 .	4·0	4·0
9·0 .	4·0	4·0
11·0 .	4·0	4·0

Fractionation of serum on diethylaminoethyl cellulose

In an attempt to find the constituent(s) of guinea-pig serum necessary for activation by Ag/Ab, a sample of serum (37 ml.) was fractionated by chromatography on diethylaminoethyl cellulose. Eleven fractions were obtained from the

chromatogram (Table IX, Fig.). However, when the serum was reconstituted from these fractions together with the euglobulins it was found to increase capillary permeability in guinea-pigs without the addition of Ag/Ab. The three main fractions, γ-globulins, α_2-globulins and albumins were dissolved in saline and investigated separately by intradermal injection. The albumins were inactive, but the α_2-globulins gave a marked response at 0·6 mg./ml. and the γ-globulins at 0·1 mg./ml. (Table IX).

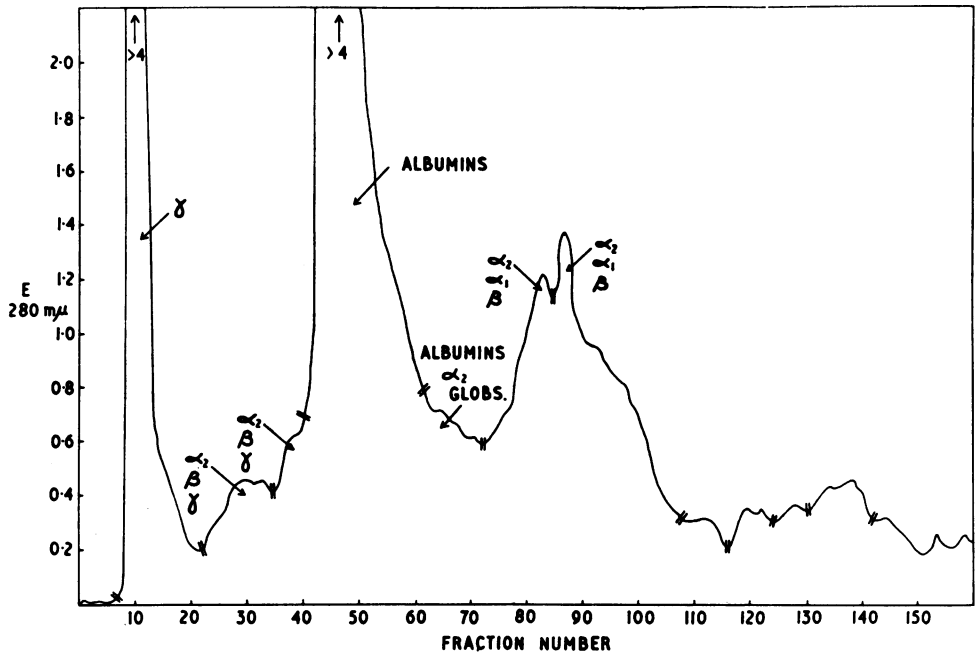

FIG.—Chromatography of guinea-pig serum on diethylaminoethyl cellulose.

TABLE IX.—*Chromatography of Guinea-pig Serum on Diethylaminoethyl Cellulose*

16 g. diethylaminoethyl cellulose giving a column 15 × 2 cm.

Fraction	Weight (mg.)	Constituents	Amount injected (μg.)	Mean diameter of blue lesion (mm.)
1	265	γ-Globulins	10	12·7
2	70	α_2-Globulins (+ trace β-globulins)	—	—
3	31	α_2 and β-Globulins	—	—
4	825	Albumins	223	6·9
5	134	Albumin + α_2-globulins	—	—
6	54	α_1-Globulins with some α_2-globulin and β-globulin	—	—
7	217	α_2-Globulins with some α_1- and β-globulin	60	11·0

Fractions 8, 9, 10 and 11 all appeared to be denatured. They weighed 12, 27, 2 and 29 mg. respectively.

Recovery from column 70 per cent.

Smooth muscle stimulants produced from PF/P and guinea-pig tissues

As stated earlier, PF/P did not release histamine from minced guinea-pig lung. At the end of the experiment the lung extracts were tested on the ileum in the presence of mepyramine (1 μg./ml.). The extract from lung with 1 : 10 guinea-pig serum and Ag/Ab caused a delayed slow contraction. When 1 ml. of the extract was added to a 20 ml. bath containing a rat uterus, again a slow contraction was obtained reminiscent of that produced by bradykinin. No contraction was obtained from lung incubated with saline, 1 : 10 serum, Ag/Ab in saline or from activated serum similarly incubated in the absence of lung tissue.

A similar activity was obtained from the interaction of PF/P and guinea-pig skin. The site of injection of 0·1 ml. of 1 : 10 guinea-pig serum, activated in the usual way with Ag/Ab, was excised from a guinea-pig 5 min. after injection and the portion of skin cut into small pieces with scissors and mixed with 2 ml. of saline. One ml. of this extract again produced a delayed slow contraction of the rat uterus. Saline extracts of skin injected with serum alone or with Ag/Ab in saline were inactive. Further work is being done on this aspect.

DISCUSSION

Inflammation, as Menkin (1956) has said, is a complex vascular reaction. This complexity has not been simplified by the present work since we appear to have added yet another permeability factor to those already known. Increased capillary permeability is a constant feature of inflammation, whatever the inciting cause. It is also technically easy to measure. For these reasons it has been extensively studied by many workers (Spector, 1958). Species differences in the response to different mediators is great (Sparrow and Wilhelm, 1957). We therefore decided to restrict our observations, at this stage, to one species.

Our own interest lay in the inflammation resulting from antigen-antibody interaction to which the guinea-pig is particularly sensitive. The system we chose for study, namely addition of Ag/Ab to serum, is one that has been extensively used elsewhere. It therefore behoves us to compare our results with those of others. Guinea-pig serum itself contains several known permeability factors. Battisto (1957) found that among a number of samples of sera from different guinea-pigs a small proportion increased capillary permeability. We have encountered this phenomenon; the activity was slight and when it occurred the experiment was rejected. Several facts show that PF/P is not a kinin; it is not dialysable, extractable by ethanol, does not contract rat uterus or relax rat duodenum. Moreover, Ag/Ab did not affect either production or destruction of serum kinin formed by dilution of guinea-pig serum. The relationship of PF/P to the globulin permeability factor PF/Dil (Miles and Wilhelm, 1955) is not yet clear but certainly its mode of formation is different in that PF/Dil is formed by merely diluting fresh guinea-pig serum 1 : 100 with saline whereas PF/P can be formed in neat serum. Formation of PF/P in fresh serum also suggests a difference from PF/Age which only occurs in aged serum. To the known ways of producing permeability factors in guinea-pig serum we must now add treatment with acid at pH 5·5. The high permeability effect of the γ-globulins isolated from the DEAE-cellulose chromatography was entirely unexpected. However, Ishizaka, Ishizaka and Campbell (1959) and Ishizaka and Campbell (1958) have reported

that when rabbit serum is separated by zone electrophoresis on starch blocks it is possible " to separate non-specific irritating substance which occurs in the fast moving components of the γ-globulin of some serums ". It seems possible that the γ-globulin isolated from guinea-pig serum by DEAE-cellulose chromatography could have contained such a component. Alternatively, it is conceivable that changes in the native γ-globulin structure could occur during absorption on to the DEAE cellulose as a result of either dissociation of the electrostatic linkages between the protein and the cellulose, alteration in the number of charges on the protein, or both. The present evidence does not allow us to decide which of these explanations is correct. The point is receiving further attention.

Permeability factors are also induced by antigen-antibody complexes themselves, under certain conditions, for example when the latter are solubilized by excess antigen (Ishizaka *et al.*, 1959, Ishizaka and Campbell, 1958 and Cochrane and Weigle, 1958). Our Ag/Ab was prepared at equivalence and was not itself active: indeed activated serum retained its effect when Ag/Ab was removed. Addition of Ag/Ab to serum is claimed to activate anaphylatoxin; the activity inhibited by mepyramine in our own experiments may be due to this substance but there is still a large effect not inhibited by mepyramine.

Further evidence that only a small amount of anaphylatoxin was formed under the conditions of our experiments was provided by the failure of serum with Ag/Ab to release histamine from guinea-pig lung or ileum.

Table X summarizes the differences between PF/P and other known permeability factors.

TABLE X.—*Permeability Factors in Guinea-pig Serum*

	Biological property					
Factor	Relaxation of rat duodenum	Contraction of g.-pig ileum	Contraction of rat uterus	Formed in neat serum	Formed in fresh serum	Inhibition by mepyramine
Kinin	+	+	+	+	+	−
Anaphylatoxin	−	+	−	+	+	+
PF/Dil	0	−	0	−	+	−
PF/Age	0	0	0	+	−	−
PF/P	−	−	−	+	+	−

\+ = Positive response.
− = No response.
0 = Unknown.

The interaction of antigen-antibody complexes with complement is, of course, well established. Recently, Osler, Randall, Hill and Ovary (1959) have shown that component 3 of complement participates in the production of rat anaphylatoxin. The first component of human serum complement (C'1) is activated by Ag/Ab to an esterase (Lepow, Ratnoff and Pillemer, 1956), which can be eluted from the Ag/Ab. This esterase is inhibited by DFP (Becker, 1956). The fact that DFP (10^{-3} M) did not inhibit the formation of PF/P makes it unlikely that C'1 esterase plays a part in the reaction. The possibility that a relationship exists between PF/P and the other components of complement is under investigation.

PF/P is formed when Ag/Ab is added to guinea-pig serum; the mechanism of its formation has not, however, been elucidated. Lack of inhibition by soya bean trypsin inhibitor suggests that it is not produced as a result of proteolysis. It is possible that a natural inhibitor to PF/P, present in serum is removed or

inactivated by Ag/Ab. Alternatively it is conceivable that the immune precipitates cause some change in a serum protein such that on either intradermal injection or incubation with lung tissue, an active substance is formed. This implies that PF/P (which is non-dialysable) is the precursor of a factor which increases capillary permeability and stimulates smooth muscle. All these possibilities are being actively pursued as is the relationship between PF/P and the permeability activity produced by acid and by fractionation on DEAE cellulose.

SUMMARY

A washed precipitate prepared from egg-albumin and anti-egg-albumin rabbit serum when added to fresh, neat or moderately diluted, guinea-pig serum leads to the formation of a factor (PF/P) which increases capillary permeability in guinea-pigs. The properties and mode of formation of PF/P suggest that it is different from other known permeability factors. Permeability-increasing factors are also produced by treating serum with acid at pH 5·5 and by fractionation on diethylaminoethylcellulose.

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