Hemorrhagic Reactions in Animals Sensitized with Ovalbumin in Adjuvants Containing Mycobacteria II. Relation to "Delayed" Hypersensitivity. (25171)

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It has been shown that in guinea pigs sensitized by injection of egg albumin incorporated in paraffin oil (incomplete adjuvant), subcutaneous injection of a very large amount (50 mg) of egg albumin results in protracted anaphylactic shock(1,2) and there is scant if any skin reaction at site of injection of antigen(3). The absence of Arthus reactions may be due to certain components of the shock syndrome, for example, fall in blood pressure(3). When similar experiments are made employing water-in-oil emulsions containing *mycobacteria* (complete adjuvants) in sensitizing the guinea pigs, both the systemic and local responses to an eliciting injection of egg albumin are different. During the very protracted systemic reaction, which may show a combination of "delayed" (DTH) and "immediate" (ITH) reactions, there is a massive hemorrhage at site of injection of egg albumin beginning in the subcutaneous tissue and spreading to the skin(4). The interaction of DTH and ITH systemic reactions is manifested by a combination of the thermal responses characteristic of these reactions. In this connection, it is of interest that Uhr and Brandriss have shown that anaphylactic shock (lowering of body temperature) counteracts the systemic DTH reaction (elevation of body temperature) in guinea pigs previously sensitized by the intracutaneous injection of very small amounts of antigen (combined with excess antibody in specific precipitates)(5). The association of the massive hemorrhagic reaction with the presence of mycobacteria in the adjuvant emulsions suggests that the hemorrhagic reaction is related to DTH(6). The following experiments were designed to shed light on the mechanism of the massive hemorrhagic reaction.

Materials and methods. Adult male or female Hartley guinea pigs were sensitized by a single dose of 2.5 mg of egg albumin (Ea). The antigen incorporated in paraffin oil adjuvants with or without mycobacteria was injected by intracutaneous, subcutaneous, intramuscular or intraperitoneal routes (sensitization schedules are included in tables). For details on the preparation of adjuvant mixtures see(6). Some of the guinea pigs were immunized with precautions taken to avoid contaminating the skin with adjuvant emulsions. For this purpose, a 15 gauge short needle was pushed through the skin in anesthetized guinea pigs and injections were made through a long (3½ inch) 18 gauge needle with regular (I.M.) or cut-off (S.C. or I.P.) end introduced through the 15 gauge needle. The long needle was wiped by pinching the subcutaneous tissue on withdrawal after S.C. injections. Serum antibody titers were determined by the passive cutaneous anaphylaxis (PCA) technic. The ear vein of the guinea pigs was split with a scalpel. Blood was taken by and diluted in a white blood cell pipette. Two or more guinea pigs were used to test each sample of plasma (2-fold dilutions; 0.1 ml of dilution injected intracutaneously) and 8-10 mm of blueing was taken as the threshold reaction. Guinea pigs were injected with the eliciting dose of 50 mg of Ea subcutaneously on the flank 4 to 6 weeks after the sensitizing injections. The temperatures of guinea pigs were taken before the eliciting injection of antigen and during the systemic reaction that followed the injection. For this purpose a thermistor thermometer (TRI-R Instruments, Long Island City, New York) was used.

Results. Local hemorrhagic reaction. It has been found necessary to incorporate the antigen in the paraffin oil emulsion containing mycobacteria in order to induce DTH to that antigen. If the antigen is injected at one site in water-in-paraffin-oil emulsion and *M. tuberculosis* in water-in-paraffin-oil emulsion is injected at a remote site the mycobacteria

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TABLE I. Effect of Injection of Mycobacteria and Egg Albumin at Separate Intracutaneous Sites during Sensitizing Procedure.

G.P. No.	Sensitization at separate sites: Egg albumin in oil (Nuchal site)	Sensitization at separate sites: *M. tuberculosis* in oil (Nuchal site)	Skin hemorrhage, mm × mm	PCA antibody titer (recip.)
1	Right*	Left*	0	6400
2			0	6400
3			0	3200
4			0	3200
5			0	ND
6			0	ND
7	Right and	Right and	40 × 30	12800
8	left	left	90 × 50	25600
9			80 × 60	"
10			50 × 50	"
11			70 × 50	6400
12			65 × 35	12800
13			0	3200
14			0	12800

* Injections made intracut. right or left of midline running parallel to spinal column.

Plan of Injection Sites

G.P. No. 1-6		G.P. No. 7-14	
Mycobact. 0.5 mg	Ea 1.25 mg	Ea 1.25 mg	Mycobact. 0.5 mg
Mycobact. 0.5 mg	Ea 1.25 mg	Mycobact. 0.5 mg	Ea 1.25 mg

do not alter the sensitization (*i.e.* do not induce DTH to the antigen)(7). Tables I and II show the data on reactions at the site of the subcutaneous injection of 50 mg of Ea in guinea pigs which had been sensitized with Ea in oil emulsion at one site and with *M. tuberculosis* in oil at another. These animals did not react with gross hemorrhages of the type seen in Fig. 1 unless both sensitizing injections were on the same side of the nuchal midline. When *M. tuberculosis* in oil and Ea in oil were injected (at separate sites) on the same side of the midline, antibody titers were somewhat higher than in animals injected with Ea on the side of the midline opposite from the *M. tuberculosis*. However, when guinea pig anti-Ea antibody was injected intravenously for passive sensitization of guinea pigs sensitized only to *M. tuberculosis* and into some animals actively sensitized to both Ea and tubercle bacilli to increase the titer of antibody, neither hemorrhagic nor inflammatory reactions were elicited when 50 mg of egg albumin were injected subcutaneously.

Injection of antigen by the intracutaneous route has been found particularly effective for inducing DTH(8,9,10). Table III shows the effect of route and site of immunization on the hemorrhagic skin reactions. Intracutaneous injection of Ea in complete adjuvant was effective in sensitization of guinea pigs whether

TABLE II. Effect of Injection of Mycobacteria at One Site and Egg Albumin at a Remote Site during Sensitizing Procedure.

G.P. No.	Sensitization at separate sites: Egg albumin in oil (Route*)	Sensitization at separate sites: *M. tuberculosis* in oil (Route*)	Skin hemorrhage, mm × mm	Passive antibody† (PCA units)	Antibody titer at time of eliciting inj. (recip.)
1	I.C.	S.C.	15 × 15‡	0	12800
2			0	0	3200
3			10 × 10‡	500,000	6400
4			0	"	12800
5	(None)		0	"	ND§
6	(")		0	"	ND§
7	I.P.	I.C.	0	0	1600
8			0	0	"
9			0	0	"
10			0	0	"
11			0	0	3200
12			0	0	"
13			0	0	"
14			0	0	"

* I.C. = intracut. inj. in nuchal area; S.C. = subcut. inj. on flank; I.P. = intraper. inj.

† Inj. intrav. just before bleeding from the ear. The eliciting inj. of 50 mg Ea followed taking of blood. A PCA unit causes blueing 8-10 mm in diameter.

‡ Resembled partially inhibited Arthus reaction (see 3). Avg size of hemorrhagic reaction is 2800 mm² in fully sensitized guinea pigs (see (4) and Table IV).

§ Phenergan (2 mg) inj. I.P. 1 hr before eliciting inj. to prevent possibility of acute anaphylactic shock (see 2). Antibody titer in circulation not determined at time of challenge.

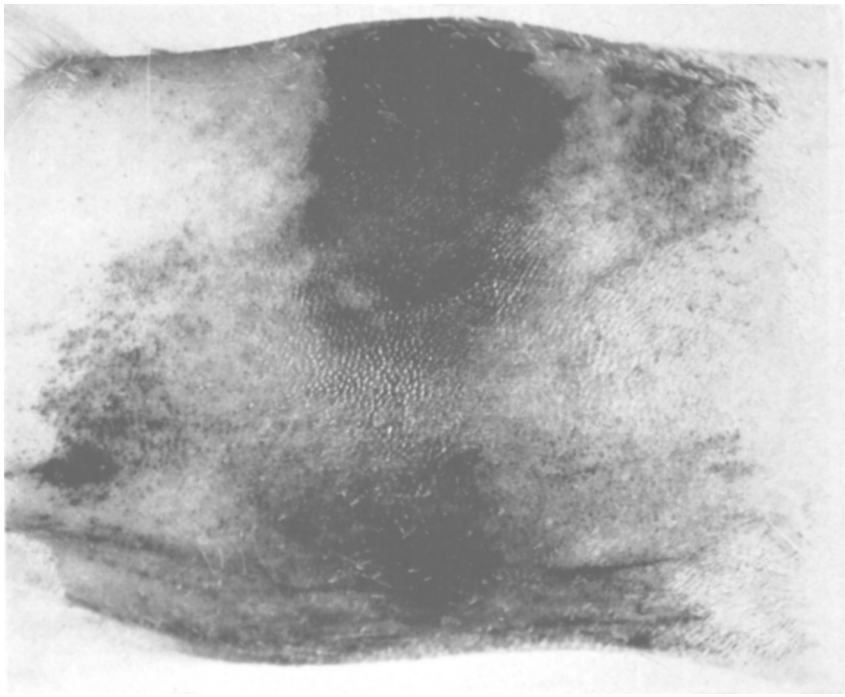

FIG. 1. Hemorrhagic reaction 24 hr after subcutaneous injection of 50 mg of egg albumin in a guinea pig sensitized with egg albumin in complete paraffin oil adjuvants containing mycobacteria.

the site chosen for intracutaneous injection was the flank (5 of 5 guinea pigs showed hemorrhages) or the nuchal area (8 of 9 guinea pigs showed hemorrhages).

TABLE III. Effect of the Site and Route of Sensitization on the Incidence of Hemorrhagic Skin Reaction.*

Route	Area	No. of G.P. Hem. skin reaction	PCA antibody titer (recip.) Mode or median	Max
S.C.	Flank	2/12	ND	ND
"	"	0/10†	3200	12800
I.C.	"	5/5	6400	"
S.C.	Nuchal	7/8 ‡	12800	"
I.C.	"	8/9	‖	‖
I.M.	"	3/5 §	12800	12800
"	Thigh	6/6 †	6400	"
I.P.		2/5	12800	25600

* All guinea pigs inj. subcut. with 50 mg Ea as eliciting dose. Sensitizing dose contained 2.5 mg Ea and 1 mg *M. tuberculosis*.

† Inj. with precautions to avoid contamination of skin with emulsion.

‡ One guinea pig died at 4½ hr. At the time, no hemorrhage in skin, but massive hemorrhage in peritoneal wall.

§ Three guinea pigs were found dead 20 hr after eliciting inj. One of these showed hemorrhagic skin reaction, the other 2, no hemorrhage in skin but massive hemorrhage in subcut. tissue and peritoneal wall.

‖ Titers in guinea pigs similarly sensitized: Mode, 12800; maximum, 25600.

Sensitization by the subcutaneous route varied with regions of injection. After sensitization with Ea combined with complete adjuvants injected subcutaneously on the flank, a subcutaneous injection of 50 mg of Ea did not elicit a massive hemorrhage in the skin, but hemorrhages were observed in the subcutaneous tissue and in the outer part of the peritoneal wall. The hemorrhagic skin reactions seen in a small percentage of guinea pigs sensitized by the subcutaneous route on the flank were not observed in animals sensitized by subcutaneous injections made with precautions to avoid contamination of the skin with emulsion. However, subcutaneous injection of antigen in the nuchal area sensitized guinea pigs so that reactions were observed in the skin as well as in the subcutaneous tissues after a subcutaneous eliciting injection.

Hemorrhagic skin reactions were elicited in most guinea pigs sensitized by the intramuscular route (thigh or nuchal area), and less frequently (2 of 5) in guinea pigs sensitized by the intraperitoneal route.

TABLE IV. Quantity of *Mycobacterium tuberculosis* and Hemorrhagic Reaction.

	Male guinea pigs			Female guinea pigs		
M. tuberculosis, mg	No. with hem. skin reaction	Size of hemorrhagic reaction in mm² Avg*	Max	No. with hem. skin reaction	Size of hemorrhagic reaction in mm² Avg	Max
.1	3/4	1800	3000	ND†	—	—
.075	3/5	"	3500	7/7	2800	4500
.04	ND	—	—	5/8	1500	3500
.03	4/5	1200	2100	3/5	1850	4500
.02	0/4	(0)	(0)	4/5	1200	3150

* Avg size of hemorrhagic area determined counting only those guinea pigs with hemorrhages in the skin.
† Not done.

It is of interest to compare the amount of killed and dried mycobacteria necessary to induce aspermatogenesis or allergic encephalomyelitis with the amount of mycobacteria required for the induction of massive hemorrhagic reactions. Table IV shows that 0.03 mg of mycobacteria in the adjuvant emulsion was effective in sensitization of male guinea pigs for eliciting hemorrhagic reactions. Females were apparently more susceptible to sensitization of this type. With 0.075 mg of mycobacteria in the sensitizing emulsion, female guinea pigs were sensitized so that maximum size reactions were elicited; in males which received this dose, reactions were not maximal. The threshold dose in males corresponded well with the 0.02 to 0.04 mg of *M. tuberculosis* necessary for inducing aspermatogenesis or allergic encephalomyelitis (only males were used in those experiments(11)).

Desensitization or decrease in reactivity. A subcutaneous injection of 50 mg of Ea 24-48 hours after eliciting of a hemorrhagic reaction in sensitive guinea pigs failed to elicit another massive hemorrhage (in animals sensitized with near threshold doses of tubercle bacilli) or produced a less severe reaction (usually in animals sensitized with relatively large amounts of mycobacteria). PCA tests of the serum of sensitized guinea pigs injected 24 hours previously with 50 mg of Ea showed the presence of at least 10% of the original antibody activity.

Tuberculin sensitivity. In guinea pigs sensitized by the intracutaneous route with Ea in complete adjuvants containing *M. tuberculosis,* subcutaneous or intracutaneous injection of relatively large amounts of purified protein derivative (PPD) (0.1 to 0.25 mg) elicited strong tuberculin reactions, but did not cause hemorrhagic responses.

Systemic reactions. The shock seen in guinea pigs sensitized with Ea in complete adjuvants by the intracutaneous route is very prolonged. However, its course with regard to rectal temperature differed somewhat from the very protracted shock observed in animals sensitized by adjuvant emulsions not containing mycobacteria. In the former group (complete adjuvants) body temperatures were normal or showed a slight rise over a 24-hour period; in the latter group (incomplete adjuvants) temperatures were normal or subnormal.

Histological observations. (Figs. 2, 3, 4, 5). In guinea pigs sensitized with Ea combined with *complete* adjuvants and challenged with subcutaneous injection of 50 mg Ea, at 24-48 hours hemorrhage and edema were conspicuous in the skin and subcutaneous tissue. The collagen and muscle fibers were swollen and in part disrupted. Inflammatory cells were conspicuous by their absence. In guinea pigs similarly treated but sensitized with Ea combined with *incomplete* adjuvants, there was edema but no hemorrhage. In addition, there was very slight infiltration of the skin and subcutaneous tissue with thrombotic blood vessels in the skin.

Discussion. These experiments show that the hemorrhagic reaction previously reported (4), which was elicited by injection of a large amount of antigen in guinea pigs sensitized using adjuvant emulsions containing mycobacteria (complete adjuvants) did not occur when the sensitizing antigen was injected

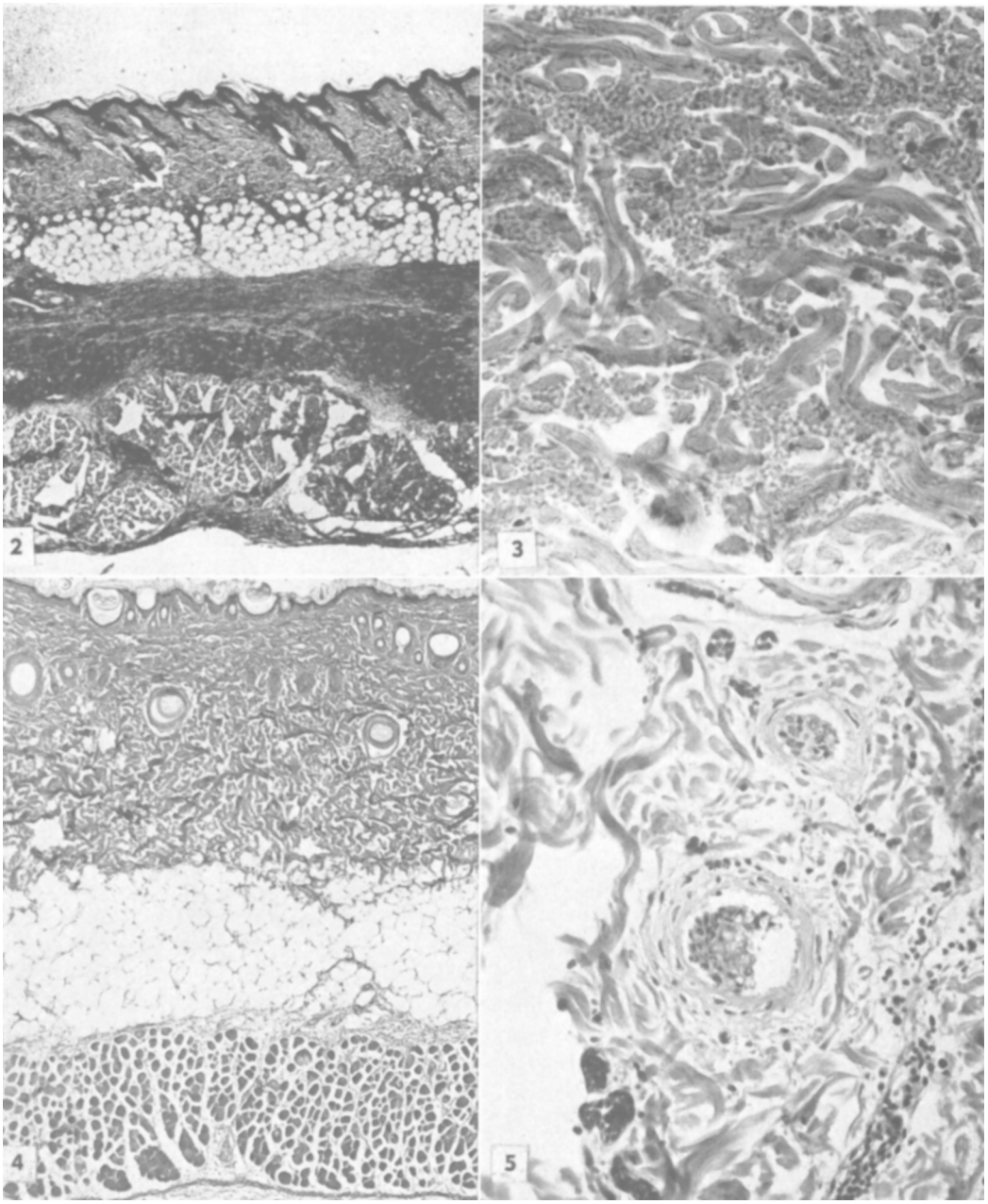

FIG. 2. Reaction in the skin of guinea pig sensitized to Ea using *complete adjuvants*. Hemorrhage and edema in skin and subcutaneous tissue. Giemsa 23 ×. Dark staining masses are RBC.

FIG. 3. Reaction in the skin and subcutaneous tissue of guinea pig sensitized to Ea using *complete adjuvants*. Hemorrhage and edema in subcutaneous tissue. Hematoxylin and eosin 235 ×.

FIG. 4. Reaction in the skin and subcutaneous tissue of guinea pig sensitized to Ea using *incomplete adjuvants*. Edema and very slight inflammatory infiltrate in skin. Hematoxylin and eosin 25 ×.

FIG. 5. Reaction in the skin of guinea pig sensitized to Ea using *incomplete adjuvants*. Thrombotic blood vessels in skin. Edema, slight and very slight infiltration. Hematoxylin and eosin 235 ×.

at one site and the mycobacteria at another site across the nuchal midline. Since DTH (*i.e.* induction of aspermatogenesis) does not occur when tissue antigens in water-in-oil

emulsion are injected at one site and mycobacteria in oil are injected at a remote site (7) there appear to be similarities between the pathogenesis of auto-allergic diseases and the hemorrhagic reaction. This observation also rules out the possibility that the hemorrhage in guinea pigs is due to increased vulnerability of blood vessels caused by the presence of mycobacterial components and suggests that the effect of killed tubercle bacilli is due to their modification of the process of sensitization to Ea. Since hemorrhagic reactions were observed in animals with mycobacteria and Ea injected at separate sites on the same side of the nuchal area, this modification may occur, at least under certain conditions, in the draining lymph nodes.

There is evidence that serum antibody is not the decisive factor in eliciting the hemorrhagic reaction. Individual animals sensitized with the Ea emulsified in oil omitting mycobacteria had titers of circulating antibody equal to those of individual guinea pigs sensitized using complete adjuvants, yet they did not react with hemorrhages. Furthermore, antibody administered passively to guinea pigs previously injected only with *M. tuberculosis* in oil did not cause sensitivity of the type described above.

Since the hemorrhagic reaction appears to be related to DTH it is of interest to determine the quantity of mycobacteria needed to sensitize for such a response. The amount, *i.e.*, 0.03 mg, is of the same order as the threshold dose for inducing aspermatogenesis and allergic encephalomyelitis (11) which are generally considered to be pathological manifestations of DTH to tissue antigens.

Guinea pigs sensitized by *subcutaneous* injection of Ea incorporated in *incomplete* adjuvants usually died during protracted anaphylactic shock elicited by subcutaneous injection of large amounts of Ea (1,2). This protracted shock is characterized by hypothermia. Guinea pigs sensitized by *intracutaneous* injection of Ea incorporated in *complete* adjuvants did not die, and showed normal temperatures or a rise in temperature. Reversal of the hypothermia of anaphylactic shock, probably caused by a systemic DTH reaction in the guinea pigs sensitized using complete adjuvants, does not explain the survival of these animals since guinea pigs sensitized by the *intracutaneous* route using *incomplete* adjuvants also survived. The causes of death after protracted anaphylactic and systemic DTH reactions, and the effects of interaction between them are still unclear, and further investigations in these areas appear necessary before further discussion of the problems involved.

In this connection the experiments of Uhr and Brandriss (5) are of interest. Guinea pigs were sensitized to diphtheria toxoid first by injection of trace amounts of toxoid combined with excess antitoxin leading to DTH (12). These animals responded to injection of toxoid by increases in body temperature. Subsequently guinea pigs which had been similarly sensitized were given guinea pig serum containing antitoxin or were stimulated to form circulating antitoxin. When these animals were tested for systemic reactions to toxoid, they responded with a drop of body temperature. Apparently the anaphylactic (ITH) response was predominant over the previously established DTH. In contrast, our experiments demonstrate a predominant DTH thermal response, perhaps reflecting a higher degree of DTH in animals sensitized using Ea incorporated in complete adjuvants (1.0 mg *M. tuberculosis*).

Summary and conclusions. 1. Massive hemorrhages in skin and subcutaneous tissue of guinea pigs sensitized with egg albumin incorporated in water-in-oil adjuvants containing *mycobacteria* caused by subcutaneous injection of large amounts of egg albumin, failed to occur in guinea pigs which had been sensitized by injection of egg albumin at one site and mycobacteria in oil at a remote site. This indicates that presence of killed tubercle bacilli at site of injection of antigen modifies the process of sensitization to ovalbumin, manifested by hemorrhage when large amounts of egg albumin were injected subcutaneously. 2. Guinea pigs sensitized by injection of egg albumin incorporated in complete adjuvants *on the flank* had hemorrhagic *skin* reactions if the sensitizing injection was intracutaneous, but not if it was made subcutaneously. Injections in the nuchal area were

effective whether intracutaneous, subcutaneous or intramuscular. 3. For inducing hemorrhagic skin reactions, 0.03 mg of killed and dried *M. tuberculosis* was necessary in the adjuvant emulsion used for sensitization of male guinea pigs. For male guinea pigs, 0.02 mg of mycobacteria was not effective, but was sufficient for female animals. 4. In guinea pigs sensitized by intracutaneous route using 2.5 mg of egg albumin and 1 mg *M. tuberculosis*, the systemic reaction elicited by subcutaneous injection of 50 mg of egg albumin was not characteristic of protracted anaphylaxis. Body temperatures were normal or slightly above normal, indicating that anaphylactic shock hypothermia is "neutralized" by concurrent systemic "delayed" reactions.

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A Fibrinogen Precipitating Factor (FPF) of Group A Streptococci. (25172)

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During experiments designed to test antigenicity of a preparation of Group A, type 1 streptococcal M protein, plasma samples from immunized rabbits were set up in capillary precipitin tubes against homologous M protein. A massive precipitate appeared in each tube 5 minutes after preparation. The magnitude of these reactions far exceeded reasonable expectation of the amount of type-specific antibody present. Furthermore, normal plasma reacted in the same manner, and sera from normal and immunized rabbits proved non-reactive. The nature of this reaction is herein examined.

Materials and methods. Bacterial strains and extracts. Streptococcal, pneumococcal and staphylococcal cultures were obtained from human sources in the course of other studies. All streptococcal cultures were typed by capillary precipitin technic of Swift, Wilson and Lancefield(1), then stored in the lyophilized state. Cultures were initiated in modified Todd-Hewitt broth† and plated for purity. Crude acid extracts were prepared by heating suspensions of bacterial cells in boiling water bath at pH 2(2). M extracts were prepared by repeated alcohol precipitation of crude extracts(3). A partially purified preparation of M protein was prepared by the method of Lancefield and Perlmann(4) from Type 1 Streptococci, strain #2788, kindly supplied, as phenolized cells, by Dr. C. P. Hegarty of Merck, Sharp, and Dohme Labs. Electrophoretic examinations of this material revealed migration as a single peak, but with sufficient boundary spreading to suggest inhomogeneity. Streptococcal T extracts were prepared by slight modification of the method of Pakula(5). Crystalline trypsin in final concentration of 0.01 mg/ml at pH 7.6 was substituted for pancreatic extract. *Enzymes.* Pepsin (Difco 1:10,000) was employed in final concentration of 1% at pH 2. Certain strains and extracts were treated with crystalline trypsin (General Biochemicals, Inc.) to

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† Lean horse meat was substituted for beef hearts.