TABLE I. Results in 55 Dogs Given a Lethal Dose of *E. coli* Endotoxin.

No. of dogs	Treatment	Survivors	Died
10	None	0	10
10	.2 mg aldosterone	2	8
10	2.0 mg aldosterone	2	8
25	.1 mg aldosterone + avg of .8 mg metaraminol	16	9

endotoxin. This viewpoint has also been expressed by Weil and Miller(3). In this laboratory reversal of shock has been most successful when a combination of adrenocorticosteroid and a pressor drug was used. It has been essential to infuse the steroid before injecting the pressor drug.

The rise in serum potassium observed with the larger doses of aldosterone may be related to the severe oliguria, or anuria, and acidosis that characterize canine endotoxin shock.

We have shown that either glucocorticoid or mineralocorticoid supplements the pressor action of metaraminol. The steroids have 2 favorable effects when used with the pressor drug. First, the amount of pressor agent necessary to sustain the systemic pressure is considerably less when preceded by administration of steroid, especially when aldosterone is employed. Second, although metaraminol will sustain the pressure when used alone, there is no significant increase in survival rate when compared to controls. Steroids not only augment the activity of pressor drug, but rate of survival is remarkably increased. The precise role shared by exogenous steroid is not known. The observations pertain only to endotoxin shock in the dog.

Summary. Lethal shock was established in 55 adult mongrel dogs with a standardized dose of *E. coli* endotoxin. The shock could not be reversed with aldosterone or metaraminol when either was used alone. However, dogs given 0.1 mg of aldosterone required approximately one-fiftieth the dose of metaraminol necessary to maintain systemic pressure, compared to the use of metaraminol alone. This action of aldosterone was about 7 times more pronounced than that obtained with hydrocortisone. The nature of the contributing effect of steroid to the pressor activity of metaraminol and to the increased survival rate is not known.

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Hemorrhagic Reactions at Sites of Passive Cutaneous Anaphylaxis in Guinea Pig after Intravenous Inoculation of Unrelated Immune Complex. (26753)

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A clear cut differentiation between anaphylactic and Arthus-type sensitivities has been afforded by the quantitative studies of Benacerraf and Kabat(1) in the guinea pig. The distinguishing features of transferred Arthus reactions are: (a) The lack of a latency period, the severity of the Arthus reaction being the same when antigen is injected immediately, 30 minutes or 24 hours after the intravenous sensitizing dose of antibody. (b) The requirement of greater amounts of antibody, *viz.*, 400 μg AbN intravenously for severe direct Arthus reactions, as contrasted with 30 μg, *i.e.*, 13 times less, the amount sufficient to convey systemic anaphylactic sensitization. In the case of passive reversed Arthus and of the local anaphylactic reaction known as "Passive Cu-

taneous Anaphylaxis" (PCA), in which antibody is injected intradermally and antigen is given intravenously, the amounts of antibody required for minimal reactions are, respectively, 10 and 0.003 μg AbN(1,2). (c) Non-precipitating rabbit antibody gives only weak Arthus responses, while being equally effective as precipitating antibody in transferring anaphylactic sensitization. (d) On the other hand, horse antibody, while able to elicit strong Arthus reactions when injected intracutaneously in the doses of 36-72 μg AbN, was found entirely ineffective in producing PCA in the guinea pig in the same doses(3).

This body of evidence led Benacerraf and Kabat(1), as well as Ovary and Bier(3) to conclude that Arthus and anaphylactic sensitivities had fundamentally different mechanisms and did not differ only in degree. While this conclusion is probably correct, previous work(4), as well as present experiments strongly indicate that concomitant PCA plays an important role in development of the Arthus reaction.

Materials and methods. 1. *Antisera.* The following antisera were employed: a) *Rabbit anti-bovine serum albumin* (anti-BSA), pools 4, 5, and 6, containing respectively 0.640, 0.560, and 0.405 mg AbN/ml. b) *Rabbit anti-egg albumin* (anti-Ea), pools 10, 11, 20 and 22, containing 0.800, 0.400, 0.450 and 0.650 mg AbN/ml respectively. c) *Rabbit (type III) anti-pneumococcal serum* (anti-SIII), which contained 0.700 mg AbN/ml. 2. *Antigens. Bovine serum albumin* (BSA), crystallized, obtained from Pentex, Kankakee, Ill.; *Chicken egg albumin* (Ea), prepared by the method of Kekwick and Cannan(5); and *SIII*, lot 202, a purified preparation kindly supplied by Dr. M. Heidelberger. 3. *Complement titrations.* Complement (C′) titers were estimated according to the technic described in(6) in serum samples obtained from blood (1-2 ml) drawn by cardiac puncture. C′3 titers were determined with R3, as described in(7). 4. *Complement fixation.* Complement-fixation titers were expressed in terms of the amount of AbN required for 50% lysis in the presence of 5 units of C′. 5. *Preparation of immune-complexes.* Immune-complexes of different composition were prepared from rabbit serum anti-BSA and crystallized BSA. Immune precipitates (IP) 1, 2, and 3 were obtained by mixing 2 ml of antiserum with amounts of antigen corresponding to 0.2, 0.5 and 1.0 the equivalent dose. Mixtures were allowed to stand overnight at 0°C and precipitates were washed 3 times with chilled saline. The amount of AbN in the precipitates was then determined by use of biuret reagent.

For preparation of soluble immune-complexes (SC) 4, 5, 6, and 7, washed precipitates obtained at the equivalence zone, as described above, were resuspended in 2 ml of saline with an excess antigen corresponding to 2, 5, 10, and 40 times the equivalent dose. Mixtures corresponding to 2 and 5 times excess antigen did not dissolve completely after 24 hour incubation in the ice box. The AbN content of their precipitates was determined under the assumption that the ratio A/G was the same as in the original precipitate and the AbN content of corresponding supernatants (SC 1 and SC 2) was estimated by difference from total antibody initially present. Precipitates resuspended in 10 and 40 times excess antigen were completely dissolved so that the AbN/ml of their supernates (SC 6 and SC 7) could be estimated on the basis of antibody content of the original precipitates. The AbN content per ml of the immune-complexes was the following: IP 1 – 0.354 mg; IP 2 – 0.570 mg; IP 3 – 0.540 mg; SC 4 – 0.110 mg; SC 5 – 0.330 mg; SC 6 and SC 7 – 0.540 mg. The IP suspensions, as well as the SC supernatants were diluted to yield 0.100 mg AbN/ml and further diluted as required for the experiments. 6. *Animals and technic of injections.* Guinea pigs of 250 ± 50 g were used throughout. Intracutaneous injections were made into the skin of previously clipped areas of the belly with a short-beveled 26 gauge needle, first introduced through the skin, then reversed in direction so as to deposit the inoculum, 0.1 ml, into the dermal layers. Intravenous injections were made also with 27 gauge needles into superficial veins of hind foot or of ear.

Results. Effect of simultaneous PCA on passive direct Arthus reaction. To investi-

TABLE I. Effect of Simultaneous PCA on Passive Direct Arthus Reactions Induced in Guinea Pigs by Different Amounts of Antibody from an Unrelated Immune System.

Anti-EaN* i.v.	Virgin site† (0.05 mg EaN)				PCA site† (Anti-BSA)				PCA site† (Anti-SIII)			
μg AbN					1.5 μg AbN				1 μg AbN			
400	+	2+	3+	4+	3+	3+	3+	2+				
	±	±	4+	4+					3+	0	+	2+
200	0	0	+	2+	3+	3+	±	±				
	0	±	±	+					3+	0	3+	3+
100	0	0	0	0	+	2+	3+	4+				
	±	0	0	0					3+	+	3+	0
50	0	0	0	0	+	2+	3+	3+				
	0	0	0	0					2+	+	2+	3+
25	0	0	0	0	+	2+	2+	3+				
	0	0	0	0					3+	3+	3+	0
12	0	0	0	0	±	+	3+	4+				
	0	0	0	0					0	4+	+	2+
6	0	0	0	0	+	+	2+	3+				
	0	0	0	0					+	+	+	±
3	0	0	0	0					±	0	+	+
0	0	0	0	0	0	0	0	0				
0	0	0	0	0					0	0	±	±

* Mixed with 0.2 mg BSA or 0.1 mg SIII.

† All reactions graded are of the Arthus type. Reactions recorded in horizontal rows in corresponding vertical columns, at virgin and PCA sites, were produced in the same animal (see text).

gate whether concomitant PCA had any effect on direct Arthus reactions elicited by unrelated immune system, guinea pigs were injected intradermally (PCA site) with 1.5 μg anti-BSA N (or 1.0 μg anti-SIII N) and 1 hour later received Ea intracutaneously at a virgin site and a mixture of anti-Ea + BSA (or SIII) by the intravenous route. Animals were killed 2 hours after challenge and the diameter of reactions measured on the internal side of skin. Intensity of reactions was graded as 4+ (more than 20 mm diameter), 3+ (15-20 mm), 2+ (10-15 mm) + (5-10 mm), ± (less than 5 mm), and 0 (no reaction). The data summarized in Table I show that severe hemorrhagic reactions developed at site of injection of Ea (virgin site) only in the animals sensitized with 0.400 mg anti-EaN. Strong reactions appeared, however, at the PCA site (anti-BSA or anti-SIII) with amounts of the Arthus-producing antibody as little as 0.012 or even 0.006 mg AbN.

Development of hemorrhagic reactions at PCA sites after i.v. *inoculation of unrelated immune complex.* The foregoing experiment suggests that Arthus-producing antigen injected into virgin site was partly absorbed and formed complexes with its corresponding antibody in circulating blood, which selectively localized at PCA site. Following experiments were performed to check this interpretation. In a first series of experiments (Table II), different amounts of a soluble immune complex prepared with Ea/anti-Ea were mixed with BSA and injected intravenously into animals previously sensitized with anti-BSA by the intradermal route. Vice versa, BSA/anti-BSA was mixed with Ea and injected intravenously in guinea pigs previously sensitized with anti-Ea.

In a second series of experiments (Table III), BSA/anti-BSA complexes of different composition, corresponding to excess antibody, equivalence, and excess antigen, were mixed with Ea and injected intravenously in animals previously sensitized with anti-Ea. In both series of experiments, hemorrhagic reactions (2 or 3+) developed at PCA sites with amounts of intravenously injected complex corresponding to relatively low levels of antibody (*cf.* Tables II and III).

Parallelism between hemorrhagic reaction,

TABLE II. Hemorrhagic Reactions Induced by Soluble Complexes of Ea/Anti-Ea at PCA Sites from BSA/Anti-BSA and *Vice versa*.

AbN/ml of immune complex	Hemorrhagic reactions at PCA sites											
	Anti-BSA						Anti-Ea					
Ea/anti-Ea												
100	4+	3+	0	2+	+	3+						
25	+	2+	2+	3+								
12	+	2+	3+	3+								
6	3+	+	2+	0								
BSA/anti-BSA												
100							3+	3+	+	0	2+	3+
25							2+	±	4+	3+		
6							3+	±	±	+	+	3+

1 ml of soluble complex inj. i.v. together with PCA antigen 2 hr after intradermal inj. of 1 μg AbN of PCA antiserum. Each reaction corresponds to a separate animal.

TABLE III. Hemorrhagic Reactions Induced at PCA Sites by Intravenously Injected Immune-Complexes of Different Composition.

Immune-complex	Amt of antigen in relation to equivalent dose	AbN/ml of immune complex, μg			μg AbN for fixation of 4 from 5 $C'H_{50}$
		100	25	6.25	
IP 1	1/5	2+	3+	±	.22
		2+	2+	0	
IP 2	1/2	+	+	0	.18
		3+	3+	+	
IP 3	1	+	+	+	.18
		+	+	+	
SC 4	2	2+	2+	+	.66
		3+	2+	+	
SC 5	5	2+	±	0	.87
		+	±	±	
SC 6	10	2+	0	0	
		+	2+	0	
SC 7	40	0	2+	±	.84
		+	±	0	

1 ml of immune-complex inj. intrav. together with 0.1 mg EaN 2 hr after intradermal inj. of 1 μg anti-EaN. Each reaction corresponds to a separate animal.

carbon granulopexis and dye accumulation. Two different phenomena occur at PCA sites —the diffusion of intravenously injected dye as a consequence of increased permeability of minute vessels and increased accumulation of colloidal particles (granulopexis) resulting from stimulation of endothelial cells by local liberation of histamine(8).

It was deemed of interest to investigate whether by using 3 different indicators, *viz.*, immune complex, Geigy Blue or colloidal carbon,* parallel results would be obtained. This is clearly shown in Table IV, either in relation to the dose of PCA-eliciting antibody, or in regard to time after challenge with PCA antigen.

Discussion. The data presented here show that severe hemorrhagic reactions (2 to 3+) of the Arthus type develop in the guinea pig skin at PCA sites induced by an unrelated immune system with amounts of intravenously injected antibody as low as 6 μg AbN (Table I). The occurrence of severe Arthus reactions after intravenous sensitization with large amounts of antibody followed by intradermal injection of antigen(1) may be associated to the rapid anaphylactic sensitization obtained with such amounts as 0.400 mg AbN. According to Ovary and Osler(8) the latent period for whole skin sensitization after intravenous inoculation of 0.500 mg AbN is reduced to 1 hour, so that the 2 hour period allowed for development of Arthus reaction in the guinea pig would be sufficient to include participation of a PCA reaction at the site of intradermal injection of Arthus-eliciting antigen. As the amount of intravenously injected antibody is decreased, the latent period for general sensitization becomes progressively greater, *e.g.*, 4 hours for a dose of 0.250 mg AbN(9). By this time the intradermally injected antigen may have been absorbed from the skin depot and formed immune complexes, which are quickly cleared from the blood stream. These complexes may, however, localize at PCA sites

* India ink. Lot C11/1431a, with particles of approximately 250 Å in diameter, obtained from Günther-Wagner, Hannover, West Germany.

TABLE IV. Parallelism between Dye Accumulation, Carbon Granulopexis and Hemorrhagic Reaction Induced by Soluble Immune Complex at PCA Sites in Guinea Pigs.

(A) In relation to dose of PCA antibody

Anti-BSA N intradermally, μg*	Hemorrhagic reactions by immune complex				Dye accumulation				Carbon granulopexis			
1.0	0	2+	3+	3+	4+	4+	4+	4+	3+	3+	4+	4+
.2	±	+	+	2+	2+	3+	3+	4+	2+	2+	4+	4+
.05	0	0	0	2+	0	2+	2+	3+	0	+	2+	2+
.01	0	0	0	0	0	0	0	0	0	0	0	+

* .2 mg BSA i.v. 2 hr later together with .5 ml .5% Geigy Blue, 8 mg colloidal carbon/100 g body wt or 400 μg EaN/100 μg anti-EaN. Reactions read 2 hr later. Each vertical line corresponds to an individual animal.

(B) In relation to time after inj. of PCA antigen

Min. between i.v. injections†	Hemorrhagic reactions by immune complex							Dye accumulation							Carbon granulopexis					
0	±	±	2+	3+	4+	4+		4+	4+	4+	4+				3+	4+	4+	4+		
15	±	±	±	+	+	2+	2+	2+	2+	3+	3+	3+	3+	3+	±	2+	2+	2+	2+	2+
30	±	±	+	+	+			0	0	0	0	0	±	+ +	0	0	0	0	0	0
60	0	0	0	±	±	±		0	0	0					0	0	0			

† Guinea pigs sensitized with 1 μg anti-BSA N intradermally and inj. i.v., 2 hr later, with 0.2 mg BSA N. Immune complex (Ea/anti-Ea), dye or colloidal carbon, inj. at intervals specified above. Reactions read 2 hr after last intrav. inj. Each reaction corresponds to an individual animal.

and there elicit hemorrhagic reactions (Table I).

The effectiveness of horse antibody in regard to the Arthus reaction is in apparent contradiction to its inability to promote PCA. However, as demonstrated by Osler *et al.*(10), horse anti-pneumococcus serum is only less active than rabbit antiserum both in regard to C′ fixation, and in conferring PCA to the rat. This is in agreement with the observations of Benacerraf and Kabat (1) that dircet Arthus reactions induced by horse antibody in the guinea pig skin are considerably less severe than those caused by equal weight of rabbit antibody.

In the experiments reported by Ovary and Bier(3), in which 36-72 μg AbN corresponding to 0.1-0.2 ml undiluted horse typhoid serum were injected intradermally, challenge with antigen plus Geigy Blue was delayed until 8 hours later to avoid non-specific effect of concentrated serum and negative PCA results were observed. While it is generally admitted that horse antibody does not fix on the guinea pig skin, the possibility can not be excluded that large amounts of horse antibody will induce immediate and transitory PCA sensitivity. However, demonstration of this specific anaphylactic sensitivity is not feasible on technical grounds because of the simultaneous non-specific irritating effect of concentrated or undiluted horse serum.

From the evidence that concomitant PCA enhances the noxious effect of immune complex it does not follow that the local anaphylactic reaction is a necessary prerequisite for development of the Arthus reaction. It is noteworthy, however, that the ability of soluble immune complexes and of aggregated gamma globulin preparations to induce hemorrhagic reactions after single intracutaneous injection in the guinea pig(11) parallels their activities in promoting C′ fixation, as well as in eliciting local accumulation of dye. Experiments are under way on the behavior of aggregated gamma globulin, from various species as to their abilities to induce hemorrhagic reactions at PCA sites when inoculated by the intravenous route.

Summary. Experimental evidence is presented pointing to the prime importance of simultaneous anaphylactic sensitivity in development of hemorrhagic reactions of the Arthus-type in guinea pigs. A final conclusion as to the mechanism by which the local anaphylactic reaction enhances endothelial

damage by intravenously injected immune complex requires further study.

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Flavokinase Activity of Rat Tissues and Masking Effect of Phosphatases.* (26754)

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Riboflavin-5'-phosphate or flavin mononucleotide (FMN) is an essential coenzyme form of the vitamin, riboflavin, and occurs widespread in plant and animal organisms (1). However, scanty information is available on the ability of animal tissues to synthesize FMN from riboflavin. It was suggested that intestinal mucosa is the site of this conversion within the mammal(2) by either a transphosphorylation catalyzed by non-specific alkaline phosphomonoesterase(3) or a phosphorylation requiring adenosine-5'-triphosphate (ATP) and the specific flavokinase(4). With the recent partial purification and characterization of flavokinase from rat liver(5), the ability of a mammalian tissue other than intestine to form the coenzyme *in situ* has been demonstrated. In this work, the recognition of interfering phosphatases which hydrolyze FMN, thereby decreasing formation of FMN with flavokinase *in vitro*, has led to the current assessment of these enzymes in animal tissues. This report demonstrates that several rat tissues possess flavokinase, and the level of apparent activity is markedly suppressed by phosphatases which are maximally active at pH 5.

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Methods. Female, 200 g Sprague-Dawley rats were sacrificed by rapid decapitation and exsanguination. Tissues were excised, rinsed and homogenized in 4 volumes of cold, 0.05 M potassium phosphate buffer, pH 7. Homogenates were centrifuged in the cold at 18.500 $\times$ g for 30 min to obtain the supernatant solutions. Protein was determined by the method of Lowry *et al.*(6).

Mixtures for determining flavokinase activity contained: 10^{-4} M riboflavin, 10^{-3} M ATP, 10^{-4} M Zn^{++}, 0.075 M potassium phosphate buffer, pH 8, and 1 mg of protein in 5 ml total volume. Trichloroacetic acid was added to stop the reaction before and after incubation was carried out in the dark at 37° C for 1 hr. The product, FMN, was determined by Kearny's adaptation(7) of the differential extraction method of Burch *et al.* (8). In addition, the product remaining after removal of excess riboflavin with benzyl alcohol was applied to Whatman No. 1 paper and the spots detected under U.V. light following development in ascending *n*-butyl alcohol/acetic acid/water (4/1/5, upper phase). Rf values agreed with those reported