

Hypersensitivity responses in monkeys

II. A comparison of several indicators in visualization of the immediate skin response to histamine, 48-80, and antigen challenge

*Samuel M. Feinberg, M.D., Alan R. Feinberg, M.D., and
Fumiko Lee, B.A., Evanston, Ill.*

Evans blue is used almost uniformly as the dye indicator of the antigen-antibody response in the monkey's skin. Because of difficulties encountered in some experiments we decided to compare other indicators with differing characteristics, such as Coomassie blue, indocyanine green, fluorescein, and riboflavin. The indicators were given intravenously and the degree and end point of skin reactions noted from intradermal injections of serial dilutions of histamine, compound 48-80, antigens, and the local challenge of sites passively sensitized with human reagin. In some experiments, blood levels of indicators were studied temporally in relation to skin reactivity. The following impressions were gained: Evans blue is reliable, stable, and advantageous for a prolonged experiment but is objectionable in some cases because of persistence of staining of skin and discoloration of serum. Coomassie blue is eliminated more rapidly and is preferable when repeated experiments are planned, but in a prolonged experiment it must be reinjected. Indocyanine green produced no visualization. Fluorescein is rapidly eliminated and is excellent for short experiments, particularly where end-point titrations are not essential. Riboflavin is not as reliable as fluorescein.

Although good skin response of the immediate type to intradermal challenges in the monkey in some instances can be recognized visually without any special aids, the slight or absent erythema and poor whealing in many require the use of an intravenous dye for good visualization. Evans blue, with or before the intravenous injection or before the intradermal injection of the challenging substance, has constituted the most popular indicator, although some investigators have used Coomassie blue. In rhesus monkeys, many of which have considerable natural blue coloration of the skin, a blue dye may present some

From the Allergy Research Laboratory, Department of Medicine, Evanston Hospital, and the Department of Medicine, Northwestern University Medical School, Chicago.

Supported in part by a grant from Abbott Laboratories, North Chicago, Ill.

Received for publication July 26, 1968.

confusion at times. Evans blue tends to persist for a longer time in the circulation, and this may be advantageous or objectionable, depending on the type of experiment involved. Reacting sites stained in one experiment may persist for a day or 2 and thus may cause interference if the nature of the investigation requires the repetition of an experiment at that interval. In experiments requiring several repetitions of intradermal injections in the same sites, bluing of these areas may persist for weeks or even months; this may result in difficulty in the interpretation of new reactions. Serum from animals having had repeated injections of Evans blue may be markedly colored for many weeks, offering obstacles to its use for passive transfer or for some other purposes.

The above problems prompted us to search for indicators which would have some differences in behavior, among which might be freedom from discoloration of the skin and serum, rapid elimination, and as good or greater sensitivity. Fluorescein seemed to offer such a possibility. The present investigation was undertaken to compare fluorescein and other fluorescing substances and Evans blue and other dye indicators with respect to their relative sensitivity, reliability, efficiency, and other advantages and disadvantages in performing intradermal challenges with histamine, histamine releaser, antigens in systemically sensitive monkeys, and antigens in passively sensitized sites. In addition, the study was planned to compare the serum levels of the indicator with the skin-test titration at the same intervals after the indicator administration.

MATERIALS AND ANIMALS

Animals

Twelve monkeys were used for these investigations. Of these, 11 were *Macaca mulatta* (9 males and 2 females), with weight ranges of 4.3 to 6.9 kilograms, most of them being around 5.5 kilograms. One animal was a female *Macaca speciosa*, weighing 4.3 kilograms.

Indicators

Indicators are: (1) Evans blue (Warner-Chilcott, Morris Plains, N. J.) 0.5 per cent, total dose of 20 mg.; (2) Coomassie blue R250 (Colab Lab. Inc., Chicago Heights, Ill.), a powder we dissolved with phosphate buffered saline to a 20 mg. per milliliter concentration. In later experiments we dissolved the Coomassie blue in distilled water, and in one experiment we used the Coomassie blue in ampuls obtained from Imperial Chemical Industries, Cheshire, England; (3) Cardio-Green (Hynson, Westcott & Dunning, Inc., Baltimore, Md.), a brand of indocyanine green, obtained in powder form in 250 mg. vials and dissolved with the supplier's solvent. The dose employed varied from 0.44 to 4.4 mg. per kilogram; (4) Fluorescein (C. F. Kirk Co., New York, N. Y.), brand of fluorescein, 5.0 per cent, total dose used being 25 mg. or in some experiments 50 mg.; (5) riboflavin and niacinamide (Eli Lilly and Co., Indianapolis, Ind.), containing 5 mg. riboflavin and 200 mg. niacinamide per milliliter. The dose administered was between 1.7 and 1.8 mg. of riboflavin content per kilogram; and (6) riboflavin phosphate sodium (Eli Lilly) obtained as a powder and dissolved with phosphate buffered saline to 5, 10, and 25 mg. per milliliter. Doses used

were 0.8, 1.6, 4, and 8 mg. per kilogram. The doses of the various indicators were decided on the basis of previous reports by others as well as by trials by us. Non-sterile solutions were filtered through a sterilizing membrane filter.

Challenging materials

The following challenging materials were used in the concentrations indicated: (1) histamine dihydrochloride, in threefold serial dilutions from 1:33,000 to 1:24,300,000 (in terms of base); (2) compound 48-80,* a histamine releaser in threefold serial dilutions from 1:11,000 to 1:8,100,000; (3) *Ascaris suis* (10 per cent extract) in fivefold serial dilutions from 1:25 to 1:48,828,125; (4) ragweed pollen (short and giant mixed, 3 per cent extract), 1:300; (5) normal human serum, in fivefold serial dilutions ranging from whole serum to 1:15,625; (6) normal monkey serum in fivefold serial dilutions from whole serum to 1:625; (7) reaginic human serum (to ragweed antigen) in fivefold serial dilutions, whole to 1:15,625; (8) the diluting fluid for the challenging solutions had the following formula, per liter: NaCl, 5.0 Gm.; KH_2PO_4 , 0.36 Gm.; $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 13.2 Gm.; phenol, 3.8 ml. liquid.

PROCEDURES

Titration of challenging solutions were made with 0.1 ml. intradermal injections in serial dilution in vertical rows, usually on the abdomen but sometimes on the chest or thighs, always including (or try to include) sufficient dilutions for one or more negative results. The indicator solutions were given either prior to, or several minutes after, the intradermal challenges. The timing of the relationship giving the best results for each indicator was finally adopted for most of the experiments. When comparisons were made, for example, the difference in response at different intervals after the injection of the indicator, titrations were made whenever possible in the same animal and in comparable locations on the opposite half of the body. Some indicators were compared in the same animal. In passive-transfer experiments, several sites were injected with 0.1 ml. of human reaginic serum in serial dilutions, intradermally; the sites were marked with a saturated solution of picric acid and challenged intradermally with 1:300 ragweed extract after 24 hours (in earlier experiments in this series a 48 hour interval between passive transfer and challenge was used).

Reactions with the dye indicators were read when they had reached the optimal reactivity, usually at 15 minutes, and the end points noted. The reactions were graded principally on the basis of size (but to some extent on intensity) of bluing as follows: + = 4 to 7 mm.; ++ = 8 to 15 mm.; +++ = 16 mm. or over. There were instances where there were marked differences in the intensity of the bluing rather than the end point. The grading of such differences is difficult to record, but sometimes this was done by photography. Either black and white Polaroid 107 film with a Wratten Filter Type A or 35 mm. Agfachrome or Anscochrome 500 (depending on the dye indicator), or both, were also used to record or to document major results of experiments. The illustrations reproduced

*Courtesy of S. Winston Singleton, M.D., Burroughs Wellcome & Co. (USA) Inc., Tuckahoe, N. Y.

in this paper are either directly from the Polaroid or from black and white reproductions of the color transparencies, as will be pointed out with each illustration.

In reading reactions with fluorescing indicators, the following procedure was adopted after preliminary variations had determined the best technique: Immediately after the intradermal challenges the monkey's skin was checked for any fluorescence with the Blak Ray lamp* in a darkened room. If any was present (from contamination of skin surface, etc.) it was washed off with water. Four to 5 minutes after the intradermal challenge the fluorescing indicator was given intravenously. In the darkened room the animal's skin was checked for fluorescence. At one to 2 minutes after the fluorescein injection, fluorescence appeared and optimal readings were recorded in 5 to 6 minutes. The grading was more or less arbitrary in terms of size and intensity. Photographs were made with the Polaroid Type 107 film with a Wratten Filter Type G or with Anscochrome 500 with a Wratten Filter Type 2A, with the use of the Blak-Ray lamp as a light source.

In order to evaluate the relationship of the serum levels of the indicator to the titer of skin reactivity, separate studies were made in several of these animals. Before and after the injection of the indicator at intervals varying from minutes to 3 days, depending on the substance used, samples of venous blood were taken and the serum centrifuged off and frozen for subsequent spectrophotometric or spectrophotofluorometric estimations. Serial skin tests with histamine dilutions were made at about the same time blood was drawn.

Dye determinations were all done in a similar manner. A standard curve was made after determining the optimum wave length for each dye. The unknowns were all done on serum diluted with normal saline. The Coleman Jr. Model 6A Spectrophotometer was used to determine Evans blue and Coomassie blue concentrations, with 620 $m\mu$ for the Evans blue and 565 $m\mu$ for the Coomassie blue. Riboflavin and fluorescein determinations were made on the Aminco-Bowman Spectrophotofluorometer. Riboflavin was done at an excitation wavelength of 470 $m\mu$ and fluorescein at 540 $m\mu$.

At the beginning of our use of Coomassie blue our first impression was that it was a satisfactory indicator. In subsequent trials about 4 weeks later the results were not as satisfactory. Since these discrepant findings were present even when the same animals were used, we began to suspect that our solution of Coomassie may have diminished in potency. To test this possibility a fresh solution of Coomassie blue was prepared and used as an indicator in titrations of histamine, 48-80, and Ascaris, when the dye was 1,-3,-10,-14,- or 17-days-old. The experiment was repeated in another animal. It was repeated also with the Coomassie blue powder we dissolved in distilled water and with the solutions in ampuls previously described.

RESULTS

Timing of indicators

Experiments in timing the relationship of indicator and challenge injection showed the following: Evans blue and Coomassie blue gave the best results when

*Blak Ray (B100) Longwave U. V. lamp, Ultra-Violet Products, Inc., San Gabriel, Calif.

the challenges were made *after* the injection of the indicator (usually 15 minutes); fluorescein and riboflavin gave the best results when the challenges were made *before* the indicator injection (about 5 minutes). Except in special experiments (to be pointed out), this timing was adopted for the investigations to be described.

Comparison of end point skin reactivity with different indicators

Table I lists the highest dilution giving a reaction with each challenging substance and indicator in the several animals. In each case, except as noted, there were one or more negative reactions in dilutions beyond the end point and also negative reactions with buffered saline. Also shown in the table are the dates and the type and dose of indicator used in each experiment. It should be noted that with some indicators a number of experiments were repeated.

Table I shows that monkey No. 1 reacted to a high dilution of human serum (about 1:15,625), while monkeys No. 7 and No. 8 reacted with serum dilutions of 1:125. These monkeys had been actively sensitized by previous injections of human serum. Both human and monkey sera possess a capillary permeability factor so that indicator leakage may produce visible reactions from intradermal injections of 0.1 ml. of these sera in dilutions up to 1:5 or 1:10 (occasionally 1:25) into normal monkeys. Reactors from higher dilutions are definitely indicative of acquired sensitivity to the serum. It should also be noted that there were animals with varying degrees of naturally acquired sensitivity to *Ascaris* (as high as 1:1,953,000). There were a number of animals not included in our data which gave no reactions whatsoever to *Ascaris* in concentrations as high as 1:25.

The data indicate that Evans blue and Coomassie blue give the most consistent end points. Fluorescein and riboflavin were less consistent, although frequently they showed higher end points. The riboflavin-niacinamide combination produced poor results, perhaps because of the inadequate dose of riboflavin administered in this manner or because of a possible general vasodilating effect by the niacinamide which could result in a lesser availability of the fluorescing substance for the skin reactions.

A single administration of Evans blue was generally sufficient for challenge tests for a period up to 2 hours. If the animal had had several injections at intervals of several days, but none for several days preceding an experiment, a single injection was capable of bluing new reacting sites from strong challenging solutions for as long as 3 days after the Evans blue injection. The sites of the reactions from the preceding day frequently still showed bluing. Toxic symptoms from the dye were not noted.

A single injection of Coomassie blue was sufficient for maximum end-point determination for only 15 to 30 minutes. Any experiment necessitating repetition after that time required another dose of the dye. When more than one dose of Coomassie blue was given within a short period, the reacting sites still showed bluing the next day. In the doses used no toxic reactions were noted from this dye. Cardio-Green showed essentially no visualization.

Fluorescein gave brilliant reactions under ultraviolet light and usually at as high or higher titer than Evans blue, although repeated experiments in the same animal were not as consistent in the end point produced. The best results

Table 1. *Highest reacting dilutions with the use of different challenging solutions and indicators in monkeys*

Ex- peri- ment No.	Date	Indicator		Histamine	48-80	Antigen		Passive transfer		
		Type	Dose (mg./ Kg.)			Type	End point	Re- agin	Antigen concen- tration	End point
Monkey No. 1, <i>M. mulatta</i> —male 5.9 Kg.										
10	9/9/67	EB	1.6		300T	HS	15,625			
11	11/24/67	Fl	3.9	900T		HS	3,125			
12	12/13/67	Fl	3.9	2,700T	100T-0	HS	625			
13	12/20/67	Fl	3.9	900T	100T	MS	5-0			
14	2/9/68	EB	3.2			HS As MS	15,625 25 25			
Monkey No. 2, <i>M. mulatta</i> —female 4.2 Kg.										
6	9/19/67	EB	2.4	900T	100T					
7	12/13/67	Fl	5.9	100T						
8	12/20/67	Fl	5.9	2,700T	100T					
Monkey No. 3, <i>M. mulatta</i> —male 5.7 Kg.										
1	9/13/67	EB	1.8	900T	100T					
6	11/24/67	Fl	3.8	300T						
7	12/6/67	Fl	7.6	900T	2,700T					
8	12/8/67	Fl	3.8	900T	100T-0					
9	1/17/68	R-N	1.8	33T-0						
12	3/28/68	EB	3.4	900T	100T	As	390T			
13	4/3/68	CB	20.0	2,700T	100T	As	1,953T			
Monkey No. 4, <i>M. mulatta</i> —male 6.1 Kg.										
1	9/13/67	EB	1.6	900T	33T					
6	11/24/67	Fl	3.7	2,700T						
7	12/6/67	Fl	7.4	2,700T	2,700T					
8	12/8/67	Fl	3.7	2,700T	100T					
9	1/12/68	R-N	1.7	100T-0	33T-0	HS MS	1-0 1-0			
10	3/6/68	Rib	4.0	8,100T+						
13	4/4/68	CB	20.0	2,700T	100T	As	390T			

This table shows the highest dilutions of intradermal challenging substances giving visible reactions with particular intravenous indicator solutions. The end points are expressed as reciprocals, and T designates 1,000's. Thus, 3,125 means 1:3,125, while 2,700T means 1:2,700,000. -0 after number implies a negative reaction at that titer. + after number indicates end point not reached. Indicator abbreviations: EB = Evans blue; CB = Coomassie blue; Fl = fluorescein; Rib = riboflavin; R-N = riboflavin with niacinamide; Card = Cardio-Green. Antigen abbreviations: HS = normal human serum; MS = normal monkey serum; As = Ascaris extract; Rag = ragweed pollen extract. Reagin abbreviations: HAb = ragweed reagent human serum (Bade); HAb₂ = ragweed reagent human serum (Kay).

*End point when challenge was made before dye injection.

†End point when challenge was made after dye injection.

‡End point when dye was injected either before or after challenges.

Table I. Cont'd

<i>Ex- peri- ment No.</i>	<i>Date</i>	<i>Indicator</i>		<i>Histamine</i>	<i>48-80</i>	<i>Antigen</i>		<i>Passive transfer</i>		
		<i>Type</i>	<i>Dose (mg./ Kg.)</i>			<i>Type</i>	<i>End point</i>	<i>Re- agin</i>	<i>Antigen concen- tration</i>	<i>End point</i>
15	4/19/68	EB	3.3	900T	100T	As	390T			
16	4/24/68	Rib	4.0 8.0	2,700T 24,300T+	100T 100T					
17	4/26/68	Rib	8.0	900T	100T					
18	5/1/68	Rib	8.0	2,700T	100T					
<i>Monkey No. 5, M. mulatta—male 5.7 Kg.</i>										
1	11/29/67	Fl	4.4	8,100T+	100T					
2	12/1/67	Fl	8.8	2,700T	8,100T+					
3	12/6/67	Fl	8.8	2,700T	2,700T					
4	12/20/67	Fl	4.4	8,100T	33T-0	MS	5			
5	1/17/68	Card	0.44	100T-0	11T-0	MS HS	1-0 1-0			
6	1/24/68	Fl	4.4	900T						
7	1/31/68	EB	3.5	900T						
8A	2/14/68	Fl	4.4	2,700T						
8B	2/14/68	EB	3.5	900T						
9	2/28/68	CB	17.2	100T* 900T†						
13A	4/17/68	Card	2.0	100T-0‡	33T-0‡					
13B	4/18/68	EB	3.5	900T	100T	As	15T			
14	4/26/68	Card	4.4	11T-0‡	33T-0‡					
<i>Monkey No. 6, M. speciosa—female 4.3 Kg.</i>										
1	11/29/67	Fl	6.4	900T	300T					
2	12/1/67	Fl	9.6	900T	300T					
3	12/6/67	Fl	9.6	900T	900T					
8	4/18/68	EB	4.6	2,700T	100T	As	1,953T			
<i>Monkey No. 7, M. mulatta—male 5.0 Kg.</i>										
10	3/27/68	EB	4.2	300T		As HS	390T 125			
15	4/19/68	EB	4.0	900T	100T	As	390T			
<i>Monkey No. 8, M. mulatta—male 4.5 Kg.</i>										
1	2/28/68	Rib	0.8 1.6	11T-0 11T-0						
2	3/1/68	CB	20.0	900T		As	25-0	HAb	Rag 300	625
5	3/26/68	EB	4.2	900T		As HS	3T 125			
7	4/2/68	CB	20.0	300T 900T	33T-0* 100T†					

Continued

Table I. Cont'd

<i>Ex- peri- ment No.</i>	<i>Date</i>	<i>Indicator</i>		<i>Histamine</i>	<i>48-80</i>	<i>Antigen</i>		<i>Passive transfer</i>		
		<i>Type</i>	<i>Dose (mg./ Kg.)</i>			<i>Type</i>	<i>End point</i>	<i>Re- agin</i>	<i>Antigen concen- tration</i>	<i>End point</i>
<i>Monkey No. 9, M. mulatta—female 4.8 Kg.</i>										
2	5/1/68	F1	5.2	900T						
3	5/8/68	Rib	8.0	8,100T						
<i>Monkey No. 11, M. mulatta—male 5.7 Kg.</i>										
3	5/3/68	EB	3.5	2,700T				HAb	Rag 300	125
								Hab ₂	Rag 300	125

were obtained when the fluorescein was given about 5 minutes after the intradermal challenges. If given 5 minutes before the intradermal injections the titer was materially reduced, while at 15 minutes there was no fluorescence. Fluorescence at the reacting sites performed at optimal conditions lasted only several minutes. Doses of about 4 mg. per kilogram produced no untoward effects, while twice that amount produced retching or vomiting in some animals.

Riboflavin also gave good fluorescing reactions and the end point was generally higher than with Evans blue, but the inconsistencies were even greater than with fluorescein. The reactions were duller than with fluorescein and were orange tinted instead of bright yellow. Although larger or smaller doses than 4 mg. per kilogram were used, the latter dose seemed to be most desirable. No toxic reactions were present. Fig. 1 shows a representative appearance of a reaction with each indicator.

Correlation of serum titer with skin reacting titer of indicator

A number of experiments were made with each indicator, but for the sake of brevity only representative experiments are described and graphed. In all these experiments histamine was used as a challenging agent.

Fig. 2 represents the findings with Evans blue as indicator and depicts the data in an animal which had not had Evans blue previously. Five minutes after dye administration the serum level was 4.4 mg. per cent. It is noted that at 6 hours after the dye administration the blood level is still over 2 mg. per cent, and at that level there is only moderate diminution of the end point of visualization of the histamine skin reaction. At 24 hours, with a serum level of 1 mg. per cent, there is still a good but lesser histamine titer. At 48 hours, with a blood level of 0.4 mg., histamine reactions cannot be visualized. In another animal which had had several previous injections of Evans blue, the serum level prior to the present injection was 2.4 mg. Five minutes after the intravenous injection of Evans blue and the histamine challenge, the serum dye level was 7.8 mg. per cent. At 6 hours the dye level was 4.8 mg., at 48 hours 2.8 mg., and at 72 hours 2.5 mg., while the histamine skin reactivity at 72 hours was still not much less than at the very beginning of the experiment.

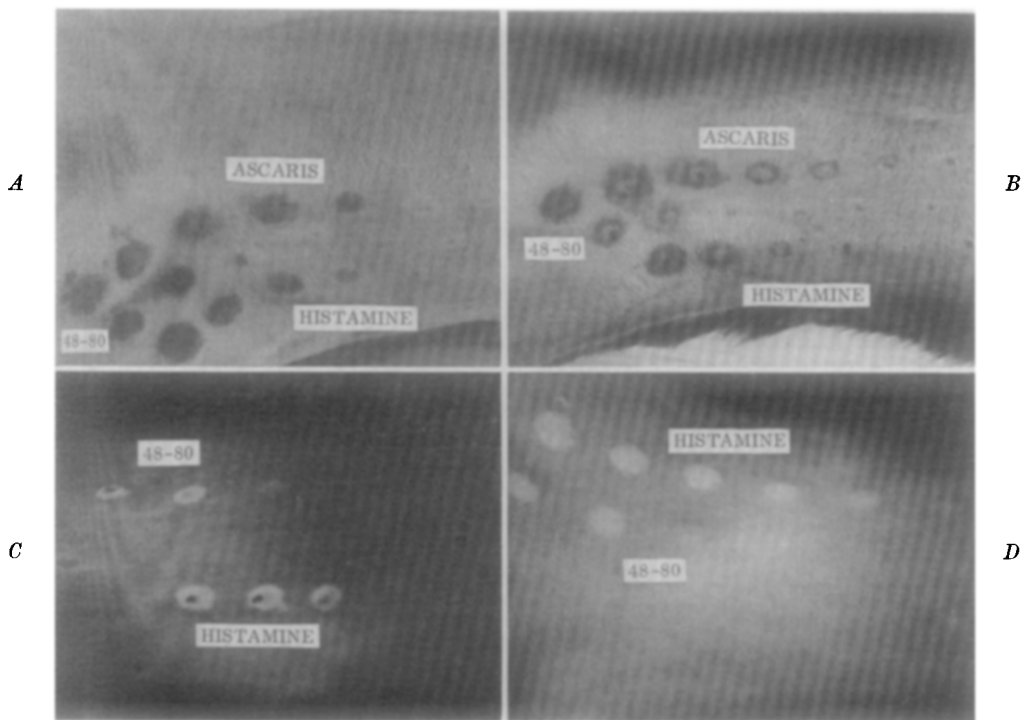


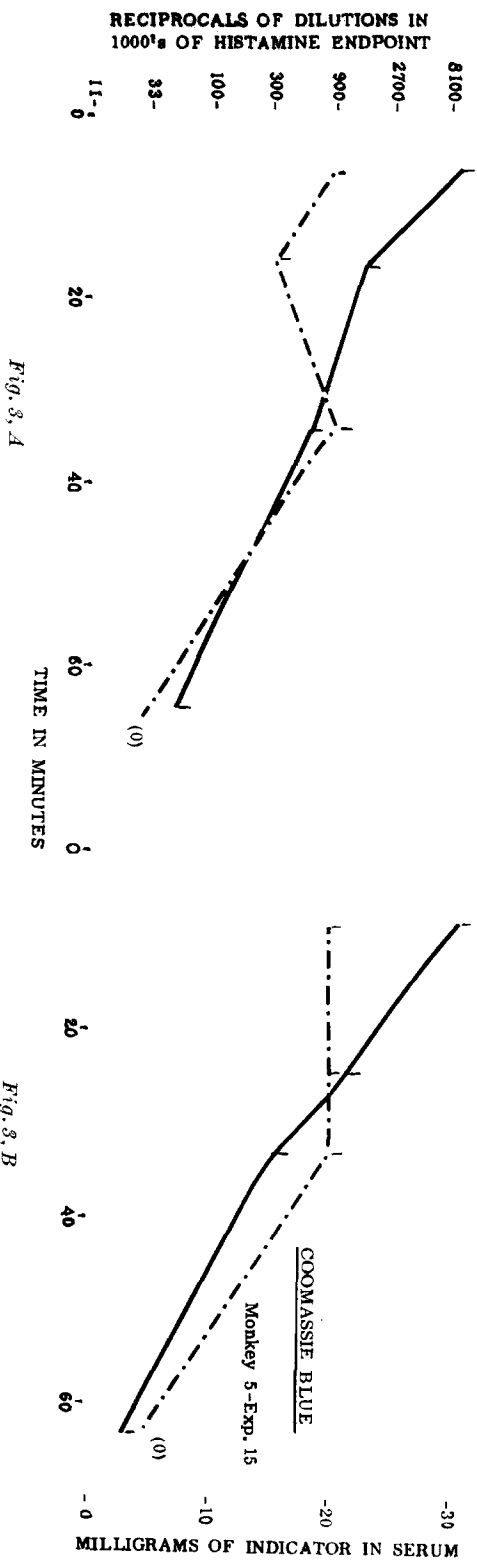
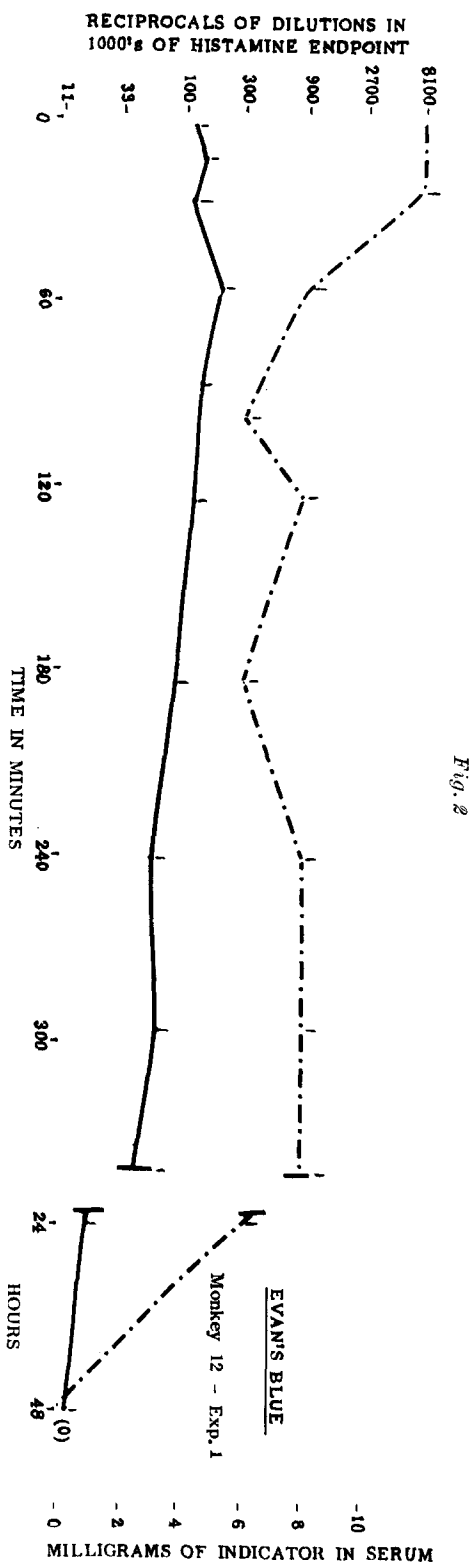
Fig. 1

A, Evans blue indicator in Monkey 4. *Ascaris* shows reactions in dilutions 1:625 to 1:390,625, 48-80 in dilutions 1:33,000 and 1:100,000, and histamine, 1:100,000 to 900,000. Polaroid photograph. *B*, Coomassie blue indicator in Monkey 4. *Ascaris* shows reactions in dilutions 1:125 to 1:390,625, 48-80 in dilutions 1:33,000 and 1:100,000, and histamine in dilutions 1:100,000 to 1:2,700,000. Polaroid copy of Agfachrome photograph. *C*, Fluorescein indicator in Monkey 6. 48-80 shows reactions 1:33,000 to 1:100,000, and histamine 1:33,000 to 1:900,000. Polaroid copy of Anscochrome photograph. *D*, Riboflavin indicator in Monkey 4. 48-80 shows reactions 1:33,000 and 1:100,000, and histamine in dilutions 1:100,000 to 1:8,100,000. Polaroid photograph.

Fig. 3 represents an experiment with Coomassie blue. Fig. 3, *A* shows that 5 minutes after the injection of the dye the serum level is 31 mg. per cent. At 34 minutes the level is 20 mg. and the skin reactivity visualization from histamine is still at a high titer, while at 64 minutes, with a level of 7 mg. per cent, 1:100,000 histamine does not show bluing. A reinjection of Coomassie blue at this point Fig. 3, *B* raised the blood level to 30.5 mg. and the histamine visualization to maximum level for over 30 minutes. At the end of an hour, with a blood level of 3 mg., 1:100,000 histamine was not visualized. It should be noted that in some animals visualization of histamine was no longer present at 30 minutes, while occasionally it was procurable for longer than an hour but at a lesser end point.

A representative finding with fluorescein is shown in Fig. 4. Five minutes after injection the serum fluorescence relative value level was 10* and histamine

*Because of some fluorescence of normal serum, fluorescence of the serum after fluorescein or riboflavin was compared with the chemical dissolved in normal saline and expressed as relative values instead of milligrams.



Figs. 2 and 3
Solid line (—) indicates serum indicator levels. Broken line (---) indicates highest dilution giving skin reaction.

skin reactivity visualization end point at 1:900,000 (the histamine being injected 5 minutes *before* the fluorescein). At 10 minutes after fluorescein injection the serum level is still high, but the histamine reaction has been reduced to 1:100,000. At 15 minutes the blood level is at 7 and histamine skin end point 1:100,000, and at 30 minutes with a blood level of 5, histamine, 1:11,000, is negative.

Fig. 5 shows a typical result with riboflavin. In this experiment the initial blood level was 75, with a high titer of histamine visualization of the skin test. Within 10 minutes the blood level had fallen to about 30, and histamine reactivity could not be visualized with a 1:100,000 dilution. In fact, reactions could only be visualized if the skin tests were made prior to the injection of the riboflavin. With both fluorescein and riboflavin the visualized reactions disappeared rapidly, lasting only about 15 minutes in the case of fluorescein and only about 5 minutes with riboflavin.

Stability of Coomassie blue solution

Fig. 6 depicts a representative finding of 8 experiments done with Coomassie blue solutions at various stages of aging. Progressive decrease in dyeing of the reactions is obvious. Not all animals nor all solutions made at different times (from the same batch of powder) showed equal deterioration, but all showed changes with time. When an animal showed diminished reactions from aged solutions of Coomassie blue, fresh solutions of Coomassie blue or Evans blue used shortly thereafter in the same animal gave reactions as in the beginning of the experiment. Solutions made with distilled water were no better, possibly even worse. A solution of Coomassie blue in sealed ampuls obtained from Imperial Chemical Industries, Cheshire, England, gave good end points in the one experiment done. This solution was not aged further since it was assumed that it already had been aged for some time in the ampuls. However, this animal had had Evans blue previously, and the bluing may have represented the combined effects of the 2 dyes.

DISCUSSION

The grading of reactions is difficult, and for the most part we do not report it. For example, the size of the reaction may depend chiefly on the depth of the injection and texture of the skin, while the intensity of the reaction may depend chiefly on the blood level of indicator as well as the individual animal's skin peculiarities. When done, grading was mainly on the basis of size of the coloration. The end point, the weakest challenging solution giving a visible reaction, was regarded as a more significant comparative measure. And yet, in some instances, such as in studies of the deterioration of Coomassie blue in solution, with age, the change in intensity of the reactions was often more striking than the decrease in the end point of the titer.

In general, good intradermal injections in monkey skin are not always accomplished satisfactorily. This is particularly true in young or small monkeys, in those very old or with a flabby skin, or with an animal that has lost fluids. The rhesus monkeys with a great deal of intense pigment may offer problems with the blue dyes. *Macacca speciosa* (Stump tail) has a light skin and the single monkey

of that species which we investigated had skin of excellent texture, permitting almost perfect intradermals in a high titer of reactivity. Our only objection to this species is its hyperactivity, but this can be combatted by proper tranquilization.

Evans blue was adjudged the best of a series of some 60 dyes investigated for circulation levels in dogs, in 1920, by Dawson, Evans, and Whipple.¹ Since then it has been used extensively and, in the last several years, almost exclusively in investigations of antigen-antibody reactions in monkeys. The dye is stable, nontoxic, and has the advantage of maintaining an effective titer for an extended

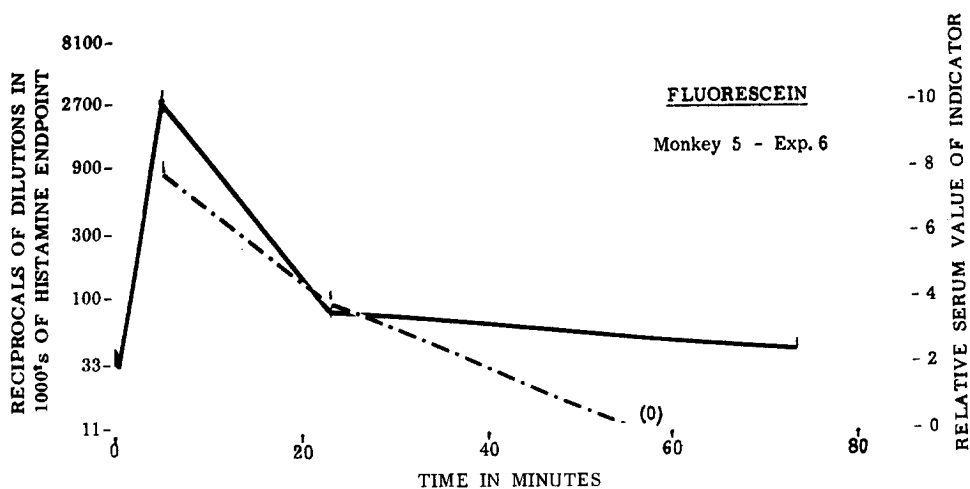


Fig. 4

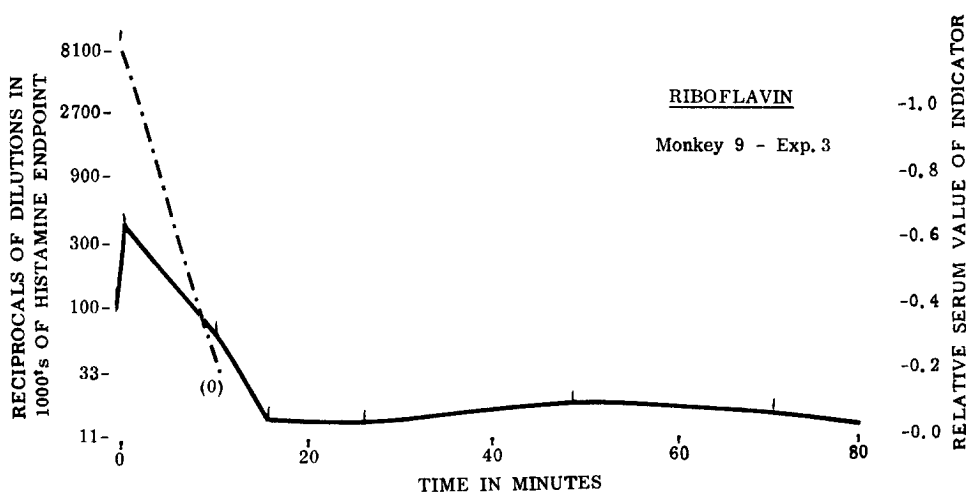


Fig. 5

Figs. 4 and 5

Solid line (—) indicates serum indicator levels. Broken line (— · — · —) indicates highest dilution giving reaction.

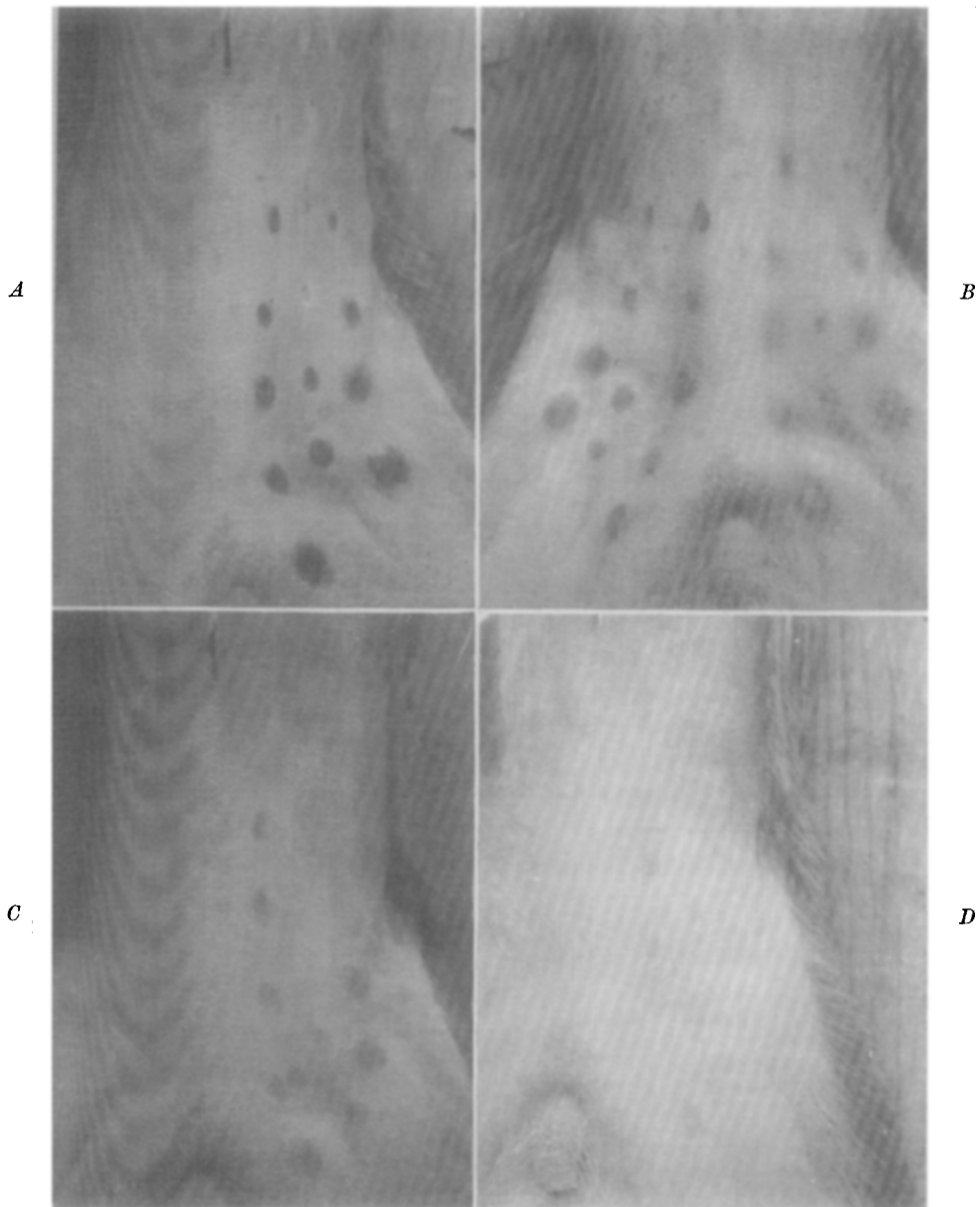


Fig. 6

The above are Polaroid photographs of reactions in Monkey 3 with Coomassie blue solution at various periods of aging. *A*, From left toward middle are reactions with histamine, 48-80, and *Ascaris* when the dye was one day old. *B*, On the right side are reactions when the same dye was 3 days old. The bluing spots on the left side are remaining discolorations from an experiment two days before. *C*, At 10 days of age dye shows considerable diminution in intensity of dyeing and some diminution in titer. *D*, When the dye was 17 days old there was virtually no bluing.

experiment (over 2 hours), in many instances without diminution in titer. However, the retention of the dye in the circulation and the cumulative effect from repeated injection cause bluing of the serum which interferes with certain studies (such as its use in passive transfer to another monkey). It may also cause a spotting of sites which may last for weeks.

Coomassie blue (sodium amazolen) was introduced in 1959 by Allen and associates² for circulation studies in man. Subsequently it was used in man by a number of investigators³⁻⁵ to study circulatory phenomena and occasionally in studies of antigen-antibody skin reactions in monkeys.⁶ Recently the solution has been taken off the market because of problems in stability and precipitate formation and is now available only in the form of the powder to be prepared and not for human use. In man, the blood level was found to be 50 per cent at 15 minutes, about 33 per cent in 30 minutes, and has completely disappeared in 12 hours.^{3, 7} Our studies in monkeys give similar results, with 30 minute levels varying from 28 to 60 per cent. In a number of instances the bluish coloration of the reactions persisted for 24 to 48 hours, thus interfering on some occasions with repeated experiments in the same area. We have not observed toxic effects from Coomassie blue. In man it has been generally held that doses of 20 to 2,000 mg. have not produced toxic effects. Hoffman and Guz,⁴ however, have reported pyrogenic effects after 3 series of injections totaling 450 to 924 mg. given in a total elapsed time of 54 minutes to 5 hours.

Reports generally indicate^{8, 9} that Coomassie blue solutions are stable. Our experience indicates that solutions in distilled water or buffered saline are quite stable up to 3 days but that 10- to 17-day-old solutions show a diminution and sometimes a total absence of bluing. A solution obtained from the manufacturer in ampuls appeared potent. We have no explanation for this difference. However, we must conclude that when Coomassie blue solutions are prepared by the investigator, they should be quite fresh when used.

In an animal which still had Evans blue in its circulation, the levels of Coomassie blue and Evans blue could be followed separately by spectrophotometric determinations. The advantages of Coomassie blue are that it does not persist as long in the body and does not color the skin or serum long enough to interfere with certain investigations. It has some disadvantages, such as the necessity for reinjection when an experiment takes more than 30 minutes and the short period of stability of the solution (at least as prepared by us).

In 1946, Lange and Boyd¹⁰ proposed the use of fluorescein (resoreinolphthalein) in the study of peripheral vascular disease. They found that the substance was completely excreted by the urine in 16 to 30 hours. In 1947, Winsor and associates¹¹ studied the comparative usefulness of several fluorescent tracer substances in determining fractional circulation times in man. Histamine phosphate 1:1,000 was injected intradermally in various parts of the body, and the fluorescing material was injected intravenously one minute later. The fluorescing substances were atabrine, theophylline, some other xanthine derivatives, acriflavine, riboflavin, and fluorescein. Riboflavin and fluorescein were the most satisfactory. Optimum fluorescence for riboflavin was calculated at about 0.5 mg. per

kilogram body weight. Möller and Rorsman¹² used intravenous fluorescein in man as an indicator of reactions to intradermal histamine dilutions.

In a previous paper¹³ we mentioned the use of fluorescein as an indicator of skin reactivity in monkeys.* In the present report we have extended our observations of the use of fluorescein in a number of monkeys as a means of demonstrating skin reactivity to histamine, a histamine releaser, and the antigen-antibody reaction. Fluorescein is helpful in all immediate types of reactions and has the advantages of rapid elimination from the skin and blood and the capability of use as a secondary indicator shortly after the use of a dye indicator. Its disadvantages include its relative quantitative inconsistencies, its too rapid disappearance when a prolonged experiment is desired, and the necessity of the use of special methods for visualization and photography. For nonquantitative short-period or repeated experiments, it would appear to be the ideal indicator.

The preparation containing riboflavin with niacinamide for human parenteral use failed to show fluorescence at the reacting skin sites in monkeys. It is possible that the riboflavin content was inadequate or that the niacinamide had some kind of neutralizing influence. In 4 to 8 mg. doses, riboflavin alone produced fluorescence and often to a high end point of reacting agent. However, as far as titer determination is concerned, it was even more inconsistent than fluorescein. Furthermore, its disappearance from the site was so fast as to make its use impracticable in many situations. The coordinated blood and skin-test studies confirmed this impression.

Indocyanine green (Cardio-Green) was suggested by Fox and Wood,¹⁴ in 1957, for circulation studies. Despite doses in monkeys several times comparatively larger than those used in man, the dye was not visible at the reaction sites. The failure of visualization may be due to the fact that this dye is taken up rapidly by the liver and bile and disappears from the circulation in about 12 minutes, as shown in dogs and monkeys.^{15, 16} Another problem is the marked deterioration of the dye solution within hours.

Demethylchlortetracycline was not investigated because of the likely toxic effects from the doses required.

*Attention is called to a typographical error in our previous paper. Instead of 30 to 60 minutes, the persistence of fluorescence is about 10 to 15 minutes.

REFERENCES

1. Dawson, A. B., Evans, H. M., and Whipple, G. M.: III. Behavior of Large Series of Dyes introduced Into the Circulating Blood, *Am. J. Physiol.* **51**: 232, 1920.
2. Allen, D., Conn, A. W., and Junkin, C. I.: Cardiac Output Determination in the Operating Room: A Microtechnic, *Canad. Anaesth. Soc. J.* **6**: 337, 1959.
3. Martin, W. B.: Coomassie Blue Used in the Estimation of Plasma Volume. A Preliminary Report. Comparison of the Dye With Other Indicators and a Report of Values Obtained in 10 Normal Adult Subjects, *Am. J. M. Sc.* **242**: 342, 1961.
4. Hoffman, J. I. E., and Guz, A.: Toxicity of Coomassie Blue, *Am. Heart J.* **6**: 665, 1961.
5. Michie, D. D., and Villareal, Y.: A Study of the Absorption and Stability of Sodium Amazolene (Coomassie Blue) Individually and in Combination With Indocyanine Green (Cardio-Green), *Brit. J. Anaesth.* **38**: 923, 1966.
6. Augustin, R.: Demonstration of Reagents in the Serum of Allergic Subjects, in Weir, D. M. editor: *Handbook of Experimental Immunology*, Philadelphia, 1967, F. A. Davis Company, p. 1102.

7. Oakley, C., Taylor, S., Wilcken, D., Hollman, A., Goodwin, J. F., and Shillingford, J.: Dye Dilution Curves in Congenital Heart Disease, *Brit. Heart J.* **22**: 533, 1960.
8. Taylor, S. H., and Shillingford, J. P.: Clinical Applications of Coomassie Blue, *Brit. Heart J.* **24**: 497, 1959.
9. Michie, D. D., Goldsmith, R. S., and Mason, A. D., Jr.: In Vitro Instability of a Commonly Used Tricarboyanine Dye, *Proc. Soc. Exper. Biol. & Med.* **111**: 540, 1962.
10. Lange, K., and Boyd, L. J.: Use of Fluorescence Method in Establishment of Diagnosis and Prognosis of Peripheral Vascular Diseases, *Arch. Int. Med.* **74**: 175, 1946.
11. Winsor, T., Adolph, W., Ralston, W., and Leiby, G. M.: Fractional Circulation Time Using Fluorescent Tracer Substances, *Am. Heart J.* **34**: 80, 1947.
12. Möller, H., and Rorsman, H.: Studies on Vascular Permeability Factors With Sodium Fluorescein. I. The Effect of Intracutaneously Injected Histamine, Human Serum and Its Protein Fractions in Normal Individuals, *Acta dermat-venereol.* **38**: 223, 1958.
13. Feinberg, S. M., Feinberg, A. R., and Lee, F.: Hypersensitivity Responses in Monkeys. Histamine Skin Response, 48-80, Passive Transfer (Man to Monkey, Monkey to Monkey) and Other Features Related to Human Reactivity, *Ann. Allergy* **26**: 99, 1968.
14. Fox, I. J., and Wood, E. H.: Application of Dilution Curves Recorded From the Right Side of the Heart or Venous Circulation With the Aid of a New Indicator, *Proc. Staff Meet. Mayo Clin.* **32**: 541, 1957.
15. Wheeler, H. O., Cranston, W. I., and Meltzer, J. I.: Hepatic Uptake and Biliary Excretion of Indocyanine Green in the Dog, *Proc. Soc. Exper. Biol. & Med.* **99**: 11, 1958.
16. Vogin, E. E., Moreno, O. M., Brodie, D. A., and Mattis, P. A.: Indocyanine Green Clearance in the Rhesus Monkey (*Macaca Mulatta*), *J. Applied Physiol.* **21**: 1880, 1966.