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**UNIVERSITY OF LOUISVILLE**

**Studies In Clinical Allergy**

**A Dissertation**

**Submitted to the Faculty**

**Of the Graduate School of the University of Louisville**

**In Partial Fulfillment of the**

**Requirements for the Degree**

**Of Master of Science**

**Department of Bacteriology**

**By**

**M. Lacefield Waterstone**

**1941**

**NAME OF STUDENT: M. LACEFIELD WATERSTONE**

**TITLE OF THESIS: STUDIES IN CLINICAL ALLERGY;**

**I. PHENOLPHTHALEIN HYPERSENSITIVITY**

**II. SULFANILAMIDE HYPERSENSITIVITY**

**APPROVED BY READING COMMITTEE COMPOSED OF THE  
FOLLOWING MEMBERS:**

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**NAME OF DIRECTOR: \_\_\_\_\_**

**DATE: May 28, 1941**

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**THE CONJUGATION OF HAPTENS IN VIVO:**

**A Study of the Development of Sensitivity  
to Phenolphthalein and to Sulfanilamide  
in Guinea Pigs and Rabbits.**

## **INTRODUCTION**



## THE CONJUGATION OF HAPTENS IN VIVO:

### I. PHENOLPHTHALEIN HYPERSENSITIVITY

### II. SULFANILAMIDE HYPERSENSITIVITY

Allergy is the term now generally used synonymously with the words anaphylaxis, hypersensitiveness, sensitization, hypersusceptibility and idiosyncrasy. There is a tendency to restrict the term anaphylaxis to the description of the condition occurring in animal experimentation in which acute and violent manifestations of specific hypersensitiveness are obtained.

It was formerly thought that proteins only were antigens, but we now know that carbohydrates, simple chemicals and possibly such physical agents as heat and cold may likewise be antigenic.

When an individual who is susceptible, or in an allergic state, is sensitized by contact with an allergen, he remains in a balanced allergic state as long as his tolerance is not overcome by sufficient exposure to the same specific allergen. Should this tolerance be overcome, the individual suffers from allergic shock, and he will manifest symptoms such as asthma, hives, or gastro-intestinal disturbances dependent upon which organ attempts to combat or absorb the shock. A study of the chart (Fig. 1) simplifies and may give a better understanding of the mechanism of allergic symptom production.

**MECHANISM OF ALLERGIC SYMPTOM PRODUCTION**

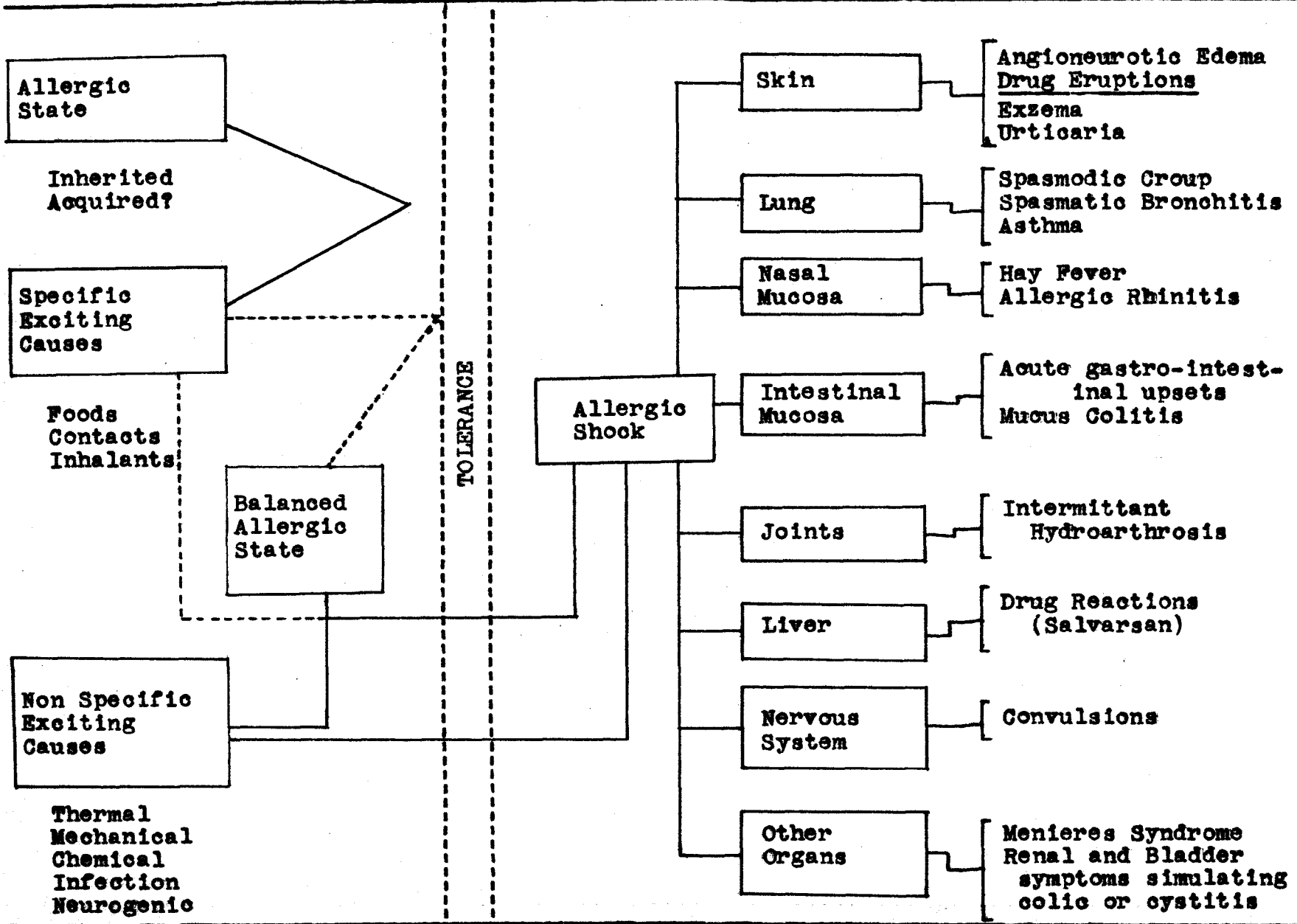


Fig. 1

In any one animal species the symptoms and localization of the pathology of anaphylactic shock are qualitatively the same in all individuals of that species, i.e. the shock organ is constant. In atopic hypersensitiveness, on the other hand, this localization of symptoms and pathology (shock organ) is variable; thus we have localization in the nose, bronchioles, skin, etc. (hay fever, asthma, eczema, etc.). With the concept of haptens we are more able to reconcile clinical allergy with protein anaphylaxis.

Early in the work on Forssman's heterophilic antigen it was disclosed that his antigen has two components, an alcohol soluble component and a protein. When the alcohol soluble component was extracted from guinea pig kidney, for example, the remaining protein failed to produce antishoop hemolysins when injected into a rabbit. Either component by itself, or both injected separately possessed little or no heterogenetic activity, yet added to an antigenic protein, the alcoholic extract restored the heterophylic antigenic property (Landsteiner). Of the two components, then, only the alcohol soluble one was specific but the protein was indispensable for immunization. Immune sera for cholesterol and lecithin were obtained by Sachs and Klopstock (1925) by injecting mixtures of lipids with serum. Likewise, Cesari was able to differentiate fatty extracts of pig heart, spleen, beef liver, etc.

Landsteiner (1936) convincingly demonstrated that haptens other than lipids could form antigens if they could be adequately attached to proteins. He was able to couple proteins to simple compounds (diazotized aromatic amino acids and aniline). These compounds were specific so that in the case of peptides, for example, he found that the specificity of the conjugated amino acids depended on the structure of the terminal amino acid carrying the free carboxyl group and, to a lesser degree, on the second amino acid.

In a similar way, Heidelberger, Goebel and Avery (1925) have produced antigenic carbohydrate proteins by first preparing an aminophenol glucoside and then coupling it to the globulin of horse serum.

Since precipitins can be obtained by immunizing with simple chemicals compounded with proteins, the question arises as to whether sensitivity to certain drugs may also result from a hapten-protein combination formed in the body. Landsteiner and Jacobs (1936) found that certain substances having labile groups (Cl or NO<sub>2</sub>, for example) combine with proteins to form sensitizing compounds. Cutaneous sensitivity to 1,2,4, chloro-dinitro-benzene, p-nitro-sodimethyl-aniline, and to a number of other aromatic compounds developed after intradermal injections of these drugs into animals.

We have to-day gone far beyond the earlier concept of allergy as being limited to protein sensitization and must recognize it as a type of reactive response not only with proteins but with non-protein substances and environmental factors such as heat, light and cold. Loveman (1938) and Sternberg reported sensitivities to oils; Levin reported sensitivity to sawdust; Goodman and Sulzberger (1938) reported hypersensitivities to simple chemicals. From the first great impetus given to the study of hypersensitivity in Kentucky by Moore, the subject matter of allergy has progressively become more complex and of such extreme interest that allergists are now seeing as many cases of drug allergy as of protein hypersensitization. Cases in point are: Allergy to dinitro-phenol (Frumess); allergy to sulfanilamide (Hageman and Blake); allergy to iodides (Happel); allergies to arsphenamines (Ellis), (Stuart), (Simon and Sulzberger 1935); allergy to aspirin (Prickmann and Buchstein), (Cooke); allergies to amidopyrine (Madison and Squier), (Strauss); allergy to phenolphthalein (Belote and Whitney); allergy to epinephrine (Cohen and Waterstone); allergy to synthetic hypnotics (Menninger). Often, indeed, only a few exposures to these completely new and foreign chemical substances are required before allergy to them develops.

Concerning hypersensitiveness to drugs of several clinical forms such as a number of clinical varieties of

skin eruptions, asthma, purpura, etc., the evidence suggests that the true allergen is a combination of the drug with body proteins (Simon, 1938). Reactions to drugs may be pemphigoid or bullous, scarlatiniform, or fixed. In a broad classification of drug eruptions one would naturally have to include all eruptions caused by drugs. The type of sensitivity, however, on which this discussion is based is limited only to those conditions included in the term *dermatitis medicamentosa*. The reactions produced by drugs should in no way be related to either the pharmacologic or the toxic action of the drug. Antibodies are not a necessary requisite in judging the allergic nature of a drug exanthema. The eruption, furthermore, should be capable of being reproduced by infinitesimal amounts of the drug, so called specific reaction (Loveman, 1939).

The successful employment of sulfanilamide (p-aminobenzenesulfonamide), and to a lesser degree two other related organic compounds, in the treatment of diseases caused by diverse micro-organisms, particularly the hemolytic streptococci, constitutes the greatest advance in chemotherapy since the discovery of salvarsan. Since there is an unprecedented breadth of coverage against several infections, the limitations of which are at present by no means established, sulfanilamide has been given a trial in the therapy of practically every known disease and infection.

Novak reported the use of the drug as a preservative of stored blood with satisfactory results.

The ingestion of sulfanilamide is followed in some patients by local reactions which at times are sufficiently severe to lead the physician to consider seriously whether the drug should be discontinued or whether it should be continued with the hope that the pharmacological benefits would overbalance the toxic or allergic manifestations. Most of the reactions to sulfanilamide have been caused by the toxic action of the drug rather than by the development of a true sensitivity. Loveman and Simon (1939) have observed a case of typical fixed eruption proved to be sulfanilamide hypersensitivity. It was Loveman's interest in this and similar cases that led him to suggest the original work which forms the basis of sulfanilamide hypersensitivity in this thesis.

The purpose of this work was to attempt the sensitization of guinea pigs and rabbits to phenolphthalein and to sulfanilamide, neither of which contains Cl or NO<sub>2</sub> groups (fig. 2 and fig. 3). It is well known that cutaneous eruptions are sometimes noted in humans following the ingestion of phenolphthalein (cf. literature cited by Abramowitz). It is a moot question whether this reaction is an allergic phenomenon or some sort of idiosyncrasy not due to sensitization. It therefore seemed quite desirable to investigate the nature of these reactions,

especially, from the standpoint of a possible allergic mechanism.

Rosenthal (1938) reported sensitization to phenolphthalein in guinea pigs and rabbits, but sensitization developed only after the drug had been conjugated in another animal. In other words, the primary animals were not successfully sensitized, but the secondary animals were sensitized after injections with the conjugated drug in the serum from the primary animals. The work presented in this thesis on phenolphthalein hypersensitivity was an attempt to sensitize the primary animals. The procedure followed here was a repetition of Rosenthal's work with some modifications in technique.



PHENOLPHTHALEIN HYPERSENSITIVITY

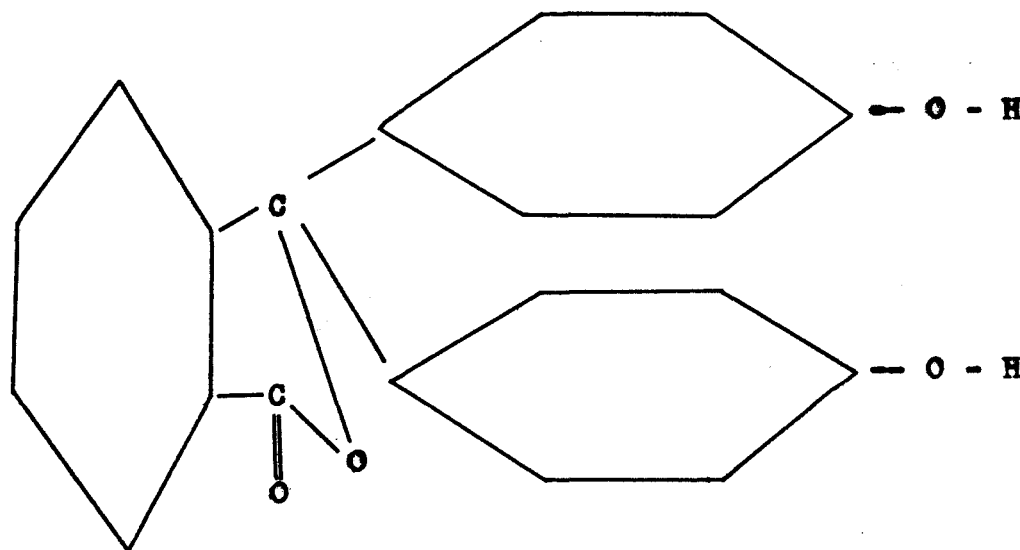


Fig. 2

Phenolphthalein has no Cl or NO<sub>2</sub> groups.

**THE CONJUGATION OF HAPTENS IN VIVO:**

**I. PHENOLPHTHALEIN HYPERSENSITIVITY**

**PHENOLPHTHALEIN HYPERSENSITIVITY**

**MATERIALS**

**and**

**METHODS**

## MATERIALS and METHODS

### ANIMALS.

White-haired guinea pigs of ordinary stock weighing from 500 gms. to 1000 gms.

White-haired rabbits of ordinary stock weighing from 1235 gms. to 1850 gms.

### PREPARATION OF COLLOIDAL PHENOLPHTHALEIN.

Colloidal phenolphthalein was prepared by the method described by Fantus and Dyniewicz. Two and one-half grams of gelatin were dissolved in 50 c.c. of water. In another flask 10 c.c. of N/1 NaOH were added to 40 c.c. of water and one gram of phenolphthalein. Both solutions were autoclaved separately for 30 minutes under 15 lbs. pressure. After sterilization the contents of one flask were poured into the other, mixed well, and CO<sub>2</sub> slowly introduced, with constant shaking, until the color changed from a deep red to an opalescent whitish tan to darker brown. The pH was usually around 7.0; if not, it was so adjusted. (The adjustment was made with acetic acid; the amount needed was calculated from the potentiometric determinations). Acetic acid was used because of its low ionization constant thereby hoping to prevent the phenolphthalein from precipitating out of solution by acting as a buffer.

## MATERIALS and METHODS

### DETERMINATION OF FREE AND OF CONJUGATED PHENOLPHTHALEIN.

Determinations were made only on guinea pigs because of the fact that figures have already been established for other animals. The method employed was as follows:

Serum was extracted with 10 parts of ether, repeating the extraction until the ethereal extract gave no color with N/10 NaOH. The amount in the extract was determined by comparing it with a known standard in the colorimeter. Conjugated phenolphthalein was determined by first extracting all the free phenolphthalein with ether and then treating the serum with con. HCl in a waterbath (60°C) for three hours. This mixture was then in turn extracted with ether and the amount of conjugated phenolphthalein determined by comparing it with known standards.

### PREPARATION OF STOMACH TUBE FOR ORAL ADMINISTRATION:

When administering drugs orally to laboratory animals, only rough approximates of the desired dosage are obtained. In order to facilitate the oral administration of drugs and to obtain accurate measurements of doses a "stomach tube" was employed. This tube was made as follows:

An 18 gauge hypodermic needle of length sufficient to pass into the stomach of the animal being used makes an ideal tube. A 100 mm. length needle is suitable for the smaller

## MATERIALS and METHODS

laboratory animals.

The beveled point of the needle was removed on an emery wheel and the last 2 cm. of the end of the needle was covered with a layer of solder several millimeters thick. This end was then shaped on a lathe until a smooth blunt tip, about 2 mm. in diameter with a gradual taper to the shank of the needle remained. The needle was then attached to a syringe of capacity sufficient to hold the dose being administered.

For adult rats, guinea pigs and young rabbits, a needle 4 inches in length allows insertion into the stomach. For mice a 20 gauge needle, 2 inches long with a ball tip of about 1 millimeter in thickness, should be used.

The needle should be passed into the mouth in line with the animal's body and to the left of the incisors with the blunt end against the palate. By a gentle lever action on the syringe forcing the head back as the end of the needle approaches the larynx, the needle passes easily over the glottis into the esophagus and enters the stomach without difficulty.

## MATERIALS and METHODS

### PREPARATION OF THE AGAR SUSPENSION OF PHENOLPHTHALEIN.

Two grams of phenolphthalein were shaken with 50 c.c. of water in a stoppered 100 c.c. cylinder. Twenty c.c. of 1 per cent hot agar solution were added and mixed. The volume was made to 80 c.c. and the suspension was thoroughly mixed. It was then cooled under running tap water until the material remained suspended.



### EXPERIMENTAL

#### SKIN REACTIONS FOLLOWING THE INJECTION OF COLLOIDAL SOLUTIONS OF PHENOLPHTHALEIN PREPARED WITH GELATIN.

Guinea pigs and rabbits were injected intradermally and subcutaneously with a 1 per cent colloidal solution of phenolphthalein. The colloidal form was used because phenolphthalein is practically insoluble in water or saline (0.03 mgm. per 100 c.c.) and because it was hoped that by adsorbing the phenolphthalein to the gelatin the larger molecule might aid in the production of an active antigen. Doses were started with 1 c.c. and were rapidly increased to 10 c.c. The interval between injections was 5 days. Two rabbits received 120 c.c. (1.20 grams of phenolphthalein) each and two guinea pigs received 50 c.c. (0.50 grams of phenolphthalein) each. Cutaneous and interfaced precipitin tests were performed eight days and one month after the last injection. Colloidal phenolphthalein, a 2 per cent agar suspension of phenolphthalein, a 1 per cent saline suspension of phenolphthalein and gelatin were used as antigens. (A saline solution was substituted for the suspension in the precipitin tests). For skin testing 0.1 c.c. was injected intradermally and readings were taken after 30 minutes, 1, 3, 24 and 48 hours.

**RESULTS:**

Both the cutaneous and precipitin reactions were entirely negative.

Employing a "stomach tube", 1.75 grams of phenolphthalein were administered orally to both sets of animals after observing the reactions for the first 24 hours.

**RESULTS:**

No effect was observed.

## EXPERIMENTAL

### SKIN REACTIONS FOLLOWING THE INJECTION OF COLLOIDAL SOLUTIONS OF PHENOLPHTHALEIN PREPARED WITH GUINEA PIG SERUM AND RABBIT SERUM.

These experiments were an exact repetition of those performed above except, that in the preparation of the colloidal solution of phenolphthalein, serum was substituted for the gelatin. The blood was drawn aseptically, centrifuged to obtain the serum, and then phenolphthalein was added to make a 1 per cent solution. The mixture was incubated at 37°C overnight in an effort to conjugate the drug.

### RESULTS:

All reactions were negative eight days and one month after the last injection. Here, as previously noted, the oral administration of phenolphthalein was without effect. By adsorbing phenolphthalein onto gelatin or serum, sensitivity to phenolphthalein was not produced. In the serum-injected animals the additional factor of sensitivity to the serum was avoided by injecting guinea pigs with the colloidal solution made with the guinea pig sera; the rabbits were injected with the colloidal solution made with rabbit sera. (Guinea pig sera and rabbit sera were substituted for the human sera which Rosenthal (1938) employed thus further eliminating the possibility of obtaining false reactions).

### CONJUGATION OF PHENOLPHTHALEIN IN VIVO

It was observed that phenolphthalein, administered parenterally, soon appeared in the blood in free and conjugated forms. For the free drug, the peak was found after one-half hour and for the conjugated form it was found after one and one-half hours (Table I). Conjugated phenolphthalein was also found in the urine.

TABLE I

## AMOUNT OF PHENOLPHTHALEIN IN THE BLOOD AFTER ENTERAL OR PARENTERAL ADMINISTRATION OF PHENOLPHTHALEIN

Animal	Route of Injections	Amount of Phenolphthalein Given Grams	Time Interval	Phenolphthalein Recovered	
				Free Mg./100 c.c.	Conjugated Mg./100 c.c.
RABBIT	Intradermally & Intrav.	0.1	1/2 hr.	0.5	Trace
	Intradermally & Intrav.	0.1	1 hr.	Trace	0.1
	Intradermally & Intrav.	0.1	1 1/2 hr.	Trace	0.2
	Intradermally & Intrav.	0.1	3 hr.	Trace	Trace
GUINEA PIG	Subcutaneously	0.1	1 hr.	0.8	3.0
	Subcutaneously	0.1	2 hr.	0.6	3.5
	Intramuscularly	0.1	1 hr.		6.0
	Intramuscularly	0.1	2 hr.	0.6	5.0
	Intravenously	0.1	1 hr.		3.0
	Intravenously	0.1	2 hr.	0.0	3.0
	Oral	0.4	2 da.	0.0	6.0
	Oral	1.2	6 da.	0.05	3.5
	Oral	12.0	2 mo.	0.0	6.0
	Oral	15.0	2 da.	0.0	2.2
RABBIT	Oral	15.0	2 mo.	0.0	4.29
CAT	Oral	70.0	1 yr.	0.0	1.4

PHENOLPHTHALEIN HYPERSENSITIVITY

**SKIN REACTIONS OF RABBITS FOLLOWING THE INJECTION  
OF SERA FROM GUINEA PIGS AND RABBITS PREVIOUSLY  
INJECTED WITH COLLOIDAL PHENOLPHTHALEIN.**

Guinea pigs were injected with colloidal phenolphthalein in 10 c.c. amounts, 8 c.c. intramuscularly and 2 c.c. intradermally. Blood was withdrawn aseptically after one and one-half hours and placed in the cold box to clot. The blood was then centrifuged in order to obtain the serum for injection into a rabbit. One rabbit was injected with colloidal phenolphthalein (15 c.c.) divided intradermally and intramuscularly and the serum obtained as above.

INJECTIONS OF SERA-CONJUGATED PHENOLPHTHALEIN:

Pooled guinea pig sera was injected intradermally and intramuscularly into a rabbit in 2 and 3 c.c. amounts. The animal was given 8 injections at 5 to 6 day intervals. A total of 20 c.c. was injected. Rabbit serum was similarly injected into another rabbit.

Tests were performed 8 days after the last injection. The following antigens were used: normal cat's serum; serum from the same cat after it had been injected with 15 c.c. of colloidal phenolphthalein divided intradermally and intravenously; colloidal phenolphthalein; gelatin plus sodium hydroxide and  $\text{CO}_2$  (adjusted to pH 7.0); a saturated suspension of phenolphthalein in saline and an agar suspension of phenolphthalein.

## PHENOLPHTHALEIN HYPERSENSITIVITY

### RESULTS: (Table II).

Cutaneous reactions were positive to both the cat serum combination (the cat's serum contained both free and conjugated phenolphthalein) and to the colloidal phenolphthalein. The strongest reactions were obtained with the serum-conjugated phenolphthalein and consisted of erythema with induration. Animal number 1 received serum-conjugated phenolphthalein from another rabbit. The greater reactions shown by this animal were probably due to the greater reaction mechanism rather than to the source of the serum. In this experiment the precipitin reactions were all negative using the same antigens as were employed in the skin reactions.

The dimensions given in Table II and the succeeding tables are in millimeters; the symbols one plus to four plus indicate the severity of the reaction whereas a negative sign indicates no reaction; the upper signs represent erythema while the lower ones represent induration.

PHENOLPHTHALEIN HYPERSENSITIVITY

TABLE II

RABBITS RECEIVING SERA OF GUINEA PIGS AND SERUM OF A RABBIT PREVIOUSLY  
INJECTED WITH COLLOIDAL PHENOLPHTHALEIN

Animal No.	Skin Reaction Hrs.	Cat Serum Phenolphthalein	Normal Cat Serum (Control)	Colloidal Phenolphthalein	Gelatin Plus NaOH Plus CO <sub>2</sub> (Control)	Saline Suspension of Phenolphthalein	Agar Suspension of Phenolphthalein
1 {	24	35 x 60 <sup>+++</sup> ++++	Neg	15 x 15 <sup>-</sup> +	Neg	10 x 10 <sup>-</sup> +	10 x 10 <sup>-</sup> +
	48	25 x 40 <sup>+++</sup> ++++	7 x 10 <sup>-</sup> +	Neg	Neg	Neg	Neg
2 {	24	15 x 20 <sup>++</sup> ++	Neg	20 x 20 <sup>+</sup> +	Neg	Neg	10 x 10 <sup>-</sup> +
	48	20 x 32 <sup>+++</sup> ++	Neg	Neg	Neg	Neg	Neg
3* {	24	Neg	Neg	Neg	Neg	Neg	Neg
	48	Neg	Neg	Neg	Neg	Neg	Neg

\* Control Animal



## PHENOLPHTHALEIN HYPERSENSITIVITY

### SKIN REACTIONS OF GUINEA PIGS FOLLOWING THE INJECTION OF RABBIT SERA-CONJUGATED PHENOLPHTHALEIN

Colloidal phenolphthalein was injected into rabbits in 10 to 15 c.c. amounts divided intradermally and intramuscularly. The sera was obtained and injected intradermally and intramuscularly into guinea pigs in 2 c.c. amounts every 5 to 6 days for a total of 12 injections. The total volume injected was 24 c.c.

#### RESULTS: (Table III).

The results were similar to those noted in the experiments with rabbits injected with guinea pig sera.

These were the first positive results in guinea pigs but the condition as it exists in humans was not being reproduced; that is, phenolphthalein was being injected rather than ingested.

It was decided to feed phenolphthalein to guinea pigs and to determine whether free or conjugated phenolphthalein appeared in the blood. By means of a stomach tube 0.2 gm. of phenolphthalein was administered daily. Phenolphthalein appeared in small amounts in the blood after 1 to 2 days (Table I), and after 6 days of feeding there were traces of free phenolphthalein and an average of 3.5 mgm. per 100 c.c. of conjugated phenolphthalein. As in the injected animals, free phenolphthalein disappeared from the blood very quickly whereas the conjugated form remained for a longer time.

PHENOLPHTHALEIN HYPERSENSITIVITY

TABLE III

GUINEA PIGS RECEIVING SERUM OF RABBITS PREVIOUSLY INJECTED WITH COLLOIDAL PHENOLPHTHALEIN

Animal No.	Skin Reaction Hrs.	Cat Serum Phenolphthalein	Normal Cat Serum (Control)	Colloidal Phenolphthalein	Gelatin Plus NaOH Plus CO <sub>2</sub> (Control)	Agar Suspension of Phenolphthalein (Control)
1	24	20 x 30 <sup>++</sup> +++	5 x 5 <sup>-</sup> +	Neg	Neg	Neg
	48	15 x 18 <sup>++</sup> +++	5 x 5 <sup>-</sup> +	8 x 8 <sup>+</sup> +	5 x 5 <sup>-</sup> +	10 x 10 <sup>+</sup> +
2	24	25 x 30 <sup>+++</sup> +++	Neg	20 x 20 <sup>+</sup> ++	5 x 5 <sup>-</sup> +	5 x 5 <sup>-</sup> ++
	48	25 x 30 <sup>++</sup> +++	Neg	10 x 10 <sup>+</sup> +	Neg	Neg
3	24	18 x 20 <sup>++</sup> ++	5 x 8 <sup>-</sup> +	15 x 15 <sup>+</sup> +	Neg	Neg
	48	20 x 15 <sup>++</sup> ++	Neg	10 x 10 <sup>+</sup> +	Neg	5 x 5 <sup>-</sup> +

## PHENOLPHTHALEIN HYPERSENSITIVITY

### COMPARISON OF THE SKIN REACTIONS OF RABBITS INJECTED WITH THE SERUM OF GUINEA PIGS THAT HAD BEEN FED AND INJECTED WITH COLLOIDAL PHENOLPHTHALEIN

Two rabbits were injected with serum from 2 guinea pigs, one pig having received injections of colloidal phenolphthalein and the other having been fed (by stomach tube) the phenolphthalein. The rabbits received 8 injections of 2 c.c. each at intervals of 4 days. The injections were divided intradermally and intramuscularly.

#### RESULTS: (Table IV).

Both animals reacted intradermally with approximately the same intensity to colloidal phenolphthalein, although the animal receiving the serum of the guinea pig which had been fed phenolphthalein developed both induration and necrosis (necrosis is indicated by N in the tables).

These findings are in accord with those of Rosenthal (1938) except that hemorrhage plus necrosis was not observed in the case of the rabbit receiving serum of guinea pig previously injected with phenolphthalein.

Having shown that it was possible to produce cutaneous sensitivity to colloidal phenolphthalein and to eat serum-phenolphthalein, the factor of sensitivity to guinea pig's serum was considered as a possible influence upon the reaction to phenolphthalein; animals sensitive to serum may sometimes respond more intensely to heterologous antigens. The following experiments were performed to evaluate this factor:

**PHENOLPHTHALEIN HYPERSENSITIVITY**

**TABLE IV**

<b>Animal No.</b>	<b>Skin Reaction Hrs.</b>	<b>Phenolphthalein* Solution With Agar</b>	<b>Gelatin* 5%</b>	<b>Colloidal Phenolphthalein</b>
<b>A. RABBIT RECEIVING SERUM OF GUINEA PIG PREVIOUSLY INJECTED WITH PHENOLPHTHALEIN</b>				
	1	Neg	15 x 15 -	30 x 20 +
2	24	Neg	15 x 15 -	35 x 40 ++
	48	Neg	Neg	10 x 12 +
<b>B. RABBIT RECEIVING SERUM OF GUINEA PIG PREVIOUSLY FED PHENOLPHTHALEIN</b>				
	1	Neg	15 x 15 -	20 x 20 +
3	24	Neg	10 x 10 -	25 x 40 ++
	48	Neg	Neg	15 x 20 +
				(N) +

\* Controls

PHENOLPHTHALEIN HYPERSENSITIVITY

COMPARISON OF THE SKIN REACTIONS OF RABBITS  
INJECTED WITH THE SERUMS OF GUINEA PIGS AND RABBITS FED  
PHENOLPHTHALEIN

One rabbit was injected with the sera of guinea pigs which had been fed phenolphthalein; a second rabbit was injected with the serum of a rabbit which had been fed phenolphthalein. Eight doses of 2 c.c. each were given at intervals of 5 days. Tests were performed with the following antigens: normal rabbit's serum, serum from a rabbit after one month of feeding of phenolphthalein, colloidal phenolphthalein, and gelatin treated with NaOH and CO<sub>2</sub> and adjusted to a pH of 7.0.

RESULTS: (Table V).

Cutaneous reactions were positive to colloidal phenolphthalein and to the serum of the rabbit which had been fed phenolphthalein for one month.

There were no striking differences in the results of the two sets of experiments. Thus the factor of serum sensitivity did not appreciably alter the results.

Interfaced precipitin reactions here, as well as in all of the other experiments, were negative.

Anaphylactic shock in all of the experiments were negative when the animals were subjected to the intravenous (intracardial) injections of 3 c.c. of colloidal phenolphthalein or 3 c.c. of serum containing phenolphthalein.

PHENOLPHTHALEIN HYPERSENSITIVITY

TABLE V

Animal No.	Skin Reaction Hrs.	Normal * Rabbit Serum	Rabbit Serum (Fed Phenolphthalein for one month)	Colloidal Phenolphthalein	Gelatin * Plus NaOH Plus CO <sub>2</sub>
<b>A. RABBIT INJECTED WITH SERUM OF GUINEA PIGS FED PHENOLPHTHALEIN</b>					
1	3	Neg	20 x 25 + ++ +++	20 x 20 + ++ +	Neg
	24	Neg	40 x 45 ++++ (N)	15 x 20 ++ (N)	Neg
	48	Neg	25 x 30 ++ (N)	10 x 10 + +	Neg
<b>B. RABBIT INJECTED WITH SERUM OF RABBIT FED PHENOLPHTHALEIN</b>					
2	3	Neg	25 x 25 ++ (N)	20 x 15 + +	Neg
	24	Neg	25 x 30 ++ (N)	20 x 20 + (N)	Neg
	48	Neg	10 x 10 + +	12 x 15 + +	Neg

\* Controls

The dimensions are given in millimeters; the symbols one plus to four plus indicate the severity of the reaction whereas a negative sign indicates no reaction; the upper signs represent erythema while the lower ones represent induration. N indicates necrosis.

## PHENOLPHTHALEIN HYPERSENSITIVITY

### REACTIONS OF GUINEA PIGS FOLLOWING MASSIVE INJECTIONS OF COLLOIDAL PHENOLPHTHALEIN PREPARED WITH GELATIN

The results of the experimental work so far carried out have not materially or markedly differed from those obtained by Rosenthal (1938).

It was decided to determine what results could be obtained by injecting guinea pigs with massive doses since the experimental work reported above indicated that the development of sensitivity to phenolphthalein was a very slow process.

Guinea pigs were injected intradermally, subcutaneously and intramuscularly with a 1 per cent colloidal solution of phenolphthalein with gelatin. Doses were started with 2 c.c. and were rapidly increased to 10 c.c. The interval between injections was 5 days. Each of two guinea pigs employed in this experiment received a total of 150 c.c. of the colloidal preparation. Such massive injections produced splitting of the epidermis, dermis and muscles overlying the abdominal wall with subsequent sloughing of the tissues. Skin testing and interfaced precipitin reactions were performed 8 days and 1 month after the last injection. Blood counts and differential counts were done to rule out the possibility of drug toxemia in the results.

PHENOLPHTHALEIN HYPERSENSITIVITY

RESULTS:

Both the cutaneous and precipitin reactions were negative.

Patch-testing was carried out employing the following preparation:

Phenolphthalein	5%
Alcohol qs sol	20 m
Water	10%
Aquaphor qs ad	½ oz.

For control testing the following was employed:

Phenolphthalein	00%
Alcohol	20 m
Water	10%
Aquaphor qs ad	½ oz.

RESULTS:

Sensitivity to phenolphthalein was very suggestive by patch-testing, but the reaction was not clear cut.

The attempt to produce anaphylactic shock was undertaken two weeks after the final injection. These two animals were injected without anesthesia in order to rule out the possibility of the anti-anaphylactic action of the anesthetic. Three c.c. of colloidal phenolphthalein and three c.c. of serum-phenolphthalein were used as antigens.



PHENOLPHTHALEIN HYPERSENSITIVITY

RESULTS:

Both pigs demonstrated shock. The symptoms included bristling of the hair, batting of the nose with their paws and generalized instability. Bronchial spasms with coughing were momentarily observed in one pig, but the symptoms in both pigs subsided in 10 minutes with complete recovery. This was the first time that the results (shock production) differed essentially from those observed by other workers.

It was decided to wait two weeks and repeat the attempt to produce shock by reversing the antigens, that is, the pig that previously received the colloidal phenolphthalein was to receive the serum-phenolphthalein and vice versa with the other pig.

RESULTS:

No shock reaction of any kind was observed.

It was decided to wait three weeks before again attempting the production of shock. The antigens were again reversed to coincide with the experimental conditions above when shock symptoms were observed.

RESULTS:

There was no evidence of shock.

**THE CONJUGATION OF HAPTENS IN VIVO:**

**II. SULFANILAMIDE HYPERSENSITIVITY**

**SULFANILAMIDE HYPERSENSITIVITY**

**MATERIALS**

**and**

**METHODS**

## SULFANILAMIDE HYPERSENSITIVITY

### MATERIALS and METHODS

#### ANIMALS.

White-haired guinea pigs of ordinary stock weighing from 500 gms. to 1000 gms.

White-haired rabbits of ordinary stock weighing from 1235 gms. to 1850 gms.

#### PREPARATION OF COLLOIDAL SULFANILAMIDE.

Colloidal sulfanilamide was prepared by a method described by Fantus and Dyniewicz. (See method of preparation of colloidal phenolphthalein).

#### PREPARATION OF CONJUGATED SULFANILAMIDE IN URINE WITH GELATIN.

Guinea pigs were injected with a saline suspension of sulfanilamide (1 gm. per kilo.) for two days and then they were given 0.4 gm. per kilo for two days (divided in two doses 6 hours apart). Three hours after the final dose the animal's urine was collected for a 48 hr. period. The urine was obtained by placing each animal in an 8 inch glass funnel, to which was attached a clean, well-stoppered collecting bottle. Wire gauze was placed in the apex of the funnel to prevent fecal contamination of the urine. A wire gauze cover was placed over the top of the funnel to prevent the animal's escape. The urines were placed in the

## MATERIALS and METHODS

cold box for 3 days in order to obtain the crystallized drug. The deposited crystals were filtered off and dried. On analysis these crystals were found to contain 3 per cent of free sulfanilamide and 97 per cent of the conjugated compound (calculated as the acetyl derivative). After 3 re-crystallizations from water the compound was found to have a constant melting-point of  $218.5^{\circ}\text{C}$  which is the accepted melting-point for p-acetyl-benzenesulfonamide. A 1 per cent colloidal solution of this substance in gelatin was prepared.

### QUANTITATIVE DETERMINATION OF SULFANILAMIDE (Marshall, 1937).

#### BLOOD:

Protein-free Filtrate: One c.c. of oxalated blood was diluted with 7 c.c. of 0.05 per cent saponin and allowed to lake. Two c.c. of 20 per cent p-toluenesulfonic acid were added, shaken, allowed to stand for 5 minutes, and then filtered. Hydrolysis: For the total sulfanilamide determination, 10 c.c. of the filtrate were measured into a test tube graduated to 10 c.c. and placed in a boiling water bath for 90 minutes. It was permitted to cool and then water was added to compensate for the loss due to evaporation. Determination: To 10 c.c. of the cooled filtrate 1 c.c. of 0.1 per cent sodium nitrite solution (freshly prepared from the G. P. salt) was added and allowed to stand for 3 minutes. Five c.c. of dimethylnaphthylamine solution (1 c.c. in 250 c.c. of 95 per cent ethyl alcohol) were then

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added. At the same time 10 c.c. of a standard sulfanilamide solution (containing 18 c.c. of 20 per cent p-toluene-sulfonic acid per 100 c.c.) were treated in the same manner. A 1.0 mg. per cent standard was found satisfactory for bloods containing from 5 to 10 mg. per 100 c.c. The solutions were then compared in a colorimeter (from 10 to 60 minutes) after the development of color.

### URINE:

Dilution: Urine was diluted so that the concentration of sulfanilamide approximated 1 mg. per cent. Hydrolysis: To 1 c.c. of undiluted urine were added 2 c.c. of NaOH solution (1 normal). The mixture was then diluted to approximately 1 mg. per cent. Determination: To 10 c.c. of diluted urine 1.0 c.c. of 20 per cent p-toluenesulfonic acid was added and the procedure was then followed as for blood.

### PREPARATION OF AGAR SUSPENSION OF SULFANILAMIDE.

Four grams of sulfanilamide (if the drug supplied was already in a very finely divided state) were shaken with 50 c.c. of water in a stoppered 100 c.c. cylinder. Twenty c.c. of 1 per cent hot agar solution were added and mixed. The volume was made to 80 c.c. and the suspension was thoroughly

### MATERIALS and METHODS

mixed. The suspension was then cooled under running tap water until the material remained suspended for some time.

If the sulfanilamide supplied was in too coarse a state, the four grams were dissolved in 45 c.c. of hot concentrated hydrochloric acid. Two or three drops of 1 per cent phenolphthalein reagent were added as an indicator to 1 or 2 c.c. of the mixture, and while still hot, the solution was neutralized with 40 per cent NaOH. From the burette readings the amount of 40 per cent NaOH required to neutralize the remainder of the mixture was calculated. The agar was then added and the volume made to 80 c.c. as before.



SULFANILAMIDE HYPERSENSITIVITY

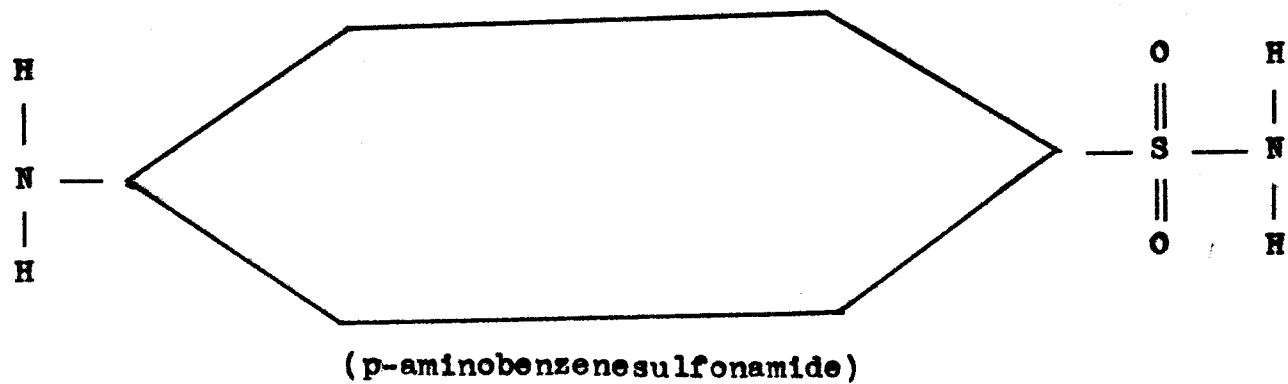


Fig. 3

Sulfanilamide has no Cl or NO<sub>2</sub> groups.

EXPERIMENTAL**SKIN REACTIONS FOLLOWING THE INJECTION OF SULFANILAMIDE  
IN SALINE SOLUTIONS AND IN AGAR SUSPENSIONS**

Guinea pigs and rabbits were injected intradermally and subcutaneously with an 0.8 per cent solution of sulfanilamide in normal saline. Another group of animals received injections of an agar suspension of sulfanilamide (5 per cent sulfanilamide in 1 per cent agar solution). An 0.8 per cent solution of sulfanilamide was employed because of the poor solubility of the drug at moderate temperatures. The following table gives the solubility of sulfanilamide at varying temperatures:

<u>Temperature</u>	<u>Per cent solution</u>
10°C	0.26
15°C	0.42
20°C	0.60
<hr/>	
25°C	0.75
30°C	1.00
35°C	1.30
<hr/>	
40°C	1.70
60°C	4.00
100°C	47.70

The interval between injections was 5 days. Doses were started with 1 c.c. and were rapidly increased to 10 c.c. The agar suspension was injected in 2 and 3 c.c. amounts.

One rabbit received 125 c.c. of the saline solution of sulfanilamide (1.00 gm. of sulfanilamide); another rabbit received 40 c.c. of the agar suspension of sulfanilamide (2.00 gm. of sulfanilamide). One guinea pig received 65 c.c. of the saline preparation of sulfanilamide (0.52 gm. of sulfanilamide); another guinea pig received 20 c.c. of the agar preparation of sulfanilamide (1.00 gm. of sulfanilamide). Cutaneous and interfaced precipitin tests were performed eight days and one month after the last injection. An agar suspension of sulfanilamide, colloidal sulfanilamide with gelatin, an 0.8 per cent saline solution of sulfanilamide and the saline solution (substituted for the suspension in the precipitin tests) were used as antigens. For skin testing 0.1 c.c. was injected intradermally and readings were taken after 30 minutes, 1, 3, 24 and 48 hours.

#### RESULTS:

Both the cutaneous and precipitin reactions were negative.

Employing a stomach tube, 1.75 grams of sulfanilamide were administered orally to both sets of animals after observing the reactions for the first 48 hours.

#### RESULTS:

No effect was observed.

## SULFANILAMIDE HYPERSENSITIVITY

### SKIN REACTIONS FOLLOWING THE INJECTION OF COLLOIDAL SOLUTIONS OF SULFANILAMIDE PREPARED WITH GELATIN

Four guinea pigs and one rabbit were injected intradermally and subcutaneously with a 1 per cent colloidal solution of sulfanilamide with gelatin. The colloidal form was used because it was hoped that by adsorbing the sulfanilamide onto gelatin the larger molecule might aid in the production of an active antigen. Doses were started with 1 c.c. and were rapidly increased to 5 c.c. Because of the numerous reports of toxicity to the drug, blood counts on these animals were made frequently to rule out the possibility of toxicity in the results obtained. Differential counts could not be made because of the fact that the leukocytes did not take the stain. The interval between injections was 5 days. The rabbit received 80 c.c. of the colloidal solution (0.80 gm. of sulfanilamide) and the guinea pigs received from 15 to 40 c.c. of the colloidal solution (0.15 gm. to 0.40 gm. of sulfanilamide).

#### RESULTS:

Cutaneous and precipitin reactions to the antigens were not made on these animals due to the fact that they all died during the progress of sensitization. The rabbit and two of the guinea pigs died some 12 - 18 hours following the last

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injection given to these animals. One of these pigs was autopsied and the findings were consistent with those observed with typical anaphylactic shock in these animals. One of the other two guinea pigs went into shock twenty minutes after receiving an intradermal and subcutaneous injection of 5 c.c. of the colloidal preparation. This was the fourth injection into this animal and represented a total of only 15 c.c. (0.15 gm. of sulfanilamide) given. The other guinea pig went into shock 5 minutes after the observation of a dark-red erythema which developed 10 minutes after the intradermal and subcutaneous injection of 5 c.c. of the colloidal preparation. This was the eighth injection into this animal and represented a total of 35 c.c. (0.35 gm. of Sulfanilamide) given. Blood counts were made on all of these animals prior to the fatal injection. Since the counts were normal it can be said that the factor of toxicity did not enter into the clinical impression of true anaphylactic shock.

## SULFANILAMIDE HYPERSENSITIVITY

### SKIN REACTIONS FOLLOWING THE INJECTION OF COLLOIDAL SOLUTIONS OF SULFANILAMIDE PREPARED WITH GUINEA PIG SERUM AND RABBIT SERUM

These experiments were an exact repetition of those performed with the colloidal preparation of sulfanilamide with gelatin except that, in preparing the colloidal solution of sulfanilamide, serum was substituted for the gelatin. The blood was drawn aseptically, centrifuged to obtain the serum, and then sulfanilamide was added to make a 1 per cent solution. The mixture was incubated at 37°C overnight in an effort to conjugate the drug.

#### RESULTS:

All reactions were negative eight days and one month after the last injection. Here, as previously noted, the oral administration of sulfanilamide to the experimental animals was without effect. The additional factor of sensitivity to the serum was avoided by injecting guinea pigs with the colloidal solution made with the guinea pig sera. Likewise, the rabbit was injected with the colloidal solution of sulfanilamide made with rabbit serum.

Ten days after testing the animals the procedure was repeated using trypan blue to accentuate the dermal response. Immediately after the intradermal injection of the colloidal

### SULFANILAMIDE HYPERSENSITIVITY

solution of sulfanilamide 4 c.c. of 1 per cent solution of trypan blue in normal saline were injected intravenously (intracardially).

#### RESULTS:

After from 30 minutes to 3 hours a concentration of the dye at the test sites was noted indicating a mild cutaneous response to the serum-prepared sulfanilamide solution. This reaction was entirely overlooked in the previous tests.

A comparison of the results obtained in the two sets of experiments clearly indicated the superior potency of colloidal sulfanilamide as an antigen.

### CONJUGATION OF SULFANILAMIDE IN VIVO

It was observed that sulfanilamide administered parenterally appeared in the blood in free and conjugated forms. No drug was found in the serum during the first two to three days because of rapid absorption and elimination. For the free drug, the peak was found after one hour, and for the conjugated form, it was three hours after the first dose and fifteen to eighteen hours after the second dose. Conjugated sulfanilamide was also found in the urine.

A study of Table VI indicates that 0.4 gm. per kilo twice daily yielded a maximum amount of conjugated sulfanilamide. A daily dose of 0.16 gm. per kilo subcutaneously divided into two doses 6 hours apart did not result in a greater concentration of free sulfanilamide than 1.4 mg. per 100 c.c. by the ninth day. A daily dose of 0.2 gm. per kilo by oral administration resulted in a concentration of 6.5 mg. per 100 c.c.; a daily dose of 1 gm. per kilo in two doses 6 hours apart resulted in a concentration of 12.5 mg. per 100 c.c. on the sixth day (free sulfanilamide). The animal succumbed on the seventh day.

One-tenth gm. per kilo twice daily intramuscularly resulted in a concentration of conjugated sulfanilamide of 14 mg. per 100 c.c. A dose of 0.4 gm. per kilo daily in two divided doses gave a maximum concentration of 29.5 mg. per



### CONJUGATION OF SULFANILAMIDE IN VIVO

100 c.c. for the conjugated form. The peak for the concentration of the conjugated form in the blood was reached approximately 3 hours after the first dose and 17 hours after the second dose of the drug.

The intramuscular route of injection appears to be the preferred method of administration of the drug since the slower rate of absorption and elimination resulted in a greater quantity of the conjugated form of the drug.

A study of Table VII indicates clearly that the smaller the injected dose the more rapidly was the drug (sulfanilamide) excreted.

SULFANILAMIDE HYPERSENSITIVITY

TABLE VI

AMOUNT OF SULFANILAMIDE IN THE BLOOD OF ANIMALS AFTER ENTERAL OR PARENTERAL  
ADMINISTRATION OF SULFANILAMIDE

Animal	Route of Injections	Amount of Sulfanilamide Per Kilo	Time	Sulfanilamide Recovered	
				Free Mg./100 c.c.	Conjugated Mg./100 c.c.
		Daily in 2 divided doses (6 hr. apart)			
RABBIT	Subcutaneous	0.16 gm.	9th. day	1.4	
	Oral	0.20 gm.	5th. day	6.5	
	Oral	1.00 gm.	6th. day	12.5	
GUINEA	Intramuscular	0.10 gm.	3 hrs.		14
PIG	Intramuscular	0.40 gm.	3 hrs.		29.5

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TABLE VII

AMOUNT OF SULFANILAMIDE IN THE URINE OF GUINEA PIGS AFTER ENTERAL OR PARENTERAL ADMINISTRATION OF SULFANILAMIDE

Animal	Route of Injections	Amount of Sulfanilamide per Kilo	Total Excretion in Urine in Per cent of Amount Given in			
			24 hrs.		48 hrs.	
			Free	Total	Free	Total
GUINEA PIG	Subcutaneous	0.1 gm.	8	65	8	73
	Intramuscular	0.1 gm.	9	76	9	78
	Intramuscular	1.0 gm.	16	67	17	82
	Subcutaneous	1.0 gm.	32	67	43	83

SULFANILAMIDE HYPERSENSITIVITY**SKIN REACTIONS OF GUINEA PIGS FOLLOWING THE INJECTION  
OF CONJUGATED GUINEA PIG SERA-SULFANILAMIDE**

Two guinea pigs were injected with an agar suspension of sulfanilamide in 8 c.c. (0.4 gm. sulfanilamide) amounts, 6 c.c. intramuscularly and 2 c.c. intradermally. The suspension was given in two doses daily, six hours apart, for 5 days. Blood was withdrawn 3 hours after the final injection and the serum obtained for injection into other guinea pigs.

Pooled guinea pig sera was injected intradermally and intramuscularly into other guinea pigs in 2 and 3 c.c. amounts. The animals were given 8 injections at 5 to 6 day intervals. A total of 20 c.c. was injected. Tests were performed 8 days after the last injection. The following antigens were used: colloidal sulfanilamide, gelatin plus NaOH and CO<sub>2</sub> (adjusted to pH 7.0), conjugated guinea pig serum-sulfanilamide and an agar suspension of sulfanilamide.

**RESULTS: (Table VIII).**

Cutaneous reactions were positive to both the guinea pig serum-sulfanilamide and to the colloidal sulfanilamide. The strongest reactions were obtained employing the colloidal preparation of sulfanilamide with gelatin and consisted of erythema and induration. In this experiment the precipitin reactions were positive.

**SULFANILAMIDE HYPERSENSITIVITY**

**TABLE VIII**

**SKIN REACTIONS OF GUINEA PIGS FOLLOWING THE INJECTION  
OF CONJUGATED GUINEA PIG SERA- SULFANILAMIDE**

Animal No.	Skin Reactions Hrs.	Colloidal Sulfanilamide	Conjugated Sera Sulfanilamide	Gelatin Plus NaOH Plus CO <sub>2</sub> (Control) <sup>2</sup>	Agar Suspension of Sulfanilamide (Control)
1	24	30 x 35 +++ +++	25 x 30 ++ -	Neg	10 x 10 + +
	48	20 x 30 +++ +++	20 x 22 ++ -	Neg	Neg
2	24	15 x 20 ++ ++	17 x 25 ++ -	Neg	Neg
	48	10 x 15 ++ ++	7 x 10 + -	Neg	Neg
3*	24	Neg	Neg	Neg	Neg
	48	Neg	Neg	Neg	Neg

\* Control Animal

The dimensions given are in millimeters; the symbols one plus to four plus indicate the severity of the reaction whereas a negative sign indicates no reaction; the upper signs represent erythema while the lower signs represent induration.

## SULFANILAMIDE HYPERSENSITIVITY

### SKIN REACTIONS OF GUINEA PIGS FOLLOWING THE INJECTION OF COLLOIDAL SULFANILAMIDE PREPARED WITH GELATIN

In the previous experiments when colloidal sulfanilamide prepared with gelatin was injected into laboratory animals, the animals died from anaphylactic shock before cutaneous or precipitin tests could be performed. It was therefore thought desirable to inject another set of animals with the same sulfanilamide preparation as was previously employed, but in this work the tests were to be made after giving the animals no more than 3 or 4 injections. It was hoped by this plan to sufficiently sensitize the animals without a fatal termination with anaphylactic shock.

#### Injections:

Two guinea pigs were injected intradermally and intramuscularly with a 1 per cent colloidal solution of sulfanilamide in gelatin. The interval between injections was 5 days. The guinea pigs received 4 injections of 3 c.c. each (0.12 gm. of sulfanilamide). Cutaneous and interfaced precipitin tests were performed eight days after the last injection. Colloidal sulfanilamide, conjugated guinea pig serum-sulfanilamide, gelatin plus NaOH and CO<sub>2</sub> and an agar

### SULFANILAMIDE HYPERSENSITIVITY

suspension of sulfanilamide were used as antigens. (A saline solution was substituted for the suspension in the precipitin tests).

#### RESULTS: (Table IX).

Both the cutaneous and the precipitin tests were positive; the stronger reactions were observed with the colloidal preparation used as antigen.

Employing the stomach tube, 1 gm. per kilo of sulfanilamide was administered after observing the reactions for the first 24 hours.

#### RESULTS:

The sites previously tested with the conjugated serum-sulfanilamide flared, but the sites previously tested with the colloidal preparation of sulfanilamide with gelatin developed central necrosis following the flare-up.

SULFANILAMIDE HYPERSENSITIVITY

TABLE IX

SKIN REACTIONS OF GUINEA PIGS RECEIVING COLLOIDAL SULFANILAMIDE WITH GELATIN

ANIMAL No.	Skin Reactions Hrs.	Colloidal Sulfanilamide	Gelatin Plus NaOH Plus CO <sub>2</sub> (Control)	Conjugated Guinea Pig Serum- Sulfanilamide	Agar Suspension of Sulfanilamide (Control)
1	24	+++ 30 x 35 +++ (N)	Neg	+++ 20 x 25 ++	+ 10 x 10 +
	48	+++ 25 x 32 +++ (N)	Neg	+++ 12 x 15 ++	Neg
2	24	++++ 40 x 60 +++ (N)	Neg	++ 15 x 20 ++	- 10 x 15 +
	48	++++ 30 x 45 ++++ (N)	Neg	+ 7 x 10 -	- 5 x 15 +
3*	24	Neg	Neg		Neg
	48	Neg	Neg		Neg

\* Control Animal

The dimensions given are in millimeters; the symbols one plus to four plus indicate the severity of the reaction whereas a negative sign indicates no reaction; the upper signs represent erythema while the lower signs represent induration. N indicates necrosis.



SULFANILAMIDE HYPERSENSITIVITYSKIN REACTIONS OF GUINEA PIGS FOLLOWING THE INJECTION  
OF URINE CONJUGATED-SULFANILAMIDE IN GELATIN

In the experiments above it was seen that cutaneous and precipitin reactions could be demonstrated in animals receiving injections of sera-conjugated sulfanilamide and also in animals receiving injections of colloidal solutions of sulfanilamide prepared with the animal's serum. Those animals receiving sera-conjugated sulfanilamide gave good reactions. It was therefore thought desirable to: (1) rule out the possibility of these animals having received free as well as the conjugated form of the drug and (2) to prove the antigenicity of the pure, conjugated form of the drug. For this purpose the conjugated form of the drug was recovered from the urines of guinea pigs receiving saline suspensions of the drug (cf. materials and methods). The crystallized drug was recrystallized three times from water. The compound then gave a melting-point which coincides with the melting-point for p-acetyl-benzene-sulfonamide. A colloidal solution of this substance with gelatin was prepared (cf. materials and methods). The urine-conjugated sulfanilamide with gelatin was then injected intradermally and intramuscularly into guinea pigs in 2 c.c. amounts. The animals were given 10 injections at 5 day intervals. A total of 20 c.c. (0.20 gm. of the substance) was injected. Tests were performed 8 days and 2 weeks after the

SULFANILAMIDE HYPERSENSITIVITY

last injection. Colloidal sulfanilamide, urine-conjugated sulfanilamide with gelatin, conjugated guinea pig serum-sulfanilamide, gelatin plus NaOH and CO<sub>2</sub> (adjusted to pH of 7.0) and an agar suspension of the drug were used as antigens.

RESULTS:

Cutaneous and precipitin reactions were essentially negative.

Patch-testing was done two days later (the patch was allowed to remain for 1 week) with the following preparation:

Sulfanilamide	5%
Alcohol qs sol	20 m
Water	10%
Aquaphor qs ad	$\frac{1}{4}$ oz.

For control testing the following was employed:

Sulfanilamide	00%
Alcohol	20 m
Water	10%
Aquaphor qs ad	$\frac{1}{4}$ oz.

RESULTS:

Cutaneous reactions were essentially negative to the patch tests.

Twenty-four hours after removing the patches, skin testing was repeated using trypan blue to accentuate the dermal response. Immediately after the intradermal injection, 4 c.c. of 1 per cent trypan blue solution (in normal saline) were injected intravenously (intracardially). Test sites were observed at 30 minutes, 1, 3, 24 and 48 hours.

RESULTS:

Cutaneous reactions were entirely negative.

**DISCUSSION**

## PHENOLPHTHALEIN AND SULFANILAMIDE HYPERSENSITIVITIES

### DISCUSSION

The evolution of this method of producing cutaneous sensitivity to combined forms of phenolphthalein was a slow one. The preliminary studies are recorded because they serve as controls and they emphasize the superior potency of the conjugated form of the drug. It was shown, however, that the opposite condition prevailed in the sulfanilamide studies. The conjugated form of the drug did not sensitize.

In the phenolphthalein experiments, the simple injection of the colloidal forms made with gelatin or with animal serum failed to incite a reaction to colloidal phenolphthalein.

In the sulfanilamide experiments, however, the simple injection of the colloidal forms made with gelatin or with animal serum incited strong reactions to colloidal sulfanilamide; the stronger reactions were obtained with the gelatin preparation employed as antigen for sensitization.

With the knowledge that body conjugated phenolphthalein and body conjugated sulfanilamide appear in the blood after enteral or parenteral introduction of the drugs and remain for a longer time than the free forms of the drugs, the use of these serum-conjugated preparations for sensitization

PHENOLPHTHALEIN AND SULFANILAMIDE HYPERSENSITIVITIES

resulted in uniform success only in the experiments dealing with phenolphthalein hypersensitivity. The phenolphthalein treated animals gave positive reactions only to colloidal phenolphthalein prepared in the test tube with gelatin and to the serum of other phenolphthalein treated animals. The experiments in which anaphylactoid reactions were observed cannot be explained. Positive results were obtained with conjugated phenolphthalein in either heterologous or homologous serum. It would seem, therefore, that the animals respond only to free or to conjugated phenolphthalein if the drug is combined with organic substances.

It was shown that sulfanilamide in the free form when combined with gelatin produced a potent antigenic substance, but the conjugated form of the drug combined with gelatin was entirely devoid of antigenicity.

It was shown that homologous-phenolphthalein-containing serum produced a cutaneous sensitivity when injected into another animal of the same or of another species, but no adequate explanation can be given to the observation that the animals receiving phenolphthalein by mouth or by injection did not develop a cutaneous sensitivity. Thus, it appears that for some unknown reason, the use of the conjugated phenolphthalein as an antigen is more active when introduced into another animal of the same or of a different

### PHENOLPHTHALEIN AND SULFANILAMIDE HYPERSENSITIVITIES

species. Conjugation of phenolphthalein with serum could not be accomplished in vitro by simple mixture and incubation. However, it was shown that simple mixture and incubation of sulfanilamide with gelatin did produce a mild antigenic combination. The antigenicity was greatly enhanced by preparing the colloidal solution as given under materials and methods.

Despite the fact that anaphylactic shock could not be produced and precipitin reactions could not be demonstrated in the experiments with phenolphthalein, it is felt that the reactions produced to phenolphthalein were allergic in nature.

The fact that sensitivity to the conjugated form of sulfanilamide could not be produced indicates that this form of the drug is probably totally inactive in the treatment of bacterial infections.

**SUMMARY and CONCLUSIONS**

## PHENOLPHTHALEIN AND SULFANILAMIDE HYPERSENSITIVITIES

### SUMMARY and CONCLUSIONS

Cutaneous sensitivity to phenolphthalein could only be produced in secondary animals. The method consists of feeding phenolphthalein to animals or injecting them with colloidal phenolphthalein and using their serums as antigens to inject into other animals of the same or of a different species. These antigens were found to contain appreciable amounts of a conjugated phenolphthalein and little or no free phenolphthalein. Cutaneous sensitivity to sulfanilamide, however, was readily produced in the primary animals.

Sensitivity to phenolphthalein was a very slow process, whereas, the development of sensitivity to sulfanilamide was a rapid process.

Skin test reactions to phenolphthalein and to sulfanilamide were greatest (intensity) during the first 24 hours and then gradually subsided. It was found that the reactions were immediate (wheal type) or delayed (tuberculin type) depending upon the degree of sensitization. This is very similar to the observations of others employing protein substances. Dienes, 1931, observed that repeated intracutaneous injections of foreign serum and egg in guinea pigs caused gradual evolution of delayed, tuberculin type reactions. With further injections, the immediate or wheal



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type reaction gradually evolved. Jones and Mote observed a similar evolution of skin sensitization, from the delayed tuberculin type to the immediate wheal, following repeated intradermal injections of rabbit serum into man. Simon and Rackemann, 1934, using guinea pig serum, confirmed these observations. Injections were given at weekly intervals to patients suffering from respiratory allergy and to others, used as controls, who gave no history of allergy. The evolution of the positive skin reaction was similar in both groups, again showing confirmatory evidence of no qualitative difference between the allergic and the non-allergic. Both groups were susceptible to artificial sensitization of the skin with guinea pig serum, the degree of sensitization being mild or early when the tuberculin type reaction was obtained and strong when the immediate (wheal) type was observed. The findings strongly suggest that both the immediate and the delayed types of allergy have a common basis and that one factor which determines which type of reaction will be elicited by skin testing is the abundance or scarcity of antibodies or reagins in the blood at the time of testing.

It is believed that the method employed for the production of sensitivity to phenolphthalein can be employed

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with other non-antigenic substances to render them antigenic. However, such substances as sulfanilamide, which apparently act only in the free state of the drug rather than in the conjugated form, would be rendered non-reactive by this method. For such substances it would seem wise to prepare colloidal solutions with gelatin as employed in the experimental work above.

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