STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

IV. Anaphylaxis Induced by Picryl Chloride and 2:4 Dinitrochlorobenzene

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In previous communications (1–4), report has been made of sensitization effects in guinea pigs following intracutaneous or superficial administration of a number of simple chemical compounds, *e.g.*, nitrosodimethylaniline and 2:4 dinitrochlorobenzene. Thus, animals treated intradermally with small quantities of the substances exhibit distinctly increased reactions to subsequent intradermal injections, and give erythematous reactions when a solution of the incitant is spread on the skin. A study of various nitrochlorobenzenes then demonstrated a parallelism between skin sensitizing capacity and chemical reactivity, consistent with the idea that sensitization effects are due to conjugated antigens formed *in vivo*. That artificially conjugated antigens—azoproteins—can sensitize (anaphylactically) to the conjugate, the reactions being specific for the substance linked to protein, had been shown previously (5; *cf.* 6), but there was no direct evidence that sensitivity to simple substances may depend upon the formation of such antigens (7). Another result was that in the case of acyl chlorides, which also are able to sensitize, skin sensitiveness and anaphylaxis were produced simultaneously upon repeated intracutaneous injections, indicating a relationship in the causation of the two effects. In the present paper, this question is further investigated.

EXPERIMENTAL

Anaphylaxis Experiments with Picryl Chloride and 2:4 Dinitrochlorobenzene.—In the experiments cited above, benzoyl chloride and

p-chlorobenzoyl chloride gave rise to both skin sensitization and anaphylaxis. The formation in these instances of conjugate antigens evidently responsible for the production of anaphylaxis is not particularly surprising, since the substances are easily decomposed by water and are highly reactive, *e.g.* with proteins. In this respect the experiments are somewhat similar to the sensitizations obtained with diazonium and diazoamino compounds (8, 9). It seemed necessary to make investigations on the possibility of producing anaphylaxis with substances that are relatively stable and are known to cause allergic phenomena in human subjects. Two substances were chosen for study, picryl chloride, previously shown to sensitize guinea pigs (1) and capable, moreover, of producing sensitization in human beings upon intracutaneous administration,[1] and 2:4 dinitrochlorobenzene, which is known to cause contact dermatitis in a large number of industrial workers handling it (10–12). Picryl chloride, although combining readily with proteins in alkaline solution, is not decomposed by water at room temperature for a considerable period of time, and can be recrystallized from boiling alcohol; it does, however, react slowly with serum proteins at serum alkalinity. The second substance, 2:4 dinitrochlorobenzene, is stable in boiling water for hours and does not combine with serum proteins to any appreciable extent without the addition of alkali.

Guinea pigs were sensitized by repeated intracutaneous administration on the dorsum, the course commonly consisting of about fifteen daily injections, six a week, each of 1/400 mg. picryl chloride in 0.1 cc. saline.[2] The commercial preparation was used after two recrystallizations from a benzene-alcohol mixture, m.p. 82° (uncorrected). For the injections, solutions of the requisite concentration were prepared by diluting in saline an alcoholic 0.3 per cent solution.

White male guinea pigs weighing 350–450 gm., mostly albinos, were used for the sensitizations. The development and the degree of skin sensitivity to picryl chloride correspond in a general way to observations reported with 2:4 dinitrochlorobenzene (1). With daily injections, the reactions being recorded 24 hours

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[1] Personal communication from Dr. Marion B. Sulzberger.

[2] Lately it has been found that very satisfactory skin sensitization can be attained by intracutaneous injection of larger quantities of picryl chloride than those used before, given simultaneously in several sites (1/50 mg. injected in each of 7 sites, test made 1 month later).

later, evidence of a heightened response would usually be noted between 6 and 8 days after the first injection, the sensitivity gradually developing to give elevated, pink areas 10 to 15 mm. in diameter, often with blanched or livid centers, at times with a necrotic spot. A period of 2 or 3 weeks was allowed between the last injection of the course and the final testing, which was made by spreading on the belly, after clipping the hair, 1 drop of a 2 per cent solution of picryl chloride in olive oil; for subsequent tests, new belly sites were chosen each time. The reactions were read 18 to 24 hours later, following use of a depilatory 2 or 3 hours before. On normal animals, similarly treated as controls, reactions were faint or absent. Of 134 pigs treated with picryl chloride by the above or a comparable method, only seven did not show evidence of sensitization; this uniformity closely resembles experiences with 2:4 dinitrochlorobenzene (4).

For the preparation of protein conjugates 1 millimol of picryl chloride in 5 cc. chloroform was shaken vigorously for 15 minutes with a mixture of 30 cc. horse serum (or guinea pig serum) and 15 cc. N/2 Na_2CO_3, the temperature being kept at about 5°C. After the removal of insoluble material by centrifugation in the cold, the supernatant was acidified to maximum precipitation, and the picryl protein sedimented by spinning. The protein compound was dissolved in water with addition of alkali to pH 8–9, the reaction adjusted close to neutrality, and a small amount of insoluble material centrifuged off. For purification, the protein was precipitated by treatment with 5 volumes of alcohol, centrifuged, and redissolved in water at slight alkalinity; it was finally precipitated with acid, washed once with saline by centrifugation, and dissolved in saline at slight alkalinity. The solution was made approximately neutral, becoming somewhat turbid thereby, and the concentration (about 2 to 3 per cent) was determined.

Recently we have found that the protein compounds can be made by gentle shaking of serum with finely ground picryl chloride; this method can also be used with 2:4 dinitrofluorobenzene (see below).

With intracutaneous injections of picryl chloride, as previously found with *p*-chlorobenzoyl chloride (2), guinea pigs were found to develop anaphylactic, in addition to skin, sensitivity. As seen in Table I, typical anaphylactic shock resulted when picryl protein conjugates were given intravenously. While with the batch of animals shown in the table 4 mg. represented approximately the limiting quantity of antigen to produce fatal shock, this value fluctuated with different series of sensitized animals. It was as low as 1/5 mg. in a group which had received six weekly intradermal injections of picryl chloride (tested 10 days later with "picryl horse serum"), and also in a lot given ten to fourteen daily injections (test made with "picryl guinea pig serum" after 4 weeks' rest); in other experiments,

only a small proportion of the animals were fatally shocked with 10 or 20 mg. doses of antigen.

As a complication, in some cases it seemed that large doses were less effective than smaller ones in demonstrating anaphylaxis (*cf.* 8). The interval between the last sensitizing injection and the intravenous injection of picryl protein may

TABLE I

Anaphylaxis in a group of guinea pigs given daily intradermal injections of 1/400 mg. picryl chloride for 15 days, and injected intravenously with picryl protein between 8 and 12 days after the last skin injection. Figures in parentheses indicate change in temperature (°C.).

No.	Amount injected	Intravenous injection of picryl horse serum
	mg.	
1	12	Slight symptoms (−1.1)
2	10	Typical anaphylaxis † 4 min.*
3	8	" " † 6 "
4	8	" " † 13 "
5	8	" " † 4 "
6	8	Severe shock, recovered (−5.9)
7	4	Typical anaphylaxis † 4 minutes
8	4	" " † 6 "
9	4	Slight symptoms (−1.2)
10	2	Coughs, eyes running, labored breathing (−1.5)
11	1	Typical anaphylaxis † 7 min.
12	1	Slight symptoms (−1.0)
13	1/5	No symptoms (+0.2)
		Controls
14	16	No symptoms (−0.6)
15	16	" " (−0.2)
16	8	" " (−0.4)
17	8	" " (−0.4)
18	4	" " (−0.6)

* The symbol † signifies death of animal; in all cases the autopsy findings have been characteristic.

be as short as a week; in one experiment in which animals were tested after intervals of 1 week and 5 weeks, the degree of anaphylactic sensitivity had diminished by the 5th week.

The presence of antibodies in the serum of guinea pigs sensitized by intradermal injections of picryl chloride was demonstrable by passive transfer, using the Schultz-Dale method (see 13).

The sensitized animals were bled between the 6th and the 14th day after the final intradermal injection of picryl chloride, and the serum, commonly in 3 cc. amounts, was injected intraperitoneally into virgin female guinea pigs weighing 180–250 gm. After 24 or 48 hours the animals were exsanguinated by heart puncture and the excised uterine horns were rinsed and mounted in a Dale apparatus in separate chambers of 20 cc. capacity, the recording being made at about threefold magnification. The oxygenated bath usually was the fluid recommended by Dale (13) (NaCl 0.9 per cent, KCl 0.042 per cent, $CaCl_2$ 0.012 per cent, $NaHCO_3$ 0.05 per cent, in glass-distilled water); infrequently the amount of calcium chloride was reduced to one-half. The antigen (1 or 2 mg., or less, of picryl protein made with horse serum) was added to the bath in a volume of 0.2 cc. or less, the resultant concentration being without effect upon the uterine horns of normal guinea pigs. If a contraction ensued, and specific desensitization was to be demonstrated, the horn was allowed to relax fully and was then washed by repeated changes of oxygenated fluid; thereupon the same dose of antigen which had caused the first contraction was added to the bath. Finally, to test the condition of the muscle, an addition of histamine was made.

A representative experiment of this sort is given in Text-fig. 1, where it is seen that the passively sensitized muscle responded to picryl protein even at 1:80,000 dilution and that when the test was repeated the uterus was seen to be desensitized. Anaphylactic antibodies were found with most batches in a large proportion of animals sensitized by intradermal injections of picryl chloride as described above, *e.g.*, with one group, eight out of ten guinea pigs, and in another experiment seven out of nine, gave positive transfers; in some lots definitely poorer effects were obtained. On the whole, these results would seem to compare not unfavorably with those obtained in homologous passive transfer experiments with sera from animals injected with proteins (14, 15).

Many of the transfers effected with 3 cc. amounts of serum resulted in maximal contractions maintained 2 to 6 minutes when the horns were exposed to picryl horse serum in the usual concentration of 1:20,000. With the most active sera, 1 cc. was adequate for sensitization. In some instances where uteri appeared to be sensitized highly, the antigen was employed in greater dilutions; in one case, there was a maximal sustained contraction to an antigen concentration of 1:2,000,000, which indicates a degree of sensitivity close to that shown by highly sensitive uteri as a result of customary protein immunization (*cf.* 13). It may be mentioned that there

seemed to be some parallelism between the intensity of skin reactions elicited by the later preparatory injections and the content of anaphylactic antibodies in the serum; in some series of animals, however, such a relationship was not seen in the degrees of anaphylactic and cutaneous sensitivities, the latter tested by applying oil solutions on the skin several weeks after the last treatment.

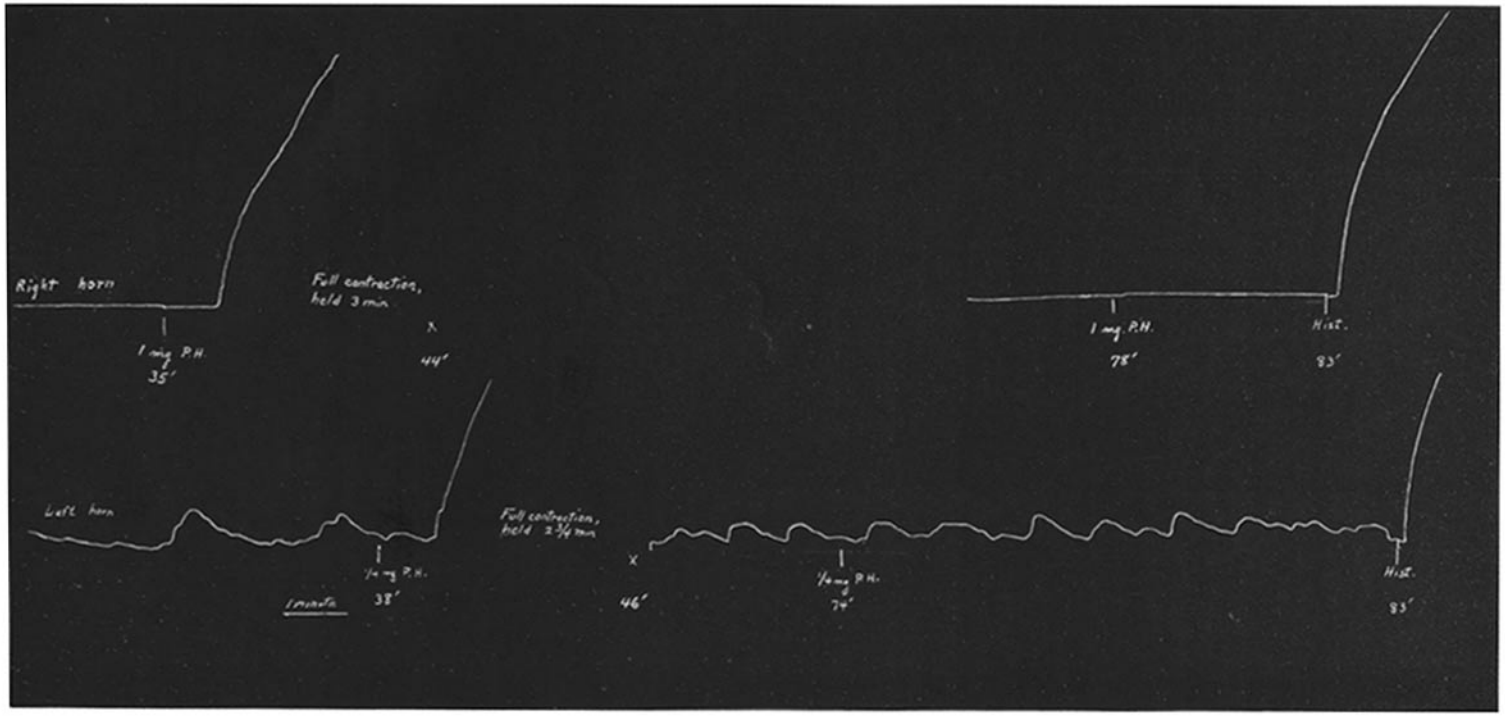
Precipitins were detected in some of the sera which were among the most active in conferring passive sensitization, picryl protein being used as antigen in ring tests. The rings, although faint and detectable only with clear sera, were definite; the optimal concentration of picryl guinea pig serum was usually 1:30,000. Attempts to transfer skin sensitiveness passively by means of sera containing anaphylactic antibodies and precipitins have so far been negative.

While in the above experiments skin sensitization and anaphylactic antibodies were developed by intradermal injections as stated, it is of interest that in a preliminary experiment anaphylactic antibodies were found also in guinea pigs sensitized by continued daily application of one drop of a 2 per cent solution of picryl chloride in olive oil to the intact skin, anaphylactic sensitivity arising in a normal guinea pig from the intraperitoneal injection of 7.5 cc. pooled serum taken from three cutaneously sensitized guinea pigs.

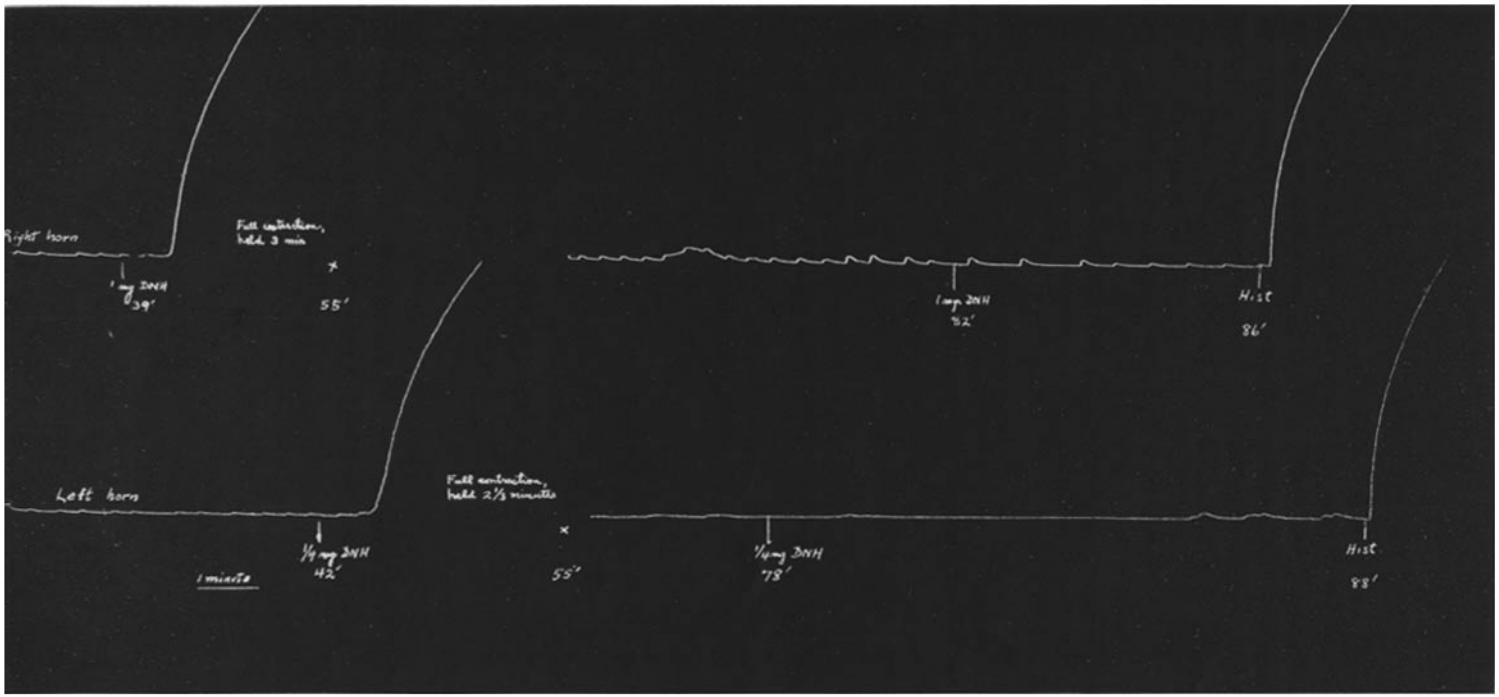
Analogous experiments were carried out on guinea pigs sensitized to 2:4 dinitrochlorobenzene by means of repeated intracutaneous injections, as in the case of picryl chloride.

The development of skin sensitivity to this substance has been described (1); cutaneous sensitivity was tested by superficial application of a 1 per cent solution in olive oil. On account of its greater reactivity as compared with the chloro compound, 2:4 dinitrofluorobenzene (16) was used in the preparation of 2:4 dinitrophenyl protein conjugates. The latter were prepared with guinea pig or horse serum, the method for picryl protein being followed in the main. The guinea pig serum preparation was made by shaking in the cold for 10 minutes; in reprecipitating the protein derivative, careful adjustment of the pH and addition of salt were necessary; the antigen was used after dialysis against isotonic saline. The horse serum preparation was obtained in a similar manner. The solution tended to become turbid when kept in the ice box at neutral reaction, and before use the amount required was considerably clarified by cautious addition of NaOH and then adjusted with HCl until almost neutral.

In Schultz-Dale experiments with actively sensitized guinea pigs, since the animals usually were rather heavy by the time of testing, and the horns showed



TEXT-FIG. 1. Anaphylactic antibodies in serum of guinea pig given fifteen daily intradermal injections of picryl chloride (see text), 7 days after the last injection, demonstrated by passive transfer. Recipient guinea pig (235 gm.) was injected intraperitoneally with 3 cc. serum, and the uterine horns were tested 2 days later. Additions of picryl horse serum (P.H.) to the bath (20 cc. Dale solution) were made at the times noted after mounting the horns; the sign X designates washing out of the chamber; histamine dihydrochloride (Hist.) was finally added (concentration 1:5,000,000 of the free base).



TEXT-FIG. 2. Anaphylactic response in a guinea pig sensitized by fifteen daily intradermal injections of 2:4 dinitrochlorobenzene (see text) and tested by Dale's method with 2:4 dinitrophenyl horse serum (D.N.H.) as antigen. 11 days after the last injection, the uterine horns of the animal (360 gm. body weight) were mounted in 20 cc. modified Dale solution (calcium chloride reduced to one-half), and the antigen was added as shown. Histamine (Hist.) was used in a concentration of 1:10,000,000.

spontaneous contractions in the above mentioned Dale solution, modified bath fluids were employed, either the Dale solution with the amount of calcium chloride reduced to one-half or one-quarter (animals weighing 325–400 gm.) or the calcium-free solution used by Bristol and Fleischner (17). For the latter (see 18), a stock solution is made with 10.5 gm. NaCl, 0.5 gm. KCl, 0.1 gm. $MgCl_2$, 5 cc. N H_3PO_4, 50 cc. water, and the bath fluid is prepared from 50 cc. stock solution, 5 cc. N Na_2CO_3, and 1000 cc. water. Along with repression of spontaneous contraction in these solutions, the specific reactions are apparently diminished and the tests less sensitive.

TABLE II

Anaphylactic response of a group of guinea pigs given 6 weekly intradermal injections of 1/400 mg. 2:4 dinitrochlorobenzene, 2:4 "dinitrophenyl guinea pig" serum being injected intravenously 4 weeks after the last skin injection.

No.	Amount injected	Intravenous injection of 2:4 dinitrophenyl protein
	mg.	
19	20	Severe symptoms (chronic type), recovered
20	20	Typical anaphylaxis, † 4 min.
21	20	No symptoms
22	20	Typical anaphylaxis, † 4 min.
23	10	Questionable symptoms
		Controls
24	20	No symptoms
25	20	" "

Guinea pigs sensitized by intracutaneous injections in the manner stated were tested after 7 to 40 days by intravenous injection with the protein conjugate. Resultant anaphylactic symptoms were variable, and the instances of fatal shock were few. Nevertheless, as seen in Table II (which presents the best result thus far obtained), the fact that the intracutaneous administration of 2:4 dinitrochlorobenzene can induce an anaphylactic state could be demonstrated beyond doubt. It would seem probable that continued study should determine experimental conditions under which the results will be more regular and passive transfer can be demonstrated.

Further evidence was forthcoming from experiments made with the Schultz-Dale method, the horns of sensitized guinea pigs being tested with 1:10,000 or 1:20,000 dilutions of 2:4 dinitrophenyl pro-

TABLE III

Anaphylactic desensitization by subcutaneous injection of picryl protein in two batches (A and B) of guinea pigs sensitized by 15 daily intradermal injections of 1/400 mg. picryl chloride. 12 days after the last skin injection, part of the animals were given 10 mg. picryl guinea pig serum subcutaneously; next day, all were tested for skin sensitiveness, and one day later picryl horse serum was injected intravenously. Figures in parentheses indicate change in temperature (°C.). The skin reactions on normal animals ranged from negative to faint pink.

Animals given desensitizing injection				Sensitized controls			
No.	Application of a 2 per cent solution of picryl chloride in olive oil on the skin	Intravenous injection of picryl horse serum		No.	Application of a 2 per cent solution of picryl chloride in olive oil on the skin	Intravenous injection of picryl horse serum	
		Amount	Symptoms			Amount	Symptoms
		mg.				*mg.*	
			Group A				
26	p., el.*	10	None**	30	dp., m.el.	10	† 4 min.
27	p., el.	10	None**	31	p., sl.swol.	10	† 11 min.
28	pp., sl.el.	10	None (+0.8)	32	pp.–p., el.	10	† 4 min.
29	pp.–p., sl.el.	5	None (−0.9)	33	p., el.	5	Moderate to severe (−0.7)
				34	pp., sl.el.	10	Moderate (−1.7)
				35	pp., sl.el.	10	Moderate (−1.9)
				36	pp.–p., sl.el.	10	† 30 min.
			Group B				
37	p., sl.el.	10	None (−0.1)	40	pp., sl.el.	10	Slight to moderate (−1.8)
38	p., sl.el.	10	None (+0.5)	41	p., sl.el.	10	† 14 min.
39	p., el.	10	None (−1.0)	42	p., sl.el.	10	Moderate (−2.1)

*The following abbreviations are used: faint pink (fp.), pale pink (pp.), pink (p.), dark pink (dp.), slightly elevated (sl.el.), elevated (el.), markedly elevated (m.el.), swollen (swol.), blanched center (bl.c.).

**Temperature change not determined.

tein compounds prepared from horse or guinea pig serum. Here again the results were inconstant, and negative with two small batches of animals, which incidentally did not exhibit a high degree of skin sensitivity; in other lots of guinea pigs, however, definitely positive reactions occurred, *viz.* with two out of eight, and in five out of a group of fifteen (tested 9 days after the last intradermal injection), and in four out of nine (examined after a rest of 32 to 50 days). The reactions ranged from relatively weak contractions to, in the majority

TABLE IV

Failure of subcutaneously administered picryl protein to desensitize the hypersensitive skin of selected guinea pigs previously given intradermal injections of picryl chloride. After the first skin test made by applying 1 drop of a 2 per cent solution of picryl chloride in olive oil to the skin of the belly, half of the animals were reserved for comparison, the others were injected subcutaneously with picryl guinea pig serum (10 mg. on the 2nd, 4th, and 8th days, 20 mg. on the 10th day), and a second skin test was made in the same way on the 12th day.

Animals given desensitizing injections			Sensitized controls		
No.	First skin test	Second skin test (after four injections of picryl protein)	No.	First skin test	Second skin test
43	p., bl.c., swol.	dp., m.el.	47	p., el.	pp.
44	pp., sl.el.	pp.	48	pp., sl.el.	pp.
45	pp.–p., sl.swol.*		49	pp–.p., sl.swol.	p., swol.
46	p.	p.	50	pp.	pp.–p.

* Animal died within a few hours after the first subcutaneous injection of picryl protein.

of cases, maximal contractions sustained for 1 to 3 minutes, specific desensitization being demonstrated regularly; such a record is shown in Text-fig. 2.

Desensitization Experiments with Picryl Protein.—With animals sensitized to picryl chloride, experiments aimed at desensitization were carried out by administering picryl guinea pig serum subcutaneously prior to the intravenous shocking injection. The subcutaneous injections were not seldom followed by local reactions, consisting of edema, more pronounced than in normal animals, and sometimes reddening of the skin. The results as presented in Table III show that anaphylactic desensitization could be achieved reg-

ularly. In contrast to this, it will be seen that the reactivity of the skin to superficial application of picryl chloride was not concomitantly abolished, and indeed even repeated subcutaneous injections of picryl protein had no noticeable influence on the degree of skin sensitiveness (Table IV). While we have not investigated the subject particularly, in an experiment with a few animals we were able to desensitize the skin by long continued daily applications of an olive oil solution of the incitant, 2:4 dinitrochlorobenzene. In a similar way Kobayashi (19), working with guinea pigs sensitized with an extract of *Rhus vernicifera*, reported desensitization by long continued painting with the extracts. By means of subcutaneous injections in human beings, Blank and Coca (20) found that a certain degree of immunity to contact with poison ivy develops.

COMMENT

The above experiments demonstrate that certain simple substances which cause human hypersensitiveness, with skin manifestations, produce upon intracutaneous injection into guinea pigs both skin sensitivity and anaphylactic sensitization. This has been shown with picryl chloride and with 2:4 dinitrochlorobenzene, which are capable of inducing cutaneous sensitization in human beings. It is noteworthy, indeed, that in these cases very small quantities of simple compounds can produce anaphylactic sensitization, evidently through combination with some substance of the animal body.

From our results, it appears that although both the compounds mentioned sensitize guinea pig skin in like manner they probably differ quantitatively in their capacity to evoke an anaphylactic state. While this distinction is one of degree only, for also with 2:4 dinitrochlorobenzene unquestionable anaphylactic effects were obtained, nevertheless the result taken in conjunction with the skin effects would indicate differences in the mode of anaphylactic sensitization and sensitization of the skin to superficial application of the incitant.[3] There are several other facts, from the experiments with picryl chloride, pointing in this direction. In the first place it has not been possible to induce skin sensitivity to the simple substance, although

[3] *Cf.* Landsteiner and Levine (6), page 353; Landsteiner (4).

an anaphylactic state is set up in this way, by injecting the protein conjugate intradermally, in contrast to the outcome of the converse experiment (Table I). Again, several attempts at passively sensitizing the skin to contact with the simple substance, by means of sera containing anaphylactic antibodies, have failed. Then in some experiments we observed a lack of parallelism in the degrees of anaphylactic and skin contact sensitizations in animals prepared by intradermal injections of picryl chloride. There are, finally, the desensitization experiments with protein conjugates (Table III) which were successful so far as anaphylaxis is concerned but were without effect on the dermal reactions produced by superficial application of the incitant to the intact skin. It would be premature to elaborate hypotheses concerning the differences in the processes leading to the two sorts of sensitization; tentatively it might be considered that the cutaneous manifestations are due to antibodies, perhaps of a special sort, produced by and fixed in the skin (*cf.* 6), or one could possibly think of the formation of various sorts of antigenic conjugates having the same "hapten component." The answer to these and other possibilities must await further study. Yet it would be most unlikely that the two specific sensitization effects induced at the same time by intradermal injections of "non-antigens," namely skin sensitivity and general anaphylaxis, are without a fundamental relationship.

That the two conditions are related is strongly indicated by the fact that both were found to be induced by substances characterized by their ability to form conjugates (2). Antibodies, it is true, have so far been demonstrated in our experiments only for the anaphylactic sensitization which results from intracutaneous (or even superficial) treatment with suitable chemical substances. However, in the experimental allergic dermatitis of guinea pigs as in human contact dermatitis the instrumentality of antibodies in the broadest sense of the word, namely specific substances formed in consequence of previous contact,[4] must be assumed *a priori* because of the phenomenon of specificity, although they have not as yet been experimentally established. Considering other cases, such as the absence of cir-

[4] *Cf.* Doerr (21, 22); Zinsser (23).

culating antibodies in later stages of the anaphylactic state, it is obvious that failure to demonstrate passive transfer of skin manifestations by means of serum is no decisive proof against the existence of antibodies, confined to the skin or perhaps circulating in small amounts and only transiently. Thus from the foregoing one may conclude that in the cases examined in this and in previous studies (1–3) the immunizing activity of conjugated antigens comes into play, this concept affording a plausible explanation for the immunological effects of simple substances.

SUMMARY

It has been shown that by the cutaneous administration of simple chemical compounds in small quantities—2:4:6 trinitrochlorobenzene (picryl chloride) and 2:4 dinitrochlorobenzene, the latter a typical incitant of contact dermatitis in man—it is possible to induce true anaphylactic sensitization in guinea pigs, demonstrable by the intravenous injection of protein conjugates and by the Dale technique, using isolated uterine horns. This furnishes strong evidence for the formation of antigenic conjugates following application of substances of simple chemical constitution. Since the anaphylactic state is induced by the same method of administration that gives rise to cutaneous sensitivity, the assumption would appear justified, when one takes into account the chemical properties of the inciting substances, that the formation of conjugated antigens offers an explanation for the skin effects also.

In the experiments with picryl chloride, anaphylactic antibodies, and occasionally precipitins, have been demonstrated.

The differences between the cutaneous and anaphylactic types of sensitivity are discussed.

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